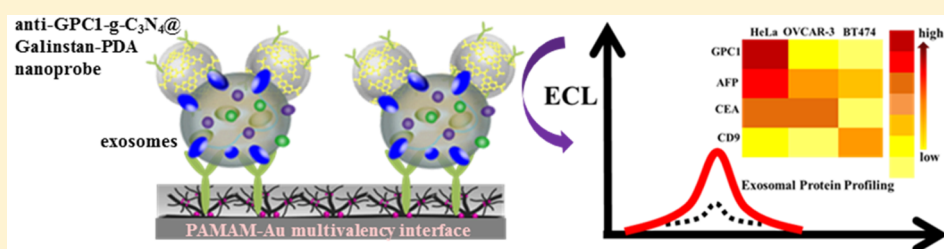


Multivalency Interface and g-C₃N₄ Coated Liquid Metal Nanoprobe Signal Amplification for Sensitive Electrogenerated Chemiluminescence Detection of Exosomes and Their Surface Proteins

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Supporting Information



ABSTRACT: Exosomes derived vesicles and their surface anchored proteins by cells are extremely vital in intercellular communication, immuno-stimulation, and so on, which are promising in potential tumor biomarkers for disease diagnosis. However, the highly sensitive detection of exosomes and their surface proteins is still challenging. Herein, we combined the g-C₃N₄ conjugated polydopamine coated Galinstan liquid metal shell–core nanohybrids (g-C₃N₄@Galinstan-PDA) nanoprobe and multivalent PAMAM-AuNPs electrode interface to realize a highly sensitive detection of exosomes and their surface proteins by electrogenerated chemiluminescence (ECL) biosensor. The antibody-modified PAMAM-Au nanoparticles (NPs) electrode interface provided a multivalent recognition platform for highly effective capture of exosomes. Meanwhile, the Galinstan NPs were applied as the nanoprobe. The antibody modified g-C₃N₄@Galinstan-PDA can recognize the exosomes specifically and exhibit stable and strong ECL signals due to the excellent features of the Galinstan NPs in facilitating electron transfer and suppressing the g-C₃N₄ passivation during electrochemical reduction procedures. In this way, high sensitivity for HeLa cell derived exosomes analysis was obtained with the limit of detection (LOD) of 31 particles μL^{-1} . Moreover, we operated the exosomes analysis in the real samples including serum, urine, and blood, and identified the multiple biomarkers (GPC1, CD9, CEA, and AFP) on the exosome surface derived from different kinds of cell lines (HeLa cell, OVCAR-3 cell, and BT474 cell). These consequences suggest that the proposed ECL biosensor has the potential to be a powerful tool for exosomes study and clinical diagnostic as well as wearable devices.

Exosomes are nanosized extracellular vesicles with 30–100 nm in diameter secreted from many cell types. Exosomes carry abundant molecular information including proteins, nucleic acids, and lipids of their parent cells, and they are enriched in genetic materials and membrane proteins, which are involved in various physiological and pathological processes such as the formation of metastatic niches, immuno-stimulation, and cell-to-cell communication.^{1–6} Impressively, there are plenty of protein markers on the exosome surface predicting the originating cells, which are promising non-invasive biomarkers applied in the disease early diagnosis.^{7–9} Hence, it is valuable to develop a high sensitivity, convenient, and economical detection strategy for exosomes to research the fundamental biochemical process and the clinic diagnostics. Currently, enzyme-linked immunosorbent assays (ELISA), flow cytometry, Western blot, and electrochemical analysis have been reported for detecting exosomes.^{10–15}

Despite the advances in detection strategies, it is still a challenge in the detection of exosomes sensitively and surface proteins on the exosomes.

Electrogenerated chemiluminescence (ECL) is the technique that combines the advantages of the electrochemistry and luminescence. Meanwhile, a light emission process was contained in a redox reaction of reactants. As compared to other electrochemical methods, the ECL assay has advantages in the wide range of concentration response and high sensitivity. What is more, it is a potential- and spatial-controlled technique. For the above-mentioned merits, ECL has the potential to be applied in the clinical samples. On the basis of ECL emitter such as graphite-like carbon nitride

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nanosheets ($\text{g-C}_3\text{N}_4\text{NSs}$), heavy-metal-containing quantum dots (QDs), tris(2,2'-bipyridyl)ruthenium(II) ($\text{Ru}(\text{bpy})_3^{2+}$) derivatives, and luminol, the applications have been developed in diverse fields including the molecular-level analysis and chemical analysis with high sensitivity and simplified setup.^{16–20} Recently, the ECL sensor was also applied for exosomes detection. For instance, Shen et al. reported a turn-off ECL aptasensor for exosomes determination on the basis of the quench of ECL signal from CD63 aptamer modified Eu^{3+} -doped CdS quantum dots.²¹ An ECL aptasensor for the detection of exosomes was also reported in our group on the basis of the CD63 aptamer electrode interface and the Ti_3C_2 nanosheets probe to improve the ECL signal of luminol in solution.⁴ These works present the feasibility for exosomes detection by ECL technique. However, the high background ECL signal from the blank samples may cause a false positive result and limited sensitivity especially for the real samples in the presence of plentiful interferences.

Multivalent recognition is a common binding action in biological systems, and it has excellent performance in enhancing binding to biological targets. The multivalent system has been proven to overcome the low affinity with 1–9 orders of magnitude relative to monovalent binding on the basis of special scaffolds. For instance, the valid way of organizing a multivalent array was provided by the dendritic scaffold with their inherent repetitive branched structures.^{22,23} Poly(amidoamine) (PAMAM) dendrimers, a new polymer with well-defined globular shape, branched treelike structure, and amounts of functional groups on its periphery, have been applied in facilitating multivalent effects.^{24,25} The capture efficiency of cells was enhanced via dendrimer-mediated circulating tumor cells capture platform.²⁶ In recent years, the multivalency strategies have been also executed on AuNPs, graphene for carbohydrate, antibody, and aptamers.^{27–29} The binding affinity can be significantly improved by “cluster effect” of the molecules on nanoscaffolds. In addition, nanoparticles have fantastic electrochemical, photonic, and magnetic characteristics, which allow them to be superior transducers for sensing design.

Gallium-based eutectic alloys such as Galinstan (68.5% gallium, 21.5% indium, 10% tin) and EGaIn (75% gallium, 25% indium) are liquid metals, which own many excellent characters such as desirable flexibility, low toxicity, high electrical conductivity, and large surface tension.^{30–32} On the basis of their excellent mechanical and fluid mechanics features, liquid metal nanoparticles (NPs) were applied in smart drug delivery systems and microfluidic devices with a promoted drug releasing performance as a light-fueled transformer for effective cargo delivery, which was the a chemical-mechanical process.³³ Moreover, the physical and chemical performances of liquid metal can also be improved by the size regulation, surface functionalization, and so on, which endow its great prospects in the establishment of biosensor and wearable devices.

In our work, we described a strategy for exosomes determination based on the multivalency interface of the GPC1 antibody (anti-GPC1) modified PAMAM dendrimer coated AuNPs (PAMAM-AuNPs) electrode, which can improve the exosomes capture efficiency because of the expression of GPC1 on exosome surfaces. Meanwhile, anti-GPC1 modified $\text{g-C}_3\text{N}_4$ nanosheet conjugated polydopamine (PDA) coated Galinstan NPs (Galinstan-PDA) nanohybrids (anti-GPC1- $\text{g-C}_3\text{N}_4$ @Galinstan-PDA) were used as the ECL

signal probes. It was found that the Galinstan NPs can significantly enhance and stabilize the ECL signal of $\text{g-C}_3\text{N}_4$, in which the Galinstan NPs can promote the electrode interfacial electron transfer and weaken the $\text{g-C}_3\text{N}_4$ passivation during the negative potential scanning, enabling the effective ECL signal amplification. The designed ECL biosensor showed excellent performances for exosomes determination with the LOD of 31 particles μL^{-1} for the exosomes derived from HeLa cell, and the linear range was over 3 orders of magnitude. Moreover, the exosomes analysis in the real samples including serum, urine, and blood, and identification of the multiple biomarkers (GPC1, CD9, CEA, AFP) on the exosome surface derived from different kinds of cell lines (HeLa cell, OVCAR-3 cell, and BT474 cell), were also conducted. These consequences illustrate that this proposed ECL biosensor is a promising platform for exosomes study and wearable analysis devices design.

EXPERIMENTAL SECTION

Materials and Reagents. The glassy carbon electrodes (GCE, diameter of 3 mm) were obtained from CH Instruments (Shanghai, China). 1-(3-(Dimethylamino) propyl)-3-ethylcarbodiimidehydrochloride (EDC) and *N*-hydroxysuccinimide sodium salt (NHS) were purchased from Alfa Aesar (Shanghai, China). Anti-GPC1 rabbit polyclonal antibody was obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The liquid metal (Ga 68%, In 22%, and Sn 10%, Galinstan) was purchased from Yantai Aibang Electronic Material Co., Ltd. (Yantai, China). Poly(amidoamine) (PAMAM) dendrimer was purchased from Chenyuan Organosilicon New Material Co., Ltd. (Weihai, China). Dopamine was obtained from Sigma-Aldrich (Shanghai, China). $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was obtained from Sinopharm Co., Ltd. (Shanghai, China). Dicyandiamide, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ were obtained from Beijing Chemical Co., Ltd. (Beijing, China). Phosphate buffer saline (PBS) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Apparatus and Characterization. Ultrasonic pulverization was operated on Scientz-IID (SCIENTZ, China). A JSM-7401 (JEOL, Japan) field emission scanning electron microscopy (SEM) system, H-7650B (HITACHI, Japan) transmission electron microscope (TEM), JEM 2100F (JEOL, Japan) high-resolution transmission electron microscope (HRTEM), and energy-dispersive spectroscopy (EDS) were used to observe the morphology of the samples. Nanoparticle Tracking Analysis (NTA) was operated on the NanoSightNS500 (Malvern Instruments, England). A UV–vis spectrophotometer (UV-3900, HITACHI, Japan) and Fourier transform infrared spectrophotometer (GX, PerkinElmer, U.S.) were used for characterizing the samples. Powder X-ray diffraction (XRD) analysis was obtained on a D8-Advance diffractometer ($\text{Cu K}\alpha$ radiation, $\lambda = 0.15406 \text{ nm}$, Bruker, Germany). X-ray photoelectron spectroscopy (XPS) analysis was operated on a PHI Quantera SXM (ULVAC-PHI, Japan). Nanometer particle size and zeta potential were recorded on SOE-070 (Brookhaven, U.S.). The cyclic voltammetry (CV, CHI660b instrument, CH Instrument Co., U.S.), electrochemical impedance spectroscopy (EIS, SP-150 potentiostat/galvanostat, Bio-Logic, France), and ECL measurements (MPI-B, Xi'an Remex Analytical Instrument Ltd. Co., China) were conducted as previously reported.⁴

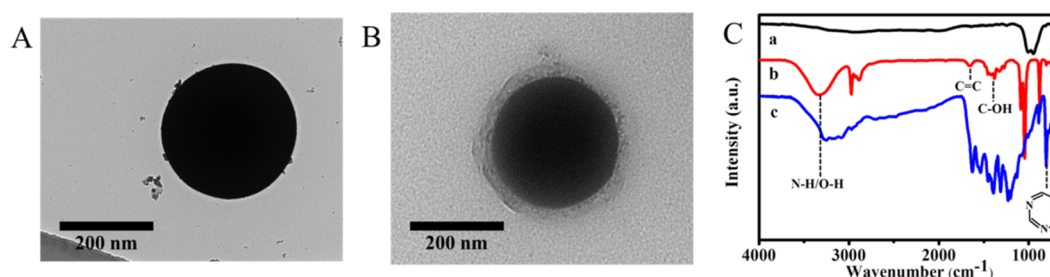


Figure 1. TEM images of (A) Galinstan NP and (B) Galinstan-PDA NP. (C) FT-IR spectra of Galinstan NPs (curve a), Galinstan-PDA NPs (curve b), and g-C₃N₄@Galinstan-PDANPs (curve c).

Cell Culture, Exosomes Extraction, and Counting.

Three different exosomes from HeLa cells line, human ovarian carcinoma cells line (OVCA-3 cells), and breast cancer cells line (BT474 cells), respectively, were prepared as previously reported.³⁴ The exosomes were counted by NTA. The size of exosomes (ca. 70 nm) and the concentration of exosomes (2.0×10^8 particles mL⁻¹) were shown in Figure S1. The exosomes were stockpiled at -20 °C for the following experiments.

Synthesis of Galinstan NPs, g-C₃N₄ NSs, and Anti-GPC1-g-C₃N₄@Galinstan-PDA Nanoprobe. Galinstan (200 mg) was placed in a 50 mL sample tube filled with 10 mL of ethanol.³⁵ Ultrasonication was operated via a conical tip sonicator for 30 min with a 6 mm ϕ probe (40% of 400 W). The temperature of the sample during ultrasonication was controlled at 0 °C in ice bath. These carboxylated g-C₃N₄ NSs were prepared as previously reported.³⁶ From the TEM image of carboxylated g-C₃N₄ NSs (Figure S2A), the size was ca. 150 nm. In Figure S2B, the characteristic diffraction peak of g-C₃N₄ XRD patterns centering at 27.6° ($d = 0.324$ nm) pertained to the obvious graphitic interlayer stacking peak (002). The peak at 13.0° can be ascribed to peak (100) of tri-s-triazine units ($d = 0.669$ nm). After the carboxylation, the peaks were at 27.8° and 12.9°, respectively, which were close to that of the g-C₃N₄, indicating the lattice structure retained after the carboxyl process.

The above Galinstan NPs were centrifuged (2000 rpm, 5 min), and the obtained supernatant was resolved in 100 mL of water. One milliliter of the above solution was added into 10 mL of 10 mM Tris-HCl solution with 2 mg of dopamine, the obtained solution was then stirred for 6 h, followed by centrifugation (2000 rpm, 5 min), and the gathered precipitation was resolved in 2 mL of water. Activated carboxylated g-C₃N₄ NSs (1 mg mL⁻¹) stock solution was obtained after EDC (400 mM) and NHS (100 mM) were added. The resulting solution then was centrifuged (8000 rpm, 20 min), and the obtained supernatant was resolved in water. Subsequently, the anti-GPC1-g-C₃N₄@Galinstan-PDA was obtained by adding 200 μ L of the g-C₃N₄ contained solution and 100 μ L of anti-GPC1 into 200 μ L of Galinstan-PDA solution with gentle shaking for 60 min, and then it was centrifuged (8000 rpm, 20 min) and the final precipitate was collected.

Synthesis of Gold Nanoparticles (AuNPs), PAMAM-AuNPs Conjugates. The AuNPs were prepared as previously reported.³⁷ 1 mL of PAMAM dendrimer solution (0.25 mM) and 1 mL of AuNPs solution (0.030%) were mixed for stirring to prepare PAMAM-conjugated AuNPs (PAMAM-AuNPs). Figure S3A shows the UV-vis absorption spectra; the peak around at 520 nm indicated that approximately 20 nm AuNPs was prepared successfully as per the previous reports.³⁷ After

the addition of PAMAM, there was a peak at 278 nm and a peak at 520 nm, which were attributable to the formation of nanocomposite. The FT-IR spectrum (curve b, Figure S3B) of PAMAM presented peaks at 1551 and 1650 cm⁻¹ corresponding to the N-H bending, C=O stretching vibration of amide I and C-N stretching vibrations of amide II. After AuNPs integrating, the peaks (curve c) shifted to 1555 and 1646 cm⁻¹, respectively, which confirmed AuNPs had been conjugated onto the PAMAM dendrimers. In addition, TEM images (Figure S4) indicated successful conjugation.

Biosensor Fabrication. The GCE was polished successfully using Al₂O₃ power. The GCE was washed ultrasonically with ethanol and DI water, respectively, and it was dried with N₂. PAMAM-AuNPs/GCE was formed by dropping the mixture of PAMAM-AuNPs (6 μ L) on pretreated GCE. Anti-GPC1 was activated by EDC (400 mM) and NHS (100 mM). Anti-GPC1/PAMAM-AuNPs/GCE was obtained by immersing PAMAM-AuNPs/GCE electrode in the 40 μ L activated solution with the anti-GPC1 at 37 °C for 120 min. The above electrode was put in 200 μ L of HeLa exosomes suspension to capture HeLa exosomes and was rinsed with DI water. Afterward, the above electrode was incubated in the solution with the anti-GPC1-g-C₃N₄@Galinstan-PDA nanoprobe for 120 min at 37 °C. Finally, anti-GPC1-g-C₃N₄@Galinstan-PDA/exosomes/anti-GPC1/PAMAM-AuNPs/GCE was used for subsequent experiments.

RESULTS AND DISCUSSION

Characterization of the Carboxylated g-C₃N₄ Nano-sheets Coated Galinstan Nanoparticles (g-C₃N₄@Galinstan-PDA NPs). Galinstan nanoprobe was produced by sonication, the homogeneous and stable gray suspension was obtained, and Figure S5 shows the SEM image. In Figure 1A, the TEM image exhibits the Galinstan NPs with an average diameter about 240 nm. The surface of Galinstan NPs was relatively smooth. After the polymerization of dopamine on the surfaces of Galinstan NPs, uniform PDA coating was observed on the surface of the Galinstan NPs, and the thickness was ca. 50 nm (Figure 1B). The increased dimension of the PDA coated Galinstan NPs (Galinstan-PDA) coating can also be confirmed by dynamic light scattering (Figure S6A). The coating of PDA on the Galinstan NPs was also confirmed by zeta potential. The charge of as-synthesized Galinstan NPs after the coating of PDA was slightly negative as compared to the positive charge of Galinstan NPs (Figure S6B) because PDA is negative at neutral condition.³⁸ These facts indicated that the PDA was successfully coated onto the Galinstan nanoparticles. FT-IR was applied to characterize the surface functional groups of the nanoparticles. Figure 1C shows the FT-IR spectra of Galinstan NPs (curve a) and Galinstan-PDA

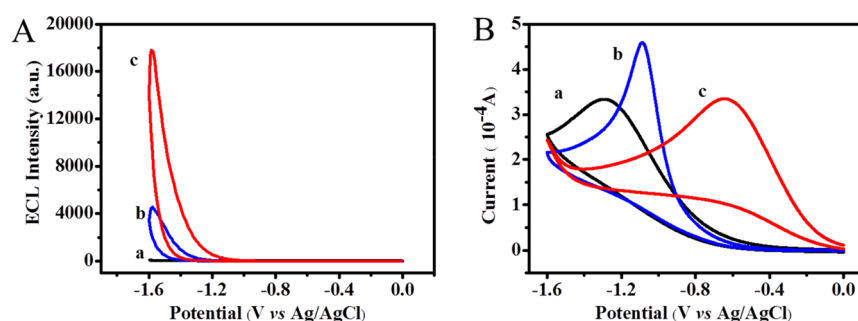
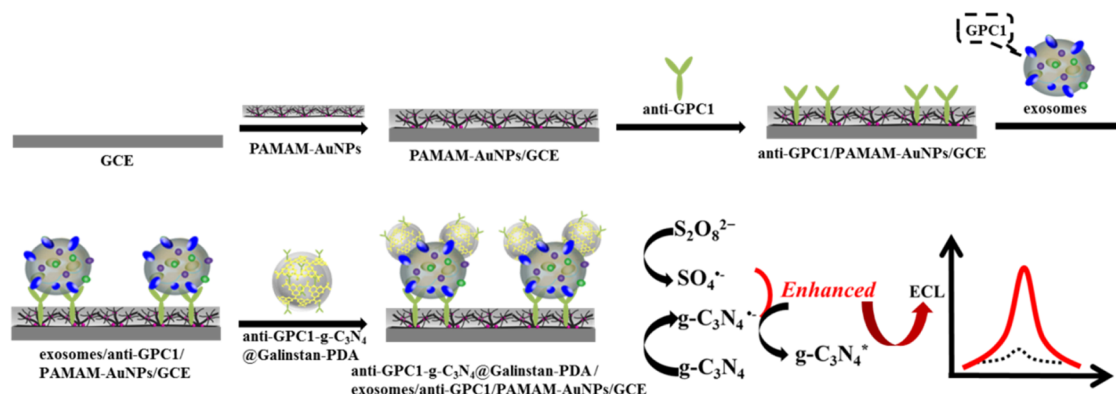


Figure 2. (A) ECL intensity–potential behavior and (B) cyclic voltammograms were obtained at bare GCE (curve a), g-C₃N₄ NSs modified GCE (curve b), and g-C₃N₄@Galinstan-PDANPs modified GCE (curve c). The experiments were operated in PBS (pH 7.4) containing 0.1 M KCl and 20 mM K₂S₂O₈ after oxygen removal reaction. The scan rates were all 0.1 V s⁻¹. The voltage of the photomultiplier tube (PMT) was set as 800 V.

Scheme 1. Schematic Illustration of the ECL Biosensor for Exosomes Based on Multivalent Recognition and Signal Amplification Strategy by Anti-GPC1-g-C₃N₄@Galinstan-PDA Nanoprobe



NPs (curve b). After the coating of PDA on the Galinstan NPs, the peaks at around 3337 and 2974 cm⁻¹ were revealed and were ascribed to N–H/O–H stretching vibrations. In addition, obvious peaks at around 1653, 1328 cm⁻¹ indicated the existence of C=C and C–OH, respectively.³⁹ We further conducted EDS mapping for the produced nanoparticles to show the distribution of gallium, indium, tin, carbon, and nitrogen, as shown in Figure S7. This fact confirmed that PDA was coated onto the Galinstan NPs. The PDA coated Galinstan NPs with abundant amino group not only provide the Galinstan NPs diverse functionality but also improve its biocompatibility and water solubility.⁴⁰ Afterward, the carboxylated g-C₃N₄ NSs conjugated Galinstan NPs-PDA (g-C₃N₄@Galinstan-PDA) was fabricated by carboxyammonia reaction between carboxyl groups on the carboxylated g-C₃N₄ NSs and amino groups on the surface of PDA. The peak at 806 cm⁻¹ of tri-*s*-triazine (curve c, Figure 1C) emerged after carboxyammonia reaction, which was further evidenced by the XPS spectra obtained for the Galinstan NPs shown in Figure S8. The peaks around at 285.6 eV were assigned to C–N of tri-*s*-triazine in the C 1s spectrum.⁴¹ The N 1s spectrum shows the peaks around at 399.1 and 400 eV assigned to imine and amine nitrogen of g-C₃N₄@Galinstan-PDA NPs, and the C 1s spectrum shows two peaks, centered at 287.4 and 288.8 eV, which corresponded to imine and amide groups. The facts confirmed that g-C₃N₄ nanosheets were conjugated onto the surface of Galinstan-PDA NPs.

ECL Behaviors of the g-C₃N₄@Galinstan-PDA Nanoprobe. GCEs were modified with g-C₃N₄ NSs and g-C₃N₄@Galinstan-PDA, respectively, to operate electrochemical and ECL experiments to illustrate the influence of Galinstan NPs

on the ECL and electrochemical performance of g-C₃N₄ NSs. As compared to bare electrode (curve a) in Figure 2A, the ECL emission was given by the g-C₃N₄ NSs modified GCE in the presence of S₂O₈²⁻ (curve b). An intensive annihilation occurred where the g-C₃N₄ NSs reduce it to g-C₃N₄^{•-}, and SO₄^{•-} was electrogenerated from S₂O₈²⁻.⁴² A bright ECL emission was given by the g-C₃N₄@Galinstan-PDA modified GCE (curve c), which was about 4 times that of the g-C₃N₄ NSs modified GCE. In comparison with bare GCE (curve a), the g-C₃N₄ NSs modified GCE had a lower cathodic peak at -1.08 V (curve b) in Figure 2B due to electrons injected into the g-C₃N₄ NSs layer. Furthermore, the peak potential at -0.65 V was given by g-C₃N₄@Galinstan-PDA modified GCE (curve c), due to an obvious electrocatalytic reduction of S₂O₈²⁻ by Galinstan NPs to offer more abundant SO₄^{•-} free radical.²⁰ Also, the strong oxidant SO₄^{•-} further reacted with g-C₃N₄^{•-} to generate the excited state g-C₃N₄^{*}. The light was emitted and detected, while g-C₃N₄^{*} fell from the excited state to the ground state.

In the presence of Galinstan NPs, a more stable and stronger ECL signal from g-C₃N₄ nanosheets can be obtained by g-C₃N₄@Galinstan-PDA modified GCE under the same potential conditions as compared to g-C₃N₄ modified GCE (Figure S9A). Obviously, the ECL emission of g-C₃N₄ NSs film gradually degraded at the potential range of 0 to -1.6 V, due to electrode passivation occurring (Figure S9D). The passivation can be availably restrained via cutting down the energy of electrons transferred to g-C₃N₄ NSs. Therefore, the potential range of -1.3 to 0 V (Figure S9E) or -1.1 to 0 V (Figure S9F) was operated to obtain the stable ECL signal, but the ECL intensities were apparently too weak. Cathodic

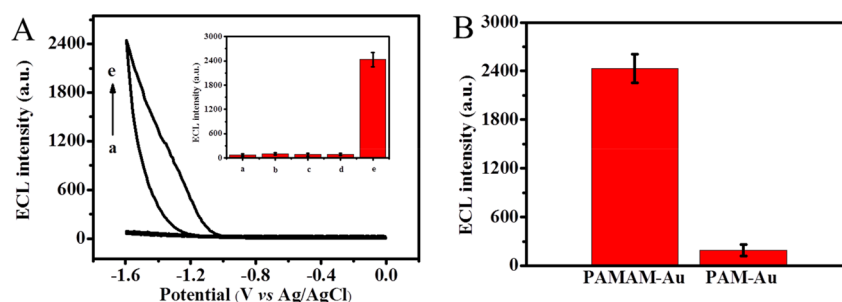


Figure 3. (A) ECL intensity–potential behaviors were obtained at bare GCE (curve a), PAMAM-AuNPs/GCE (curve b), anti-GPC1/PAMAM-AuNPs/GCE (curve c), exosomes/anti-GPC1/PAMAM-AuNPs/GCE (curve d), and anti-GPC1-g-C₃N₄@Galinstan-PDA/exosomes/anti-GPC1/PAMAM-AuNPs/GCE (curve e). (B) ECL intensities of anti-GPC1-g-C₃N₄@Galinstan-PDA/exosomes/anti-GPC1/PAMAM-AuNPs/GCE (pillar a) and anti-GPC1-g-C₃N₄@Galinstan-PDA/exosomes/anti-GPC1/PAM-AuNPs/GCE (pillar b). The experiments were operated in PBS (pH 7.4) containing 0.1 M KCl and 20 mM K₂S₂O₈. The scan rates were all 0.1 V s⁻¹. The voltage of the photomultiplier tube (PMT) was set as 800 V. The concentration of HeLa exosomes was 1 × 10³ particles μL⁻¹.

passivation occurred caused by overinjection of highly energetic electrons into the conduction bands of g-C₃N₄, where a low conductive layer was formed and hindered the electron move at the electrode.²⁰ An analogical cathodic passivation phenomenon has been reported where AuNPs played the role in stabilizing the ECL emission.²⁰ Besides its excellent fluid mechanical characteristics, the intrinsic metallic feature of Galinstan NPs can also endow it to seize and reserve electrons from the conduction band of g-C₃N₄ NSs, leading to the improved and stabilized ECL signal. The electrochemical impedance spectra were further investigated. The electron transfer resistance (*R*_{et}) transformed lower obviously after the g-C₃N₄@Galinstan-PDA NPs (curve d) modified at bare GCE (Figure S10) as the Galinstan NPs can facilitate electron transfer among g-C₃N₄. Moreover, the Galinstan NPs functionalized with PDA can suppress g-C₃N₄ NSs falling off the electrode at subsequent potential scans by the covalent binding generated from the carboxyl ammonia reaction between the carboxylated g-C₃N₄ and PDA on the surface of Galinstan NPs. Thus, the g-C₃N₄@Galinstan-PDA has been identified as an excellent ECL nanoprobe for biosensor fabrication.

Construction of ECL Biosensor for Exosomes Analysis. Scheme 1 presents the ECL biosensor for exosomes detection that was fabricated on the basis of the multivalent recognition interface of antibody conjugated PAMAM-AuNPs modified electrode and the multiple amplification of g-C₃N₄@Galinstan-PDA nanoprobe. Taking advantage of high bio-specific recognition of antibody–antigen, anti-GPC1 was considered for capturing exosomes to demonstrate the principle. GPC1 is a membrane-anchored protein that is overexpressed in several types of tumors including cervical, glioma, and pancreatic.^{43–45} Amounts of surface functional groups can be afforded by PAMAM dendrimer-conjugated AuNPs to accommodate multiple capture probes of anti-GPC1. Anti-GPC1 on the PAMAM-AuNPs can selectively capture exosomes based on efficient immunorecognition. After the adsorption of exosomes, the antibody modified g-C₃N₄@Galinstan-PDA nanoprobe can also bind to the exosomes, forming a sandwich-type system. Because of unique features of liquid metals such as outstanding electron transfer ability, the large surface area, and so on, the ECL signal of g-C₃N₄ in the presence of potassium persulfate can be amplified significantly, allowing the sensitive detection of exosomes. Meanwhile, the surface proteins on exosomes can be evaluated by tuning the specific antibodies in the ECL biosensing system.

Electrochemical Characterizations of the Biosensor.

The step-by-step assembly process of the modified electrode was characterized by CV and EIS using Fe(CN)₆^{4-/3-} as probe (Figure S11). The reversible redox peaks are observed for the bare GCE in curve a. The peak currents of PAMAM-AuNPs/GCE then increased (curve b), which was due to the outstanding electrical conductivity and the large surface area of dendrimer-conjugated AuNPs. After the anti-GPC1 was incubated on the electrode, the signal decreased slightly (curve c). The peak currents further decreased after the capture of HeLa exosomes (curve d) because the electronically inert feature of anti-GPC1 and exosomes blocked the electron transfer and mass transfer of Fe(CN)₆^{4-/3-} ions on the electrode interface. Finally, after the incubation with the anti-GPC1-g-C₃N₄@Galinstan-PDA solution, peak currents decreased slightly (curve e). Figure S11B displays the EIS curves. The *R*_{et} was represented by the diameter of the semicircle. The PAMAM-AuNPs modified GCE (curve b) revealed lower *R*_{et} than that of bare GCE (curve a) because of the good electronic transfer ability of AuNPs. Afterward, the diameter of the semicircles increased with the sequential assembly, showing the increase of *R*_{et}. These results were similar to the results in CVs, demonstrating the successful assembly of biosensor.

ECL Behaviors of the Biosensor. In this design, the ECL signals from anti-GPC1-g-C₃N₄@Galinstan-PDA nanoprobe were closely relevant to the exosomes captured on the electrode. The ECL behaviors of the biosensor were studied in the 0.1 M KCl and 20 mM K₂S₂O₈. The responses of the series of control experiments were shown in Figure 3A to testify the feasibility of this ECL sensor. Slight ECL emissions are observed at bare GCE, PAMAM-AuNPs/GCE, anti-GPC1/PAMAM-AuNPs/GCE, and exosomes/anti-GPC1/PAMAM-AuNPs/GCE. Nevertheless, the ECL intensity increased sharply after incubating exosomes/anti-GPC1/PAMAM-AuNPs/GCE in the solution containing the anti-GPC1-g-C₃N₄@Galinstan-PDA nanoprobe. The anti-GPC1-g-C₃N₄@Galinstan-PDA was adsorbed specifically on the electrode depending on the specific binding between anti-GPC1 and GPC1 on exosomes derived from HeLa cell due to the high biospecific affinity between antibody and proteins on the exosomes. On the basis of the super ECL behaviors of anti-GPC1-g-C₃N₄@Galinstan-PDA nanoprobe, the ECL signal increased significantly, which can be applied for exosomes detection with high sensitivity.

To confirm the effect of multivalent recognition based on the dendritic scaffold, PAM, a chain polymer, was chosen as a

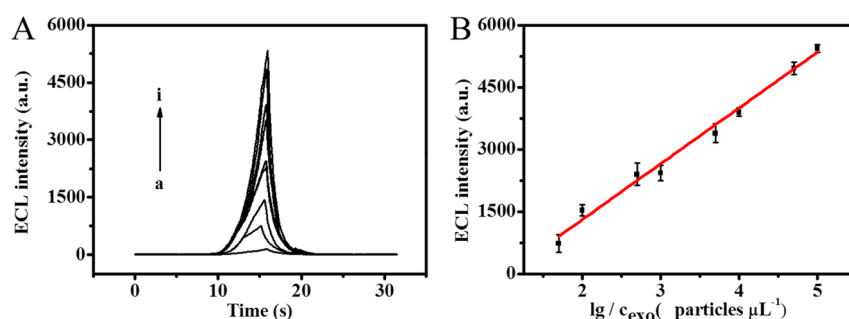


Figure 4. (A) ECL intensities of assay versus different concentrations exosomes derived from HeLa cell (from a to i was 0, 5.0×10^1 , 1.0×10^2 , 5.0×10^2 , 1.0×10^3 , 5.0×10^3 , 1.0×10^4 , 5.0×10^4 , and 1.0×10^5 particles μL^{-1} , respectively). (B) Plot of ECL intensity versus the logarithm value of the exosomes concentrations. The experiments were operated in PBS (pH 7.4) containing 0.1 M KCl and 20 mM $\text{K}_2\text{S}_2\text{O}_8$. The scan rates were all 0.1 V s^{-1} . The voltage of the photomultiplier tube (PMT) was set as 800 V.

control sample to fabricate biosensor in the same conditions as those of PAMAM. In the experiments, anti-GPC1/PAMAM-AuNPs/GCE and anti-GPC1/PAM-AuNPs/GCE were fabricated, and then they were incubated in exosomes precursors with the same concentration, followed by the adsorption of the anti-GPC1-g- C_3N_4 @Galinstan-PDA nanoprobes. The ECL signal intensity from the PAMAM-AuNPs modified electrode is 6 times higher than that of PAM-AuNPs modified electrode in Figure 3B. The fact can be ascribed to the multivalent recognition effect on the PAMAM interface and fewer recognition sites on PAM chain polymer electrode interface. These results demonstrate that the PAMAM-AuNPs multivalent electrode interface is beneficial to fabricate highly sensitive biosensor for exosome analysis.

Optimization of the Measurement Conditions. The incubation temperature and time are crucial for the activity of the exosomes. The incubation temperature and time for exosomes in the assay were optimized as shown in Figure S12A,B. The optimal exosomes incubation temperature was 37 °C. It can be observed that the maximum intensity was reached at 120 min with increasing exosomes incubation time. The ECL response of the biosensor was significantly influenced by the concentration of anti-GPC1 and $\text{K}_2\text{S}_2\text{O}_8$, which served as a coreactant in the ECL system of g- C_3N_4 NSs. The ECL intensity of the biosensor using 10 $\mu\text{g mL}^{-1}$ anti-GPC1 reached the maximum value (Figure S12C). The ECL signal increased when the concentration of $\text{K}_2\text{S}_2\text{O}_8$ was promoted to 20 mM (Figure S12D). Therefore, 10 $\mu\text{g mL}^{-1}$ of anti-GPC1 and 20 mM of $\text{K}_2\text{S}_2\text{O}_8$ as the optimum conditions for the biosensor were adopted in the biosensor.

Analytical Performance of the Biosensor. The ECL biosensor for exosomes was fabricated with the optimal condition to detect exosomes derived from HeLa cell in PBS (pH 7.4) containing 0.1 M KCl and 20 mM $\text{K}_2\text{S}_2\text{O}_8$. Figure 4A shows the ECL signal intensities from the systems with different concentrations of the exosomes. The ECL intensity was enhanced gradually with promoting concentrations of exosomes, and it was linear with the $\log(C_{\text{exo}})$ in the range of $50\text{--}10^5$ particles μL^{-1} . The linear equation is I (the ECL intensity) = $1345.171 \log[C_{\text{exo}} (\text{exo } \mu\text{L}^{-1})] - 1380.793$ with a correlation coefficient of 0.990 ($n = 3$) (Figure 4B). The LOD was calculated on the basis of $\log(\text{LOD}) = \frac{3\theta - b}{k}$ (θ = the background standard deviation, $b = 1380.793$, $k = 1345.171$). The LOD was calculated to be 31 particles μL^{-1} ($\text{S/N} = 3$), which was superior to previous reported literature.^{13,15,46} The promotion of the sensitivity benefits from the multivalent

recognition effect based on the PAMAM dendrimer-conjugated AuNPs and multiple ECL signal amplification of the anti-GPC1-g- C_3N_4 @Galinstan-PDA nanoprobes.

Moreover, the reproducibility and stability of the biosensor were studied. The relative standard deviation (RSD) for HeLa exosomes (10^4 and 10^5 particles μL^{-1}) were calculated, and the results were 2.9% ($n = 3$) and 1.8% ($n = 3$), respectively, representing excellent reproducibility. Meanwhile, Figure S13 exhibits the ECL responses of this biosensor with successive scanning and stable ECL signal, indicating the ECL biosensor had excellent cycling stability. Therefore, the as-designed biosensor could be efficient to detect exosomes with a low detection limit, a broad detection range, and excellent reproducibility.

Analysis of the Different Exosomes, Protein Markers on Exosome Surface, and Practical Samples. The proteins on the surface of exosomes play important roles in cell communications and regulate their biological function. In this strategy, the ECL signal was not only associated with the concentration of exosomes but also related to the level of surface protein expressed on the exosomes. Thus, this biosensor can be used for profiling the surface proteins such as GPC1, AFP, CEA, and CD9 across different exosomes derived from HeLa, OVCAR-3, and BT474 cell lines. These exosomes with the same concentration were operated for fabrication of biosensor. Depicted in Figure 5, the relative expression levels of the proteins of exosomes derived from HeLa, OVCAR-3, and BT474 cell lines were summarized as a

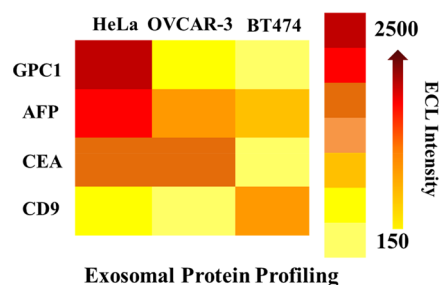


Figure 5. Expression levels of four kinds of cancer markers, including GPC1, AFP, CEA, and CD9, on exosomes derived from HeLa, OVCAR-3, and BT474 cells. The concentration of exosomes was 10^3 particles μL^{-1} . The experiments were operated in PBS (pH 7.4) containing 0.1 M KCl and 20 mM $\text{K}_2\text{S}_2\text{O}_8$. The scan rates were all 0.1 V s^{-1} . The voltage of the photomultiplier tube (PMT) was set as 800 V.

heat map. It was observed that exosomes from HeLa cell presented a higher GPC1 level than that derived from the other cell lines. What is more, the GPC1 level on exosomes derived from HeLa cell was obviously higher than that of AFP, CEA, and CD9, and was expressed less on the exosomes derived from OVCAR-3 and BT474 cell, which was similar to those reported.^{3,47,48} More AFP and CEA were expressed on exosomes derived from OVCAR-3 cell than those of GPC1 and CD9. The expression levels of CD9 and AFP from BT474 cell were higher than those of GPC1 and CEA.^{49–51} The facts demonstrate that the ECL biosensing strategy is capable to screen and profile the protein markers on exosomes derived from different cell lines, which is promising in the exosomes studies and clinic diagnostics.

To verify the performance of the as-designed biosensor in clinical samples, we operated recovery experiments in serum, urine, and blood sample. The recovery ranges from 91% to 109% were shown in Table 1. The consequences indicate that

Table 1. Exosomes Activity Analysis in Serum, Urine, and Blood Using the Biosensor^a

sample	added (lg particles μL^{-1})	found (lg particles μL^{-1})	recovery (%)
serum	2	2.18	109
serum	3	2.72	91
serum	4	4.07	102
urine	2	2.04	102
urine	3	2.77	92
urine	4	3.92	98
blood	2	1.83	92
blood	3	2.78	93
blood	4	3.97	99

^aThe experiments were operated in PBS (pH 7.4) containing 0.1 M KCl and 20 mM $\text{K}_2\text{S}_2\text{O}_8$. The scan rates were all 0.1 V s^{-1} . The voltage of the photomultiplier tube (PMT) was set as 800 V.

the biosensor owns an excellent capacity of resisting disturbance and selectivity in complex conditions and has potential in the clinical samples assay.

CONCLUSIONS

In summary, an innovative ECL biosensor for highly sensitive exosome determination was developed on the multivalent recognition of PAMAM conjugated AuNPs electrode interface using $\text{g-C}_3\text{N}_4$ NSs conjugated with Galinstan NPs as the nanoprobe. Capture of exosomes with high efficiency and the significant ECL signal responses can be fulfilled by the nanoprobe and PAMAM-AuNPs electrode interface. The anti-GPC1- $\text{g-C}_3\text{N}_4$ @Galinstan-PDA as the nanoprobe exhibits strong and stable ECL emission on account of the excellent electrochemical properties of liquid metal. By the integration of the signal amplification strategy as well as antibody specific recognition, the constructed biosensor was successfully used for exosomes detection sensitively with the LOD of 31 particles μL^{-1} . Furthermore, this ECL biosensor is feasible to evaluate different proteins on the surface of exosomes derived by various cell lines and detect exosomes in real samples, which presents great prospect in exosomes study, clinic diagnostics, as well as wearable devices fabrication.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.9b03427.

Figures for exosomes characterization, $\text{g-C}_3\text{N}_4$ NSs characterization, PAMAM-AuNPs characterization, Galinstan NPs characterization, $\text{g-C}_3\text{N}_4$ @Galinstan-PDA NPs characterization, biosensor characterization, and sensor conditions' optimization (PDF)

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Notes

The authors declare no competing financial interest.

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