Flexible electrochemical biosensors based on interfacially assembled metal nanocrystals and graphene paper

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**FLEXIBLE ELECTROCHEMICAL BIOSENSORS
BASED ON INTERFACIALLY ASSEMBLED METAL
NANOCRYSTALS AND GRAPHENE PAPER**

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This study focuses on the fabrication of hybrid flexible electrodes to detect molecules related to human health. The hybrid electrodes were built upon flexible graphene paper and novel noble metal nanostructures. The size, morphology and surface chemistry of noble metal nanoparticles were well tailored using wet chemical method. Moreover, functionalized noble nanostructures were assembled at the interface between water and organic solvents and transferred on the flexible graphene paper via dip-coating. The fabricated free-standing flexible hybrid electrodes were further applied to detect biomolecules (such as dopamine and nitric oxide) released from living cells under external stimuli and heavy metals released from degraded quantum dots uptake inside living cells. Owing to the high loading density of 2D-assembled nanocrystals, remarkable electrocatalytic activity of nanocrystals and good conductivity of flexible graphene paper, the constructed sensors exhibited excellent performances toward detection a range of targets. Thus, this work illustrated a simple and novel approach to the further development of point-of-care electrochemical devices.

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Journals

1. **Zan, X.L**; Fang, Z; Wu, J; Xiao, F; Huo, FW; Duan, HW (2013); "Freestanding graphene paper decorated with 2D-assembly of Au@Pt nanoparticles as flexible biosensors to monitor live cell secretion of nitric oxide" *Biosensors and Bioelectronics*, 49, 71-78. (IF: 6.451)
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3. Bai, HW ; **Zan, X. L**; Juay, J; Sun, DD (2015); "Hierarchical heteroarchitectures functionalized membrane for high efficient water purification" *Journal of Membrane Science*, 475, 245-251. (IF: 4.908)
4. Wu, J; **Zan, X. L**; Li, SZ ; Liu, YY ; Cui, CL; Zou, BH; Zhang, WN ; Xu, HB ; Duan, HW. et al., (2014); "In situ synthesis of large-area single sub-10 nm nanoparticle arrays by polymer pen lithography" *Nanoscale*, 6, 749-752. (IF: 6.739)
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9. H. W. Bai, **Zan X. L.**, Z. Y. Liu and D. D. Sun, "Three-tier Hierarchical Clusters of Carbon-Coated $\text{Li}_4\text{Ti}_5\text{O}_{12}$ Single Crystals for High Power and High Energy Lithium-ion Batteries Anode", ChemElectroChem, **Accepted**.
10. H. W. Bai, L. L. Zhang, **Zan X. L.**, and D. D. Sun, "Multi-functional CNT/ZnO/TiO₂ Nanocomposite Membrane for Concurrent Filtration and Solar Photocatalytic Degradation", Separation & Purification Technology, **Accepted**.

Manuscripts

1. 2D-Assembled Novel Dendritic Pt Nanoparticles on Flexible Graphene Paper to Detect Dopamine Secreted from Live Cells
2. 2D-Assembled Au@PDA Nanocrystals on Flexible Graphene Paper for the Detection of Multiplex DNA
3. A Dual-Modality Method to Monitor Intracellular Degradation of Quantum Dots
4. Entrapping Peroxidase-Loaded Hybrid Nanoflowers into Graphene Hydrogel for High-Performance Enzymatic Biosensors
5. Flexible Electrochemical Biosensors Based on Novel Nanomaterials

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2. Zan, X. L, Symposium CBE 2014, 17-18, Nov, 2014, School of chemical and biomedical engineering, NTU, Singapore.

3. **Zan, X. L.**, Symposium on Chemical & Biotechnology Engineering 2014 (SCBE2014), NTU, Singapore.
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9. **Zan, X. L.**, Join us for the *Small* Sciences Symposium: Frontiers in Nanomedicine, December 10-12, 2012 Mandarin Orchard Hotel, Singapore.
10. **Zan, X. L.**, Nanoengineering and medicine workshop, 27 & 28 February 2012, Resorts World Sentosa, Singapore.

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LIST OF ABBREVIATIONS

1D	one dimensional
2D	two dimensional
3D	three dimensional
PDA	polydopamine
CNT	carbon nanotube
GO	graphene oxide
rGO	reduced graphene oxide
RGON	reduced graphene oxide/Nafion
NMNPs	noble metal nanoparticles
EIS	electrochemical impedance spectroscopy
TMB	3,3',5,5'-tetramethylbenzidine
QDs	quantum dots
SAM	self-assembled monolayer
LBL	layer-by-layer
LB	Langmuir-Blodgett
BTX-2	brevetoxin B
GGNRs	guanine-assembled graphene nanoribbons
BTX-2-BSA	bovine serum albumin-BTX-2
BGGNRs	GGNS conjugated BTX-2-BSA
ELISA	enzyme-linked immuno sorbent assay
CVD	chemical vapour deposition
CV	cyclic votammetry
PVP	polyvinyl pyrrolidone
DA	dopamine
HI	hydroiodic acid
APBA	3-aminophenylboronic acid
TBE	Tris-borate-EDTA
FESEM	field emission scanning electronic microscope

TEM	transmission electronic microscope
HRTEM	high resolution transmission electronic microscope
AFM	atomic force microscope
XRD	X-ray diffraction
TGA	thermo gravimetric analysis
DPV	differential pulse voltammetry
TCEP	tris(2-carboxyethyl) phosphine
PC 12	pheochromocytoma 12
HUVECs	human umbilical vein endothelial cells
BBE	bovine brain extract
CCK-8	cell counting Kit-8
DMEM	Dulbecco Modified Eagle Medium
HBSS	Hank's buffered saline solution
PMA	Tetradecanoylphorbol-13-acetate
AA	ascorbic acid
UA	uric acid
DTBE	2, 2'-dithiobis [1-(2- bromo-2-methyl- propionyloxy)] ethane
EASA	electrochemically active surface area
ROS	reactive oxygen species
RNS	reactive nitrogen species
NO	nitric oxide
SPR	surface plasmon resonance
SWASV	square-wave anodic stripping voltammetry
SDS	sodium dodecyl sulfo
PANI	polyaniline

CHAPTER 1 INTRODUCTION

1.1 Background

Healthcare is to protect human beings away from any disease by integration of diagnosis, treatment, and prevention tactics, which is now being constantly improved by developing more and more efficient, rapid, useful point-of-care devices and portable instruments as molecular diagnostic equipments.¹⁻⁴ In the past decade, lots of attentions have been focusing on the development of efficient, rapid, user-friendly, flexible and miniaturized electrochemical biosensors in view of their good properties, such as easy operation, high sensitivity, good selectivity and broad applications in detecting and monitoring specific target.⁵⁻⁹ Flexible, free-standing biosensors have well met the above mentioned characteristics and therefore become a topic of considerable research interests.

Free-standing graphene paper composed of a two-dimensional crystal (one-atom-thick) nanosheets of high crystal quality and excellent electronic conductivity, a rapidly rising star of conductive support, has attracted great interest to be employed as the flexible substrate of hybrid electrodes.^{10, 11} Compared with other carbon materials, it is the high electron transfer ability and large specific surface area that ensure graphene to be an excellent transducer material in biosensors. Another advance is the flexible graphene paper based hybrid materials, especially

nanocrystals-decorated graphene composites, have been widely used as electrode materials with an increasing number of electrochemical biosensors.^{12, 13}

The emerging nanotechnology and nanoscience create many opportunities for the development of innovative non-enzymatic electrochemical biosensors. Moreover, the fast development in wet-chemical synthesis further enhanced the feasibility in systematically tailoring the structural parameters (such as size, morphology, chemical configuration and exposed facets) of metal nanocrystals and stimulated the great interest in electrochemical catalytic study. Thus, nanocrystals decorated hybrid flexible electrodes with high loading density and more active sites are critical for their prominent catalytic activities, which can be obtained by the following two routes. Firstly, various nanostructures of metal nanocrystals, such as dendritic, core-shell and dual core-shell nanoparticles (NPs), nanotubes, nanowires and porous nanomaterials have been synthesized and interfacially self-assembled at the oil-water phase to form a uniform film. Secondly, interfacially self-assembled colloidal particles films can be loaded on the surface of fixed graphene paper substrate. Another reason is that the well developed electrocatalysts with various nanostructures are helpful to solve the problems related to non-enzymatic electrodes such as poor selectivity and surface fouling. Following such considerations, this PhD project will discuss the contribution of flexible graphene paper based hybrid electrochemical biosensors to the advancements in point-of-care testing and pose an emphasize on their applications and performance for the detection of numerous analytes of clinical interest and their analytical challenges.

1.2 Motivations

Although a variety of nanomaterial-facilitated non-enzymatic electrochemical biosensors have been extensively studied and widely applied, it remains a challenge to develop flexible free-standing hybrid and high performance electrochemical biosensors. In addition, surface modifications play an important role for interfacial self-assembly of synthesized nanocrystals, which further influence the morphologies of nanofilm loaded on flexible graphene paper. Thus, the author is greatly motivated to further enhance the catalytical performances toward biomolecules by designing and fabricating nanocrystals with desirable structures and more reactive sites.

1.3 Objectives

The objectives of this research covers three aspects in a gradual in-depth logical manner. (1) To design and synthesize nanocrystals with desirable structures and surface properties; (2) To Fabricate different free-standing hybrid electrodes composed of synthesized nanocrystals and graphene paper substrate; And (3) to explore the catalytic properties of fabricated hybrid electrodes toward different detection targets.

1.4 Organization

This dissertation explores the fabrication of free-standing flexible electrochemical biosensors based on flexible graphene paper and synthesized

nanocrystals and performances toward biomolecules as well as electro-active targets released from living cells. Briefly, this dissertation is organized in a logical manner as below.

Chapter 1 defines the study areas and oversees the motivations and objectives of PhD research. Chapter 2 retrospects the previously published researches and advances in the related field, such as development of flexible graphene carbon substrate, the hybridization approaches of nanocrystals with graphene electrode substrates and the commonly detected targets for academic researches as well as the progress of current practical applications of one dimensional (1D), two dimensional (2D) and three dimensional (3D) self-assembled graphene nanostructure based electrochemical biosensors. Chapter 3 explains the detailed experimental approaches, describes the materials and instruments applied throughout the whole PhD period research work. Chapter 4 elaborates the synthesis of 2D-assembled dendritic PtNPs on flexible graphene paper electrodes and investigates their performance on the detection of dopamine (DA) secreted from living cells. Chapter 5 further demonstrates 2D-assembled Au@Pt NPs decorated free-standing graphene paper electrodes to monitor live cell secretion of nitric oxide (NO). Chapter 6 discusses flexible graphene paper based electrochemical biosensors decorated with 2D-assembled Au@polydopamine (PDA) nanocrystals for the detection of multiplex DNA targets. Chapter 7 unveils a dual-modality method to monitor intracellular degradation of QDs. Chapter 8 summarizes the main findings and recommends future outlooks.

CHAPTER 2 LITERATURE REVIEW

2.1 Overview

The advances on nanotechnology as well as its unique feature to be customized into bio-recognition component greatly stimulates the development of useful and reliable non-enzymatic biosensor devices, which has built up a solid foundation to solve the problems related to enzymatic biosensors including weak stability of the immobilized enzymes for a long time and the poor selectivity arising from high over potentials.^{14, 15} Firstly, we review different types of nanomaterials like carbon nanomaterials especially 2D-assembled flexible graphene nanosheets and various noble metal nanostructures for hybrid free-standing electrochemical electrodes. Secondly, semiconductor quantum dots (QDs) were also reviewed due to its applications in cell-imaging and signal amplification before summarizing the methods of conjugation noble metal nanostructures on graphene substrate. Then the research progress of self-assembled graphene nanostructure based non-enzymatic electrochemical biosensors with different dimensions (1D, 2D and 3D) will be discussed.

2.2 Nanomaterials for Hybrid Non-enzymatic Electrochemical Biosensors

2.2.1 Carbon Based Nanomaterials

Carbon nanomaterials have been widely applied for the fabrication of electrodes in the field of electroanalytical chemistry because of their attractive features in

terms of (1) relatively wide potential window in aqueous media, (2) low cost, (3) low background current, (4) high electrical conductivity, (5) biocompatibility, (6) robust mechanical strength and (7) good chemical inertness in most electrolyte solutions.¹⁶⁻¹⁹ Carbon based nanomaterials morphologies are critical to enable their functionality and stable operation in the design of efficient electrochemical sensors, which will then affect their electron transport kinetics.²⁰⁻²⁴ Very recently, multi-dimensional carbon nanomaterials were found to attract enormous attentions because of their unique properties.^{10, 25, 26}

Besides these carbon materials (0D buckyballs and 1D carbon nanotube (CNT)) (Figure 2.1), the discovery of free-standing graphene in 2004 by Geim and Novoselov,^{27, 28} diversified the family of carbon materials and ignited the graphene "gold rush" throughout the world. Noticeably, superior features of graphene like large theoretical specific surface area ($2630 \text{ m}^2 \text{ g}^{-1}$) and excellent electron conductivity make it potential to become a valuable candidate for many electrochemical applications, such as fuel cells, supercapacitors, batteries and electrochemical sensors.²⁹⁻³³

Figure 2.1 A schematic diagram showing the formation of 0D buckyballs, 1D nanotubes and stacked 3D graphite by graphene sheets.³⁴ Reprinted with permission from ref. 34. Copyright 2007 Nature Publishing Group.

Graphene has great potentials to be widely used in biosensors, electrocatalysts, and even biological applications because of its physicochemical properties as a result of its truly 2D planar nanostructure.³⁵⁻³⁸ It is highly demanded to clearly reveal the theory of functional groups on single atomic plane and deeply understand the fundamentals of robust assembly techniques, this will greatly enhance the feasibility to integrate the 2D planar structure of graphene. This will also greatly enhance the possibilities to assemble GO nanosheets into macroscopic architectures with different macroscaled shapes (i.e., 1D graphene fibers/ribbons, 2D graphene films/papers, 3D network-structured graphene monoliths) through a variety of assembly techniques under different interaction forces (Figure 2.2). For a further step, the macroscopic graphene assemblies can become the ideal scaffolds to incorporate functional materials so as to make full use of the hybrid structure with specific desired functionalities, which further broaden its applications in biosensors.

Figure 2.2 Principles, strategy illustration of graphene assembly and merits of macroscopic assemblies and architectures.³⁹ Reprinted with permission from ref. 39. Copyright 2014 Royal Society of Chemistry.

The successful fabrication of free-standing paper-like or foil-like graphene in 2007 is a milestone for 2D assembly.⁴⁰ Results indicated that well-exfoliated GO nanosheets can be self-assembled into well-ordered macroscopic structures with adjustable thickness (from 1 to 30 μm) and excellent mechanical, optical and chemical properties (Figure 2.3). 2D-assembled graphene paper-like scaffold has been synthesized and demonstrated to be an attractive candidate to many electronic devices.⁴¹ The developed graphene paper has some good properties such as light weight, robust mechanical properties and good electrical conductivity.^{42, 43} Assembled graphene paper with different well-controlled shapes, large surface areas are beneficial for being incorporated with various functional guest materials, such as metals nanostructures, semiconductor nanostructures and even polymers, which

enrich their functionalities and pave the way for the practical biosensor applications.^{44, 45}

Figure 2.3 Images of GO paper. (A-D), digital camera images of GO paper. (A), ~ 1 μm -thick; (B), folded ~ 1 μm -thick semi-transparent film; (C), folded, ~ 25 μm -thick strip; (D), strip after fracture from tensile loading. (E-G), low-, middle- and high-magnifications SEM cross-view images of ~ 10 μm -thick sample.⁴⁰ Reprinted with permission from ref. 40. Copyright 2007 Nature Publishing Group.

2.2.2 Noble Metal Nanoparticles

Noble Metal Nanoparticles (NMNPs), especially gold nanoparticles (AuNPs), platinum nanoparticles (PtNPs) and their alloy (Au-Pt) nanostructures possess many merits in terms of good conductivity, high electrocatalytic activity, large surface-to-volume ratio and good biocompatibility, have been extensively used for developing

new electrochemical sensing platforms with good performances.⁴⁶ With the help of distinct surface modification strategies (e.g. interfacial self-assembly, layer-by-layer, *in-situ* growth, hybridization and sol-gel technology), NMNPs can be well hybridized on the electrode substrates.¹² This is in favour of well controlling over the microenvironment of biological molecules retaining their activity, and facilitating the electron transfer between the redox centre of biomolecules and electrode.⁴⁶

AuNPs, owing to their delicately tailored size ranging from ~ 1nm to ~ 100 nm, superior stability and complete recovery in biochemical redox processes, have been widely recognized as ideal supports for fabrication of electrochemical biosensors and applied as catalysts in numerous biosensing applications.^{47, 48} A graphene/AuNPs modified electrode was developed to detect DNA targets by applying electrochemical impedance spectroscopy (EIS) as signal amplification method (Figure 2.4A).⁴⁹ When target DNA hybridized with the hairpin probe to form a double stranded DNA. Owing to its unique enzymatic property, a sequence independent exonuclease (Exo) III was applied the sensing system to specifically cleaves the open, hybridized hairpin DNA and release the target DNA. This will further play a catalytic role in the stepwise removal of mononucleotides from the blunt or recessed 3'-hydroxyl termini of a duplex DNA and was inactive to single stranded DNA. The released target DNA upon enzymatic cleavage of the probe will again hybridize with the remaining hairpin DNA probe and trigger another target recycling cycle. This will substantially decrease the electron transfer resistance (R_{et}) monitored by EIS as a result of the decreased steric hindrance between hairpin

structured probe DNA and less negative charges on the phosphate backbone of the surface immobilized hairpin probes. Typical Nyquist plots of the sensors displayed dependence upon the concentration of target DNA. Results indicated that along with the decreased concentration of the target DNA (from a to g) (Figure 2.4B) lead to an increase of R_{et} value accordingly (from g to a). With the involvement of the target recycling signal amplification and good conjugation of probe DNA with AuNPs, this developed DNA biosensor exhibited a dynamic linear range from 50 fM to 5 nM and the detection limit was estimated to be 10 fM, which was 20-500 fold lower than other EIS detection strategies.⁵⁰

Figure 2.4 (A) Schematic diagram revealing the enzyme-assisted target recycling for amplified EIS detection of DNA on a graphene/AuNPs modified electrode. (B) Nyquist diagrams for sensors incubated with different concentrations of target DNA: (a) 5 nM, (b) 500 pM, (c) 50 pM, (d) 5 pM, (e) 500 fM, (f) 50 fM, (g) blank (0 fM target). And (C) the resulting calibration plot of $\log C$ vs. ΔR_{et} for the target DNA over the 50 fM to 5 nM range (error bars: SD, $n=3$).⁴⁹ Reprinted with permission from ref. 49. Copyright 2011 Royal Society of Chemistry.

Similarly, NMNPs such as PtNPs, are also drawing more and more attentions and are widely used in a variety of biomedical applications due to their size and shape differences.^{15, 48, 51} An efficient enzyme mimetic H₂O₂ biosensor was fabricated by using GO nanocolloids to disperse conductive carbon supports of CNTs before and after the loading of Pt nanocatalysts.⁵² Colorimetric investigations (Figure 2.5A) of peroxidase-like catalysis activities of well dispersed GOCNT–Pt nanocomposites (Figure 2.5B) indicated that they had greatly enhance peroxidase-like catalysis with 3,3',5,5'-tetramethylbenzidine (TMB)-H₂O₂ substrate and electrocatalysis activities toward H₂O₂ in water (Figure 2.5C). In this study, the supported Pt nanoparticles (Pt nanos) were supposed to be the dominated factor for the catalysis event. Particularly, the apparent Michaelis constant (K_m) value of the GOCNT-Pt (1.82 mM) with H₂O₂ substrate was lower than those GO-Pt (4.24 mM), CNT-Pt (6.24 mM), and HRP (3.7 mM) with TMB substrate. The GOCNT-Pt modified electrode displayed 10 times larger redox response to H₂O₂ than those of the commonly used CNT modified electrodes. Accordingly, a linear range from 2.5×10^{-6} to 1.0×10^{-2} M H₂O₂ was achieved (Figure 2.5C). Thus, the as-developed Pt nanocatalysts can fully exploit catalysis performances in comparison with natural peroxidase. These suggest that when Pt nanocatalysts used in the non-enzymatic biosensors, they have some merits such as much higher peroxidase-like catalysis and electrocatalysis activities than some inorganic nanocatalysts commonly used. Therefore, Pt nanocatalysis displayed great potential in the non-enzymatic electrochemical biosensors in terms of higher catalysis efficiency, electron transfer, aqueous stability and environmental robustness.

Figure 2.5 (A) Colorimetric comparison of catalytic redox reactions for the TMB- H_2O_2 substrate among CNTs, GOs, Pt Nanos, and the nanocomposites of GO-Pt, CNT-Pt, and GOCNT-Pt consisting of 0.05 mg mL^{-1} CNT or GO and /or 0.05 mg mL^{-1} Pt Nanos. (B) Typical TEM images of GOCNT-Pt nanocomposites in GO nanocolloids (inset: the amplified part of GOCNT-Pt (left top) and the photograph of suspension solution (right top)). (C) Typical current-time responses of the GOCNT-Pt electrode to H_2O_2 successively added with a step of 0.2 mM at an applied potential of -0.2 V . Inset was the steady-state calibration curve of amperometric responses of the GOCNT-Pt sensor to H_2O_2 with different concentrations.⁵² Reprinted with permission from ref. 52. Copyright 2014 Royal Society of Chemistry.

2.2.3 Semiconductor Nanomaterials

Semiconductor QDs have excellent optical properties, such as high quantum yields, broad absorption spectra, narrow and symmetric size-tuneable emission, and strong resistance to photobleaching.⁵³ These unique merits of QDs make them to be widely used for biosensing applications over traditional fluorophores. After

modification with specific affinity ligands (antibody, peptide and aptamer), QDs act as desirable fluorescent labels for biomolecular recognition.⁵⁴ This makes it possible to be used for the detection and quantification of target analytes. For example, Figure 2.6 showed a target-specific imaging of trans-membrane receptors by using quinonyl glycosides functionalized QDs.⁵⁵ A “switch-on” biosensor based on quinonyl glycosides functionalized QDs was developed and used for the specific targeting and imaging of trans-membrane glycoprotein receptors on the surface of cancer cells. The rationale behind the quinonyl glycosides is that the quinone moiety acts as a quencher of QDs and the glycoside moiety acts as a biospecific ligand for targeting a receptor. The quenched photoluminescence of the quinone glycosides functionalized QDs were significantly recovered by a specific lectin selectively bond to the glycosides clustering QDs. However, negligible effect was observed by a panel of nonspecific lectins. At the same time, quinonyl galactoside functionalized QDs were found to be able to optically image the asialo glycoprotein receptors of a hepatoma cell line in a target oriented manner. Thus, this study is very important to fabricate photoluminogenic biosensors in order to analyze the universal ligand–receptor recognitions in nature.

Figure 2.6 Schematic revelation of SnO_2 QDs/RGO (SRGO) composite as an urea biosensor.⁵⁵ Reprinted with permission from ref. 55. Copyright 2014 American Chemical Society.

Different from the above mentioned QDs, many other semiconductors such as metal oxides, noble metal-doped metal oxides, metal oxide-CNTs nanocomposites, and metal oxide-polymer composites have also been employed as non-enzymatic biosensors.⁵⁶ Xiao *et al.* reported a non-enzymatic electrochemical glucose sensor based on coral-like PtAu-MnO₂ nanocomposites.⁵⁷ The coral-like PtAu-MnO₂ nanocomposites were anchored on the substrate through one-step template-free

electrodeposition, which results in the intimate contact between the PtAu alloy and MnO₂ matrix. The flexible electrode has a specific structure and electrochemical properties such as better uniformity, larger active surface areas, and faster electron transfer over the control electrode prepared by tandem growth of MnO₂ network and PtAu alloy by a two-step method. While for non-enzymatic amperometric detection, PtAu–MnO₂ binary nanostructure-modified graphene paper showed remarkably enhanced sensing performance such as wide linear range (0.1 mM to 30.0 mM), high sensitivity (58.54 mA cm⁻² mM⁻¹), low detection limit (0.02 mM, S/N = 1/3), good selectivity, good reproducibility and stability, as well as good tolerability to mechanical stress. The strategy of co-growth of NMNPs and semiconductor metal oxides on freestanding carbon substrates creates new possibilities to fabricate high-performance flexible electrochemical sensors.

2.3 Methods for The Fabrication of Hybrid Electrodes

In a strategic view to fabricate high performance electrochemical biosensors. It is fundamentally important to modify the electrode surface with molecular assembly. It is also necessary to design and tailor nanomaterials with desirable chemical and physical properties to result for a sensitive electrode. More importantly, the post-treatment method used for properly modify the synthesized nanomaterials on substrate electrodes plays a significant role in increasing the immobilization efficiency of biological molecules and accelerating the electron transfer rate on electrode surface.^{58, 59} Here we retrospect and summarize several typical surface modification methods about NMNPs, which are either directly assembled onto

electrode surface, or integrated with other materials to form good performance of electrodes.

2.3.1 Hybridization

The hybridization of NMNPs with single or multiple species of organic, inorganic nanomaterials and polymers were found to affect the modification effect of electrode to enhance its conductivity, catalytic performance and biocompatibility.^{31, 60} For instance, Pt-CNT nanocomposite, resultant from the uniformly entrap of PtNPs on the surface of CNT, has remarkable immobilization area. What's more, the synergistic effect of PtNPs and CNTs have greatly benefited H_2O_2 -based catalysis happened on the electrode surface and reduced its over-potential from 0.6 V to 0.02 V, which is beneficial to the sensitive detection of H_2O_2 .⁶¹

2.3.2 Sol-Gel Technology

The sol-gel process, which involves the formation of the gel matrices and entraps NMNPs into the network, has been widely used to integrate different reagents for biosensors development.^{12, 46} This developed sol-gel method has some advantages such as well dispersion of NPs, providing a stable environment of the composite, facilitating the electron transportation between the nanomaterials and surroundings.

2.3.3 *In-Situ* Growth

In-situ growth of nanomaterials on electrode substrate has been applied in the hybridization of electrodes for a long period. It mainly includes *in-situ* polymerization and *in-situ* electrodeposition. For instance, *In-situ* polymerization of polyaniline (PANI) nanowire has been carried out for the detection of H₂O₂ and glucose.^{62, 63} *In-situ* electrodeposition of nanomaterials has also created many nanostructures such as NPs, nanowires, nanotubes and so on, which further facilitate the electron transfer process.⁶⁴⁻⁶⁶ A kind of PtNi NPs-graphene nanocomposites has been synthesized for non-enzymatic detection of glucose.⁶⁷ The prepared electrode displayed a synergetic effect of PtNi alloy NPs as well as the reduced graphene oxide (rGO).

However, it is still worthy to note some drawbacks from *in-situ* growth of nanomaterials for non-enzymatic biosensors like poor size and uniformity control of synthesized nanostructures, which might have a great impact on the electrocatalytic effect.

2.3.4 Self-Assembled Monolayer

Self-assembled monolayer (SAM) is a method for immobilizing NMNPs onto the surface of electrodes substrate. The premise of SAM lies in molecules which should possess a head group that has strong affinity to the substrate and anchors the molecules to it. Figure 2.7 shows a typical SAM consisting of a head group, tail and functional end group. The commonly used head groups include thiols, silanes, phosphonates, etc.

The general formation of SAMs can be approached by chemisorptions of "head group" onto a substrate from either vapor or liquid phase followed by a slow organization of "tail group". The formation route shown in Figure 2.7 is a typical process happened in solution phase. Briefly, the molecular density is low on the surface. At the very initial stage, adsorbate molecules are easy to form either a disordered mass of molecules or form an ordered 2D "lying down phase". While in the last stage after a period of minutes to hours, the concentration of molecular coverage becomes high, this will result in the adsorbate forming 3D crystalline or semicrystalline structures on the substrate surface.

The well-organized assembly layer allows a good control of the composition and thickness of the transducer surface due to the specific and strong chemisorptions functional groups on the surface of electrodes.¹² The "head groups" are easier to assemble together on the substrate than the "tail groups". As thus areas of close-packed molecules nucleate and grow until the surface of the substrate is covered in a single monolayer. The popularly used SAMs in the electrochemistry field are greatly dependent on the specific affinity between functional groups such as thiols and amines with the surface of noble metals (such as Au, Pt nanostructures).^{58, 59, 68} Consequently, the well-ordered NMNPs monolayers formed by S-/NH- noble metal bonds in favor of immobilizing biological molecules. This has led to a high degree of control over the molecular architecture of the recognition interface.^{69, 70} The possibility of changing particle size and density by tailoring particle synthesis parameter further enhances the attractiveness of NMNPs-modified interfaces for sensing applications.⁷¹ Also, adsorbate molecules adsorb readily because of the lower surface free-energy on the substrate and are stable due to the strong

chemisorption of the "head groups." These synergic functions would create more monolayers than the physisorbed bonds of Langmuir–Blodgett films.^{70, 72} This SAM method is usually used for the formation of single layer structures on surfaces and this method is of great importance for modifying the surface of various materials and devices but it is not effective for multilayer formation.

Figure 2.7 Schematic illustration of SAM process happened at the air-water interface and assembled on the surface of solid substrate.

2.3.5 Layer-by-Layer Assembly

The layer-by-layer (LBL) assembly technique develops a complicated yet highly-ordered molecular architecture by precisely controlling the composition, number of layers and thickness of films at a molecular level.⁷³ The LBL assembly integrates a variety of matrixes with distinctive nature, size and topology. Their properties could be delicately tailored by selecting different configurations, types of components and numbers of layers. A variety of techniques such as immersion, spin, spray, electromagnetism, etc. can be used to achieve this. To clearly explain this in

a simple way, we can use "+", "-" and "W" to define two oppositely charged polyions and defining the wash step as "W".

A LBL film with three bilayers can be made via depositing "W+W-W+W-W+W-W" on the electrode surface, which is specially in the form of "+ - + - + -". Meanwhile, both the electrostatic attraction and some other forces such as hydrophobic attraction are actively involved in the interaction between layers. The bilayers and wash steps can be performed in many different ways, including dip-coating, spin-coating, spray-coating and flow based techniques. The preparation method has greatly influence on the properties of the resultant films and its different applications. Among these, NMNPs have been widely used to form multilayer assembly on the electrode surface, as a result of strong electron conductivity, adsorption ability as well as the biocompatibility.⁷⁴⁻⁷⁶ A multilayer structure based on catalase-encapsulated AuNPs which were electrostatically assembled with anionic and cationic polyelectrolyte was reported.⁷⁷ This AuNPs multilayer allowed electrostatic charge reversal and structural transformation dependent on pH value. Besides, it was capable of increasing the loading density of catalase as well as promoting electron transfer with the electrode. Although this method seems to be able to precisely control the thickness of layers and allow for better retention of the conformation and bioactivity of proteins, both positively and negatively charged materials are necessary needed.

Figure 2.8 Schematic illustration of LBL assembly process on the surface of solid substrate. (A) displayed LBL assembly steps including washing of substrate before the first layer deposition, polycation layer deposition, washing, polyanion and next cycle of washing. (B) Scheme of the deposited ions with both negative ions and positive ions.

2.3.6 Langmuir-Blodgett Method

The Langmuir-Blodgett (LB) method is a traditional method named after Irving Langmuir and Katharine B. Blodgett, who invented LB method has been applied for film application at the interface between liquid and air, as it is beneficial for the control of unique film thickness as well as the molecular orientation.^{72, 78} Normally, a LB film has one or more monolayers of a kind of special organic material, which is deposited from the surface of a liquid onto a solid by an immersing approach. The vertically assembled monolayers are usually composed of amphiphilic molecules

with a hydrophilic head and a hydrophobic tail (Figure 2.9). The tendency is orientated by surface-energy considerations. Since the tails are hydrophobic, it is easier to exposure air over water. Similarly, since the heads are hydrophilic, it will have a favorable interaction with water than air. The overall effect is the reduction in the surface energy or surface tension of water. Polyaniline nanotubes were deposited by LB method on solid ITO glass supports by loading polyaniline nanotubes in chloroform with sodium dodecyl sulfate as the stabilizer.⁷⁹ The LB assembled polyaniline nanotubes were electroactive and displayed potential applications on electrochemical biosensors. Although LB method can offer much more uniformity in fabricating nanofilm on electrode substrate, a hydrophobic functionalized nanocrystals surface is its guarantee.

Figure 2.9 Schematic illustration of LB assembly process. After adding a certain amount of amphiphilic sample into water. It quickly dispersed uniformly on the surface of air-water (step1) with the hydrophobic tail exposed in the air and hydrophilic head in the water. After immersing a substrate into the surface of air and water (step 2), the well dispersed amphiphilic sample attached on the surface of substrate.

2.3.7 Interfacial Self-Assembly

Since discovered in 2004, self-assembly of nanostructures at oil–water interfacial has become a widely applied bottom-up approach for development of nanofilm because of the low cost and high efficiency for all low-dimensional nanostructures.⁸⁰ Interestingly, it has become more and more popular to develop functional nanofilms by the self-assembly of nanostructures and applied in a large fields, such as high-performance display units, biosensors, thin-film capacitors and transistors, and essential components in the microelectronic devices.⁸¹ Albeit the traditional solution-based routes for assembling nanoscale building blocks into functional films, such as LB deposition,^{82, 83} layer-by-layer sequential adsorption,^{84, 85} and covalent attachment,^{58, 86} can significantly decrease the manufacturing cost, their wide applications are still hindered by the needed special equipment and its time-consuming drawback. What's more, high-quality thin films could be achieved from these wet-chemical methods but only used for some peculiar nanostructures. For instance, a hydrophilic nanostructure surface will greatly facilitate the assembly transfer to a solid substrate at the water/air interface (Figure 2.10), so as to make then float on water surface.^{83, 87} Therefore, the developed oil–water planar interface was considered to be an ideal template for self-assembly of lots of nanostructures into functional nanofilms, and thus have received intensive attentions in recent years.^{88, 89} This assembly strategy not only successfully opens the door for self-assembling of hydrophilic nanostructures into closely-packed nanofilms but also paves the way to fabricate nanofilm-based devices for promising applications such as photodetectors, electrochemical biosensors device.⁸¹

Figure 2.10 Schematic illustration of interfacial self-assembly process at the interface of oil-water. A monolayer of self-assembled Au nanoparticles can be loaded onto a solid substrate forming a closely packed monolayer.

2.4 Progress on the Development of Hybrid Biosensors Based on Different Dimensional Assembled Graphene

2.4.1 Electrochemical Biosensors Based on 1D Graphene Nanoribbon

The developed 1D graphene nanostructures mainly include graphene fibers and graphene ribbons, which have been attracting considerable research interests because of their particular properties and potential to be applied in biosensors.^{66, 90-}

⁹³ Here is one example of developed 1D graphene nanoribbon based electrochemical immunoassay for neurotoxin (brevetoxin B: BTX-2) by using guanine-assembled graphene nanoribbons (GGNRs) as molecular tags on a home-made magnetic carbon paste electrode.⁹⁴ As indicated in Figure 2.11A, the recognition elements were prepared by chemically modifying bovine serum albumin-BTX-2 conjugate (BTX-2-BSA) with the GGNRs. And then the immunosensing probes for the capture of BTX-s were prepared by covalently

immobilization of monoclonal mouse, while anti-BTX-s antibodies were covalently immobilizing on the surface of magnetic beads (Figure 2.11B). Because of the competition between the added BTX-2 and GGNS conjugated BTX-2-BSA (BGGNRs), the resultant magnetic immunocomplex was precipitated and integrated on the electrode with an external magnet, subsequently be determined in pH 6.5 PBS containing 2 μM $\text{Ru}(\text{bpy})_3\text{Cl}_2$ (Figure 2.11C). In this study, many guanine nucleobases can be conjugated onto the GNRs due to the specific hydrophobic and π -stacking interactions between the nucleobases and graphene,⁹⁵ As a result of the large number of conjugated guanines on the large surface area of GNRs network and the aid of $\text{Ru}(\text{bpy})_3^{2+}$ to enhance the oxidation of guanines, the electrochemical signal was significantly amplified. Results indicated this immunoassay displayed a well linear relationship between 1.0 pg mL^{-1} to 10 ng mL^{-1} with a detection limit of 1.0 pg mL^{-1} , which was lower than the reported competitive-type enzyme-linked immuno sorbent assay (ELISA) (0.6 ng mL^{-1}). In conclusion, this developed 1D graphene nanoribbons based immunoassay protocol showed a high sensitivity and good selectivity toward detection of BTX-2, which paves a way to exploit the novel non-enzyme electrochemical biosensors with a signal amplification.

Figure 2.11 Synthetic routes of (A) guanine-functionalized graphene nanoribbons conjugated with BTX-2-BSA and (B) anti-BTX-2 antibody-functionalized magnetic beads, and (C) competitive-type immunoassay protocol and measurement method.⁹⁴ Reprinted with permission from ref. 94. Copyright 2015 Elsevier.

2.4.2 Free-Standing Electrochemical Biosensors Based on 2D Graphene Nanostructure

Free-standing 2D assembled hybrid electrochemical biosensors focused on carbon-based films, mainly including “Bucky paper” and “Graphene paper”.⁹⁶⁻¹⁰⁶ In the past years, our research group has made some contributions to the flexible graphene film based electrochemical biosensor (Figure 2.12A).⁹⁶ For example, GO film was fabricated by evaporation method. Free-standing GO film has good hydrophilic property after a simple peeling off process. Bipolymer modified AuNPs easily self-assembled at the interface between water and oil (Figure 2.12B). By

fixing GO film on a solid plate, the self-assembled AuNPs were transferred on the GO film to naturally result the formation of a hybrid flexible electrode with a monolayer of densely packed AuNPs. This integrated hybrid electrode was further reduced by electrochemical method to restore the ordered structure and electron transfer property of graphene. Thus, the combination of 2D-assembled metal nanocrystals and flexible graphene paper has paved the way for fabrication of portable and miniaturized non-enzymatic biosensors.

Figure 2.12 Schematic illustration (A) of development of hybrid working electrode with post-assembly AuNPs on GO film by dip-coating method and further reduced GO film by electrochemically method, and (B) of the AuNPs by coupling biopolymer on it and optical image of the assembled AuNPs at the interface between oil and water.⁹⁶ Reprinted with permission from ref. 96. Copyright 2012 American Chemical Society.

2.4.3 Free-Standing Electrochemical Biosensors Based on 3D Graphene Nanostructure

The development of macroscopic graphene materials with 3D nanostructures has increasingly attracted attentions in view of expanding the significance of graphene, both in fundamental sciences and in practical applications.^{39, 107-109} Due to their attractive properties in term of light weight, high porosity, large surface area, high electrical conductivity, and stable mechanical property, 3D macroscopic graphene has been widely applied in electrochemical biosensors.¹¹⁰⁻¹¹⁵ A novel monolithic graphene foam (Figure 2.13A(a)) was synthesized by chemical vapour deposition (CVD) method and employed as electrochemical electrodes to detect DA.¹¹⁶ This graphene foam exhibits well-defined macroporous structure and it offers a smooth and large specific surface area (Figure 2.13(b) and (c)). Electrochemical experiments demonstrated that compared with glass carbon electrode, 3D graphene electrode displayed a pair of redox peaks in DA solution (Figure 2.13B(d)). Typical amperometric response (at 0.177 V) (Figure 2.13C(e)) showed that the graphene foam electrode displayed a wide linear range from 25 nM to 25 μ M and a high sensitivity of 619.6 μ A mM⁻¹ cm⁻². From the above example, we can see that the graphene foam provides a promising platform for the development of electrochemical biosensors.

Figure 2.13 (A) (a) Photo of a 3D graphene electrode. (b, c) SEM images of 3D graphene foam at different magnifications. (B) cyclic voltammetry (CV) plots of 3D graphene electrode and glass carbon electrode in PBS (I) without and (II) with 20 μM DA. Scan rate = 20 mV/s. (C) The average amperometric dose-response curve from three electrodes (at 0.177 V). The inset shows the amperometric response to 25 nM DA.¹¹⁶ Reprinted with permission from ref. 116. Copyright 2012 American Chemical Society.

CHAPTER 3 MATERIALS AND METHODOLOGIES

3.1 Materials and Instruments

3.1.1 Materials

Hydrogen hexachloroplatinate (IV) hydrate ($\text{H}_2\text{PtCl}_6 \cdot 4\text{H}_2\text{O}$, purity: 99.9 %), hydrogen tetrachloroaurate (III) hydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, purity: 99.9 %), graphite powder (<150 μm , 99.99 % trace metals basis), NO gas (purity: ≥ 99.5 %), Polyvinyl Pyrrolidone (PVP), uric acid (UA), L-ascorbic acid (AA), reagent-grade NaClO, NaOH (> 98%), $\text{Na}_2\text{S}_2\text{O}_3$, KI (99%), tris(2-carboxyethyl) phosphine (TCEP), phorbol 12-myristate 13-acetate (PMA), H_2O_2 (30% w/v, A.C.S reagent grade), DA hydrochloride, Tris(hydroxymethyl)aminomethane, sodium dodecyl sulfonate (SDS), Tris-borate-EDTA (TBE), hydroiodic acid (HI, 55% in water), 3-aminophenylboronic acid (APBA), were obtained from Aldrich. 0.1 μm millipore filters were obtained from Pall Corporation. DA, (3-hydroxyltyramine hydrochloride, Aldrich) stock solutions were prepared by dissolving DA in HClO_4 and stored in refrigerator at 4 °C. Standard solutions of DA with different concentrations from 5 nM to 10 mM were obtained by diluting its stock solutions with phosphate buffer solution (PBS pH 7.4). The bath solution contained 140 mM NaCl, 2 mM CaCl_2 , 4.2 mM KCl, 0.7 mM MgCl_2 , 1 mM NaH_2PO_4 and 10 mM HEPES with a pH of 7.4. The stimulus was K^+ solution (pH=7.4) containing 40 mM NaCl, 105 mM KCl, 6 mM CaCl_2 , 1 mM MgCl_2 and 10 mM HEPES. Ultrapure deionized (DI) water was made from the Millipore Ultrapure water machine at a conductivity resistance of 18 M Ω used to prepare all the above solutions.

All other chemicals used were of analytical reagent grade. 0.1 M PBS, phosphate buffer (PB) (10 mM, pH 8.0), HClO and H₂O₂ solutions were prepared freshly with DI water before usage and their concentrations were determined by measuring the absorbance value at 292 nm and 230 nm by UV-vis spectroscopy, respectively. Basically, ϵ_{292} and ϵ_{230} were taken to be 350 M⁻¹ cm⁻¹ and 81 M⁻¹ cm⁻¹, respectively. The acetate buffer solution (NaAc-HAc, 0.1 M pH 5.0) was prepared by mixing 0.1 M NaAc and HAc.

3.1.2 Instruments

A JEOL JSM-6700 field emission scanning electronic microscope (FESEM) equipped with energy dispersive X-ray (EDX) detector was applied to measure the topography and composition information of samples.

A JEM 2010 microscope was used to obtain the transmission electronic microscope (TEM) images of samples.

The topology and height profiles were characterized and obtained by atomic force microscopy (AFM, SPM 3100, Veeco Instruments Inc., USA).

KSV DC dip-coater was applied for the dip-coating of self-assembled densely packed NPs onto rGO paper.

Zeta-potential was performed by BIC PALS Zeta Sizer.

X-ray diffraction (XRD) (Bruker AXS, Germany) provided the XRD pattern and FTA2000 (First Ten Angstroms, Inc., USA) was used to measure the contact angle at ambient temperature.

The thermal properties were determined by thermogravimetric analysis (TGA, Perkin Elmer Pyris Diamond thermogravimetric analyzer, USA).

A UV-1800 spectrophotometer was applied to record the UV-spectra of samples.

Fluorescence properties of QDs were characterized fluorometer (FluoroMax-3).

Gel electrophoresis was carried out in agarose gels with 1 × (TBE; pH 7.4) as the running buffer at 100 V for 30 min (Thermo Scientific EX 300XL).

Electrochemical Workstation (CHI 660D, CH Instruments) was employed to the measurement of electrochemical performances with a conventional three-electrode system.

Olympus71 inverted microscope was used to capture the optical bright field images of living cells with a PIXIS: 100B spectroscopy CCD camera.

3.2 Materials Synthesis

3.2.1 Preparation of Flexible Graphene Paper

GO solution was synthesized by a modified Hummers method from graphite powder.¹¹⁷ Freestanding GO paper was prepared by a mold-casting method.¹¹⁸ The merit of this method is that both the size and thickness of GO paper could be freely controlled by applying casting molds with different sizes and dropping dispersed GO solution with different volumes (Figure 3.1 A&B). GO paper was reduced into rGO paper by immersing it into HI solution for 1 h at room temperature (Figure 3.1 C).¹¹⁹ Residual HI was washed with DI water and the resulted electrode was dried naturally. As indicated in Figure 3.1C, GO paper and rGO paper can be fabricated in a large scale and displayed good flexibility. Moreover, rGO paper exhibited very low electrical transfer resistance (around 15 Ω) (Figure 3.1D), indicating the good

conductivity of rGO paper. Figure 3.1E displayed the SEM image of distinguished layer-by-layer flexible rGO paper structures.

Figure 3.1 Evolution fabrication process of graphene paper from (A) the dispersed GO solution to (B) flexible GO paper after evaporation and (C) flexible rGO paper after chemical reduction in HI solution. (D) Multimeter detection of conductivity of flexible reduced graphene paper and (E) SEM image of flexible rGO paper cross-section structure.

3.2.2 Synthesis of 2, 2'-dithiobis [1-(2- bromo-2-methyl- propionyloxy)] ethane (DTBE)

DTBE was prepared by acrylation of bis(2-hydroethyl) disulfide. In a typical experiment, 2-bromo-2-methylpropionyl bromide (14.8 mmol) was added dropwise to a mixture of the disulfide (6.17 mmol) and triethylamine (31.5 mmol) in dichloromethane (150 mL) at 0°C under an argon atmosphere. The solution was stirred at 0°C for 1h and then for another 2 h at room temperature. After the

precipitates were filtered off, the organic phase was extracted with 2N Na_2CO_3 solution saturated with NH_4Cl to remove the excess bromides. Subsequent removal of dichloromethane yielded DTBE.¹²⁰

3.3 Electrochemical Techniques

Several electrochemical techniques including CV, differential pulse voltammetry (DPV), amperometric i-t, AC impedance and anodic stripping voltammetry were used in this project.

CV is a type of potential dynamic electrochemical technique, which is widely applied to study the electrochemical properties of an analyte in a solution. The analyte studied by CV is usually redox active within the experimental potential window. The current at the working electrode is plotted versus the applied voltage to trace the cyclic voltammogram in a CV scan (Figure 3.2A). This inversion can be multiple times in a single experiment. And these cycles of CV are highly repeatable up to many times as desired. Both anodic current (i_{pa}) and cathodic current (i_{pc}) are recorded as the function of potential for each cycle. In a typical CV curve, the working electrode potential is ramped linearly versus time and ramping is known as the experiments' scan rate (V s^{-1}) (Figure 3.2B). In a standard three-electrode system, the potential is applied between the working electrode and the reference electrode while the current is measured between the working electrode and the counter electrode. The potential is scanned from the initial potential toward the final potential (either a high potential or a low potential). If an increasing oxidizing potential is applied at the initial forward scan (from t_0 to t_1), the anodic current will increase over this time period. At some point when the oxidation potential of the

analyte is reached, the anodic current will decrease because of depletion of the oxydic anayte. During a reverse scan (from t_1 to t_2), which redox couple is reversible, the oxidized analyte will be reduced gradually, giving rise to a current of reverse polarity (cathodic current) to the original state. In a well-reversible analyte solution, the shape of oxidation peak and reduction peak are highly similar, which can be characterized by the ratio of peak currents at analyte $i_{pa}/i_{pc}=1$. (https://en.wikipedia.org/wiki/Cyclic_voltammetry///accessed on June, 20, 2015).

Figure 3.2 (A) Typical CV curve where i_{pa} and i_{pc} show the peak anodic and cathodic current respectively for a reversible reaction. (B) CV waveform.

DPV can be considered as a derivative of linear sweep voltammetry or staircase voltammetry, with a series of regular voltage pulses superimposed on the potential linear sweep or stair steps. The base potential is increased from initial voltage toward final voltage. A potential pulse is applied and the current before the potential pulse and at the end of the potential pulse are sampled. The difference of these two current samples is recorded as the function of potential. The current is measured immediately before each potential change, and the current difference is plotted as a function of potential (Figure 3.3). Actually, the DPV usually displays lower detection limit and is much more sensitive to the current change near a redox potential than that of CVs. The measurements of DPV is similar to that of voltammetry with three-electrode system. The potential between the working electrode and the reference electrode is changed as a pulse from an initial potential to an inter-level potential and remains at the inter-level potential for about 5 to 100 milliseconds. Then it changes to the final potential, which is different from the initial potential. The pulse is repeated, changing to the final potential, and a constant difference is kept between the initial and the inter-level potential. The value of the current between the working electrode and counter electrode before and after the pulse are sampled and their differences are plotted versus potential. (https://en.wikipedia.org/wiki/Differential_pulse_voltammetry///accessed on June, 20, 2015).

Figure 3.3 Typical DPV curve with a specific oxidation peak current.

In amperometric i - t measurement, a constant potential (usually an oxidation or reduction potential of a redox reaction) is applied and the current is recorded as the function of time. Generally, the current change is dependent on the concentration change of the analyte which can be monitored and a calibration can be generated. Figure 3.4 showed that after each addition of analyte, the current was generated and recorded showing as each staircase. It is worth to note that the data sample interval should be chosen according to the length of the experiment. As shown in Figure 3.4, from the amperometric i - t curve, a calibration curve (Figure 3.4 inset) can be obtained to show the linear range of the detection analyte. (<https://en.wikipedia.org/wiki/Amperometry> accessed on June, 20, 2015).

Figure 3.4 Typical amperometric i - t curve with successive addition of analyte (arrow at the staircase) and the linear range of the detection analyte (inset).

EIS is the measurement of the ability of a circuit to resist the flow of electrical current. Electrochemical impedance is usually measured by imposing an alternative potential signal with different frequency wave, which is the ratio between alternative potential and current changing along frequency wave (ω) or impedance phase angle (Φ) changing with ω . With the understanding of electrochemical impedance, the dynamic process of electrode, double electric layer and diffusion can be analyzed. For a further step, the mechanism of electrode materials, solid electrolyte, conductive macromolecule and corrosion prevention can be investigated. In the practical application, the EIS electrochemical system is simplified to an Randles equivalent circuit (the inset of Figure 3.5), where R_s is solution resistance of the electrochemical system, R_{ct} is Faradic charge transfer resistance, C_{dl} is the constant phase element and W is the Warburg impedance. In a given system, two limiting cases, the relationship of R_{ct} and Warburg determines the EIS curve. Specifically, as indicated in Figure 3.5, if the reaction on the electrode is kinetic

controlled, it will have a large R_{ct} indicated by the semicircle at high frequencies. If the reaction on the electrode is mass transfer controlled, then the Warburg impedance (the slope of the curves at a low frequency) will be displayed. In the Nyquist diagram, the diameter of the semicircle reflects the R_{ct} of redox conversion of the electroactive marker $[\text{Fe}(\text{CN})_6]^{3-/4-}$ on the electrode at certain applied potential. The process is strongly dependent upon any modification to the electrode surface. (https://en.wikipedia.org/wiki/Dielectric_spectroscopy accessed on June, 20, 2015).

Figure 3.5 Nyquist plots of EIS. Inset is the equivalent circuit.

Anodic stripping voltammetry is a voltammetric method for quantitative determination of specific ionic species. Several steps involved in the process. They are cleaning step, electroplating step, equilibration step and stripping step. One is the deposition step, in which the analyte of interest is electroplated on the working electrode. And the electroplated species are oxidized from the electrode during the stripping step. During the stripping step, the current is measured. The oxidation of species is registered as a peak in the current signal at the potential at which the

species begins to be oxidized. The stripping step can be either linear, staircase, square wave, or pulse. (https://en.wikipedia.org/wiki/Anodic_stripping_voltammetry accessed on June. 20. 2015).

3.4 Cell Culture

Pheochromocytoma 12 (PC 12) cells were selected as the model cell to study regulated neurotransmitter secretion from neurons. PC 12 cells were seeded into the 24-well microplate with a density of 1×10^6 cells/mL and maintained at 37 °C in a humid atmosphere with 5 % CO₂. The cell culture medium of RPMI-1640 was mixed with 5% Horse Serum, 10 % Fetal Bovine Serum (FBS), and 1% Penicillin–Streptomycin.

Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC (USA) and the cells were maintained in Endothelial Cell Growth Medium (EGM), which contained bovine brain extract (BBE) supplement. EGM® is a modified MCDB 131 formulation and is supplemented with 10 µg/ml hEGF (human recombinant Epidermal Growth Factor), 1.0 mg/ml Hydrocortisone, 50 mg/ml gentamicin, 50 µg /ml amphotericin B, 3 mg/ml BBE (2ml), and 2% v/v fetal FBS. The HUVECs seeded on flexible paper electrodes in 24-well plate were maintained at 37 °C in a humid atmosphere with 5% CO₂ and reached 60% confluence before experiments. The viability of the cells was evaluated using a standard cell counting Kit-8 (CCK-8) assay in a 96-well plate. A piece of Au@Pt-rGO paper electrode was placed to the tested well and incubated with HUVECs of 2 h, 4 h and 6 h respectively. Subsequently, CCK-8 solution was added to each tested well, and the

absorbance of every well at 450 nm was measured using a microplate reader after 2 h incubation. The cell viability was calculated based on the ratio of the absorbance of the sample well to that of the control cell and expressed as a percentage.

MDA-MB-435 (435) cells, mouse macrophage RAW 264.7, MCF-7 and 3T3-L1 cells were obtained from ATCC and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 % FBS and 1 % penicillin/streptomycin in an incubator at 37 °C with 5 % CO₂ according to ATCC recommendations. The cells were seeded in a 24-well microplate with a concentration of 1×10^6 cells/mL.

For cell imaging and stimulated QDs quenching experiments, cells growth medium was replaced by Hank's buffered saline solution (HBSS) with calcium and magnesium but without phenol red. The reaction was carried out in a 24-well microplate. QDs-PBA (10 nM) PMA was firstly incubated with cells for 30 min and then PMA (10 μ M) was added. Control experiments were conducted by pre-incubation cells with 5 mM NaN₃, thereafter with a further addition of 10 μ M PMA.

3.5 Electrochemical Measurements

Electrochemical measurements were carried out using CHI 660D electrochemical workstation with a three-electrode system, employing a platinum wire as the counter electrode, a silver/silver chloride as the reference electrode, and the fabricated flexible free-standing hybrid electrode as the working electrode. For detection of analytes secreted from living cells, three electrodes were immersed into 24-well microplate.

CHAPTER 4 2D-ASSEMBLED DENDRITIC Pt NANOPARTICLES ON FLEXIBLE GRAPHENE PAPER TO DETECT DOPAMINE SECRETED FROM LIVE CELLS

4.1 Introduction

As one of the most widely studied monoaminergic neuroreceptor, DA has been playing a significant role in modulating many aspects of the brain circuitry of mammals.¹²¹⁻¹²³ It has been reported that intractable psychiatric and neurodegenerative diseases such as Huntington, Alzheimer, Schizophrenia and Parkinson's diseases are associated with abnormalities of DA.^{124, 125} It is highly desirable to improve the capability to monitor DA levels *in vitro* and *in vivo* so as to lead to greater understanding of neuronal function for the development of diagnostic tools.¹²⁶ Until now, a variety of methods including fluorimetry, ionic chromatography, capillary electrophoresis, ultraviolet-visible spectroscopy and electrogenerated chemiluminescence have been developed for the sensitive detection of DA.¹²⁷⁻¹³¹ In comparison with these well established methods, electrochemical method has been popularly used to detect DA since it possesses some outstanding properties such as easy operation, rapid response and high sensitivity in biological systems over other methods.^{132, 133}

The electrochemical response intensity for DA is extremely dependent on the materials composition and surface properties of the constructed working

electrode.^{130, 134} Additionally, it is much challengeable to eliminate interferences from coexists such as UA and AA, which have similar oxidation potentials and higher concentration.^{132, 135-137} Based on these situations, many nanomaterials and biological recognition species such as noble metals and metal oxides,^{138, 139} conductive polymers,^{140, 141} carbon materials modified with small molecules,^{139, 142, 143} RNA aptamer¹²⁹ and enzyme¹³² have been introduced as the modifying components for DA biosensors with improved sensitivity and selectivity. These modifications could inhibit the interference reactions or promote DA oxidation through effective electrocatalysis,^{138, 139} electrostatic attraction^{135, 137, 140} or specific recognition between DA and enzyme (such as tyrosinase) or RNA aptamers.^{129, 144} However, most of these modified electrodes are inflexible and not suitable to construct flexible sensors/devices with very high sensitivity. Considering the bright application prospects and great miniaturization potential of flexible biosensors, some new sensing platforms with excellent flexibility are highly demanded. Free-standing graphene paper, deriving from the layer-by-layer stack of single graphene nanosheets, has emerged as a novel class of supporting scaffolds for hybrid flexible electrode.^{135, 144} Previous researches in our group have demonstrated that the hybrid of graphene paper and metal nanocrystals is a kind of ideal flexible electrode materials, which has displayed good performance toward detection of biomolecules released from living cells.^{118, 145} The good performance could be attributed to the well-tailored nanocrystal size, morphology, enhanced loading density, also the excellent mechanical strength, structural uniformity, and electrical conductivity of flexible graphene paper based substrate.^{57, 146}

Herein, we developed a novel hybrid electrode by structurally integrating 2D-assembled dendritic PtNPs on flexible graphene paper to broaden its application in detecting DA released from living cells.^{147, 148} Dendritic PtNPs are selected among various Pt nanostructures such as nanowire, nanotube, polyhedral and multipod because of their larger surface area which provides enough adsorption sites for molecules within a confined space.¹⁴⁹ Moreover, through an effective self-assembly and accumulation at the interface between oil and water,^{31, 32} monolayer dendritic PtNPs with high density and excellent uniformity could be formed on the surface of graphene paper. Thus, the high catalytic efficiency of dendritic PtNPs could be fully exploited and revealed. As illustrated in Figure 4.1, 2D-assembled dendritic PtNPs were transferred onto the fixed graphene paper to form flexible hybrid electrode through the dip-coating approach.⁹⁶ This strategy has endowed an attractive advantage for a new kind of biosensor by integrating the optimized metal nanostructures and flexible graphene paper into the specifically designed electrode. The developed functional electrodes have effectively circumvented the problems such as low loading density of NPs and poor control over the NPs structures encountered in the existing methods.^{148, 150, 151} Results indicated that this flexible composite electrode exhibited ultra-high sensitivity, wide linear range as well as very low detection limit toward the detection of DA. These interesting findings collectively enable such an electrochemical biosensor to be potentially applied as an implantable, portable and miniaturized smart device in living tissues.

Figure 4.1 Schematic illustration for the fabrication of hybrid electrodes and their electrochemical sensing towards DA released from living PC 12 cells.

4.2 Preparations of Dendritic PtNPs-rGO Paper Electrode

4.2.1 Synthesis of Dendritic PtNPs

Dendritic PtNPs were synthesized with referring to the previous report.^{152, 153} Briefly, 13.4 mL H_2O , 70 mg PVP and 13.6 mL (100 mM) AA were well mixed and heated to 90 °C, and then 13 mL (20 mM) H_2PtCl_4 was added into the above solution with stirring for 3 h at 90 °C. After 3 h sufficient reaction, dendritic PtNPs were purified by removing the extra PVP and re-suspended in DI water.

4.2.2 Interfacial Assembly of PtNPs Array on rGO Paper

Dendritic PtNPs were capped with DTBE through ligands exchange reactions.¹⁵⁴ Briefly, 500 μ L DMF containing 10 mg DTBE was added into 100 mL prepared dendritic PtNPs solution. After reaction for 12 h, the NPs were purified by centrifugation and stored in DMF for further usage. The oil-water interface assembly of dendritic PtNPs was performed by introducing PtNPs into a mixture of water and hexane (V/V, 1/1). After gently mixing the two-phase system for 5 min and leaving it undisturbed, many metallic lusters appeared at the interface between water and hexane, which is the indication of the 2D-assembled dendritic PtNPs. The hybrid electrode (Pt-rGO paper) was fabricated by dip-coating 2D-assemblies of PtNPs onto fixed rGO paper. All hybrid paper electrodes were cut into fixed size, washed and dried under room temperature.

4.3 Morphology and Characterizations of PtNPs-rGO Paper Electrode

4.3.1 Morphology Characterizations

DTBE conjugated dendritic PtNPs spontaneously assembled into 2D monolayer film at the interface between water and hexane, which were displayed by a low magnification TEM image (Figure 4.2A) with a large scale on a Cu grid. The uniformity and monolayer property of PtNPs film could be observed more clearly through the high magnification TEM image (Figure 4.2B). For a further step, high resolution TEM (HRTEM) (Figure 4.2C) was used to examine the morphology of synthesized NPs. HRTEM revealed that the dendritic PtNPs (~ 20 nm) were

composed of small uniform Pt crystals (~ 3 nm). A typical distance of 0.22 nm is observed continuously and can be ascribed to the lattice fringe of Pt (111) face.^{152, 155, 148} It is worth indicating that the ligands exchange process is a reliable strategy for the synthesis of 2D-assembled dendritic PtNPs film.¹⁵⁶

Figure 4.2 (A) and (B) are TEM images of 2D-assembled film of dendritic PtNPs at low and high magnifications, respectively. (C) HRTEM image of dendritic PtNPs.

The cross section FESEM image in Figure 4.3A revealed the layer-by-layer structured shiny metallic rGO paper with uniform thickness. Through a simple dip-coating process at the oil-water interface, dendritic PtNPs were uniformly anchored on the surface of rGO paper (Figure 4.3B). The formation of a well-defined

dendritic PtNPs monolayer on rGO paper could be observed more clearly in the top view image of the hybrid flexible electrode (Figure 4.3C). This was speculated to be as a result of the hydrophobic force between PtNPs and rGO paper.¹⁵⁴ XRD was used to further investigate the crystal structure of PtNPs-rGO paper in comparison with that of the pure rGO paper. As it can be seen from Figure 4.3D, a typical characteristic (002) diffraction peak ($2\theta = 24.4^\circ$) was observed both for rGO paper and Pt-rGO paper, suggesting the unchanged crystal structure of rGO paper before and after modification with PtNPs.¹⁴⁸ On the other hand, four characteristic diffraction peaks ascribed to the (111), (200), (220), and (311) planes of face-centred cubic (fcc) structure of Pt appeared for Pt-rGO paper, located at 2θ values around 39.9° , 46.3° , 67.7° and 81.4° , correspondingly, indicating that the successful anchoring of PtNPs on rGO paper.

Figure 4.3 (A) and (B) cross-sectional SEM images of dendritic PtNPs decorated rGO paper electrode at low and high magnifications. (C) Top view SEM image of dendritic PtNPs decorated rGO paper electrode. (D) XRD patterns of rGO and Pt-rGO paper electrodes.

4.3.2 Electrochemical Characterizations of Pt-rGO Paper Electrode

Figure 4.4A displayed the CV curves of Pt/rGO paper electrode and bare Pt foil electrode in 1.0 M H_2SO_4 with a scan rate of 100 mV s^{-1} . Both CV curves had two peaks appearing in the potential range between -0.2 V and 0.2 V for forward and reverse scanning, which was attributed to the typical profiles of hydrogen desorption and adsorption on the Pt electrode surface.¹⁵⁷ The electrochemically active surface area (EAS) was estimated using hydrogen adsorption and desorption peaks on the CV curves by assuming a $210 \mu\text{C cm}^{-2}$ as a monolayer hydrogen on a

smooth Pt electrode.^{158, 159} The EAS values were 236 and 47.2 cm² mg⁻¹_{Pt} for Pt-rGO electrode and Pt foil electrode, respectively. The remarkably enlarged active surface area of Pt-rGO paper electrode (236 cm² mg⁻¹_{Pt}), which was larger than that of some previous reported electrodes (PtNPs/CNT array 143 cm² mg⁻¹_{Pt}, mesoporous Pt powders 60 cm² mg⁻¹_{Pt}), could be explained as a result of the high density loading of dendritic PtNPs on rGO paper.^{160, 161} The charge transfer resistance of Pt-rGO paper electrode (90 Ω) was much smaller than that of rGO paper electrode (325 Ω) (Figure 4.4A inset), thus electron transfer became easier at the hybrid electrode. Besides the high sensitivity caused by enlarged EAS, the hybrid electrode exhibited good selectivity. Figure 4.4B showed the DPV curves of DA in the presence of AA and UA. It is clear that the voltammetric peak intervals of DA to AA and UA are 189 mV and 156 mV, respectively, in line with the previous report.¹⁶² This indicated that the electrochemical response of DA could be distinguished and quantified by Pt-rGO paper electrode even under the existence of potential interferences (e.g. AA and UA) with higher concentration in biological environment. The reproducibility and stability of the developed biosensors were also acceptable. After 4 weeks storage, the peak current density retained 95% of its initial response. Five repeated measurements using the same electrode yielded a relative standard deviation (RSD) of 4.5%.

Figure 4.4 (A) CV curves of hybrid Pt-rGO paper electrode and Pt foil electrode in 1 M H₂SO₄. Scanning rate: 100 mV s⁻¹. Inset is the impedance plots of rGO and hybrid Pt-rGO paper electrode in 0.1 M KCl containing 1.0 mM K₃Fe(CN)₆ and 1.0 mM K₄Fe(CN)₆. (B) DPV curves of selectivity detection of hybrid electrode in blank bath solution and bath solution containing 0.15 mM AA, 0.003 mM DA and 0.15 mM UA.

4.4 Electrochemical Performance of Pt-rGO Paper Electrode

The electrocatalytic activity of different electrodes was investigated by applying DA as the model analyte. Obviously, Pt-rGO paper electrode had better electrochemical catalytic activity toward DA oxidation than rGO paper electrode because of the increased current density and negatively shifted oxidant potential (Figure 4.5A). Both the cathodic and anodic current densities of DA were found to vary linearly with scan rates (Figure 4.5B), indicating the electrochemical redox process of DA at the electrode surface was adsorption-controlled. In order to avoid memory effect, the Pt-rGO paper electrode was cleaned prior to the next detection. To be specific, the electrode undergone several cyclic voltammetry scans in blank PBS (pH 10.0) after every measurement, and the results confirmed that the

adsorption of DA could be well eliminated and the electrode surface could be successfully recovered. Figure 4.5C displayed the DPV responses of DA under various concentrations at the Pt-rGO paper electrode, and the DPV peak intensity was demonstrated to be proportional to DA concentration over the range of 87 nM-100 μ M (Figure 4.5D), with an excellent sensitivity of 2 μ A μ M⁻¹ cm⁻².^{129, 142} The detection limit was about 5 nM. Further comparison for detection limit and linear range with other reports is given in **Table 4.1**.

Table 4.1 Performance comparison of the DA biosensors based on different electrode materials

Electrode	Sensitivity (μ A μ M ⁻¹ cm ⁻²)	Linear Range (μ M)	Detection Limit (μ M)	Ref.
RNA Aptamer	0.06	0.10-5	0.1	129
NiNPs@P-1,5-DAN	0.88	1-6000	0.011	163
RuO ₂ /CNTs	0.08	0.60-3600	0.06	164
Self-assembled peptide	0.22	5.0-40	2.8	140
pyrolytic carbon films	0.20	18-270	2.30	165
oligo(phenylene ethynylene)s/graphene		0.01-60	0.005	143
silica /CNTs	0.17	0.50-6.00	0.014	138
3D graphene foam	0.62	0.025-25	0.025	142
2D-assembly Dendritic Pt/Graphene paper	2.00	0.087-100	0.005	This work

Figure 4.5 (A) DPV curves of rGO and Pt-rGO paper electrode in PBS (0.1 M, pH 7.4) containing DA. (B) CVs of Pt/rGO paper electrode at different scan rates in PBS (0.1 M, pH 7.4) containing 10 μM DA. The inset shows the dependence of redox peak current density on the potential sweep rates. (C) The DPV responses of various concentrations of DA at the Pt-rGO paper electrode. The inset shows the DPV curves at low concentration of DA. It is necessary to clarify that we intend not to show all the concentrations curves here to make it to be clarified easily. (D) The relationship between the DPV current densities and the concentrations of DA. The inset is the equivalent curve for the low concentrations of DA.

The above results revealed the high performance of the hybrid Pt-rGO paper electrode toward DA, which provided a high feasibility for tracking DA released from living cells. Before the application of detecting DA released from living PC 12 cells, the biocompatibility of the hybrid Pt-rGO paper electrode was confirmed by a

cell viability test with the CCK-8 assay. Figure 4.6A showed the quantitative results of the living cells. It indicated that after co-cultured with hybrid Pt-rGO paper electrode for 8 h, more than 95% of PC 12 cells still stayed alive, witnessing the good biocompatibility of Pt-rGO paper electrodes. Based on the above merits of the flexible hybrid Pt-rGO paper electrode, it was employed to detect secreted DA from living PC 12 cells. After PC 12 cells were cultured on the 24-microplate well for more than 24 h, the cells reached 85% confluence. Then high K^+ saline solution was added into the well as stimulus and DPV was carried out to detect DA released by PC 12 cells. It has been confirmed that addition of high K^+ saline solution will result in cell membrane depolarization to further open Ca^{2+} ion channels.¹⁶⁶ In turn, the Ca^{2+} influx through these opened channels to stimulate several sets of proteins to immobilize and fuse large dense core secretory vesicles.^{46, 47} In such a way, DA inside the vesicle fused with plasma membrane was discharged into the extracellular space.^{166, 167} Figure 4.6B (a) showed that no peak current was detected in the absence of stimulus (high K^+ solution). Upon the addition of certain amount of K^+ solution, peaks belong to the oxidation of DA appeared. The intensity of DPV response was related to the concentration of existing stimuli (Figure 4.6B (b-d)). In the presence of 105 mM high K^+ solution, the most used concentration level as stimulus, a response value of $24 \mu A cm^{-2}$ was obtained. These resultant multiple current densities were due to the oxidation of DA from individual exocytotic events and the electrical signal was equivalent to an extracellular value of 110 zmol transmitter molecules in a single cell, which is consistent with previous reports.¹⁶⁸⁻

Figure 4.6 (A) Cell viability testing of the hybrid Pt/rGO paper working electrode after co-cultured with PC 12 cells for several hours. (B) DPV responses (from a to d) of Pt/rGO paper electrode in microplate well with cultured PC 12 cells in the presence of 0 mM (a), 35 mM (b), 85 mM (c) and 105 mM (d) K⁺ solution.

4.5 Summary

A hybrid, freestanding, and stable dendritic PtNPs decorated flexible graphene paper electrode was successfully fabricated through dip-coating of fixed graphene paper into a biphasic solution of water and hexane, where self-assembled 2D array of dendritic PtNPs was formed at the interface. Owing to the remarkable electrocatalytic activity, high loading density of dendritic Pt nanocrystals and good conductivity of flexible graphene paper, the constructed sensor exhibited excellent performances such as very high sensitivity, outstanding selectivity and good stability toward detection of DA. These integrated features also allowed for its application in detection of DA secreted from live PC 12 cells. This work illustrated a simple and novel approach for the development of electrochemical sensors based on thin monometallic PtNPs film decorated flexible paper electrodes, which could

pave the way to fabricate bimetallic NPs such as Au@Pt nanofilm decorated rGO paper electrode for reactive oxygen species (ROS) detection.

CHAPTER 5 2D-ASSEMBLY OF Au@Pt NANOPARTICLES ON GRAPHENE PAPER AS FLEXIBLE BIOSENSORS TO MONITOR LIVE CELL SECRETION OF NITRIC OXIDE

5.1. Introduction

Biosensors capable of monitoring molecular events in living systems are important tools for fundamental biomedical research and practical applications in clinical diagnosis and high-throughput drug screening.^{171, 172} Detecting specific biomolecules in such complex environments containing a large number of interfering species is technically challenging.¹⁷³ Among the wide spectrum of biomolecules, ROS and reactive nitrogen species (RNS) are recognized as essential messengers for cellular signaling pathways and physiological processes such as neurotransmission and immune response.^{174, 175} And misregulated levels of ROS/RNS are commonly observed for cells under oxidative stress and have been linked with major human diseases such as neurological disorder and cancer.^{176, 177} This fact creates the need for the accurate detection of ROS/RNS. Particularly, NO, as an important member of the ROS/RNS family participating in various physiological and pathological processes, shows limited diffusion distance due to its short lifespan and high reactivity, and therefore the sensitive detection of NO necessitates analytical techniques that can generate real-time and in situ response.^{178, 179} A diverse range of techniques such as chemiluminescence,

ultraviolet spectrometry, laser absorption spectroscopy, and electrochemical methods have been utilized for the direct measurements of NO or downstream reaction products such as nitrite and nitrate.¹⁷⁹⁻¹⁸² Electrochemical analysis has received considerable interest because of the combination of rapid detection, low cost, easy operation, and high selectivity, afforded by measuring redox reactions of NO at specific potentials.^{183, 184} Performance of electrochemical sensors is highly dependent on the electrocatalytic activity of their working electrodes.^{185, 186} Constant efforts have been made to optimize the structural and surface properties of working electrodes for improved sensitivity, stability and selectivity.¹⁸⁷⁻¹⁸⁹ Importantly, recent work by several groups has led to the development of electrodes with a cell-adhesive coating and microelectrodes that allow for close contact with living cells for real-time monitoring of cell secretion of NO.^{190, 191}

Here we report the development of flexible graphene paper loaded with closely-packed core-shell Au@Pt NPs as a freestanding electrochemically-active cell culture substrate and its use for real-time monitoring of NO released by living cells. Flexible electrodes offer unique opportunities for developing miniature electrochemical sensors, which are in great demand for better interfacing with living systems in confined spaces and performing localized detection down to cellular level.^{64, 192} Recent advances in directed assembly of graphene and its chemical derivatives have given rise to flexible paper-like substrates with excellent mechanical stability and electrical conductivity.^{193, 194} Our work has shown that graphene paper with flat surfaces can serve as the loading substrate for 2D assembly of NPs at the oil-water interface and form stable binding through non-covalent

interactions between its surface and the nanoparticle coating. As illustrated in Figure 5.1, the hybrid electrode was fabricated through a modular approach in which the nanoparticle assembly was transferred onto graphene paper through dip-coating. We previously have shown that the hybrid electrode of 2D-assembly of closely packed AuNPs and graphene paper provides sensitive and selective detection of glucose and H_2O_2 secreted by living cells.⁹⁶ In this study, we have compared the performance of graphene paper electrodes loaded with 2D-assemblies of Au and core-shell Au@Pt NPs in NO detection. More importantly, we have further engineered the surface property of the NPs to allow for stable adhesion of HUVECs on the electrodes. Our results have demonstrated that the use of the hybrid electrode for amperometric detection of NO led to a linear dynamic range of 400 nM to 673.9 μM and a detection limit of 100 nM. This enables the real-time monitoring of NO secretion by HUVECs grown on the paper electrode.

Figure 5.1 Schematic illustration of the fabrication of the freestanding hybrid electrode from 2D-assembly of Au@Pt NPs and graphene paper.

5.2 Fabrication of Au@Pt-rGO Paper Electrode

5.2.1. Synthesis of Au and Au@Pt NPs

AuNPs of 14 nm were synthesized by citrate reduction of HAuCl_4 . Briefly, 200 mL of 1 mM HAuCl_4 under stirring was brought to a reflux. Subsequently, 5 mL of a 155.2 mM trisodium citrate solution was added quickly, resulting in a color change of the solution from pale yellow to deep red. After the color changed, the solution was kept under refluxation for another 15 min and cooled down at room temperature, and stored at 4 °C. Au@Pt NPs were synthesized according to literature reports.^{153, 195} 125 mL of the 14 nm Au solution was mixed with 125 mL H_2O and heated to 100 °C, and 12.5 mL of 1% H_2PtCl_6 (20 mM) was added under stirring. Afterwards, 10 mL of 100 mM L-AA was dropped into the solution in 2 min, and the reaction mixture was heated for 20 min to yield the Au@Pt core-shell NPs.

5.2.2. Interfacial Assembly of Au@Pt NPs Array on rGO Paper

DTBE was coated on Au NPs and Au@Pt NPs through ligands exchange reactions. Briefly, 10 mg of DTBE was dissolved in 500 μL DMF and added into 100 mL Au and Au@Pt solution. After 12 h reaction, the NPs were purified by repeated centrifugation and redispersion to remove free DTBE and finally were dispersed in DMF for further use. To prepare the 2D-assembly of NPs at oil-water interfaces, the nanoparticle DMF dispersion was mixed with equal volume of water, and hexane (hexane/DMF = 2:1, v/v) was introduced. The two-phase system was gently mixed for 5 min and left undisturbed until the 2D assembly with metallic luster was formed at the interface. To fabricate the hybrid electrode (Au@Pt-rGO), 2D-assemblies of NPs were transferred on rGO paper by dip-coating (KSV DC dip-

coater). All the modified paper electrodes were washed carefully with redistilled water and dried at room temperature.

5.3 Characterizations of Au@Pt-rGO Paper Electrode

5.3.1 Morphology Characterizations

As shown in Figure 5.2A, AuNPs solution exhibits deep red color because of the distinct surface plasmon resonance (SPR) centered at 519 nm. Coating a layer of Pt on AuNPs by seeded growth^{153, 195} led to an eminent color change into black. The absorption peak decayed and blue-shifted because the SPR absorption of Pt nanostructures locates in UV range and the Pt shell is lack of characteristic absorption in the visible spectral range. TEM observation (Figure 5.2B) demonstrates that Au@Pt NPs have uniform size and urchin-like morphology. High-resolution TEM (Figure 5.2B inset) reveals that these Au@Pt NPs have a typical core-shell structure. The average diameter of the Au@Pt NPs is about 20 nm, and the thickness of the Pt shell is roughly 3 nm, based on the size (14 nm) of Au seed NPs. The crystal lattice of (111) face of Pt shell and Au core are 0.22 nm and 0.235 nm, which are in line with what was reported previously.^{152, 155} Our recent work has shown that NPs coated with ligands containing 2-bromopropionate end group can spontaneously accumulate at the oil-water interface to form closely-packed 2D-assemblies.¹⁵⁶ The results in this study suggest that this strategy is also applicable for Au@Pt NPs. After the core-shell Au@Pt NPs were coated with DTBE with a disulfide anchoring group, the assembly with metallic luster appeared at the interface of hexane and the mixture of water and DMF. Figure 5.2C displays

the TEM images of 2D-assembly of Au@Pt NPs deposited on a carbon-coated copper grid, showing a large area of 2D-assembly of closely attached core-shell NPs. Similar results were also observed for the assembly of AuNPs (Figure 5.2D).

Figure 5.2 (A) UV-vis spectra of Au and Au@Pt NPs dispersed in water and photographs of the two dispersions (inset); (B) TEM and HRTEM (inset) images of Au@Pt NPs; (C) TEM images of 2D-assembly of Au@Pt NPs. Inset in Figure 5.2C is a photograph of the 2D-assembly Au@Pt NPs formed at the interface. (D) TEM images of 2D-assembly of Au NPs.

The rGO paper obtained by reducing GO paper in HI solution displays shiny metallic color. SEM was applied to observe the surface structure of rGO paper. The characteristic wrinkle feature is clearly visible on its surface (Figure 5.3A). The 2D-

assembly core-shell Au@Pt NPs were transferred on the rGO paper fixed on a solid substrate through dip-coating, forming a uniform closely-packed monolayer on the entire rGO paper (Figure 5.3B). The components of the paper electrode are confirmed by EDX (Table 5.1). We also observed the nanoparticle coating remained stable in varieties of buffer solutions, which is probably due to the strong hydrophobic interaction of the DTBE coating and the rGO paper.

Table 5.1 Components of different paper electrodes calculated from EDX analysis.

Paper electrodes	Element	Weight (%)	Atomic (%)
GOP electrode	C	51.73	58.81
	O	48.27	41.19
Au@Pt-rGOP electrode	C	20.27	72.40
	O	4.11	11.02
	Pt	51.80	11.39
	Au	23.82	5.19

Figure 5.3 (A) SEM images view of rGO paper. (B) SEM image of core-shell Au@Pt-rGO paper.

5.3.2 Electrochemical Characterizations

Figure 5.4A shows the CV curves of different paper electrodes in 0.5 M H₂SO₄ solution. The peaks associated with the reduction of oxide species of the electrode

surface can be used to determine the surface composition. The reduction peak starting from 0.4 V corresponds to Pt species, whereas the one at 0.9 V is associated with Au species.¹⁹⁶ For Au@Pt-rGO paper, the characteristic Pt-oxide and Au-oxide reduction peaks are clearly visible. More importantly, the hydrogen adsorption/desorption peaks and oxide formation/stripping peaks of polycrystalline bulk Pt were also readily seen for Au@Pt-rGO paper, indicative of the high electrochemical activity of the Au@Pt-rGO paper electrode. The EAS of the paper electrode was estimated by the cathodic or anodic charge in the hydrogen adsorption/desorption region, which display that Au@Pt-rGO paper electrode has a high active surface area of $18.83 \text{ m}^2 \text{ g}^{-1}$, much larger than that ($10.70 \text{ m}^2 \text{ g}^{-1}$) of the flat Pt foil electrode. This demonstrates that our strategy for coating 2D-assembly of Au@Pt alloy NPs on graphene paper give rise to catalysts with enhanced active surface area, which is expected to improve its electrocatalytic performance.

$[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe was chosen to further investigate the intrinsic electrochemical property of different hybrid paper electrodes. As shown in Figure 5.4B, a quasi-reversible Faradic currents associated with the well-defined redox waves of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ couple in 0.1 M KCl is observed on rGO paper. The observed electrochemical activity should originate from the improved electron transport property of rGO with partially recovered sp^2 -hybridized network after chemical reduction of GO. The coating of 2D assembly of AuNPs on rGO paper caused an increase in the Faradic current densities of $[\text{Fe}(\text{CN})_6]^{3-/4-}$, which again confirms the improved EAS by coating rGO paper with metallic nanocatalysts. More importantly, after coated with a 2D-assembly of Au@Pt NPs, the peak current

densities of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ increased dramatically. Apparently, the Au@Pt NPs that densely loaded on the electrode are expected to increase the reactive sites of the electrode, which finally lead to an increased real surface area of the electrode^{197, 198}. Based on the Randles-Sevcik equation:¹⁹⁹ $i_p = 269n^{3/2}AD^{1/2}v^{1/2}c$. The electroactive surface areas are 4.37 cm², 2.05 cm² and 1.26 cm² (average of three measurements) for Au@Pt-rGO paper, Au-rGO paper and rGO paper, respectively. Heterogeneous electron transfer rate constants (k), which are based on the peak separation (ΔE_p) between the forward and reverse scans in a cyclic voltammogram are typically calculated using the Nicholson method²⁰⁰. The heterogeneous electron transfer rate constants of ferricyanide are 0.48×10^{-3} , 2.24×10^{-3} , and 3.58×10^{-3} cm s⁻¹ for rGO paper electrode, Au-rGO paper electrode and Au@Pt-rGO paper electrode, respectively²⁰¹. This improved electron transfer rate constant of Au@Pt-rGO paper electrode corresponds well with the enhanced current density and reduced ΔE_p in comparison with other control electrodes. The EIS curve in Figure 5.4C further shows that Au@Pt-rGO paper electrode has a low charge transfer resistance of 50 Ω , which is much smaller than those of Au-rGO paper electrode (100 Ω) and rGO paper electrode (325 Ω), confirming a faster electron transfer rate of Au@Pt-rGO paper electrode. We have found that for all the paper electrodes, the peak current (i_p) is linear to the square root of the scan rate ($v^{1/2}$) in the scan rate range studied (i.e. 2 mV s⁻¹ to 100 mV s⁻¹ Figure 5.4D), which represents a diffusion-controlled electron transfer process. Taken together, our results suggest that the loading of densely packed 2D-assembly of Au@Pt NPs gives rise to paper electrodes with enhanced catalytic activity.

Figure 5.4 (A) CV curves of Au@Pt-rGO paper electrode and Au-rGO paper electrode in deaerated 0.5 M H_2SO_4 . Scan rate: 50 mV s^{-1} . (B) CV curves of Au@Pt-rGO, Au-rGO and rGO paper electrodes in 0.1 M KCl containing 1.0 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 1.0 mM $\text{K}_4\text{Fe}(\text{CN})_6$. Scan rate: 10 mV s^{-1} . (C) Impedance plots of three electrodes in 0.1 M KCl containing 1.0 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 1.0 mM $\text{K}_4\text{Fe}(\text{CN})_6$. (D) CV of electrochemical behavior of Au@Pt-rGO paper electrode with the increasing of scan rate in 0.1 M PBS containing 1.0 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 1.0 mM $\text{K}_4\text{Fe}(\text{CN})_6$. Inset is the relationship between the peak current and the square root of different scan rate.

5.4 Electrochemical Behavior of NO at Au@Pt-rGO Paper Electrode

In order to investigate the electrocatalytic activity of different paper electrodes against NO, NO ($50 \mu\text{M}$) was detected by these paper electrodes (i.e., rGO paper

electrode, Au-rGO paper electrode, and Au@Pt-rGO paper electrode). It was found that Au@Pt-rGO paper electrode exhibited higher electrochemical catalytic activity toward NO oxidation than rGO paper and Au-rGO paper electrode, indicated by the increased current density and decreased over-potential (Figure 5.5A). Due to its high performance, Au@Pt-rGO paper electrode was further tested for the amperometric detection of NO. For sensing applications, electrocatalysts are generally evaluated by measuring currents at fixed potentials and time after addition of the analyte. The current-time (i - t) plots and calibration curve (with the equation of $I \text{ (mA cm}^{-2}\text{)} = 3.653 [\text{NO}] \text{ mM} + 0.06$, $R^2=0.998$) for the sensor under the optimized experimental conditions are depicted in Figure 5.5B and Figure 5.5C. The sensor responds linearly to NO concentration up to 673.9 μM with a detection limit of 100 nM ($S/N = 3$) and a detection sensitivity of $3.653 \text{ mA cm}^{-2} \text{ mM}^{-1}$, suggesting a high sensitivity of the as-prepared paper electrode, which is essential for the real-time monitoring of NO secretion by living cells. The stability and reproducibility of the hybrid electrode are also important performance parameters. Three repeated measurements of 50 μM NO using the same electrode yield relative standard deviation (RSD) of 4.0%, and as shown in Figure 5.5D, the current retains over 85% of its initial value after one and a half months post-preparation. Moreover, the RSD of the current responses across five sensors is 4.5%. The above results demonstrated a good stability and accuracy of the sensor for long-term repeated uses.

Figure. 5.5 (A) DPV curves of Au@Pt-rGO, Au-rGO and rGO paper electrode in PBS (pH 7.4) containing $50 \mu\text{M}$ NO. (B) Typical amperometric response of Au@Pt-rGO paper electrode to the successive addition of NO with different concentration in a stirring PBS (pH 7.4), and inset is the current change in response to low concentrations of NO (applied potential: 0.75 V). (C) Linear relationship between peak current vs. concentration of NO. Inset curve is the corresponding calibration curve in the low concentration of NO. (D) Stability and reproducibility test of the working electrode.

DPV method was used to individually analyze NO and common interferences such as AA, DA, UA, and NO_2^- and the results show that the analytes have different electrochemical oxidation potential at 0.75 V (NO), -0.09 V (AA), 0.1 V (DA), 0.234 V (UA), and 0.6 V (NO_2^-), respectively (Figure 5.6A). Amperometric i-t curve with the applied potential of 0.75 V was further employed to examine

whether NO₂⁻ affects the detection of NO. Detection of NO (50 μM) in the presence of NO₂⁻ (5 mM) of 100 times more concentrated than NO only led to 5.1% increase in current response in comparison with that of the control sample without NO₂⁻ (Figure 5.6B). We further tested the potential interference of a list of electroactive species by amperometric *i-t* method and the results confirmed the high selectivity of Au@Pt-rGO paper electrode (Table 5.2). Notably, our flexible hybrid electrodes outperform a large number of electrodes previously reported for NO detection (Table 5.3).

Figure 5.6 (A) DPV curve of Au@Pt-rGO paper electrode of AA, DA, UA, NO₂⁻ and NO samples, respectively. (B) Amperometric *i-t* curve of interference detection of NO₂⁻.

Table 5.2 Influence of foreign species on the determination of 0.05 mM NO with Au@Pt-rGO paper electrode in 0.1 M PBS (pH 7.4).

Foreign species	Concentration spiked (mM)	Change of current response (%)	RSD (%) [a]
UA	0.2	1.5	3.5
AA	0.2	2.6	3.4
DA	0.1	2.2	3.2
Cl ⁻	0.1	-1.3	2.0
NO ₃ ⁻	0.1	-1.5	3.7
SO ₃ ²⁻	0.1	2.7	3.3
SO ₄ ²⁻	0.1	1.7	2.9
Ca ²⁺	0.05	-3.8	2.3
K ⁺	0.05	-3.0	2.6
Na ⁺	0.05	-2.0	2.9
Mg ²⁺	0.05	2.9	4.6
Cd ²⁺	0.05	-3.8	2.6
Fe ²⁺	0.05	-4.2	2.6
Co ²⁺	0.05	3.8	2.8
Ni ²⁺	0.05	-3.8	3.6
Mn ²⁺	0.05	2.7	3.0
Hg ²⁺	0.05	-1.9	2.6
Pb ²⁺	0.05	-3.2	2.4
Zn ²⁺	0.05	2.7	2.1
Fe ³⁺	0.05	1.9	4.2
NO ₂ ⁻	5	5.1	3.2

[a] n=10

Table 5.3 Summary of some other carbon-related materials of NO sensors

No.	Electrode	Linear Range	Detection Limit	Reference
1	Electrocatalytic film composed of dihexadecyl hydrogen phosphate, platinumNPs, and acetylene black on glass carbon electrode	0.18 to 120 μ M,	50 nM	202
2	Hemoglobin adsorbed on the gold colloids-modified carbon paste electrode	0.9 to 300 μ M	100 nM	203
3	Electrochemically reduced grapheme-modified electrode	0.72 to 78 μ M	200 nM	204
4	Gold nanoparticle arrays directly grown on nanostructured indium tinoxide electrodes:	1-500 μ M	650 nM	205
5	Au@Pt graphene flexible electrode	0.4-673.9 μ M	100 nM	This work

5.5 In-Situ Monitoring of NO Secretion from Living Cells

For the electrochemical monitoring of NO secretion of living cells, it is essential to maintain an intimate contact between the cells and the working electrode because of the limited diffusion distance of NO.^{179, 206, 207} In this study, HUVECs were directly grown on the surface of the flexible Au@Pt-rGO paper electrode to ensure the intimate contact. Calcein-AM is known to only stain living cells. The fluorescence microscopy images show that the stained HUVECs cells on flexible Au@Pt-rGO paper electrode were healthy and remained well-spread (Figure 5.7A). The hybrid electrode of graphene paper and core-shell Au@Pt NPs coated with DTBE molecules showed a good cell-adhesion property, which is not available for our previously reported paper electrode coated with AuNPs with amphiphilic polymeric coating containing cell-repellent poly (ethylene glycol).⁹⁶ Quantitative cell viability test further confirmed that cells maintained over 95% viability after

cultured with Au@Pt-rGO paper electrode for 6 h, indicative of the good biocompatibility of Au@Pt-rGO paper electrode (Figure 5.7B).

The amperometric responses were measured at the Au@Pt-rGO paper electrode with cultured HUVECs in pH 7.4 PBS after injection of 1.0 mM acetylcholine (Ach). Ach induces a Ca^{2+} influx by binding to the Ach receptors on the surface of HUVECs,¹⁷⁴ and Ca^{2+} activates endothelial NO synthase to generate NO. As shown in Figure 5.7C, when Ach injected into PBS solution with the cultured HUVECs (Figure 5.7C black line), the current density increases to 6 μA within 5 s, which reveals the electrochemical oxidation of NO released from the HUVECs. In comparison (Figure 5.7C red line), the addition of the same amount of Ach does not cause an increase of the current for the cells pretreated with 10 μM N_ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME), which is known as a NO-synthase inhibitor. This confirms that the amperometric signal is indeed generated from NO secreted by HUVECs. Moreover, the control group (Figure 5.7C blue line) containing no HUVECs or L-NAME does not generate any signal response to the addition of Ach. These results demonstrate the possibility of using the flexible hybrid electrode for real-time monitoring live cell secretion of electroactive substances.

Figure 5.7 (A) Fluorescence microscopy image of the calcein-stained HUVECs on flexible Au@Pt-rGO paper electrode. (B) Quantitative cell viability results. (C) Amperometric responses of the HUVECs-bearing Au@Pt-rGO paper electrode in 0.1 M PBS (pH 7.4) at different conditions.

5.6 Summary

In summary, we have prepared a new type of flexible electrode based on graphene paper loaded with closely-packed core-shell Au@Pt NPs. The free-standing graphene paper assembled from individual graphene nanosheets exhibits a combination of mechanical robustness, good electrical conductivity, and stability in electrochemical environment. 2D-assembly of NPs with well-defined structures were transferred on the graphene paper through dip-coating, leading to a high loading density of NPs. The resultant free-standing electrochemically-active cell culture substrate allows for the intimate contact of the paper electrode with living cells and real-time detection of NO secreted by living cells. We envision that our modular approach for designing high-performance flexible electrodes with tailored surface properties to well conjugated with detection targets would offer new insights to realize multiplex detection of targets.

CHAPTER 6 2D-ASSEMBLED Au@PDA NANOCRYSTALS ON FLEXIBLE GRAPHENE PAPER FOR MULTIPLEX DETECTION OF DNA

6.1 Introduction

Since their significant role in more and more areas such as (1) genetic diseases diagnostics, (2) infectious bacteria monitoring, (3) forensic samples analysis, and (4) bioterrorism agents screening, DNA sensors have been attracting an increasing number of attentions throughout the world.²⁰⁸ Thus, sensitive and multiplex DNA detection is highly demanding in the fields of molecular biology and clinical medicine, because it is greatly beneficial for identification of disease and pharmacogenomics.²⁰⁹⁻²¹¹ Apparently, the signal variation amplitude of a hybridization event is the detrimental factor for the detection sensitivity of a DNA sensor.^{212, 213} Electrochemical DNA sensors well integrate the advantages of (1) electrochemical transducers (including the speed, portability, high sensitivity, and low cost) and (2) DNA targets (including high affinity and specificity), and as thus have been paid more and more attentions.^{214, 215} Very recently, amount of tactics have been used to increase the detection signal toward DNA hybridization.^{216, 217} For instance, there are many public reports about the enhancement of detection sensitivity either by enzyme-based incorporation of many redox labels in a single DNA strand or replacement of small organic redox molecules.^{218, 219} It was also reported that modification of electrodes surface with self-assembled monolayer chemicals such as mercaptohexanol and dithiothreitol can dramatically improve the

signal-to-noise characteristics of electrochemical DNA hybridization.^{214, 220} DNA sensors based on NPs protocol for detecting DNA hybridization by electrochemical stripping detection of metal tags have also been reported.^{214, 221, 222} However, the associated low conjugation of probe DNA and confined modification on Au electrode tends to limit the detection of DNA targets, leading to the reduced activity. Thus, effective modification of electrode with nanocrystals and selecting a proper substrate is still a great technological challenge to be addressed.

Graphene, as the substrate of electrode, has attracted great interest in a wide range of areas because of its unique electronic, optical, thermal and mechanical properties.^{10, 20, 21, 223} Recently, flexible electrochemical biosensors combined with rGO paper and nanocrystals are under researching.^{118, 154, 194, 224} This flexible electrode has combined the good conductivity of rGO paper substrate and high catalytic properties with well-tailored size and morphology of nanocrystals. To be specific, the mussel-inspired self-polymerized polydopamine (PDA) exhibits strong adhesion on virtually any organic and inorganic substrates.^{225, 226} Researches indicated that PDA coated nanocrystals could form a uniform thin film through self-assembly between water and oil and can be loaded on the surface of flexible rGO paper substrate, creating high density nanocrystals decorated electrodes with a large active surface areas.²²⁷

Herein, we have developed a flexible biosensor for multiplex detection of DNA targets with core-shell Au@PDA nanocrystals decorated rGO paper electrode. As illustrated in Figure 6.1, "sandwich" DNA sensor was realized through the

following steps. **Pre-step.** In order to capture report DNA-tagged semiconducting metal sulfide QDs by electrodes, the electrodes were prepared by loading the self-assembled 50 nm Au@PDA nanocrystals (self-assembled at oil-water interfacel) onto the surface of rGO paper through dip-coating method. The 50 nm Au@PDA-rGO paper electrodes were immersed into PB solution containing capture DNA and stirred for 2 h to allow the conjugation of capture DNA with 50 nm Au@PDA. Then the capture DNA anchored 50 nm Au@PDA-rGO paper electrodes were washed and dried. The surface of 50 nm Au@PDA substrate was then blocked by a 10 min treatment with 6-mercapto-1-hexanol (0.1 M, 100 μ L) and rinsed with DI.

Step 1. Capture DNA conjugated 50 nm Au@PDA-rGO paper electrodes were further immersed into DNA targets solution with stirring for 1 h to conjugate with target DNA. Thus, in this step, sandwich DNA hybridization was formed by further combination between target DNA and semiconducting metal sulfide QDs-tagged reported DNA. In the sandwich structure, capture DNA and report DNA flank target DNA sequence.

Step 2. The well-formed sandwich structure of electrodes were immersed into acid solution to decode semiconducting metal sulfide QDs, releasing the corresponding electroactive heavy metal ions, such as Zn^{2+} , Cd^{2+} , Pb^{2+} , and Cu^{2+} .

Step 3. Electrochemical signals were generated by the dissolved semiconducting metal sulfide QDs those specifically bind to target DNA, indicating the concentration of DNA targets.²²⁸ The combination of several key techniques provides the feasibility for the construction of the multiplexed DNA sensor. One is the design of a functional PDA layer on the surface of Au NPs. The mussel-inspired self-polymerized PDA shell of 50 nm Au@PDA exhibits strong adhesion on virtually any organic and inorganic substrates, facilitating robust binding of the

interfacial assembly of metal nanocrystals on solid substrates, such as glass and free-standing rGO paper.^{226, 227, 229} The other interesting finding is that metal ions released from etched semiconducting metal sulfide QDs have high affinity ability with PDA layer through specific coupling effect, facilitating the accumulation of metal ions. Another thing is that porous hydrogel-like structure PDA has provided enough conjugation sites for capture DNA, which further increase the specific hybridization strands of target DNA. In particular, the 50 nm Au@PDA nanocrystals loaded rGO paper flexible DNA electrode represents a new class of DNA biosensor that combine the good mechanical stability and conductivity of free-standing flexible rGO paper with the high catalytic performance of 50 nm Au@PDA nanocatalysts.

Figure 6.1 Schematic illustration of flexible biosensors and the detection process of multiplex DNA targets.

6.2 Preparation of Flexible Au@PDA-rGO Paper Electrode

6.2.1 Synthesis of Au@PDA Nanocrystals

The synthesis of 50 nm Au@PDA was achieved by the following steps. In the first step, 20 nm AuNPs were synthesized as the seeds of 50 nm AuNPs. 20 nm Au NPs were synthesized by quickly injecting 182.4 mg (in 2 mL H₂O) citrate sodium to 160 mL boiling HAuCl₄ 3H₂O (1%). The solution was boiling for another 30 min and cool down to room temperature. In the second step, 50 nm AuNPs were synthesized by adding 12 mL 20 nm AuNPs with the adsorption of 0.9 (UV=522 nm) into 88 mL H₂O with stirring for 3 min. Then 1.2 mL 0.2 M NH₂OH HCl was added into the above mixture solution with stirring for another 3 min. 12 mL HAuCl₄ 3H₂O (0.1%) was drop wise added into the mixture solution. After finishing drop wise, 2 mL citrate sodium (1.5 mM) was added into the reaction solution to stabilize the synthesized AuNPs. The synthesized 50 nm AuNPs were collected and the UV absorption of 2.12 was determined at the wavelength of 533 nm. In the third step, 50 nm Au NPs with a UV absorption of 70.4 was stabilized with 0.5% SDS solution and collected into 1 mL vial. In the fourth step, 50 nm Au@PDA NPs were synthesized by dispersion 190 μ L of the SDS stabilized 50 nm Au NPs (A=70.4) into 5 mL DI H₂O with stirring for 3 min. Then 30 mL Tris buffer (10 mM, pH 8.5) was added into the dispersed 50 nm Au NPs solution. Following by drop wise 4 mL DA solution with the final concentration of 0.1 mg/mL. The thickness of synthesized PDA layer can be controlled by the amount of dissolved dopamine solution. After overnight reaction, the synthesized 50 nm Au@PDA NPs

were collected into 1 mL by centrifuge (850 rcf, 12 min) and stored in the room temperature.

6.2.2 Decorating Au@PDA Nanocrystals on Flexible rGO Paper

50 nm Au@PDA decorated rGO paper electrode was obtained through the following steps. Firstly, core-shell 50 nm Au@PDA NPs was self-assembled at the interface of chloroform and water by gently shaking the mixture for one minute. A closely packed monolayer of self-assembled 50 nm Au@PDA NPs film was formed because of the total free energy reduction in the biphasic system.¹⁵⁶ Secondly, self-assembled core-shell 50 nm Au@PDA NPs film was transformed on the surface of rGO paper with a fixed substrate and dried at room temperature through dip-coating method.⁹⁶ The obtained hybrid dual core-shell 50 nm Au@PDA NPs film decorated rGO paper was cut into fixed size and further applied as a flexible freestanding working electrode.

6.3 Preparation of "Sandwich" DNA Biosensor

6.3.1 Conjugation between Capture DNA and Au@PDA Nanocrystals

Capture DNA were received with disulfide protecting groups. Disulfide groups need to be cleaved into thiol group by the following protocol. The disulfide-protected nucleotides were diluted in DI water to 100 μ L and treated with TCEP (20 fold molar concentration higher of disulfide-protected nucleotides) for 1 h and stored in 4 °C. Before the hybridization, capture DNA has been conjugated with 50 nm Au@PDA on the surface of rGO paper electrode in PB buffer (10 mM, pH 8.5) According to the previous report, thiolated DNA can be specifically conjugated to

PDA layer.²³⁰ In order to better control the reaction ratio between 50 nm Au@PDA NPs and DNA, reaction was firstly performed in the solution with stirring. Results indicated that when the ratio between 50 nm Au@PDA to capture DNA reached 1:10000, 50 nm Au@PDA nanocrystals were fully conjugated with capture DNA, which was the saturated state. Thus, in the conjugation between 50 nm Au@PDA NPs on rGO paper hybrid electrode, a certain concentration of 50 nm Au@PDA NPs was applied to self-assembled into the film, which was fully loaded onto rGO paper. Then 20000 fold higher concentration of capture DNA was added into the reaction with stirring in PB buffer for 2 h. The capture DNA conjugated hybrid electrode was washed twice with DI water to remove the extra capture DNA.

6.3.2 Preparation of Semiconducting Metal Sulfides QDs Nanocrystals

QDs of semiconducting metal sulfides nanocrystals (ZnS, CdS, PbS and CuS) were prepared based on a previously reported (slightly modified) procedure.²³¹⁻²³³ Briefly, 4 mL OLA and 2 mmol metal precursors (ZnCl₂, CdCl₂, PbCl₂, and CuCl₂ 2H₂O) were loaded into a three-necked flask and the oxygen was removed by purging argon. The sulfur precursor solution was prepared by heating 3 mmol sulfur and 8 mL OLA at 80 °C and it was quickly injected into the metal-oleylamine precursor solution at 180 °C. Dark brown colour appeared immediately after injection, and the temperature was decreased to 150 °C. The reaction solution was stopped for another 30 min at 150 °C and quickly cooled to room temperature by fluxing cold hexane. The resultant solid products were purified by centrifugation (7000 rcf, 4 min) with ethanol and hexane to remove the extra ions and ligands. The final product was stored in 10 mL hexane. Hexane stored semiconducting metal sulfides QDs (10 mL) was transferred into water-soluble QDs by drop wise MPA

(100 μ L) and methanol (2 mL) into it and stirred for 2 h. QDs were collected by centrifugation (8000 rcf, 4 min) and dispersed into 10 mL DI H₂O. For the preparation of TEM sample for synthesized QDs, residual MPA was removed by adding extra KOH and centrifuged (5000 rcf, 1 min) to discard the suspending part.

6.3.3. Preparation of QDs-Report DNA Conjugates

The report DNA were attached onto the carboxyl/mercaptopropionic-capped water-soluble QDs most commonly based on cross-linking reaction between amine and carboxylic acid groups (catalyzed by carbodiimide) according to the previous report.^{234, 235} Briefly, in the first step, the MPA capped QDs was activated by EDC/NHS, followed by coupling of the 40 folds higher concentration of 3'-C6-amine-modified 35-mer report DNA to QDs surface carboxylic group via the formation of an amide-linker in borate buffer (50 mM, pH 7.4). The reaction was terminated after 4 h reaction with stirring and the samples were purified with 0.2 μ m Millipore centrifugation filter (3000 rcf, 5 min) three times. The purified sample was stored at 4 °C.

6.3.4 Analytical Procedure of Sandwich Assay

The sandwich assay involved a dual hybridization event. Each capture DNA with the concentration of 10000 fold higher than 50 nm Au@PDA NPs was added into the PB buffer (10 mM, pH 8.5), with 50 nm Au@PDA-rGO paper electrode immersed into it. The resulting hybrid-conjugated capture DNA-50 nm Au@PDA-rGO paper electrodes were then washed twice with DI water. DNA is prone to non-specifically adsorb on graphene via π - π stacking interaction, leading to poor

detection specificity of the CNT- or graphene-based nanocrystals. Therefore, non-specific blocking on the surface of 50 nm Au@PDA-rGO electrode was performed by 6-mercapto-1-hexanol. The blocked capture DNA-50 nm Au@PDA-rGO paper electrode was washed and employed in the first hybridization. The corresponding amount of each target (p16: T1, p21: T2, p53: T3 and p73: T4) was added into the hybridization buffer containing the capture DNA-50 nm Au@PDA decorated hybrid rGO paper electrode and stirred for another 30 min. The resultant target DNA-capture DNA-50 nm Au@PDA-rGO paper electrode was washed twice in DI water to remove the extra DNA. In the second hybridization, the corresponding QDs-report DNA was added into the hybridization buffer solution with stirring for another 30 min and washed twice with DI water. The fabricated sandwich hybrid electrode was immersed into 1 M HNO₃ for 10 min to dissolve the conjugated QDs, resulting the dissolved heavy metals. The acid solution containing the dissolved QDs was transferred into 1 mL 0.1 M NaAc (pH 6.0) solution. Square-wave anodic stripping voltammetry (SWASV) measurements of the dissolved QDs were carried out at an in-situ 50 nm Au@PDA-rGO paper electrode. After 60 s accumulation at -1.2 V, subsequent stripping from -1.2 V to 0.8 V was carried out with 10 sec quiet period (without stirring), using an amplitude of 20 mV and a frequency of 25 Hz. Background correction of the resulting voltammograms was performed using the moving average mode according to the CHI software.

6.4 Characterizations of Au@PDA-rGO Paper Electrode

6.4.1 Morphology Characterizations of Semiconducting Metal Sulfide QDs

The synthesized metal sulfide nanocrystals (ZnS, CdS, PbS and CuS) were firstly characterized by TEM, with corresponding TEM image shown in Figure 6.2A ~D, respectively. Obviously, TEM images revealed that the synthesized four types of metal sulfide nanocrystals have similar and round shape, and each kind of nanocrystal has the uniform particle size of about 11 nm. This uniform and well-dispersed metal sulfide nanocrystals allows and guarantees its further modification with report DNA.

Figure 6.2 TEM images of (A) ZnS; (B) CdS; (C) PbS and (D) CuS nanocrystals.

6.4.2 Physical Characterizations of Report DNA Conjugated Semiconducting Metal Sulfide QDs

MPA capped QDs were conjugated with report DNA by the specific reaction between carboxyl group and amino group with the activation of EDC and NHS. In order to distinguish four DNA targets, the well corresponding four kinds of report DNA of R16, R21, R53 and R73 are employed to conjugate with four different semiconducting metal sulfide QDs of ZnS, CdS, PbS and CuS, correspondingly. The optical properties of metal sulfide QDs nanocrystals before and after conjugated report DNA were characterized by UV-vis absorption (Figure 6.3).

Results showed that after reaction with report DNA, a specific absorption peaks appear at the wavelength around 260 nm, which belong to the absorption of report DNA. And the absorption band of ZnS (from 326 nm to 328 nm), PbS (from 500 nm to 501 nm) and CuS (from 506 nm to 508 nm) (Figure 6.3A, C and D) and the absorption peak of CdS (from 460 nm to 462 nm) (Figure 6.3B) displayed 1~2 nm red shift in comparison with the control groups (without reaction of report DNA). This is postulated to be an indirect proof that report DNA was successfully conjugated to metal sulfide nanocrystals, with referring to previous report.^{236, 237}

Figure 6.3 UV-vis absorption spectra of (A) ZnS; (B) CdS; (C) PbS and (D) CuS nanocrystals before and after conjugation with corresponding report DNA.

To further explore the interactions between metal sulfide nanocrystals of QDs and report DNA, zeta potential was employed to measure the change of surface charge. As shown in **Table 6.1**, the average zeta potential of QDs-report DNA decreased significantly, which is attributed to the conjugated of more negative charged DNA.^{238, 239} This suggested the effective covalently binding between QDs and report DNA and the surface charge was changed.

Table 6.1 Comparison of zeta potential of metal sulfide nanocrystals of QDs before and after conjugation with report DNA.

Sample Mean Zeta Potential mV	ZnS	ZnS- R16	CdS	CdS- R21	PbS	PbS- R53	CuS	CuS- R73
	-43.61	-53.21	-38.5	-59.38	-48.25	-64.1	-35.37	-55.37

6.4.3 Physical Characterizations of Au@PDA-rGO Paper Electrode

UV-vis spectra (Figure 6.4A) show the localized surface plasmon resonance (LSPR) red-shifted from 522 nm (20 nm Au NPs) to 533.5 nm (50 nm AuNPs) and finally to 556 nm (50 nm Au@PDA nanocrystals), indicating the successful synthesis of 50 nm Au NPs and the growth of 50 nm Au@PDA nanocrystals. It is known that bare 50 nm Au NPs are not stable in NaCl solution. Thus, the stability detection of 50 nm Au@PDA nanocrystals was carried out. UV-vis spectra (Figure 6.4B) indicate that 50 nm Au@PDA nanocrystals are very stable in NaCl solution, facilitating the conjugation of capture DNA to 50 nm Au@PDA nanocrystals. UV-vis spectra (Figure 6.4C) further indicate that DNA conjugated 50 nm Au@PDA nanocrystals continued experiencing red-shift from bare 50 nm Au@PDA nanocrystals to captured DNA conjugated 50 nm Au@PDA nanocrystals, following the hybridization of target DNA and report DNA. Electrophoresis result (Figure 6.4C inset) further verified the well-conjugated 50 nm Au@PDA nanocrystals. The

mobility speed (from a to g) is becoming lower because of the increased size of 50 nm Au@PDA nanocrystals after conjugation with different ratio of capture DNA (a-1:0; b-1:2000; c-1:5000; d-1:8000; e-1:10000; f-1:15000 and g-1:20000). TEM observation (Figure 6.4D) reveals that 50 nm Au@PDA nanocrystals has two-layer structure, which corresponds to a dark 50 nm Au core and transparent layer PDA coating of 10 nm in thickness surrounding the 50 nm Au core surface. The cross-section of the graphene paper based hybrid electrode displayed distinguished layer-by-layer structure of self-assembled graphene nanosheets (Figure 6.4E). FESEM observation (Figure 6.4F) further reveals the surface morphology of 50 nm Au@PDA nanocrystals with high loading density and uniform structure on rGO paper.

Figure 6.4 (A) UV-vis spectra of 20 nm Au, 50 nm Au and 50 nm Au@PDA nanocrystals. (B) Stability monitoring of 50 nm Au@PDA nanocrystals at various concentration of NaCl solutions. (C) UV-vis spectra of bare 50 nm Au@PDA nanocrystals, captured conjugated, target DNA hybridized and reported DNA conjugated 50 nm Au@PDA nanocrystals. Inset is the electrophoresis result of various ratio of DNA modifications of 50 nm Au@PDA nanocrystals. (D) TEM images of 50 nm Au@PDA nanocrystals. (E) Cross-section image of graphene paper based hybrid electrode. (F) FESEM image of the interfacial assembly of 50 nm Au@PDA nanocrystals on rGO paper.

6.4.4 Optimization of Detection Conditions of Au@PDA-rGO Paper Electrode

The adsorptive stripping analysis includes two steps: a non-electrochemical accumulation step at open circuit and an anodic stripping voltammetric step. To optimize the best condition for electrochemical signals, report DNA conjugated QDs were dissolved in 100 μL 1 M HNO₃ for 10 min and re-suspended in 1 mL supporting electrolyte (NaAc). In the present study we demonstrate that the electrodiverse population of inorganic QDs nanocrystal tags yields distinct four-potential voltammetric signatures for four different DNA cancer markers. For this purpose, ZnS, CdS, PbS and CuS have been linked to report DNA of p16, p21, p53 and p73,

respectively (Table 6.2). For the accumulation step, the 50 nm Au@PDA nanocrystals modified rGO paper electrode was immersed into 1 mL of supporting electrolyte (NaAc) containing the dissolved heavy metal target analyte (Zn^{2+} , Cd^{2+} , Pb^{2+} , and Cu^{2+}), to allow the pre-concentration of the ions at open circuit potential with stirring. The SWASV parameters were -1.2 V deposition potential, 25 Hz frequency and 20 mV potential amplitude. After optimization, it revealed that when the mixture DNA targets of T1, T2, T3 and T4 (each with the concentration of 800 nM) (Figure 6.5) were employed as the example. Their signal can be amplified by the specific peak of dissolved distinguished heavy metal ions in the supporting electrolyte. Result indicated that the best condition of supporting electrolyte of NaAc is at the concentration of 0.10 M and at the pH of 6.

Figure 6.5 Typical stripping voltammograms for dissolved QDs in (A) different concentrations of NaAc and (B) different pH values for 0.10 M NaAc.

Table 6.2 Oligonucleotide sequences for multidetection of four types of tumor-suppressor genes (p16, p21, p53 and p73) by using the graphene based flexible biosensors.

oligonucleotides	sequences
Capture DNA (p16)	5'-SH -C6-TTTTTTTTTT -CTG CGG GGG GCA CCA G-3'
Target DNA T1 (p16 gene segment)	5'-3' GCG GGC ATG GTT ACT G CCT C TGG TGC CCC CCG CAG
Target DNA T1' (single-base mismatch p16 gene segment)	5'-3' GCG GGC ATG GTT AC TGCCTCT GG TTC CCC CCG CAG
noncomplementary DAN T1''	C6-CCG GCACC CA AGG AGC TGG AAG GCT GGG
Report DNA (p16)	5'- CAGT AAC CAT GCC CGC-TTTTTTTTTT-C6-NH-3'
Capture DNA (p21)	5'-SH-C6-TTTTTTTTTT-CTC CAA GAG GAA GCC C-3'
Target DNA T2 (p21 gene segment)	5'-3' GCT TCC TGT GGG CG GA TTA GG GC TTC CTC TTG GAG
Target DNA T2' (single-base mismatch p21 gene segment)	5'-3' GCT TCC TGT GGG CG GATT AGG GC TTC GTC TTG GAG
noncomplementary DAN T2''	C6-CCG GCACC CA AGG AGC TGG AAG GCT GGG
Report DNA (p21)	5'-TCCG CCC ACA GGA AGC-TTTTTTTTTT-C6-NH-3'
Capture DNA (p53)	5'-SH-C6-TTTTTTTTTT-AGG CTC CTC GCT GCCT-3'
Target DNA T3 (p53 gene segment)	5'-3' CAT GGG CCT CTA CG GC ACC AG GC AGC GAG GAG CCT
Target DNA T3' (single-base mismatch p53 gene segment)	5'-3' CAT GGG CCT CTA CG GCACCAG GC AGC GAT GAG CCT
noncomplementary DAN T3''	C6-CCG GCACC CA AGG AGC TGG AAG GCT GGG
Report DNA (p53)	5'-G CCG-TAG-AGG-CCC-ATG-TTTTTTTTTT-C6-NH-3'
Capture DNA (p73)	5'-SH-C6- TTTTTTTTTT -CCC TCC AGC TCT GGCC-3'
Target DNA T4 (p73 gene segment)	5'-3' ACC TTC GCT GGG CT AA CTG GG CC AGA GCT GGA GGG

Target DNA T4' (single-base mismatch p73 gene segment)	5'-3' ACC TTC GCT GGG CT AACTGGG CC AGA GCA GCA GGG
noncomplementary DAN T4''	C6-CCG GCACC CA AGG AGC TGG AAG GCT GGG
Report DNA (p73)	5'-TT AG-CCC-AGC-GAA-GGT-TTTTTTTTTT-C6-NH-3'

6.4.5 Hybridization of DNA Targets and Cross Interferences Investigation

For the multiplexed detection of four DNA targets, the adding sequence of DNA targets may have some interference between each other. Thus, different adding order of four DNA targets was performed. Results indicated that no matter which DNA target was added first, the conjugation of four DNA targets (each with the concentration of 500 nM) can be obtained (Figure 6.6). The intensity of the corresponding metal peaks provides the desired quantitative information on the target DNA. For instance, Figure 6.6A revealed that when T1 DNA target was added first into the reaction, the signal of dissolved ZnS nanocrystals was slightly weakened after addition of T4 DNA, although addition of T2 and T3 didn't affect the signal of T1. Therefore, to obtain the least cross-interference between each dissolved metal signal in the multiplex DNA detection, T2 will be added firstly to get the four-resolved peaks.

Figure 6.6 Cross Interference detection of four different targets. From A to D are the SWASV graphs of first adding with T1, T2, T3 and T4, correspondingly.

6.5 DNA Targets Detection by Au@PDA-rGO Paper Electrode

6.5.1 Single DNA Target Detection

To investigate the selectivity and sensitivity of each DNA target, 50 nm Au@PDA-rGO paper electrode was employed to capture each DNA target, one-base mismatch DNA target, non-complementary DNA target and the blank solution with only supporting electrolyte (zero target). SWASV response of T1, T2, T3 and T4 DNA target (Figure 6.7A, B, C and D) revealed that after hybridized with each DNA target, the distinct voltammetric stripping patterns of Zn^{2+} , Cd^{2+} , Pb^{2+} and

Cu^{2+} at distinguishable potentials with controllable current intensities offer excellent encoding capability for the prepared electrochemical QDs. Additionally, SWASV curve for T1-a, T2-a, T3-a and T4-a are the corresponding one-base mismatch DNA targets response. Although the concentrations of one-base mismatch DNA target are 500 fold higher than that of single DNA target, the current density is still much lower. For a further step, measurements of non-complementary DNA targets (T1-c, T2-c, T3-c and T4-c) and blank solution (T1-d, T2-d, T3-d and T4-d) with 500 fold higher concentrations than that of target DNA was performed. SWASV results indicated that non-complementary DNA targets displayed nearly the same current density as that of blank solution. The quantitative aspects are documented by a calibration experiment over a concentration range of 0.001-1000000 nM target DNA. Insets calibration curve in Figure 6.7A, B, C and D indicated that the voltammetric stripping response of the dissolved QDs tags was proportional to $\log[\text{DNA}]$ in the range from 3.16 nM to 1 μM of T1 target DNA, 300 pM to 1 μM of T2 target DNA, 50 pM to 100 nM of T3 target DNA and 100 pM to 100 nM of T4 target DNA, with a detection limit of 1 nM, 100 pM, 17 pM and 33 pM, correspondingly. From the detection of single DNA target, it is clear that the fabricated flexible 50 nm Au@PDA-rGO paper electrode displayed high sensitivity and excellent selectivity toward each DNA target. These results indicated that the fabricated flexible 50 nm Au@PDA-rGO paper electrode could effectively distinguish target DNA from non-complementary and one-base mismatch DNA. Such discrimination between target and non-target DNA suggested the inherent high selectivity of the 50 nm Au@PDA-rGO paper electrode as well as the minimized non-specific adsorption by the surface blocking and washing.

Figure 6.7 (A) SWASV response of T1 DNA target. T1-a, b, c and d are SWASV curve for target T1, one-base mismatch target T1, non-complementary target T1 and zero target T1, correspondingly. Inset is the calibration curve of target T1 DNA. (B) SWASV response of T2 DNA target. T2-a, b, c and d are SWASV curve for target T2, one-base mismatch target T2, non-complementary target T2 and zero target T2, correspondingly. Inset is the calibration curve of target T2 DNA. (C) SWASV response of T3 DNA target. T3-a, b, c and d are SWASV curve for target T3, one-base mismatch target T3, non-complementary target T3 and zero target T3, correspondingly. Inset is the calibration curve of target T3 DNA. (D) SWASV response of T4 DNA target. T4-a, b, c and d are SWASV curve for target T4, one-base mismatch target T4, non-complementary target T4 and zero target T4, correspondingly. Inset is the calibration curve of T4 DNA target.

6.5.2 Multiplexed DNA Targets Detection

Simultaneous detection of DNA targets is important for molecular diagnostics. For example, early phase cancers have been proven to be associated with multiple tumor-suppressor genes.²⁴⁰ Large surface area of 50 nm Au@PDA nanocrystals have provide more active sites to conjugate multiple capture DNA strands, thus affording the possibility to design multiplex detection. Indeed, four kinds of DNA capture probes were assembled on the surface of 50 nm Au@PDA nanocrystals. Afterwards, four target DNA was hybridized with capture DNA with the sequence of T2>T1>T3>T4 in our experiment. In the hybridization process of QDs tagged report DNA to target DNA, forming a sandwich structure with capture DNA and report DNA flank on the sides of target DNA. Electrochemical signals (Figure 6.8A) were measured by dissolving QDs conjugated with DNA targets. Upon the addition of four specific DNA targets, the corresponding electrochemical signals of Zn^{2+} , Cd^{2+} , Pb^{2+} and Cu^{2+} at distinguishable potentials (-0.94 V, -0.55 V, -0.38 V and +0.12 V) is significantly enhanced. Notably, in the mixture detection of four target DNA, each of them displayed calibration curve versus logarithmic DNA concentrations (Figure 6.8B). The linear range for T1, T2, T3, and T4 target DNA are from 5 nM to 1 μM , 1 nM to 1 μM , 300 pM to 100 nM and 10 nM to 10 μM , with a detection limit of 2 nM, 300 pM, 100 pM and 3 nM, correspondingly. For the blank solution, there is no linear change. Selectivity detection of hybrid 50 nm Au@PDA-rGO paper electrode was performed by electrochemical measurements of 500 fold higher concentrations of mixture targets with four kinds of one-base mismatch target DNA, non-complementary target DNA and blank solution. Figure 6.8C demonstrates that even in the multiplex DNA detection, the flexible electrode

still displayed the excellent selectivity. The reproducibility of the developed DNA biosensors were detected by employing 5 parallel electrodes to measure the sample concentration of mixture DNA targets. The peak current density retained 98% of its initial response (Figure 6.8D). And five repeated measurements using the same electrode yielded a relative standard deviation (RSD) of 4.5%.

Figure 6.8 (A) SWASV spectra obtained in the addition of four specific target DNA with different concentrations. (B) Logarithmic calibration curve of current density versus multiplex target DNA. (C) Selectivity detection of hybrid electrode toward the multiplex target DNA. (D) Reproducibility testing of the hybrid Au@PDA-rGO paper electrode. Inset is the corresponding curve of variations for each repeatability test.

6.6 Summary

To summarize, we have presented a novel flexible hybrid DNA biosensor by using core-shell 50 nm Au@PDA nanocrystals and free-standing rGO paper substrate. Significantly, 50 nm Au@PDA nanocrystals displayed excellent stability in high salt solution and high conjugation ability to capture DNA. Our design further demonstrate that multiplex target DNA can be detected simultaneously with high sensitivity and low detection limit. Furthermore, inorganic nanocrystals of QDs were employed as the signal amplification code, which greatly improve the detection sensitivity. Therefore, we expect that the flexible core-shell Au@PDA nanocrystals decorated rGO paper electrode will become a promising and practical tool to be widely applied in detection of other biomolecules.

CHAPTER 7 A DUAL-MODALITY METHOD TO MONITOR INTRACELLULAR DEGRADATION OF QUANTUM DOTS

7.1 Introduction

QDs, a preferred class of fluorescent nanomaterials, have stimulated significant research in sensing and biological imaging applications.²⁴¹⁻²⁴⁵ The rationale behind this is their superior optical advantages over conventional organic fluorescent dyes, including exceptional photo-stability, higher quantum yields, broader excitation spectrum, brighter and narrower emission bands.^{246, 247} Moreover, QDs can be easily functionalized either by polymers or small molecules to render their specific binding onto living cell membrane and reinforce their endocytosis into cells,²⁴⁸⁻²⁵¹ resulting in their effective intracellular accumulation.²⁵²⁻²⁵⁶

In spite of the exceptional performance of QDs in cellular imaging and disease diagnosis, the long term fate and potential toxicity of internalized QDs are getting more and more attention these years.^{241, 257-259} Researches showed that QDs could be oxidatively damaged by extracellular or intracellular ROS such as H_2O_2 , superoxide (O_2^-), HClO and hydroxyl radical ($\cdot\text{OH}$), leading to fluorescence quenching and chemical component releasing.^{243, 259, 260} In living organisms, HClO is synthesized from H_2O_2 and chloride ions in a chemical reaction catalyzed by the enzyme myeloperoxidase (MPO), which is localized mainly in leukocytes,

including neutrophils, macrophages, and monocytes.^{261, 262} Moreover, internalized QDs in living system was employed as a cell imaging fluorescent and a HClO-selective probe. Previous research also indicated that low concentrations (10-20 μ M) of HClO damages proteins on cell membranes and induce cell lysis.²⁶³ Thus, simultaneous monitoring of fluorescence intensity change and the detection of released components from internalized QDs are imperative. This is because the released components of QDs will further imply the long-term fate and potential toxicity. Although several examples have been focused on the studies of intracellular QDs with single-modality fluorescence method,^{259, 264} it is an ideal approach to develop a dual-modality method with strong capability in (I) real-time monitoring fluorescence quenching event of intracellular QDs with fluorescence method and (II) well detecting its degraded by-products (e.g. heavy metals and HClO) with electrochemical method.^{265, 266}

Herein, small molecule APBA conjugated QDs (QDs-PBA) were exploited for real-time monitoring of living cell imaging owing to the specific and fast binding on cell membrane. Cell membrane anchored QDs-PBA were further internalized into living cells through endocytosis (Figure 7.1 step a). With the addition of stimuli of ROS, the fluorescence intensity of internalized QDs decreased and some elements such as heavy metal ions were released (Figure 7.1 step b). Evidence indicated that degradation process of QDs could be monitored effectively through fluorescence intensity changing and electrochemical signal varying as most ROS and degradation products of QDs are electroactive. In a typical degradation process, an increasing electrochemical response corresponding to degradation products and a decreasing

fluorescence signal of internalized QDs-PBA are expected. To sensitively monitor electrochemical signal change of HClO and degraded QDs-PBA, a new class of rGO paper based hybrid flexible electrode was employed. This hybrid electrode was composed of rGO paper substrate and heterogonous nanocrystals with mussel-inspired PDA layer and bimetallic structure of core-shell Au@Pt NPs, which integrated high electrochemical catalytic activity of bimetallic structure and the excellent mechanical property of free-standing rGO paper.²²⁷ Thus, tracking fluorescence intensity change of QDs-PBA in living cells and the quantitative detection of Cd^{2+} as well as HClO lay solid foundations for a novel sensitive dual-modality mode to realize real-time monitoring of internalized QDs-PBA oxidative degradation (Figure 7.1 step c).

Figure 7.1 Schematic illustration of QDs-PBA conjugation and two detection models after stimulation inside cells. (a) Internalization process of specifically anchored QDs-PBA on cell membrane. (b) The reaction after adding stimuli into living cells. (c) Dual-modality measurements of quenched QDs-PBA.

7.2 Preparations and Measurements of QDs-PBA

7.2.1 Preparations of QDs-PBA

Core-shell QDs (CdSe/ZnS) were synthesized with referring to a previously described method.²⁵⁰ QDs-PBA conjugates were prepared according to the developed method in our report.^{267, 268} The conjugated QDs-PBA with over 90% yields were obtained through ultra-filtration to remove the un-reacted APBA. In this study, the QDs modified by APBA are easy and fast to bind on cell membrane because of their specific conjugation with over-expressed glycans. The spectroscopic properties (absorption profile, emission wavelength, and quantum yield) of QDs-PBA characterized by UV-vis spectroscopy and fluorometer (FluoroMax-3) have little change after reaction (Figure 7.2 A). The concentration of QDs-PBA was determined on the basis of a molar extinction coefficient of $2.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at the wavelength of 610 nm. The electrophoresis mobility of QDs-PBA carried out in 1% agarose gels with $1 \times$ (TBE; pH 7.4) as the running buffer at 100 V for 30 min (Thermo Scientific EX 300XL) was lower than that of QDs-copolymer of maleic anhydride and octadecene (QDs-PMO) (inset in Figure 7.2 A), resulting from decreased surface charges after conjugation with PBA ligands. The suspended solution concentration was confirmed to be 0.14 mM as indicated in Figure 7.2 B marked by the red cycle. Thus, 0.12 mM APBA ($0.26 \text{ mM} - 0.14 \text{ mM} = 0.12 \text{ mM}$) has taken part in the reaction process. In the next step, the fluorescence intensity and UV absorption of collected QDs-PBA were conducted. The concentration of QDs-PBA was 134 nM. Therefore, the average number of PBA ligands on each QDs was about $0.12 \text{ mM} / 134 \text{ nM} = 900$. The average number of PBA ligands on each QD is about 900 (Figure 7.2 B).

Figure 7.2 (A) Optical absorption and fluorescence emission spectra of QDs-PMO and QDs-PBA. The inset in Figure 7.2 A showed the electrophoresis mobility of QDs-PBA (lane b) is lower than that of QDs-PMO (lane a). (B) The calibration curve of average number of PBA ligand on each QDs.

7.2.2 Fluorescence Quenching Measurements of QDs-PBA

H_2O_2 and HClO were selected as the etching agent for QDs-PBA owing to their neutral charge and good stability under physiological condition.^{33,34} They were able to diffuse across some coating layers, causing chemical oxidation of sulphur and selenium atoms on the QDs surface.^{246, 260, 269} Time influence of the quenching process was performed by adding H_2O_2 (10 μM) or HClO (2 μM) into 10 nM diluted QDs-PBA (Figure 7.3A and B). The results indicate that the fluorescence intensity of QDs-PBA dropped to 20 % after incubating with 2 μM HClO in the

first 3 min and there was only slight change after 60 min fluorescence intensity of the mixture was measured after being gently mixed and incubated for different time. Similarly, influence of H_2O_2 (0-10 mM) and HClO (0-16 μM) concentrations on QDs fluorescence intensity was investigated at a given time (Figure 7.3C and D). Quantitative measurements in Figure 7.3C and D revealed that QDs-PBA fluorescence intensity decreased to 15 % when the concentration of HClO was 10 μM , whereas fluorescence intensity was still over 50 % when the final concentration of H_2O_2 was at 100 μM (10 times higher than that of HClO). The insets of Figure 7.3 are corresponding fluorescence intensity curves of QDs-PBA. The above series of comparison experiments were conducted to suggest that QDs-PBA are much more sensitive to HClO than to H_2O_2 . The result also revealed that the prepared QDs-PBA exhibited substantially quenched fluorescence with the increasing concentration of both HClO and H_2O_2 extracellular.

Figure 7.3 Fluorescence spectral responses of QDs-PBA to the given concentration of 2 μM HClO (A) and 10 μM H₂O₂ (B) with different incubation time; and Fluorescence spectral responses of QDs-PBA at a given time of 3 min toward different concentrations of HClO (C) and H₂O₂ (D). Insets are the corresponding fluorescence intensity curves.

7.3 Preparations of Au@Pt@PDA-rGO Paper Electrode

7.3.1 Synthesis and Characterizations of Au@Pt@PDA Nanocrystals

A hybrid flexible free-standing electrode built up with dual core-shell Au@Pt@PDA nanocrystals anchored on rGO paper was prepared as the following three steps.^{154, 224, 227} Firstly, core-shell Au@Pt bimetallic NPs were synthesized

through one step growth method on 14 nm AuNPs (the solution absorbance for Au NPs was 1.6).¹⁵⁴ The synthesized Au@Pt nanocrystals were stabilized in 1 mL 0.5% SDS solution (Au@Pt-SDS). Dual core-shell Au@Pt@PDA nanocrystals were synthesized by dispersing Au@Pt-SDS nanocrystals stock solution (1 mM with respect to the Au precursor concentration) into 10 mL Tris buffer (pH 8.5), followed by dropping DA (0.6 mg/mL) into the mixture solution. The thickness of PDA was optimized by adjusting the amount of DA (Figure 7.4). After 6 h reaction, the synthesized dual core-shell Au@Pt@PDA nanocrystals was purified by centrifugation and stored at room temperature. Secondly, dual core-shell Au@Pt@PDA nanocrystals was self-assembled at the interface of chloroform and water by gently shaking the mixture. A closely packed monolayer of self-assembled Au@Pt@PDA nanocrystals film was formed and observed because of the decreased free energy the biphasic system.¹⁵⁶ Thirdly, self-assembled dual core-shell Au@Pt@PDA nanocrystals film was transferred on the surface of rGO paper fixed on a solid substrate through dip-coating method, and then dried at room temperature.⁹⁶ The obtained hybrid rGO paper decorated with dual core-shell Au@Pt@PDA nanocrystals film was cut into a fixed size for further application as the flexible free-standing working electrode.

Figure 7.4 TEM images of Au@Pt@PDA nanocrystals with different scale thickness of PDA (A) 4 nm, (B) 10 nm, (C) 20 nm and (D) 25 nm.

7.3.2 Optimization of PDA Layer and rGO Paper Thickness on the Influence of Electrochemical Catalytic Performance

The performance of hybrid working electrode composed with the synthesized Au@Pt@PDA nanocrystals of different thickness (4 nm, 10 nm, 20 nm and 25 nm) and rGO paper (0 μm , 4 μm , 8 μm , 10 μm and 20 μm) was carried out by detecting

a certain concentration of Cd^{2+} . Results (Figure 7.5 A and B) indicated that hybrid working electrode with 10 nm PDA layer and 10 μm rGO paper displayed best electro-catalytic performance toward a certain concentration of Cd^{2+} . Thus, 10 nm PDA layer surrounded Au@Pt nanocrystals and 10 μm rGO paper substrate were chosen as the best composition of hybrid working electrode.

7.3.3 Characterizations of Au@Pt@PDA-rGO Paper Electrode

10 nm PDA layer surrounded Au@Pt nanocrystals were chosen as the nanocrystals to be applied in the detection experiment. The high density and uniform distribution of Au@Pt@PDA nanocrystals film on rGO paper could be observed in FESEM images (Figure 7.5A). The cross section images of rGO paper with dual core-shell Au@Pt@PDA nanocrystals film (Figure 7.5B) were also clearly observed. It also displayed closely packed layer-by-layer structure of rGO paper sheets at a large scale.

Figure 7.5 (A) SEM image of self-assembled dual core-shell Au@Pt@PDA nanocrystals on rGO paper. (B) Cross section images of rGO paper with decoration of dual ore-shell Au@Pt@PDA nanocrystals.

7.4 Measurements of Au@Pt@PDA-rGO Paper Electrode

To evaluate electrocatalytic activity of dual core-shell Au@Pt@PDA-rGO paper electrode, different concentrations of Cd^{2+} were applied for electrochemical measurements. Figure 7.6A showed the DPV responses of the hybrid dual core-shell Au@Pt@PDA-rGO paper electrode toward Cd^{2+} from 10 nM to 0.2 mM in 0.1 M NaAc buffer (pH 5.0) with an accumulated time of 60 s at -1.2 V for each measurement. To remove memory effect, the hybrid electrode was cleaned by

setting the potential at 0.8 V for 120 s with the stripping mode after each detection. All of the DPV curves have peaks appearing at the potential around -0.40 V, which was attributed to the typical profiles of the oxidation of Cd^{2+} . As seen from the calibration plot (Figure 7.6A inset) of Cd^{2+} , the peak current density increased linearly versus the concentration of Cd^{2+} from 0.05 to 8 μM with a relatively low detection limit of 30 nM and a high sensitivity of $4.85 \text{ mA } \mu\text{M}^{-1} \text{ cm}^{-2}$. This high sensitivity of dual core-shell $\text{Au}@Pt@PDA\text{-}rGO$ paper was owing to the excellent absorption capacities of porous hydrogel-like PDA layer to heavy metals through the electrostatic, bi-dentate chelating, hydrogen bonding interactions, heterogeneous catalytic activity of core-shell $\text{Au}@Pt$ bimetal as well as the good conductivity of rGO paper substrate.^{229, 270}

Furthermore, H_2O_2 also could be generated from living cells after stimulated. Thus the selectivity of $\text{Au}@Pt@PDA\text{-}rGO$ paper electrode was examined by using the mixed solution of H_2O_2 and HClO as probe solution. As indicated in Figure 7.6B, there is no peak current response when only in blank NaAc buffer (black line) or H_2O_2 (red line) solution, whereas obvious peak current appeared for HClO at the potential position around 0.16 V (blue and pink line). This revealed that hybrid $\text{Au}@Pt@PDA\text{-}rGO$ paper electrode has high selectivity toward HClO . Figure 7.6B inset further displayed the peak current corresponding calibration curve of HClO , which is linear from 1.2 μM to 40 μM with the sensitivity of $0.535 \text{ mA } \mu\text{M}^{-1} \text{ cm}^{-2}$ and a detection limit of 0.2 μM . This hybrid flexible biosensor and its good performance guarantee its further applications in biological detection system.

Figure 7.6 (A) DPV responses at the hybrid electrode after 60s' accumulation with increasing concentration of Cd^{2+} ; inset was the corresponding calibration plot of Cd^{2+} . (B) DPV response for the selectivity of HClO and H_2O_2 . Inset was the calibration plot of HClO . For all electrochemical detections, the working electrode was hybrid Au@Pt@PDA-rGO paper electrode and the analyst was NaAc buffer solution at pH 5.0.

7.5 Properties and Measurements of Intracellular QDs-PBA

7.5.1 Fluorescence Monitoring of Intracellular QDs-PBA Degradation.

As for the superior fluorescence properties, the fate of internalized QDs-PBA could be monitored by the changing of fluorescence imaging (qualitatively) and

fluorescence intensity (quantitatively). Here, three kinds of cells (mouse macrophage RAW 264.7, MCF-7 and 3T3-L1 cells) were selected as the model. All cells were cultured with a cell confluence of 80%. After that, cell culture medium was replaced by HBSS, and QDs-PBA (10 nM) was added into each kind of cell for 30 min incubation. Interestingly, QDs-PBA experienced a fast internalization emerging into all three kinds of cells even within 5 min. Then stimulus, PMA (10 μ M), was added to the wells, to generate “oxidative bursts”.^{260, 271} As indicated in Figure 7.7A, panel a1, a2, a3 and a4 are fluorescence quenching images of macrophage RAW 264.7 cells after co-cultured with 10 nM QDs-PBA for 30 min and stimulated by 10 μ M PMA for 0 min, 1 min, 5 min and 10 min, correspondingly. QDs-PBA inside macrophage RAW 264.7 cells exhibited a quick quenching within 1 min after adding 10 μ M PMA and completed quenching after 10 min. In contrast, Figure 7.7B and C showed that QDs-PBA fluorescence inside both MCF-7 and 3T3-L1 cells have no obvious change, although under the same stimulation. The corresponding fluorescence intensity curves of single cell in Figure 7.7A, B and C was shown in Figure 7.7D. It was noticeable that only macrophage RAW 264.7 cell exhibited obvious fluorescence intensity decrease by 85% after adding PMA within 5 min.

Figure 7.7 (A) (B) and (C) were QDs-PBA fluorescence imaging change inside macrophage RAW264.7, MCF-7 and 3T3-L1 cells after the addition of PMA, correspondingly. Images were recorded before (a1, b1 and c1) and after addition of stimulus at 1 min (a2, b2 and c2), 5 min (a3, b3 and c3) and 10 min (a4, b4 and c4). (D) Corresponding fluorescence intensity curves of a defined single cell in (A) (B) and (C).

In order to further elucidate HClO is the main factor for QDs-PBA fluorescence quenching, 5 mM NaN_3 , an inhibitor of HClO production, was added to each cell well after co-incubation with QDs. The co-treatment with NaN_3 greatly reduced the amount of QDs fluorescence quenching for macrophage RAW 264.7 cells even after 30 min (Figure 7.8A and D). Similar results were also found for MCF-7 cells

(Figure 7.8B and E) and 3T3-L1 cells (Figure 7.8C and F). Thus, the quenched fluorescence of QDs-PBA could be ascribed to HClO releasing from macrophage RAW 264.7 cells.

Figure 7.8 (A), (B) and (C) were fluorescence imaging change of QDs-PBA recorded after 30 min inside macrophage RAW264.7, MCF-7 and 3T3-L1 cells after the addition of NaN₃, correspondingly. (D), (E) and (F) were the corresponding fluorescence intensity curves of a defined single cell in (A) (B) and (C).

7.5.2 Electrochemical Measurements of Quenched QDs-PBA after Intracellular Oxidative Burst.

To further examine the released products after QDs-PBA was oxidatively damaged, we have investigated the electrochemical signal changes in the presence and absence of intracellular QDs. Figure 7.9A was the detection result of macrophage RAW 264.7 cells. In the presence of PMA and the absence of internalized QDs-PBA, only one voltammetric peak (black line) is detected and corresponds to 15 μ M HClO. After PMA is injected into the wells with pre-

incubated QDs-PBA, the electrochemical currents were recorded at different time interval. The electrochemical signal intensity of Cd^{2+} and HClO increased along with time, while the fluorescence intensity decreased. For example, the concentration of Cd^{2+} was calculated to be $0.05\ \mu\text{M}$, $0.12\ \mu\text{M}$ and $0.6\ \mu\text{M}$ at 1 min, 5 min and 10 min, correspondingly; the concentration of HClO was calculated to be $1.5\ \mu\text{M}$, $3\ \mu\text{M}$ and $12\ \mu\text{M}$ at 1 min, 5 min and 10 min, correspondingly. In the presence of NaN_3 and QDs-PBA, PMA is added into the wells of RAW 264.7 cells, there is no obvious peak current (green line).

Figure 7.9 Electrochemical detection of released HClO and elements of degraded QDs-PBA after quenching in RAW 264.7 cells under various conditions.

Parallel experiments were performed in MCF-7 cells and 3T3-L1 cells. The results showed that no matter whether there is co-cultured QDs-PBA or not, no obvious peak current density for Cd^{2+} and HClO could be detected (Figure 7.10). The above observations strongly suggest that it is HClO generated from living macrophage RAW 264.7 cells that leads to fluorescence quenching of QDs-PBA, which further degrades QDs-PBA, generating Cd^{2+} in turn. It was also interesting to note that with the higher degradation velocity of QDs-PBA, the more Cd^{2+} was

detected. Thus, it was a concurrent process for the enhancement of electrochemical signal intensity and the decrease for the stimulated fluorescence quenching of internalized QDs-PBA.

Figure 7.10 Electrochemical detection of released HClO and elements of degraded QDs-PBA after quenching in MCF-7 and 3T3-L1 cells under various conditions.

7.5.3 Cell Viability Evaluation

For the past several years, QDs cytotoxicity has been extensively investigated in various models.²⁷² However, many factors has influenced the toxicity of QDs such as their difference in concentrations, core/shell composition, size, shape and also surface chemistry.²⁷³ In this work, the toxicity of QDs-PBA was evaluated using a standard cell CCK-8.²⁶⁸ Our results (Figure 7.11A-a1) indicate that three kinds of cells after incubation with QDs-PBA for 4 h with different concentrations 5 nM, 10 nM and 20 nM. All the cells maintained over 90% viability when the concentration of QDs-PBA is 5 nM and 10 nM, respectively. However, MCF-7 and 3T3-L1 only have less than 70% cell viability with the presence of 20 nM QDs-PBA, which indicated that when the concentration of QDs-PBA exceeds 20 nM, it could be toxic

to living cells. In order to further confirm the cytotoxicity of fluorescence quenched QDs, another cytotoxicity experiment was conducted for cells group treated by PMA (10 μM) and QDs-PBA (10 nM). Compared with other two kinds of cells (Figure 7.11A-a2), only the macrophage RAW 264.7 cells incubated with QDs-PBA and addition of PMA displayed less than 70% cell viability. For other groups of experiments, three kinds of cells still keep almost 90% cell viability. During the etching process in the presence of ROS, QDs (CdSe/ZnS) will be stimulated to show quenched fluorescence.²⁶⁰ Such a reaction would cause lattice defects of QDs-PBA and it would be degraded into soluble Cd, Zn, S and Se species. Therefore, to clearly understand the toxicity after QDs-PBA was etched in the presence of HClO, one more experiment was carried by adding different concentrations of Cd^{2+} or HClO into living RAW 264.7 cells. Results indicated that at a certain concentration of Cd^{2+} (more than 0.05 μM) and HClO (more than 0.1 μM) would be toxic to living cells (Figure 7.11B). These results suggested that the cytotoxicity of QDs-PBA could come from the releasing of Cd^{2+} and HClO intracellular after "oxidative burst".

Figure 7.11 (A) Cell viability analysis of QDs-PBA (5 nM, 10 nM and 20 nM) co-incubated with living cells for 4 h (a1) and (a2) cytotoxicity of QDs-PBA at 10 nM without or with "oxidative burst". (B) Cell viability analysis with different concentrations of Cd^{2+} or HClO into living RAW 264.7 cells.

7.6 Summary

We developed a dual-modality mode with fluorescent and electrochemical methods to realize monitoring of intracellular oxidative degradation of QDs-PBA. Such a design provides a facile approach to track the internalizing process of well modified QDs and its fluorescence quenching after stimulated oxide burst. For a further step, the amount of soluble Cd^{2+} from degraded QDs-PBA and HClO from

oxidative burst in living cells has been electrochemically quantified by hybrid flexible Au@Pt@PDA-rGO paper electrode. The specific features of dual core-shell Au@Pt@PDA nanocrystals greatly favour their good selectivity and high sensitivity measurements. Both the model of monitoring fluorescence intensity of QDs and electrochemical detection of etched QDs came to reveal the same fact. This intentionally designed dual-modality mode has overcome the disadvantages (such as incompatible with living systems or incapable to detect heavy metals etched from QDs at the same time) in the already well-known single-modality methods. In view of these advantages, this study is very meaningful to exploit the intracellular fate of QDs and pave the way to determine whether QDs could be used for injection into human patients.

CHAPTER 8 CONCLUSION AND RECOMMENDATIONS

8.1 Conclusion

One of the primary objectives behind this accomplishments of this research is the design and synthesis of nanocrystals with desirable structures and surface properties to obtain a high reactive area and more reactive sites. In the first system (Chapter 4), a kind of dendritic monometallic PtNPs were synthesized, showing uniform size and morphology. In the second system (Chapter 5), in order to obtain a better catalytic performance, a bimetallic core-shell Au@Pt NPs were fabricated. Here 14 nm AuNPs are in the core, while 3 nm small PtNPs are surrounded as the shell. Except the combination of pure noble metal NPs, the surface coating on noble metal NPs have also been obtained. Thus, in the third system (Chapter 6), another kind of core-shell Au@PDA with the mussel-inspired PDA surface was synthesized. The core were 50 nm Au NPs and the shell was transparent smooth PDA layer. A dual core-shell Au@Pt@PDA nanocrystals were synthesized with well-tailored method in the last system (Chapter 7) and displayed uniform morphology.

The second interesting finding in this study was that all the synthesized nanocrystals can be self-assembled at the interface between water and oil. For example, dendritic PtNPs and core-shell Au@Pt NPs can be self-accumulated at the interface between water and hexane to form a uniform macroscale thin film. Similarly, Au@PDA and Au@Pt@PDA nanocrystals can be self-assembled at the interface between water and chloroform.

Another objective was to fabricate the hybrid electrode with nanocrystals and flexible substrates. Thus, the self-assembled nanostructures and flexible graphene paper were combined by dip-coating method to obtain a high density loading of nanocrystals with uniform structures and morphologies.

For a further step, the developed flexible hybrid electrode of Pt-rGO paper, core-shell Au@Pt-rGO paper, Au@PDA-rGO paper and dual core-shell Au@Pt@PDA-rGO paper were employed to detect various targets of neurotransmitters (DA), RNS (NO), cancer biomarkers (DNA) and heavy metals (Zn^{2+} , Pb^{2+} , Cd^{2+} , and Cu^{2+}), correspondingly. Experimental results indicated that the developed hybrid Pt-rGO paper electrode has demonstrated a high sensitivity of $2 \mu\text{A cm}^{-2} \mu\text{M}^{-1}$, a low detection limit of 5 nM and a wide linear range of 87 nM to 100 μM , which collectively enable ultrasensitive detection of DA released from PC 12 cells and it is promising to use the electrode as the electro-active cell growth substrate. Therefore, the close-packed Au@Pt-rGO paper electrode was employed as a freestanding cell culture substrate for real-time monitoring cell secretion of NO. With the special design of mussel-inspired PDA coating on the surface of Au nanocrystals, a simultaneously detection of target DNA was achieved. Moreover, the 50 nm Au@PDA-rGO paper electrode was able to distinguish the complementary DNA from one-base mismatched DNA and noncomplementary DNA. Moreover, a unique dual modality model to investigate the activity of internalized semiconductor QDs in living cells. The results reveal that internalized QDs can be selectively etched by intracellular stimulated ROS and QDs fluorescence quenched process on living cell could be monitored by fluorescence intensity change. It was also found that some of the chemical elements of degraded

QDs and ROS can be electrochemically detected with dual core-shell Au@Pt@PDA-rGO paper electrode.

This research work not only provides the basis for design and synthesis nanocrystals for the development of graphene paper based flexible hybrid electrode with high electrochemical biosensing performance, but also expands in-depth fundamental knowledge of the effects of nanomaterials properties on biomolecules behaviours, as well as pave the way for future practical applications for point-of-care devices.

8.2 Recommendations

The current studies about free-standing flexible electrochemical biosensors have made certain breakthroughs to overcome some problems about template based flexible biosensors. For example, the fabrication process is simplified and less time consuming with good repeatability and stability. The catalysts loading density is increased by applying self-assembly of nanostructures and dip-coating method. This is especially applicable for the 2D flexible substrates (such as graphene paper and bucky paper) based electrodes. The adhesion between 2D substrates and catalysts is well stabilized by employing the typical molecule on either substrates or catalysts.

However, there is still a far way to go forward for the successful application of free-standing flexible electrochemical biosensors. For example, a well recognized and standard evaluation system is necessary to compare the current generated from different free-standing biosensors. From the point of catalysts, the thickness of semiconductor oxides based 3D network free standing electrode also needs to be optimized for good performances. Moreover, although this project has fabricated several kinds of nanomaterials with unique properties, including modifications with polymers or small molecules on it, the catalytic mechanism of the nanomaterials modified graphene paper based flexible electrode needs to be fully understood. Another problem for the future work is the fabrication of the small sized biosensors, which is beneficial for the detection of neurotransmitters.

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