

Highly Sensitive Endotoxin Assay Combining Peptide/Graphene Oxide and DNA-Modified Gold Nanoparticles

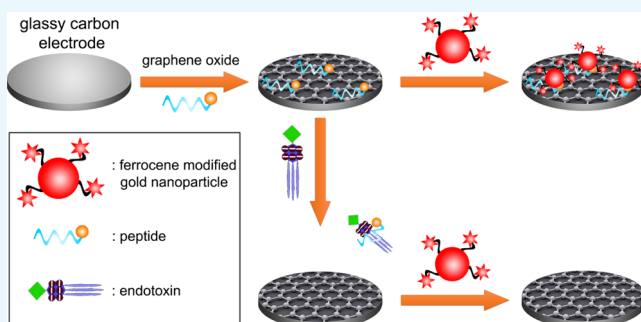
Nong Yu,[†] Xiaozhong Zhang,[†] Yuan Gao,[†] Honglan You,[†] Jingzhong Zhang,^{*,‡,§} and Peng Miao^{*,‡,§}

[†]People's Hospital of Suzhou New District, Suzhou 215010, P. R. China

[‡]Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Suzhou 215163, P. R. China

[§]Tianjin Guokeyingong Science and Technology Development Company Limited, Tianjin 300399, P. R. China

ABSTRACT: Endotoxin is a highly toxic stimulator originated from the outer membrane of Gram-negative bacteria, which should be monitored sensitively and selectively for human health concerns. Traditional detection methods mainly rely on limulus amoebocyte lysate assay. However, it suffers drawbacks like the narrow detection range, and the results may be environment-dependent. In this work, we have developed a sensitive electrochemical biosensor for endotoxin assay. Peptide is first designed as specific recognition element toward endotoxin. Graphene oxide and DNA-modified gold nanoparticles are then used to enhance the electrochemical signal. The analytical performances are excellent with the limit of detection as low as 0.001 EU mL⁻¹. This method has also been successfully applied in endotoxin assay in complex biological samples, which may have great potential use.



INTRODUCTION

Endotoxin is the main ingredient of the outer membrane of Gram-negative bacteria.¹ It acts as an extremely strong stimulator of innate immunity. Although it is beneficial at low concentrations, higher levels of endotoxin may cause symptoms like diarrhea, fever, multi organism failure, septic shock, and even death.² Exposure to endotoxin may occur through injection, dialysis, and polluted drinking water.^{3–5} For example, dialysis patients are typically exposed to an annual 20 000 to 30 000 L of dialysis fluid, and the opportunity to experience an inflammation due to endotoxin contamination is significantly increased.⁶ Considering the health risks of endotoxin, sensitive and convenient methods for endotoxin assay is critical and have attracted much attention.^{7,8}

Although there have been well-established methods for the determination of endotoxin like the limulus amoebocyte lysate (LAL) assay as the golden method, many drawbacks should be overcome.⁹ For instance, the linear range of LAL assay is narrow and the detection result is highly pH and temperature dependent. The detection of endotoxin can also be interfered by beta-glucan. Therefore, the development of enzyme-free assays should be emphasized. Different affinity molecules have been explored including aptamer,¹⁰ peptide,¹¹ protein,¹² polymycin B,¹³ and so on. In addition, many nanomaterials have also been employed for the generation and amplification of obtained signals.^{14,15} For example, An and Jang immobilized a specific aptamer on glass for optical sensing and separation of endotoxin.¹⁶ Lim et al. employed peptide-assembled graphene oxide in a fluorescent sensing system for sensitive detection of endotoxin.¹⁷ Brosel-Oliu et al. fabricated a label-free

impedimetric sensor for endotoxin assay based on concanavalin A recognition.¹⁸ However, because of the increasing demands for endotoxin assay, there still exists an urgent need for more convenient, sensitive, and reliable methods for the practical detection of endotoxin.

In this work, we have integrated peptide-assembled graphene oxide and DNA modified gold nanoparticles (AuNPs) in an electrochemical sensing system for endotoxin assay. The thiol group of the peptide is able to bind to gold, localizing AuNPs on the electrode surface. The labeled ferrocene (Fc) molecules offer intense electrochemical responses. However, in the presence of endotoxin, specific interaction between endotoxin and peptide releases the thiol-containing peptide away from the electrode and the recorded signal of Fc is decreased. This novel method achieves a wide linear range from 0.005 to 1 EU mL⁻¹ with a quite low limit of detection (LOD) of 0.001 EU mL⁻¹.

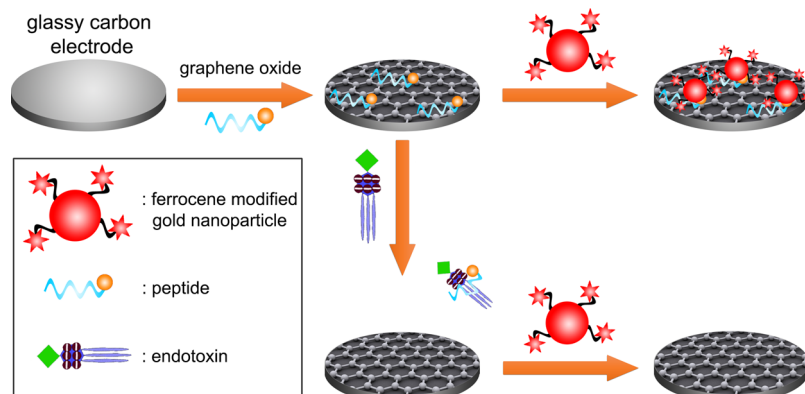
RESULTS AND DISCUSSION

Detailed sensing principle is illustrated in Scheme 1. Generally, a glassy carbon electrode is first modified with negatively charged graphene oxide. The positively charged endotoxin binding peptide (pI = 2) is able to be absorbed on the electrode via electrostatic interactions. The specific recognition overcomes the potential interference like beta-glucan, nanoparticles, and so on. The thiol group of cysteine at the C-

Received: July 2, 2019

Accepted: August 12, 2019

Scheme 1. Illustration of the Electrochemical Biosensor for the Detection of Endotoxin



terminal of the peptide allows the attachment of AuNPs on the electrode surface via gold–sulfur chemistry. Because Fc-labeled DNA probe is previously modified on the surface of AuNPs, significant Fc response could be recorded. Nevertheless, the peptide can be released from the graphene oxide by interacting with target endotoxin, negatively charged AuNPs cannot be facilely localized on the electrode, and the electrochemical signal is reduced. By analyzing the peak current of the electrochemical system, the initial endotoxin level can be evaluated. The proposed method has several merits including high selectivity and high sensitivity. The applied peptide not only allows specific recognition but also helps recruit numerous electrochemical species. The high sensitivity is attributed to two aspects. First, the good electric-conductivity of graphene oxide and AuNPs promise the electrochemical response with high intensity; second, because of the large specific area of AuNPs, a huge number of DNA molecules which are labeled with Fc are loaded on each AuNP, which assist the improvement of the sensitivity.¹⁹

The adsorption of AuNPs on graphene oxide can be intuitively determined by transmission electron microscopic (TEM) images. Sphere AuNPs can be observed with the diameter about 13 nm (Figure 1A). Without the assembled peptide, nearly no AuNPs can be decorated on the surface of

graphene oxide because of electrostatic repulsion (Figure 1B). On the contrary, the added peptide provides abundant thiols for specific gold–sulfur interaction and a large number of AuNPs can thus be located on the graphene nanosheets (Figure 1C). These results demonstrate that the peptide in this work not only functions as the recognition element but also helps the localization of AuNPs with electrochemical species.

We have further studied the effect of endotoxin on the electrochemical system. As depicted in Figure 2, the square

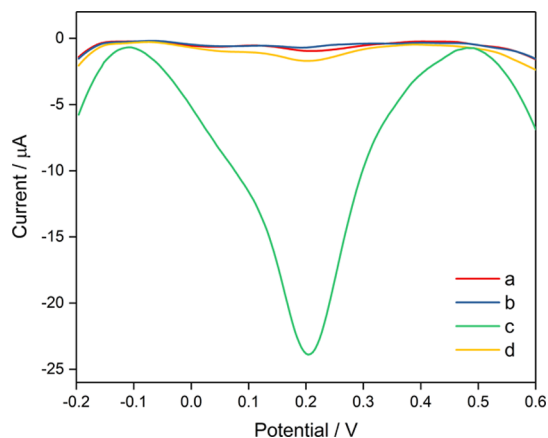


Figure 2. SWV curves of (a) graphene oxide modified electrode, (b) peptide modified electrode, after incubation with DNA modified AuNPs in the (c) absence and (d) presence of endotoxin. Modulation amplitude: 25 mV, the frequency: 70 Hz, step potential: 4 mV.

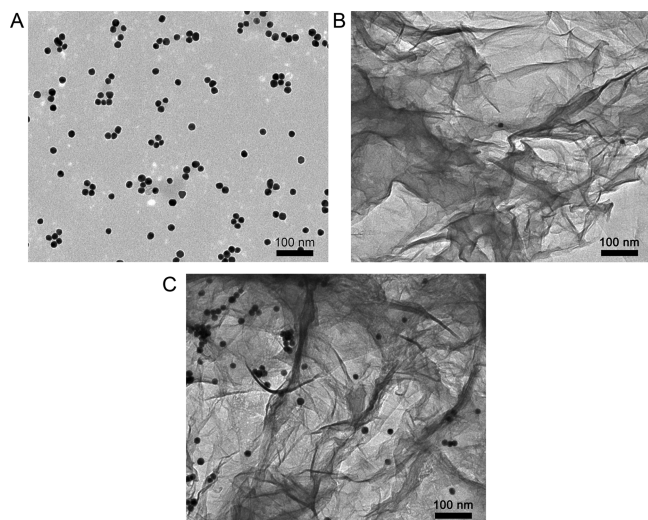


Figure 1. TEM images of (A) bare AuNPs, graphene oxide mixed with AuNPs in the (B) absence and (C) presence of peptide.

wave voltammetry (SWV) curves of graphene oxide modified electrode before and after the assembly of peptide show no current peaks of Fc. After the capture of AuNPs, a strong peak is observed. However, if endotoxin exists in the sensing system, the disassociation of the peptide makes the immobilization of AuNPs impossible and the Fc signal disappears. The SWV responses demonstrate the feasibility of the system for endotoxin assay.

To achieve the best analytical performances, we have optimized the reaction times. Longer incubation of AuNPs with the electrode and longer incubation time of endotoxin with the peptide contributes to more adequate localization of Fc and release of the peptide. Also, the peak currents reach saturation after 90 and 10 min, respectively (Figure 3). Therefore, we have chosen the reaction times in the following quantitative experiments. As shown in Figure 4a, with larger

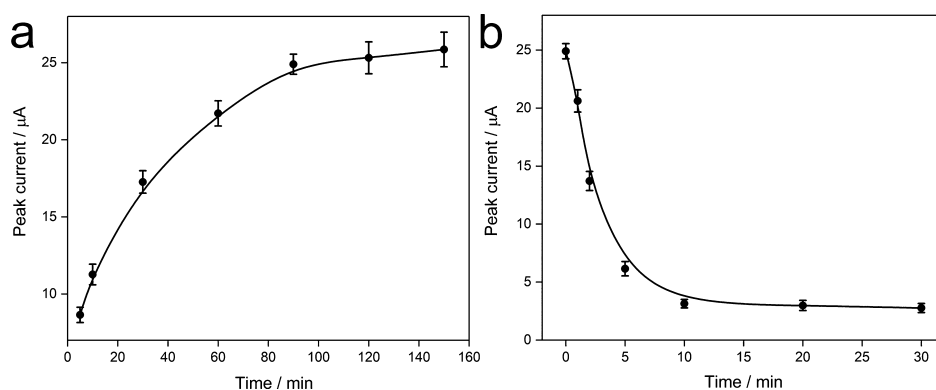


Figure 3. Relationships between SWV peak currents and (a) AuNPs incubation time, (b) endotoxin reaction time. (Error bars stand for SD s of three independent measurements.)

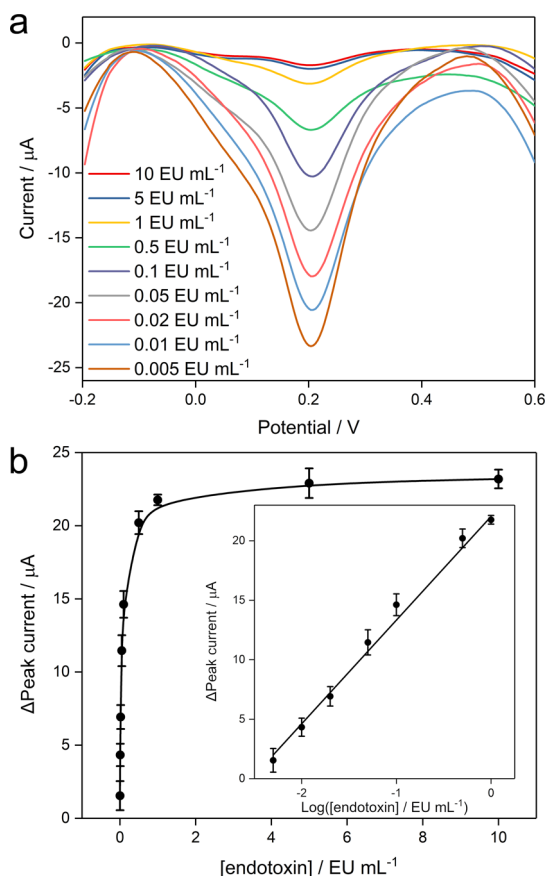


Figure 4. (a) SWV curves of the peptide-modified electrode for the detection of endotoxin (0.005, 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, and 10 EU mL⁻¹, from bottom to top). (b) Calibration curve representing the relationship between variation of SWV peak current and endotoxin concentration. Inset shows the linear range (Error bars stand for SDs of three independent measurements.)

amount of endotoxin, the SWV peak intends to be decreased. Detailed relationship between the variation of peak current and the endotoxin concentration is summarized in Figure 4b. A linear range is found to be 0.005 to 1 EU mL⁻¹ with the equation of $y = 22.04 + 8.71x$, in which x is the logarithmic endotoxin concentration and y is the decreased SWV peak current. LOD is calculated as low as 0.001 EU mL⁻¹ based on 3 standard deviation (SD)/slope of calibration. The average relative SD is less than 10%, which is satisfactory. The

analytical performances of this method are also excellent after compared with some representative reports (Table 1).

Table 1. Comparison of the Strategies and Analytical Performances of Representative Electrochemical Methods for the Detection of Endotoxin

technique	strategy	LOD (EU mL ⁻¹)	ref
DPV ^a	recombinant factor C zymogen-based assay	1	20
amperometry	competitive assay	0.07	21
electrochemical impedance spectroscopy	aptasensor	0.05	22
turbidimetry	kinetic turbidimetric LAL assay	0.01	Lonza BioScience
DPV	<i>p</i> -nitroaniline-based LAL assay	0.01	23
DPV	peptide-based capture and assay	0.04	24
amperometry	electrochemical LAL assay	0.03	9
elastography	elastography-based LAL assay	0.002	25
amperometry	redox cycling in a nanocavity using the LAL reagent	0.0005	26
substitutional stripping voltammetry	<i>p</i> -aminophenol conjugated peptide	0.0005	27
SWV	peptide/GO and Fc-modified AuNPs	0.001	this work

^aDifferential pulse voltammetry.

The selectivity of this assay is then evaluated by detecting SWV peaks in the presence of several potential coexisting interferences. The results shown in Table 2 imply that after employing bovine serum albumin (BSA), ligase, phi29 DNA polymerase, glucose, random peptide, immunoglobulin G (IgG), glutathione (GSH), some cations, and AuNPs, the electrochemical responses are similar with the blank case. After being spiked with a certain amount of endotoxin, the peak decreases significantly, demonstrating the specific interaction between the released peptide and target endotoxin. To test the practical utility of this method, we have further spiked standard endotoxin into Dulbecco's modified eagle medium (DMEM) and serum samples. The detected values are listed in Table 3, which indicate that the complicated samples do not interfere

Table 2. Selectivity Investigation: Detected Peak Currents of Potential Interferents Before and After being Spiked with Endotoxin

samples	peak current (μA)	peak current after spiking endotoxin (μA)
Blank	24.90	4.74
BSA	23.62	5.86
Ligase	24.12	5.49
phi29 DNA polymerase	23.92	4.93
glucose	23.37	3.58
IgG	24.00	4.98
random peptide	23.59	4.90
GSH	23.93	4.73
Ca^{2+}	23.01	3.83
Mg^{2+}	23.22	4.87
AuNPs	23.52	5.03

Table 3. Results of the Endotoxin Assay in the Samples of Cell Culture Medium and Human Serum

sample	spiked (EU mL^{-1})	detected (EU mL^{-1})	recovery (%)	relative error (%)
DMEM	0.2	0.21	105.0	3.9
	0.5	0.47	94.0	4.2
	1	1.08	108.0	5.0
Serum	0.2	0.22	110.0	4.7
	0.5	0.53	106.0	3.8
	1	0.96	96.0	2.6

accurate detection of endotoxin. The recoveries are from 94.0 to 110.0% and all relative errors are no larger than 5%.

CONCLUSIONS

In summary, we have fabricated a sensitive and selective electrochemical method for endotoxin assay combining peptide-modified graphene oxide and DNA-modified AuNPs. Because of excellent electric-conductivity of graphene oxide and AuNPs, significant electrochemical response can be obtained. The specificity of this method relies on the peptide-based recognition. The method achieves high sensitivity and it can be practically utilized for endotoxin assay in complicated biological samples, which has potential use for endotoxin monitoring in near future.

EXPERIMENTAL SECTION

Materials and Instruments. Endotoxin was obtained from Chinese Horseshoe Crab Reagent Manufactory (Xiamen) Co., Ltd. (China). BSA, glucose, GSH, gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), tris(2-carboxyethyl)phosphine hydrochloride, and ethylenediaminetetraacetic acid were purchased from Sigma-Aldrich (USA). Ligase and phi29 DNA polymerase were from New England Biolabs Ltd. (Beijing, China). IgG was purchased from Sino Biological Inc. (China). DMEM was purchased from Gibco (Gaithersburg, USA). Graphene oxide was purchased from Nanjing XF-NANO Materials Tech. Co., Ltd. (Nanjing, China). The specific endotoxin binding peptide with the sequence of KKNYSISSIHC and random peptide (QPVEISRGDH) were synthesized and purified by China Peptides Co., Ltd. (Shanghai, China). The endotoxin binding peptide was chosen according to a previous report, in which Suzuki et al. selected the peptide using the phage-display method.²⁸ The oligonu-

cleotide was synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). The specific sequence was 5'-ferrocene-TCCATGTACCTT-3' SH. Human serum samples were obtained from local hospital (Suzhou, China). The other chemicals or reagents were of analytical grade as received.

Preparation of DNA Modified AuNPs. Bare AuNPs were synthesized by means of citrate reduction of HAuCl_4 according to a previous report.²⁹ First, 100 mL of HAuCl_4 solution (0.01%, w/v) and 3.5 mL of trisodium citrate solution (1%, w/v) were prepared. Second, trisodium citrate was added to HAuCl_4 refluxing solution under stirring and boiling for 15 min. Third, the mixture was stirred for another 30 min before cooled down to room temperature. Next, 30 μM DNA probe was prepared in 10 mM phosphate buffer with 0.25 M NaCl (pH 7.4). DNA was then blended with AuNPs with the ratio of 1:9. After 1 min, the solution was adjusted to pH 3.0 using citrate buffer. Excess reagents were then removed by centrifuging at 12 000 rpm for 30 min. After that, the precipitate was redispersed.

Preparation of Peptide-Modified Electrode. The substrate glassy carbon electrode (3 mm) was pretreated with piranha solution (98% H_2SO_4 /30% H_2O_2 = 3:1) for about 5 min (Caution: highly corrosive). Next, the electrode was carefully rinsed and polished on P5000 silicon carbide paper to a mirror-like surface. After sonicated in ethanol and distilled water, respectively, the electrode was incubated with 10 μL of graphene oxide (500 mg L^{-1}) and then dried overnight at room temperature. The modified electrode was further treated with 2 μM peptide for 15 min in order to achieve the assembly of the peptide on the electrode surface.

Quantification of Endotoxin. Standard endotoxin with a series of concentrations was prepared and the peptide modified electrode was immersed in the solution for 10 min. Subsequently, the electrode was rinsed and then incubated with DNA-modified AuNPs for 90 min.

Electrochemical Measurement. We applied an electrochemical analyzer (CHI660D, CH Instruments) for all electrochemical experiments. A three-electrode system was employed, which was consisted of the peptide-modified electrode as the working electrode, a platinum auxiliary electrode, and a saturated calomel reference electrode. SWV was carried out in 20 mM Tris-HCl (pH 7.4) containing 140 mM NaCl and 5 mM MgCl_2 .

AUTHOR INFORMATION

Corresponding Authors

*E-mail: zhangjz@sibet.ac.cn. (J.Z.).

*E-mail: miaopeng@sibet.ac.cn. Phone: +86-512-69588279 (P.M.).

ORCID

Peng Miao: 0000-0003-3993-4778

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by the Key Research and Development Program of Tianjin (grant no. 18YFZCSY01290), the Scientific Research Instrument Developing Project of the Chinese Academy of Sciences (grant no. YJKYYQ20170067), and the Key Research and Development Program of Jiangsu Province (grant no. BE2017669).

REFERENCES

- (1) Raetz, C. R. H.; Whitfield, C. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **2002**, *71*, 635–700.
- (2) He, Q.; Gao, H.; Xu, L.-m.; Lu, Y.; Wang, C.; Rui, J.; Fan, H.; Wang, X.-y.; Wang, J.-z. Analysis of IL-6 and IL-1 release in cryopreserved pooled human whole blood stimulated with endotoxin. *Innate Immun.* **2018**, *24*, 316–322.
- (3) Hindman, S.; Carson, L.; Favero, M.; Petersen, N.; Schonberger, L.; Solano, J. Pyrogenic reactions during hemodialysis caused by extramural endotoxin. *Lancet* **1975**, *306*, 732–734.
- (4) Beutler, B.; Rietschel, E. T. Innate immune sensing and its roots: the story of endotoxin. *Nat. Rev. Immunol.* **2003**, *3*, 169–176.
- (5) Arteel, G. E.; Guo, L.; Schlierf, T.; Beier, J. I.; Kaiser, J. P.; Chen, T. S.; Liu, M.; Conklin, D. J.; Miller, H. L.; von Montfort, C.; States, J. C. Subhepatotoxic exposure to arsenic enhances lipopolysaccharide-induced liver injury in mice. *Toxicol. Appl. Pharmacol.* **2008**, *226*, 128–139.
- (6) Mishra, R. K.; Robert-Peillard, F.; Ravier, S.; Coulomb, B.; Boudenne, J.-L. beta-Hydroxymyristic acid as a chemical marker to detect endotoxins in dialysis water. *Anal. Biochem.* **2015**, *470*, 71–77.
- (7) Das, A. P.; Kumar, P. S.; Swain, S. Recent advances in biosensor based endotoxin detection. *Biosens. Bioelectron.* **2014**, *51*, 62–75.
- (8) Miao, P. Electrochemical sensing strategies for the detection of endotoxin: a review. *RSC Adv.* **2013**, *3*, 9606–9617.
- (9) Miao, P.; Han, K.; Qi, J.; Zhang, C.; Liu, T. Electrochemical investigation of endotoxin induced limulus amebocyte lysate gel-clot process. *Electrochem. Commun.* **2013**, *26*, 29–32.
- (10) Niu, J.; Hu, X.; Ouyang, W.; Chen, Y.; Liu, S.; Han, J.; Liu, L. Femtomolar detection of lipopolysaccharide in injectables and serum samples using aptamer-coupled reduced graphene oxide in a continuous injection-electrostacking biochip. *Anal. Chem.* **2019**, *91*, 2360–2367.
- (11) Saravanan, R.; Holdbrook, D. A.; Petrlova, J.; Singh, S.; Berglund, N. A.; Choong, Y. K.; Kjellstrom, S.; Bond, P. J.; Malmsten, M.; Schmidtchen, A. Structural basis for endotoxin neutralisation and anti-inflammatory activity of thrombin-derived C-terminal peptides. *Nat. Commun.* **2018**, *9*, 2762.
- (12) Ohto, U.; Fukase, K.; Miyake, K.; Shimizu, T. Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 7421–7426.
- (13) Liu, T.; Gao, L.; Zhao, J.; Cao, Y.; Tang, Y.; Miao, P. A polymyxin B-silver nanoparticle colloidal system and the application of lipopolysaccharide analysis. *Analyst* **2018**, *143*, 1053–1058.
- (14) Liu, F.; Mu, J.; Wu, X.; Bhattacharjya, S.; Yeow, E. K. L.; Xing, B. Peptide-perylene diimide functionalized magnetic nano-platforms for fluorescence turn-on detection and clearance of bacterial lipopolysaccharides. *Chem. Commun.* **2014**, *50*, 6200–6203.
- (15) Liu, C.; Xu, J.-y.; Qi, J.-f.; Liu, Y.-j.; Kang, J.-w.; Wang, H.-y.; Chen, S.-y.; Huang, Z.; Lu, B. A novel nanobiocomposite sandwich immunosensor based on molecularly imprinted nano-membrane for endotoxin detection. *Sens. Actuators, B* **2019**, *290*, 1–8.
- (16) An, Z.; Jang, C.-H. Simple and label-free liquid crystal-based optical sensor for highly sensitive and selective endotoxin detection by aptamer binding and separation. *Chemistryselect* **2019**, *4*, 1416–1422.
- (17) Lim, S. K.; Chen, P.; Lee, F. L.; Mochhala, S.; Liedberg, B. Peptide-assembled graphene oxide as a fluorescent turn-on sensor for lipopolysaccharide (endotoxin) detection. *Anal. Chem.* **2015**, *87*, 9408–9412.
- (18) Brosel-Oliu, S.; Galyamin, D.; Abramova, N.; Muñoz-Pascual, F.-X.; Bratov, A. Impedimetric label-free sensor for specific bacteria endotoxin detection by surface charge registration. *Electrochim. Acta* **2017**, *243*, 142–151.
- (19) Miao, P.; Ning, L.; Li, X. Gold nanoparticles and cleavage-based dual signal amplification for ultrasensitive detection of silver ions. *Anal. Chem.* **2013**, *85*, 7966–7970.
- (20) Inoue, K. Y.; Ino, K.; Shiku, H.; Matsue, T. Electrochemical detection of endotoxin using recombinant factor C zymogen. *Electrochem. Commun.* **2010**, *12*, 1066–1069.
- (21) Priano, G.; Pallarola, D.; Battaglini, F. Endotoxin detection in a competitive electrochemical assay: Synthesis of a suitable endotoxin conjugate. *Anal. Biochem.* **2007**, *362*, 108–116.
- (22) Kim, S.-E.; Su, W.; Cho, M.; Lee, Y.; Choe, W.-S. Harnessing aptamers for electrochemical detection of endotoxin. *Anal. Biochem.* **2012**, *424*, 12–20.
- (23) Inoue, K. Y.; Takahashi, S.; Ino, K.; Shiku, H.; Matsue, T. Development of an electrochemical Limulus amebocyte lysate assay technique for portable and highly sensitive endotoxin sensor. *Innate Immun.* **2012**, *18*, 343–349.
- (24) Liu, T.; Meng, F.; Cheng, W.; Sun, H.; Luo, Y.; Tang, Y.; Miao, P. Preparation of a peptide-modified electrode for capture and voltammetric determination of endotoxin. *ACS Omega* **2017**, *2*, 2469–2473.
- (25) Sun, H.; Miao, P.; Tang, Y.; Wang, B.; Qian, J.; Wang, D. An elastography analytical method for the rapid detection of endotoxin. *Analyst* **2015**, *140*, 4374–4378.
- (26) Ito, K.; Inoue, K. Y.; Ino, K.; Matsue, T.; Shiku, H. A highly sensitive endotoxin sensor based on redox cycling in a nanocavity. *Analyst* **2019**, *144*, 3659–3667.
- (27) Takano, S.; Inoue, K. Y.; Takahashi, S.; Ino, K.; Shiku, H.; Matsue, T. Electrochemical sensor with substitutional stripping voltammetry for highly sensitive endotoxin assay. *Analyst* **2014**, *139*, 5001–5006.
- (28) Suzuki, M. M.; Matsumoto, M.; Yamamoto, A.; Ochiai, M.; Horiuchi, Y.; Niwa, M.; Omi, H.; Kobayashi, T.; Takagi, T. Molecular design of LPS-binding peptides. *J. Microbiol. Methods* **2010**, *83*, 153–155.
- (29) Miao, P.; Tang, Y.; Mao, Z.; Liu, Y. Adamantane derivatives functionalized gold nanoparticles for colorimetric detection of miRNA. *Part. Part. Syst. Charact.* **2017**, *34*, 1600405.