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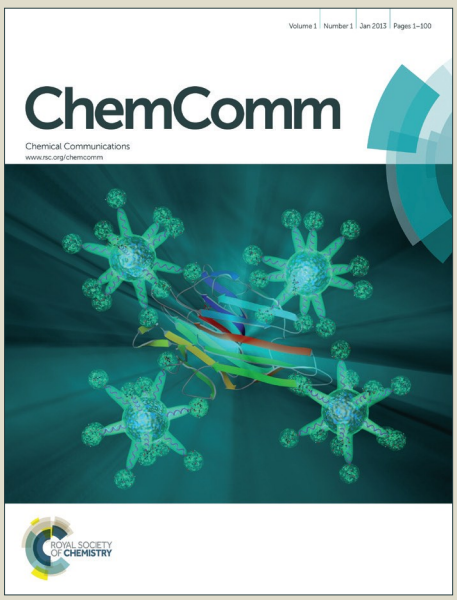

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ARTICLE TYPE

A Highly Sensitive Electrochemiluminescence Biosensor for Detection of Organophosphate Pesticides Based on Cyclodextrin Functionalized Graphitic Carbon Nitride and Enzyme Inhibition

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A signal on electrochemiluminescence (ECL) biosensor using β-cyclodextrin (CD) functionalized graphitic carbon nitride (g-C_3N_4) as luminophore was constructed for sensitive organophosphate pesticides (OPs) detection based on the enzyme inhibition of OPs that the consumption of coreactant triethylamine (Et_3N) decreased with lessening the acetic acid (HAc) *in situ* generated by enzymatic reaction.

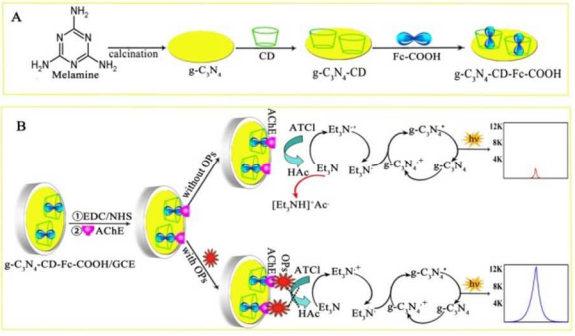
Organophosphates (OPs), acutely toxic substances, raise serious human health and environmental concerns due to irreversible inhibition of acetylcholinesterase (AChE) [1-3]. The conventional measurement techniques for OPs, such as gas or liquid chromatography and mass spectrometry, are accurate and reliable[4-6]. However, these methods need expensive instrument, complex operation, and trained personnel. Therefore, a simple, low-cost, and effective OPs analysis technique is urgently needed. Electrochemiluminescence (ECL) is an attractive technique because of its simplified optical setup, low background noise and high sensitivity detection[7-9].

As a new ECL luminophore, graphitic carbon nitride (g-C_3N_4) presents charming advantages because of its stable s-triazine ring structure and excellent photochemical properties compared with other ECL luminophores[10,11]. However, a conjugated, two-dimensional polymer of s-triazine tends to form a π-conjugated plane, and the stacking with optimized Van der Waals interactions between the single layers of g-C_3N_4 make it deposit in most solvents[12,13]. Therefore, developing a better-performing method to improve the dispersity of g-C_3N_4 is of great interesting.

β-Cyclodextrin (CD) is toroidal in shape with a hydrophobic inner cavity and a hydrophilic exterior containing lots of -OH groups[14]. The interesting characteristics can enable CD to selectively bind various guest molecules.[15] Furthermore, the functional materials modified with CD could possess supramolecular recognition, good dispersion and high luminous efficiency[16-18]. Therefore, it is conceivable that using CD to functionalize g-C_3N_4 can successfully achieve the improvement of dispersity, amplification of signal and recognition of host-guest as a result.

Enzyme-based inhibition biosensors have emerged as a promising alternative to rapidly detect OPs, where AChE as an indicator could hydrolyze the acetylthiocholine (ATCl) into thiocholine and acetic acid (HAc)[19-21]. In previous reports, most of OPs detections were based on the amount change of thiocholine [22,23] However, few researches have been reported about using HAc as a quantitative entry point for OPs detection. And as we know, acid-base reaction has been widely applied due to its simple, fast and stable chemical property. Therefore, making acid-base reaction apply in OPs determination based on HAc *in situ* generated by enzyme reaction would lead an efficient and sensitive detection.

Herein, CD as an enhancer was chemically decorated onto the surface of g-C_3N_4 to form a new nanocomposite of g-C_3N_4-CD. The host-guest inclusion complex (g-C_3N_4-CD-Fc-COOH) obtained by supramolecular recognition between ferrocenecarboxylic acid (Fc-COOH) and g-C_3N_4-CD could effectively immobilize AChE via amidation reaction between carboxyl of Fc-COOH and amino of enzyme. The resultant g-C_3N_4-CD-Fc-COOH/AChE was used to construct a signal on ECL biosensor for OPs detection by enzyme inhibition. When OPs were absent, AChE hydrolyze the substrate acetylthiocholine (ATCl) to *in situ* generate acetic acid (HAc) which could react with triethylamine (Et_3N), a typical coreactant for the ECL of g-C_3N_4, around the electrode surface, resulting in an obviously decreased ECL signal. When OPs were present, OPs could inhibit the activity of AChE, and then the HAc yield decreased accompanied with lessening consumption of coreactant Et_3N, inducing an enhanced ECL signal. The schematic diagram of preparation process of nanocomposite and response mechanism was shown in Scheme 1.



Scheme 1. (A) Preparation process of g-C_3N_4-CD-Fc-COOH nanocomposite. (B) Schematic description of the fabrication of the proposed biosensor and response mechanism.

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The stability of the g-C_3N_4-CD was supported by the fact that the g-C_3N_4-CD suspension (Fig. 1B) remained homogeneous for 10 days under ambient condition, which was much more stable than that of g-C_3N_4 suspension (Fig. 1A). In addition, the contact angle experiment was monitored on glass slide with the water droplet of g-C_3N_4 and g-C_3N_4-CD. The contact angel for g-C_3N_4-CD was 44.1° (Fig. 1D), which was smaller than that of g-C_3N_4 (54.6°, Fig. 1C) due to the rich hydrophilic hydroxyl group of CD. The results revealed that the incorporation of CD could improve the hydrophilia of g-C_3N_4.

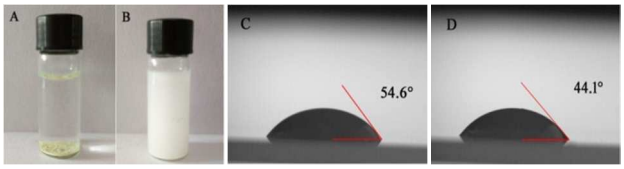

Fig. 1 Photographs of (A) g-C_3N_4 and (B) g-C_3N_4-CD dispersed in water stand for 10 days. The shape of a water droplet on the glass slide of (C) g-C_3N_4 and (D) g-C_3N_4-CD.

TEM was used to investigate the morphologies of g-C_3N_4, g-C_3N_4-CD and g-C_3N_4-CD-Fc-COOH nanocomposite. The g-C_3N_4 (Fig. 2A) showed the layers of stack bulk structure, corresponding with literature[24]. The black part of g-C_3N_4 indicated that the electron beam hardly penetrated through the layers because of thick structure. In contrast, g-C_3N_4-CD (Fig. 2B) exhibited ultrathin sheet structure with edge warping caused by the extreme lack of thickness, leading to a high surface-area-to-volume ratio. The reason might be as follows: Firstly, hydrophilic exterior of CD could improve the dispersity of g-C_3N_4 and lead to the stretch of bulk structure; Secondly, CD molecule could prevent aggregation of g-C_3N_4. In the Fig. 2C, the g-C_3N_4-CD-Fc-COOH also presented the similar structure to g-C_3N_4-CD. Meanwhile, SEM also was applied in the morphology characterization of nanocomposites, which was in good agreement with the TEM observation. The UV-vis and FT-IR (Fig. S1) were used to further support the successful synthesis of g-C_3N_4-CD-Fc-COOH nanocomposite.

In order to investigate the ECL signal of g-C_3N_4-CD-Fc-COOH, different modified electrodes were prepared, including g-C_3N_4/GCE, g-C_3N_4-CD/GCE and g-C_3N_4-CD-Fc-COOH/GCE. As shown in Fig. 3A, ECL signal of g-C_3N_4/GCE was observed obviously (curve a). The luminous mechanism of g-C_3N_4 was concluded as follows[25]:

$$g\text{-}C_3N_4 - e \rightarrow g\text{-}C_3N_4^{\bullet+} \quad (1)$$

$$Et_3N - e \rightarrow Et_3N^{\bullet+} \quad (2)$$

$$Et_3N^{\bullet+} - H^+ \rightarrow Et_3N^{\bullet} \quad (3)$$

$$g\text{-}C_3N_4^{\bullet+} + Et_3N^{\bullet} \rightarrow g\text{-}C_3N_4^{*} + Et_3N_{ox} \quad (4)$$

$$g\text{-}C_3N_4^{*} \rightarrow g\text{-}C_3N_4 + h\nu \quad (5)$$

As shown in Eq (1), when the applied potential is more positive than the valence band of g-C_3N_4, g-C_3N_4 might be electro-oxidized to form the positively charged g-C_3N_4 (i.e., g-$C_3N_4^{\bullet+}$). The cation ($Et_3N^{\bullet+}$) could be produced from the electro-oxidation of coreactant Et_3N (Eq (2)), which released a proton to form a radical ($Et_3N^{\bullet}$), as shown in Eq (3). The electro-oxidized semiconductors g-$C_3N_4^{\bullet+}$ could react with radical $Et_3N^{\bullet}$ to generate the excited state g-$C_3N_4^{*}$ via electron transfer (Eq (4)). The excited state g-$C_3N_4^{*}$ decayed back to the ground state g-C_3N_4, leading to an intense emission (Eq (5)). When CD functionalized g-C_3N_4 was modified on GCE, the ECL response of g-C_3N_4-CD/GCE (curve b) was four times higher than that of g-C_3N_4/GCE. The reason might be as follow. Firstly, CD possessed rich hydroxyl groups which as electron-donating groups tend to increase ECL intensity[26]. Secondly, when the hydroxy group and amine were coexistent, the ECL response is much higher because the hydroxy group could catalyze the oxidation of amines[27]. The ECL response of g-C_3N_4-CD-Fc-COOH/GCE (curve c) was slightly less than curve b, which maybe attributed to existence of Fe(III). The results revealed that the incorporation of CD improved luminous efficiency of g-C_3N_4.

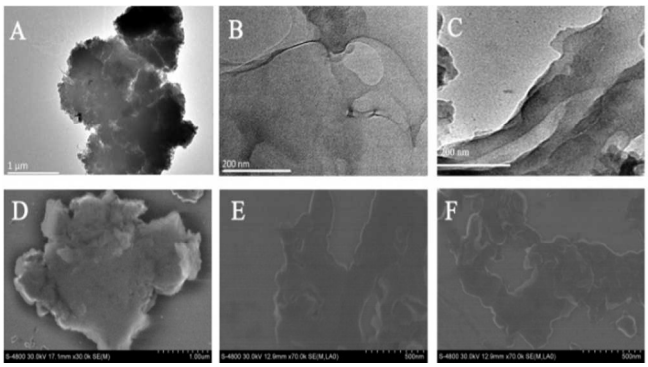

Fig. 2 TEM image of (A) g-C_3N_4 (B) g-C_3N_4-CD (C) g-C_3N_4-CD-Fc-COOH. SEM image of (D) g-C_3N_4 (E) g-C_3N_4-CD (F) g-C_3N_4-CD-Fc-COOH.

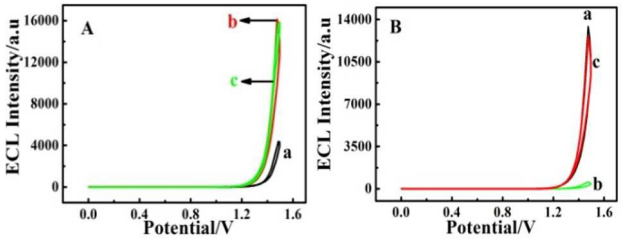

Fig. 3 (A) ECL response of (a) g-C_3N_4/GCE (b) g-C_3N_4-CD/GCE (c) g-C_3N_4-CD-Fc-COOH/GCE in 0.1 M PBS containing 50 mM Et_3N. (B) ECL response of AChE/g-C_3N_4-CD-Fc-COOH/GCE (a) without 100 μM ATCl and 0.5 μM OPs, (b) with 100 μM ATCl and (c) with both100 μM ATCl and 0.5 μM OPs.

In order to confirm the fabrication process of the modified electrode, ECL and CV characterization were investigated. The results were exhibited in Fig. S2.

The mechanism of OPs detection was studied in detailed based on enzyme inhibition in PBS (0.1 M, pH 7.0) with 50 mM Et_3N. As seen in Fig. 3B, the ECL response of AChE/g-C_3N_4-CD-Fc-COOH/GCE was strong (curve a). When 0.1 mM ATCl was dropped into detecting cell, the ECL response decreased obviously (curve b). The reason might be that AChE can catalyze the hydrolyzation of ATCl to *in situ* generate HAc which could react with the coreactant Et_3N around the electrode surface, resulting in an obvious decrease of the ECL signal. However, when OPs were present, the ECL signal increased obviously (curve c) because OPs could inhibit enzyme activity and then reduce the consumption of Et_3N.

Under the optimum detection conditions (Fig. S3), the performance of the proposed biosensor was evaluated by incubating the standard OPs solution with different

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concentrations. As depicted in Fig. 4, the ECL intensity gradually increased with increasing the concentration of OPs. The reason might be that OPs inhibited the enzyme activity and induced a low yield of HAc. The consumption of Et_3N decreased with the yield of HAc decreasing, leading to a signal on ECL. The corresponding linear equation was I(a.u.)=1968.7lg(c/pM)+1368.8 (where I was the ECL intensity and c was the concentration of OPs), with a correlation coefficient R^2=0.996. Compared with other biosensor for OPs detection (Table S1[28-31]), the proposed biosensor exhibited a lower detection limit of 0.3 pM (S/N=3), and the linear range was from 1.0 pM to 0.5 μM, achieving six orders of magnitude, which might hold a new promise for highly sensitive and ultratrace detection of OPs. In addition, the excellent stability and reproducibility of the biosensor were exhibited in Fig. S4.

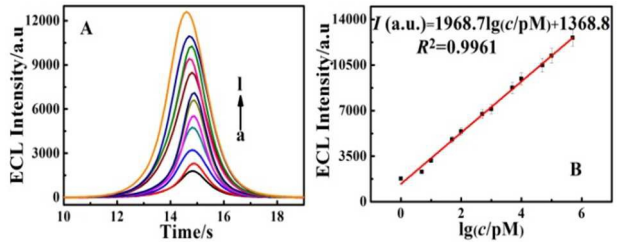

Fig. 4 (A) ECL response of the biosensor to ethyl paraoxon with the concentration: (a) 1×10^{-12} (b) 5×10^{-12} (c)1×10^{-11} (d) 5×10^{-11} (e) 1×10^{-10} (f) 5×10^{-10} (g) 1×10^{-9} (h) 5×10^{-9} (i) 1×10^{-8} (j) 5×10^{-8} (k) 1×10^{-7} (l) 5×10^{-7} M. (B) The calibration curve for ethyl paraoxon.

To further evaluate the practicality of the proposed electrode in the detection of real-life samples, the recovery experiment was performed in the supernatant of different vegetables samples by a standard addition method. The recoveries were presented in Table S2 from 94.2% to 108.4%. The results demonstrated that the ECL biosensor had a potential application to analyze practical samples.

In conclusion, a novel signal on ECL biosensor was applied for OPs detection based on CD functionalized g-C_3N_4 and enzyme inhibition reaction. Compared with pure g-C_3N_4, g-C_3N_4 modified with CD possessed good dispersity, stability, supramolecular recognition and luminous efficiency. Since HAc *in situ* generated by enzymatic reaction could rapidly consume coreactant Et_3N, the biosensor based on the proposed enzyme inhibition reaction exhibited the advantage of high sensitivity, low background signal and rapid response. Therefore, such a new ECL biosensor opened up a new direction for fast and immediate OPs detection in various samples.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [experimental section; characterizations of different nanocomposites; ECL and CV characterization of stepwise fabrication of the electrode; optimization of experimental conditions; stability and reproducibility]. See DOI:10.1039/b000000x/

1 X. J. Du, D. Jiang, N. Hao, Q. Liu, J. Qian, L. M. Dai, H. P. Mao and K. Wang, *Chem. Commun.,* 2015, **51**, 11236-11239.
2 G. Aragay, F. Pino, A. Merkoci, *Chem. Rev.,* 2012, **112**, 5317-5338.
3 M. Ali, S. Nasir, I. Ahmed, L. Fruk and W. Ensinger, *Chem. Commun.*, 2013, **49**, 8770.
4 J. Lee and H. K. Lee, *Anal. Chem.*, 2011, **83**, 6856-6861.
5 P. Payá, M. Anastassiades, D. Mack, I. Sigalova, B. Tasdelen, J. Oliva and A. Barba, *Anal. Bioanal. Chem.*, 2007, **389**, 1697-1714.
6 T. Zhou, X. H. Xiao and G. K. Li, *Anal. Chem.*, 2012, **84**, 5816-5822.
7 X. Y. Jiang, Y. Q. Chai, H. J. Wang and R. Yuan, *Biosens. Bioelectron.*, 2014, **54**, 20-26.
8 L. Dennany, R. J. Forster and J. F. Rusling, *J. Am. Chem. Soc.*, 2003, **125**, 5213-5218.
9 L. L. Liu, Q. Ma, L. Yang, Z. P. Liu and X. G. Su, *Biosens. Bioelectron.*, 2015, **63**, 519-524.
10 L. C. Chen, X. T. Zeng, P. Si, Y. M. Chen, Y. W. Chi, D. H. Kim and G. N. Chen, *Anal. Chem.*, 2014, **86**, 4188-4195.
11 X. Ou, X. R. Tan, X. F. Liu, Q. Y. Lu, S. H. Chen and S. P. Wei, *Biosens. Bioelectron.*, 2015, **70**, 89-97.
12 X. C. Wang, S. Blechert and M. Antonietti, *ACS Catal.*, 2012, **2**, 1596-1606.
13 Y. Wang, X. C. Wang and M. Antonietti, *Angew. Chem. Int. Edit.*, 2012, **51**, 68-89.
14 A. Douhal, *Chem. Rev.*, 2004**, 104**, 1955-1976.
15 K. Shang, X. D. Wang, B. Sun, Z. Q. Cheng and S. Y. Ai, *Biosens. Bioelectron.*, 2013, **45**, 40-45.
16 H. Dai, C. P. Yang, X. L. Ma, Y. Y. Lin and G. N. Chen, *Chem. Commun.*, 2011, **47**, 11915-11917.
17 Q. Chen, H. Chen, Y. Y. Zhao, F. Zhang, F. Yang, J. Tang and P. G. He, *Biosens. Bioelectron.*, 2014, **54**, 547-552.
18 Y. J. Guo, S. J. Guo, J. T. Ren, Y. M. Zhai, S. J. Dong and E. K. Wang, *ACS Nano*, 2010, **4**, 4001-4010.
19 C. Zhai, X. Sun, W. P. Zhao, Z. L. Gong and X. Y. Wang, *Biosens. Bioelectron.*, 2013, **42**, 124-130.
20 L. H. Hess, A. Lyuleeva, B. M. Blaschke, M. Sachsenhauser, M. Seifert, J. A. Garrido and F. Deubel, *ACS Appl. Mater. Interfaces*, 2014, **6**, 9705-9710.
21 D. S. Guo, V. D. Uzunova, X. Su, Y. Liu and W. M. Nau, *Chem. Sci.*, 2011, **2**, 1722-1734.
22 H. Liang, D. D. Song and J. M. Gong, *Biosens. Bioelectron.*, 2014, **53**, 363-369.
23 L. Zhang, A. D. Zhang, D. Du and Y. H. Lin, *Nanoscale*, 2012, **4**, 4674-4679.
24 J. Q. Tian, Q. Liu, C. J. Ge, Z. C. Xing, A. M. Asiri, A. O. Al-Youbicd and X. P. Sun, *Nanoscale*, 2013, **5**, 8921-8924.
25 Y. T. Liu, Q. B. Wang, J. P. Lei, Q. Hao, W. Wang and H. X. Ju, *Talanta*, 2014, **122**, 130-134.
26 X. Q. Liu, L. H. Shi, W. X. Niu, H. J. Li and G. B. Xu, *Angew. Chem. Int. Ed.*, 2007, **46**, 421-424.
27 L. H. Zhang, X. Q. Zou, E. B. Ying and S. J. Dong, *J. Phys. Chem. C*, 2008, **112**, 4451-4454.
28 R. K. Mishra, R. B. Dominguez. S. Bhand and R. Munoz, J. Marty, *Biosens. Bioelectron.*, 2012, **32**, 56-61.
29 Y. Q. Yang, H. Y. Tu, A. D. Zhang, D. Du and Y. H. Lin, *J. Mater. Chem.*, 2012, **22**, 4977-4981.
30 Y. Q. Yang, A. M. Asiri, D. Du and Y. H. Lin, *Analyst*, 2014, **139**, 3055-3060.
31 Y. Ivanov, I. Marinov, M. Portaccio, M. Lepore, D. G. Mita and T. Godjevargova, *Biotechnol. Biotec. Eq.*, 2012, **26**, 3044-3053.