Preparation of Nanomaterial-Based Artificial Enzymes Using Integrated Graphitic Carbon Nitride Nanosheet-encapsulated Metal-Organic Framework Optimized by Central Composite Design for Enhanced Electrochemiluminescence Biosensing of Gene-Specific Methylation in Thyroid Cancer Patients' Plasma

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Abstract: Herein, a sensitive electrochemiluminescence immuno-DNA sensor was designed to analyze DNA methylation using sandwiching the target methylated DNA between the magnetic nanoparticles/Anti-5-methylcytosine monoclonal antibody bioconjugate and luminol-loaded within phosphorylated DNA capture probe-immobilized graphitic carbon nitride nanosheets @UiO-66 core@shell nanozyme. The suggested method was successfully utilized to evaluate methylated DNA in human blood plasma, demonstrating the platform's potential for disease diagnostics and biochemistry research. Our method can differentiate methylation levels as low as 0.1%.

Key words: C_3N_4@UiO-66 core@shell, electrochemiluminescence, nanomaterial-based artificial Enzymes.

1. Introduction

Plasma cell free DNA (cfDNA) circulating has been considered as a non-invasive cancer biomarker due to its tumor origin [1], [2]. DNA methyltransferases-catalyzed DNA methylation cause to covalent addition of a methyl group to the C-5 position carbon of the cytosine, 5-methylcytosine , within CpG dinucleotides in clusters called CpG islands, altering the activity of the DNA segment without varying the nucleotide sequence [3]. whereas the difference in DNA methylation of genes contrasted to control studies in cancer-related genes is more than 50%, this variation in other non-communicable diseases is about 5%. Thus, low abundance determination of 5-mC DNA methylation is very important. rolling circle amplification [4], enzyme-free supersandwich DNA structure-based assembly [5], 3D matrixed DNA self-nanocatalyzer via AuNPs [6], and labeling of single antibody with multiple horseradish peroxidase [7], are some enzyme-based electrochemical signal amplification methods that have been progressed for DNA methylation detection.

Affected the activity of the enzymes with changing the conformation of the enzyme, time-consuming, high

cost, and strict experimental conditions cause to restriction of their efficency in clinical laboratory and as point-of-care devices. ECL methods have various merits such as visual imaging capabilities, wide dynamic response, high sensitiveity because of use the internal gain PMT detector [8], [9] and low background signals due to spatially separated excitation source and detection [10], [11].

As far as we know, just a few report about the using the ECL technique to detect DNA methylation has appeared in literature [12]-[17]. In this immuno-DNA sensor owing to nanoscale proximity of OH-generating reaction of C_3N_4 NS from H_2O_2, ECL tracer signal of confined luminol in void space inside the UiO-66, high density coordinated capture probe on the zirconium-based UiO-66, and effective surface area of the rGO/PGE in high-efficiency electron acceleration, ultrasensitive detection of DNA methylation RASSF1A promoter region has been achieved.

2. Experimental section

2.1. Materials and Apparatus

Phosphate buffer (PB, pH=9.5) containing 0.1M K_2HPO_4 and 0.1 M KH_2PO_4 was used to prepare luminol. Anti-5-methylcytosine monoclonal antibody (anti-5-mC) was obtained from Abcam. All used synthetic oligonucleotides for this study were obtained from Eurofins/MWG/Operon (Germany) with the following sequences (5' to 3'):

Capture probe RASSF1A: Phosphate-GGACCCTCTTCCTCTAGCACA

Target 5-mC RASSF1A: CC(M)GGAC(M)GGCCACAAC(M)GAC(M)GGC(M)GACC(M)GCAAAGCACCAC(M)GC(M)GGAGATACCC(M)GTGT TTC(M)GTGGC(M)GGCCAGCTTTACTGTGCTAGAGGAAGAGGGTCC.

The parts relating to materials, and the equipment used for topographical and structural investigations of the nanomaterial are provided in Supplementary Information file to conserve space and profit from the results.

2.2. Preparation of C_3N_4 NS@L/UiO-66 core@shell nanoparticles

According to previous study the rGO/PGE, carboxylate-modified MNPs [10], MNPs/Anti-5mc bioconjugate [18], UiO-66 [19] and C_3N_4 NS [20] was prepared. 37.5 mg $ZrCl_4$ and 5 mg C_3N_4 NS were dissolved in 18 mL DMF for 15 minutes with agitation. After addition of 29 mg of terephthalic acid into the aforesaid mixture, resulting mixture was heated in a Teflon-lined stainless-steel autoclave for 12 hours at 100°C for 24 hours. The luminol was loaded into the C3N4 NS@UiO-66 core@shell for 24 hours in the dark in 0.1 M PB (pH=9.5) using 1 mL of varied luminol concentrations and 1 mg C_3N_4 NS@UiO-66. Finally, the extra luminol was removed from separated C_3N_4 NS@L/UiO-66 by centrifugation and thorough washing with PB.

3. Result and Discussion

Herein, High sensitive detection of RASSF1A methylation has been introduced using various signal amplification including, i) Acceleration properties of graphene in electron transfer, ii) Unsaturated Zirconium on MOF surface and phosphorylated oligonucleotides are used to functionalize the UiO-66 surface with oligonucleotides at high density, iii) promotion of signal-to-noise using MNPs *via* magnetically separated of methylated target from complex media, , iv) generation of high active $^{\bullet}OH$ radicals from core of peroxidase activity of C_3N_4 NS, and v) reduce the distance between the hydroxide radical produced by the luminol before it disappears by loading the luminol inside the UiO-66. The resulting immuno-DNA sensor was added on the rG/PGE and ECL transduction was performed in 0.1 M PB (pH=9.5) using the luminol/H_2O_2 system.

3.1. Characterization of synthesized nanomaterials

Similar XRD pattern of C_3N_4 NS@L/UiO-66 with other nanomaterilas confirm the crystal structure of UiO-66 does not change with the addition of C_3N_4 NS and luminol (Fig 1A). Besides, TEM image of C_3N_4 NS@UiO-66 demonstrated the formation of 20 nm UiO-66 shell on the diameter of ~100 nm C_3N_4 NS core (Fig 1B).

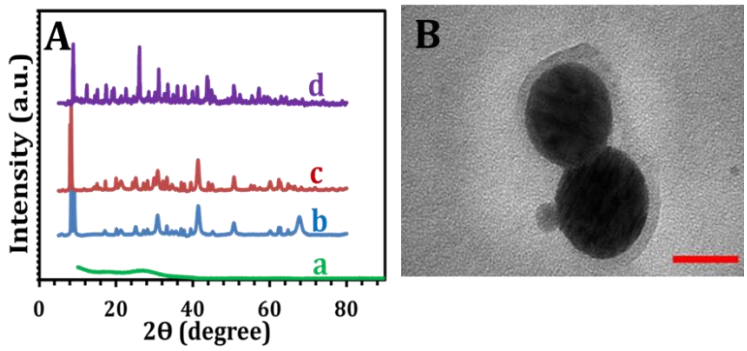

Fig. 1. A) The XRD patterns of (a) C_3N_4 NS, (b) UiO-66, (c) C_3N_4 NS@UiO-66, and C_3N_4 NS@L/UiO-66. B) TEM images of C_3N_4 NS@L/UiO-66.

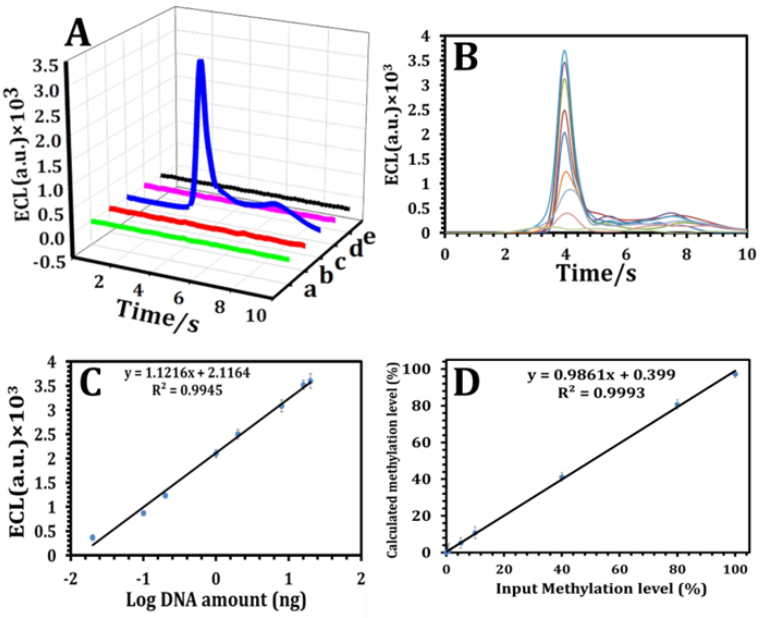

Fig. 2. A) the ECL intensity of various steps of rGO/PGE modification in 0.1 M PB (pH=9.5)+H_2O_2 (3.5 mmol L^{-1}). The rGO/PGE (trace a), MNPs/anti-5mc-rGO/PGE (trace b), the sandwich-type immuno-DNA sensor in the presence of 10 ng RASSF1A target (trace c), in a mixture of MNPs/anti-5mc and RAS-C_3N_4 NS@L/UiO-66 without RASSF1A (trace d), and in the sandwich-type between dsDNA-C_3N_4 NS@UiO-66 and MNPs/anti-5mc (trace e), without loaded luminol. B) The ECL responses of sandwich-type immune-DNA sensor versus different DNA amount in PB solution, (C) Calibration curve in plot B. (D) Plot of levels of calculated methylation *vs.* input methylation levels.

3.2. ECL Behavior of the Different Platforms

While no ECL responses was observed in the rGO/PGE (Fig. 2A, trace a) and MNPs/anti-5mc-rGO/PGE (Fig. 2A, trace b), in 0.1 M PB (pH=9.5)+H_2O_2 (3.5 mmol L-1), in the presence of 10 ng RASSF1A target, owing to hybridization reaction between the dsRASSF1A-C_3N_4 NS@L/UiO-66 and high affinity of anti-5mc to methylated sites, enhanced signal was achieved (Fig. 2A, trace c). To prove that the generated signal is

due to the presence of the target, after mixing of the MNPs/anti-5mc and RAS-C_3N_4 NS@L/UiO-66 in absence of RASSF1A, the ECL of resulting product was recorded (Fig. 2A, trace d). The absence of ECL signal indicates that not only no nonspecific absorption is observed but also the signal recorded is due to the interaction of methyl groups with the anti-5mc sample. Moreover, featureless ECL signal dsRASSF1A- C_3N_4 NS@UiO-66 and MNPs/anti-5mc (Fig. 2A, trace e), without encapsulated luminol, Indicates that the signal is observed only in the presence of luminol located in the UiO-66. In order to investigate the ability of the immunosensor in quantitative various concentrations of specific methylations, ECL responses was recorded in the range of 0-20 ng (Fig. 2B). Logarithmic concentrations in the dynamic range of 20 pg to 20 ng versus ECL intensity, with low detection limit down to 10 pg as depicted Fig. 2C. Regards to amount of circulating DNA in plasma cancer patients, 15 ng, our method could be used to assess cfDNA in clinical samples. Since small amounts of DNA methylation must be detected in the presence of non-methylated DNA in real samples, the accuracy of the proposed method was investigated. According to the calibration curve, there is a very good correlation between the calculated and inputted methylation percentage (Fig. 2D). These results indicate that this method is able to detect methylated DNA in the amount of 0.1%. It is greater than several previously published qMSP-based studies [21].

4. Conclusion

In this study, sensitive biosensing of cfDNA methylated has been developed using nano-proximity of C_3N_4 NS and luminol, the separation/enrichment tool of MNPs, and high enrichment loading of phosphorylated RASSF1A DNA probe on the UiO-66. Moreover, increase ECL signal of the luminol/ H_2O_2 on high conductive rGO onto the PGE results further in an increased ECL intensity. It can detect 10 pg of methylated genomic DNA under the optimal conditions without PCR amplification or bisulfite, and discriminate methylation levels as low as. 01%, demonstrate the attractive features with clinical diagnosis.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Seyyed Mehdi Khoshfetrat did the works on project administration, supervision, investigation, methodology; Farzaneh Moradnia did the works on methodology, formal analysis, writing – original draft; Salman Jalalifar did the works on methodology, writing – review & editing.

References

[1] De Mattos-Arruda, L., Cortes, J., Santarpia, L., Vivancos, A., Tabernero, J., Reis-Filho, J. S., & Seoane, J. (2013). Circulating tumour cells and cell-free DNA as tools for managing breast cancer. *Nat. Rev. Clin. Oncol.*, *10*, 377-389.

[2] Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics, 13,* 484-492.

[3] Egger, G., Liang, G., Aparicio, A., & Jones, P. A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature, 429,* 457-463.

[4] Liu, H., Luo, J., Fang, L., Huang, H., Deng, J., Huang, J., Zhang, S., Li, Y., & Zheng, J. (2018). An electrochemical strategy with tetrahedron rolling circle amplification for ultrasensitive detection of DNA methylation. *Biosens. Bioelectron., 121*, 47-53.

[5] Feng, Q., Wang, M., Qin, L., & Wang, P. (2019). Dual-signal readout of DNA methylation status based on

the assembly of a supersandwich electrochemical biosensor without enzymatic reaction. *ACS Sensors, 4*, 2615-2622.

[6] Zhu, Q., Yang, H., Luo, J., *et al.* (2021). 3D matrixed DNA self-nanocatalyzer as electrochemical sensitizers for ultrasensitive investigation of DNA 5-methylcytosine. *Anal. Chim. Acta., 1142*, 127-134.

[7] Povedano, E., Ruiz-Valdepeñas M. V., & Gamella, M., (2020). amperometric bioplatforms to detect regional DNA methylation with single-base sensitivity. *Anal. Chem., 92*, 5604-5612.

[8] Fazlali, F., Hashemi, P., Khoshfetrat, S. M., *et al.* (2021). Electrochemiluminescent biosensor for ultrasensitive detection of lymphoma at the early stage using CD20 markers as B cell-specific antigens. *Bioelectrochemistry, 138*, 107730.

[9] Ahmadi, A., Khoshfetrat, S. M., Kabiri, S., Dorraji, P. S., Larijani, B., & Omidfar, K. (2021). Electrochemiluminescence paper-based screen-printed electrode for HbA1c detection using two-dimensional zirconium metal-organic framework/Fe_3O_4 nanosheet composites decorated with Au nanoclusters. *Microchimica Acta, 188*, 296.

[10] Khoshfetrat, S. M., Khoshsafar, H., Afkhami, A., Mehrgardi, M. A., & Bagheri, H. (2019). Enhanced visual wireless electrochemiluminescence immunosensing of prostate-specific antigen based on the Luminol Loaded into MIL-53(Fe)-NH_2 accelerator and hydrogen evolution reaction mediation. *Anal. Chem., 91*, 6383-6390.

[11] Khoshfetrat, S. M., Ranjbari, M., Shayan, M., Mehrgardi, M. A., & Kiani, A. (2015). Wireless electrochemiluminescence bipolar electrode array for visualized genotyping of single nucleotide polymorphism. *Anal. Chem., 87*, 8123-8131.

[12] Kurita, R., Arai, K., Nakamoto, K., Kato, D., & Niwa, O. (2012). Determination of DNA methylation using electrochemiluminescence with surface accumulable coreactant. *Anal. Chem., 84*, 1799-1803.

[13] Li, Y., Huang, C., Zheng, J., Qi, H. (2012). Electrogenerated chemiluminescence biosensing method for the detection of DNA methylation and assay of the methyltransferase activity. *Biosens. Bioelectron., 38*, 407-410.

[14] Zhao, H.-F., Liang, R.-P., Wang, J.-W., & Qiu, J.-D. (2015). One-pot synthesis of GO/AgNPs/luminol composites with electrochemiluminescence activity for sensitive detection of DNA methyltransferase activity. *Biosens. Bioelectron., 63*, 458-464.

[15] Zhang, H., Li, M., Fan, M., Gu, J., Wu, P., & Cai, C. (2014). Electrochemiluminescence signal amplification combined with a conformation-switched hairpin DNA probe for determining the methylation level and position in the Hsp53 tumor suppressor gene. *Chem. Commun., 50*, 2932-2934.

[16] Jiang, W., Yin, H., Zhou, Y., Duan, J., Li, H., Wang, M., Waterhouse, G. I. N., & Ai, S. (2018). A novel electrochemiluminescence biosensor for the detection of 5-methylcytosine, TET 1 protein and β-glucosyltransferase activities based on gold nanoclusters-H_2O_2 system. *Sens. Actuators B Chem., 274*, 144-151.

[17] Wang, H., Liu, M., Bai, W., Sun, H., Li, Y., & Deng, H. (2019). A convenient electrogenerated chemiluminescence biosensing method for selective detection of 5-hydroxymethylcytosine in genomic DNA. *Sens. Actuators B Chem.*, *284*, 236-242.

[18] Povedano, E., Montiel, V. R.-V., Valverde, A., *et al*. (2019). Versatile electroanalytical bioplatforms for simultaneous determination of cancer-related DNA 5-Methyl- and 5-hydroxymethyl-cytosines at global and gene-specific levels in human serum and tissues. *ACS Sensors, 4*, 227-234.

[19] Chen, R., Zhang, J., Wang, Y., Chen, X., Zapien, J. A., & Lee, C. S. (2015). Graphitic carbon nitride nanosheet@metal-organic framework core-shell nanoparticles for photo-chemo combination therapy. *Nanoscale, 7*, 17299-17305.

[20] Chen, L., Zeng, X., Si, P., Chen, Y., Chi, Y., Kim, D.-H., & Chen, G. (2014). Gold Nanoparticle-Graphite-Like

C_3N_4 Nanosheet Nanohybrids Used for Electrochemiluminescent Immunosensor. *Anal. Chem., 86*, 4188-4195.

[21] Stephen, J. K., Chen, K. M., Merritt, J., Chitale, D., Divine, G., & Worsham, M. J. (2015). *Methylation markers for early detection and differentiation of follicular thyroid cancer subtypes*. *Cancer and Clinical Oncology, 4(1)*.

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