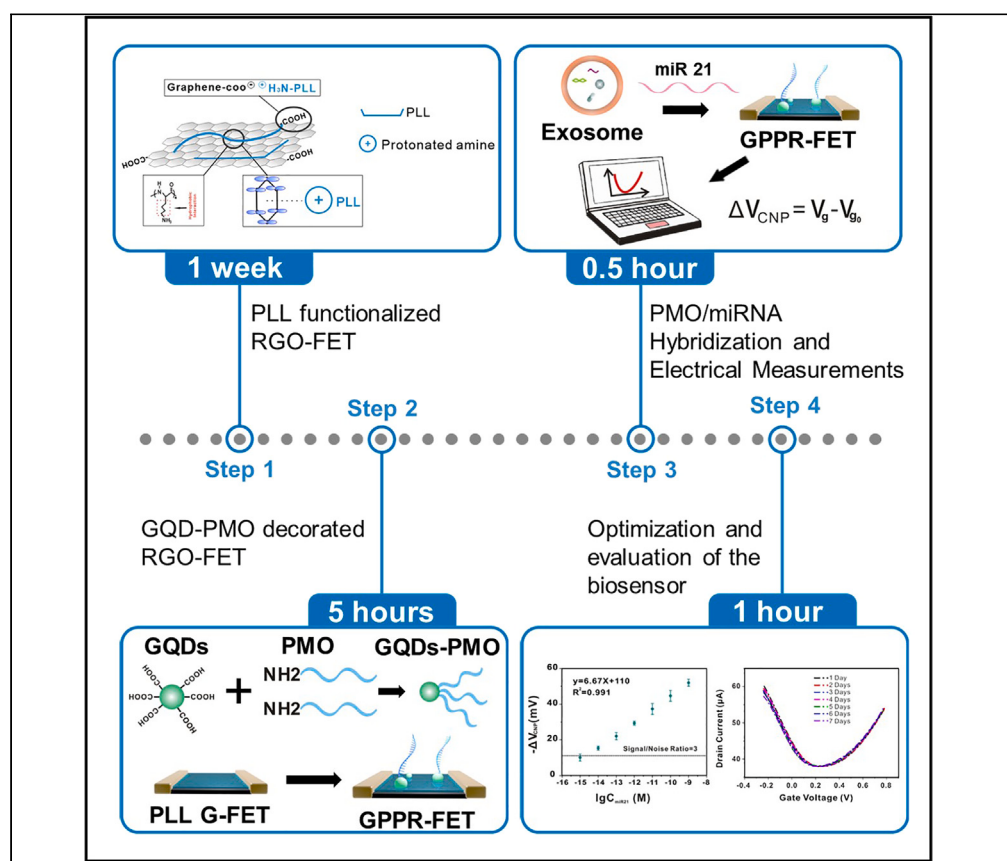


Protocol

Protocol for constructing GQD-PMO functionalized FET-biosensor for ultrasensitive exosomal miRNA detection



Here, we present a protocol for constructing an ultrasensitive biosensor for exosomal-miRNA detection. We describe steps for preparing graphene quantum dot-phosphorodiamidate morpholino oligomer hybrids, depositing them onto the reduced graphene oxide field surface, hybridizing analyte miRNA with the sensor probe, and capturing and calculating electrical signals. We also detail procedures for optimizing biosensor construction and evaluating performance. By quantifying plasma exosomal miRNA21, this protocol can identify cancer patients from healthy individuals.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to construct
GQD-PMO RGO-FET
biosensors to detect
exosomal miRNA

Steps to construct
PLL-functionalized
RGO-FET biosensor

Details to construct
PMO-GQD
decorated RGO-FET
sensor

Steps to hybridize
analyte miRNA and
measure electrical
signals

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Protocol

Protocol for constructing GQD-PMO functionalized FET-biosensor for ultrasensitive exosomal miRNA detection

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SUMMARY

Here, we present a protocol for constructing an ultrasensitive biosensor for exosomal-miRNA detection. We describe steps for preparing graphene quantum dot-phosphorodiamidate morpholino oligomer hybrids, depositing them onto the reduced graphene oxide field surface, hybridizing analyte miRNA with the sensor probe, and capturing and calculating electrical signals. We also detail procedures for optimizing biosensor construction and evaluating performance. By quantifying plasma exosomal miRNA21, this protocol can identify cancer patients from healthy individuals.

For complete details on the use and execution of this protocol, please refer to Li et al.¹

BEFORE YOU BEGIN

Institutional ethics permission

The protocol requires obtaining approval from the clinical ethics committee before conducting the study. In our work, the Hubei Provincial Hospital of Traditional Chinese Medicine (Wuhan, China) provided blood samples from cancer patients and healthy individuals and obtained approval from the hospital Ethics Committee (ethics code: HBZY2019-C30-02).

Preparation one: Select a target for testing

⌚ Timing: 1 day

To achieve early cancer diagnosis through miRNA detection with a biosensor, we need to review and analyze literature data, including *in vitro*, animal, and clinical research. This approach aims to identify the suitable miRNA biomarker, and clarify its base sequence, expression level range, *ex-vivo* stability, and clinical diagnostic significance. We can utilize miRNA databases, such as miRBase: <http://www.mirbase.org>, to simplify this process. In this work, we chose plasma exosomal miRNA21 as the biomarker for breast cancer analysis.

Preparation two: Synthesis of PMO probes

⌚ Timing: 1 week



1. The Shanghai Biotech Company synthesizes the HPLC-purified PMO probes that target miRNA21 and contain 22 nucleotides. The PMO probes consist of amine-modified and Cy5 fluorescent-labeled varieties, the sequences of which are displayed in [Table 1](#). The company completes the production, purification, and lyophilization of the PMO probes within a time frame of approximately one week. Subsequently, the company packages the probe products in aluminum foil and ships them to our facility at a temperature of -20°C .
2. Upon receiving the synthetic PMO probes, divide them into aliquots and store them in a laboratory refrigerator at a 4°C temperature. Quantify the concentration of the solutions using the NanoDrop™ Spectrophotometer. Ensure the laboratory provides an RNase-free environment to avoid any negative impact on miRNA stability by RNase. Prior to introducing analyte miRNAs, prepare sensor surfaces with RNaseZap and treat all buffer solutions and de-ionized water with 0.1% DEPC while sterilizing via autoclaving.

Preparation three: Synthesis of oligonucleotides

⌚ Timing: 1 week

Takara Biotechnology (Dalian) supplies synthetic miRNA21 mimic oligonucleotides of 22-mer in length based on the provided sequences, including miRNA21 (wild-type), OM-miRNA21 (single-base mismatch), TM-miRNA21 (three-base mismatch), and miRNA10b (random RNA). The company processes and delivers the ordered product within one week.

Preparation four: Sensor chip manufacturing

⌚ Timing: 1 h

To manufacture the semiconductor chip in our project, we use photolithography and electron-beam evaporation techniques. The process starts by creating a mask plate based on the desired circuit design. We first apply the positive adhesive photoresist 5350 onto a silicon dioxide (SiO_2) wafer, achieving a thickness of 285 nm through spin coating and baking at 110°C for 3 min. Next, we subject the SiO_2 surface to deep UV exposure for 2.6 s, followed by immersion in Developer AR 300-26 solution for 17 s to develop the film. We then wash the film with water and dry it using nitrogen. Upon completing the film, the next step involves fabricating gold electrodes on a Si/SiO_2 (285 nm) substrate. We employ macro-nano processing technologies to fabricate the source and drain electrodes, ensuring a separation distance of $4\text{ }\mu\text{m}$ between them. To enhance adhesion, we apply a 5 nm thick Ti layer between the Au and Si wafer. The sensor chip measures $6 \times 4.5\text{ mm}$, and the process is complete once the electrodes have been successfully created.^{2,3}

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Graphene oxide (GO, 99.99995%)	XFNANO	CAS: 7440-44-0
Poly-L-lysine solution	Macklin	CAS: 25988-63-0
Bovine serum	Sigma-Aldrich	CAS: 9048-46-8
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 98%)	Sigma-Aldrich	CAS: 25952-53-8
N-hydroxysulfosuccinimide (sulfo-NHS, 98.5%)	Sigma-Aldrich	CAS: 6066-82-6
Piranha solution (7:3 v/v H_2SO_4 /35% H_2O_2)	Generay Biotech Co. Ltd.	CAS: 1405-41-0
Hydrazine (98%)	Aladdin Co. Ltd.	CAS: 1289007-61-9
Carboxylated GQDs	XFNANO	CAS: 7440-40-0
1-Pyrenebutanoic acid succinimidyl ester (PASE)	Sigma-Aldrich	CAS: 114932-60-4

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
MCF-10a (human mammary epithelial cell line)	Procell Co., Ltd	
MCF-7 cell (human mammary carcinoma cell)	ATCC, Shanghai	
Oligonucleotides		
PMO probe	NH ₂ -TCAACATCAGTCTGATAAGCTA	
PMO-Cy5 probe	NH ₂ -TCAACATCAGTCTGATAAGCTA-Cy ⁵	
miRNA21	UAGCUUAUCAGACUGAUGUUGA	
OM-miRNA21	UAGCUUAUCGACUGAUGUUGA	
TM-miRNA21	UUGCUUAUCGACUGAUCUUGA	
miRNA10b	UACCCUGUAGAACCGAAUUUGUG	
Software and algorithms		
Origin	OriginLab Corporation (https://www.originlab.com/)	
Other		
NanoDrop 2000	Thermo Fisher Scientific	
Analytical Ultracentrifuge	Beckman Coulter	
Keithley 4200-SCS (or a semiconductor parameter analyzer)	Tektronix	
EverBeig BD-6 (or a probe station)	Everbeing Int'l Corp.	
7500 Real-Time PCR System	Thermo Fisher Scientific	
JEM-2100 Plus-transmission electron microscope (TEM)	LaB6TEM-Jeo USA	
Nanoparticle tracking analysis (NTA)	ZetaView Ltd., Germany	
GeneGnome XQR chemiluminescence imaging system	Synoptics Ltd, England	
Millipore (a 0.22-μm filter membrane)	Merck Millipore	

STEP-BY-STEP METHOD DETAILS

To preserve the stability and integrity of the sensing interface, we conduct all biosensor manufacturing procedures in a Class 100 Laminar Airflow Clean Lab or Workbench. We carry out these procedures at 25°C unless specified otherwise.

Construction of PLL-functionalized RGO-FET sensor

⌚ **Timing:** 1 week

- Preparation of stable RGO suspensions.
To prepare a stable RGO suspension solution, we followed the method presented by Tung et al.⁴
 - Weigh 1.5 mg of GO compound in a 2 mL microtube and add 1 mL of 98% hydrazine solution.
 - Sonicate the tube for 10 min to obtain a black suspension of hydrazinium graphene (HG).
 - Gently shake the suspension at 4°C for 1 week to prevent GO aggregation, yielding a stable reduced-GO suspension solution.
- Fabrication of RGO-FET biosensors.
 - Deploy a 0.2 μL suspension solution of RGO (0.2 mg/mL), prepared in step 1, by drop-casting onto the chip sensing channel.
 - Impose 150°C heat upon the RGO-paved sensor chip for 1.5 h to immobilize the RGO.
 - Plunge the chip into a Piranha solution (7:3 v/v, H₂SO₄/35% H₂O₂) followed by sonication for 30 s, resulting in a few-layer RGO-FET sensor chip.
 - Rinse the chip with deionized water three times and dry the RGO-FET sensor chip with nitrogen flow.

⚠ **CRITICAL:** Experimenters should handle the Piranha solution diligently and wear appropriate personal protective equipment during preparation, as it is highly corrosive. To prepare the solution, we combine 70 mL of concentrated H₂SO₄ with 30 mL of 35% H₂O₂ in a

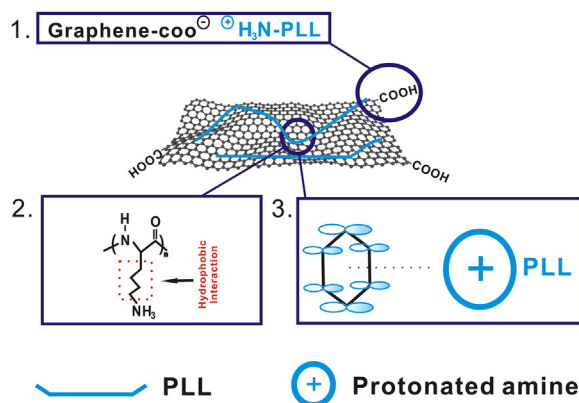


Figure 1. Synthetic scheme of PLL-mediated RGO-FET functionalization

beaker while gently stirring the mixture with a glass rod. It is essential to perform the entire procedure in a well-ventilated area, preferably within a fume hood.

3. Paving PLL film on top of the RGO-FET sensor.
 - a. Dilute the PLL stock solution (1 mg/mL) to a concentration of 0.5 mg/mL using purified deionized water.
 - b. Dispense 0.5 μ L of the PLL solution (0.5 mg/mL) dropwise onto the RGO-FET sensor surface (Figure 1).
 - c. Heat the sensor chip at 80°C for 2 h.
 - d. Sequentially rinse the PLL functionalized-RGO-FET sensor 3 times with 1 $\times$ phosphate-buffered saline (PBS) and ultra-pure water.
 - e. Dry the sensor using nitrogen.
4. Characterization of the PLL-RGO-FET sensor by X-ray photoelectron spectroscopy (XPS), Raman spectrometer (Raman), and Transmission Electron Microscopy (TEM).
 - a. TEM: PLL-RGO hybrid should reveal a flat sheet with wrinkled, folded structures.
 - b. XPS: The N1s (399.9 eV) peak of RGO-PLL should have a significant increase compared to RGO.
 - c. Raman spectra: I_D/I_G ratio of RGO and PLL-RGO increases from 1.20 to 1.28.

Note: The strong binding between RGO and PLL is the result of (1) electrostatic attraction (2) hydrophobic interaction (3) protonated amine-p electron interaction.^{5,6}

Synthesis of GQDs-PMO hybrids

⌚ Timing: 2 h

5. GQD activation and GQD-PMO hybrids formation.

To achieve stable GQDs-PMO hybrids, we activate the carboxyl groups on the surface of GQDs using EDC (50 μ L, 50 mg/mL) and NHS (50 μ L, 12.5 mg/mL) solutions. Subsequently, we co-incubate NH₂-modified PMO probes with the activated GQDs, forming the desired hybrids. The interaction between the activated carboxyl groups on GQDs with NH₂-modified PMO probes leads to the formation of stable GQDs-PMO complexes (Figure 2).

 - a. Take 50 mg of EDC and 12.5 mg of NHS into separate 2 mL microtubes. Add 1 mL of deionized water into each tube and thoroughly mix to create stock solutions of EDC and NHS.
 - b. Dissolve 1 mg of GQDs product in 1 mL of deionized water.
 - c. Mix 50 μ L of EDC stock solution, 50 μ L of NHS stock solution, and 100 μ L of GQDs solution in a new microtube.
 - d. Cover the mixed solution tube with tin foil to shield it from light exposure. Place the tube onto a shaker at 25°C for 30 min with mild shaking to activate the GQDs (500 μ g/mL).
 - e. Dilute the PMO stock solution into a concentration of 1 μ M.

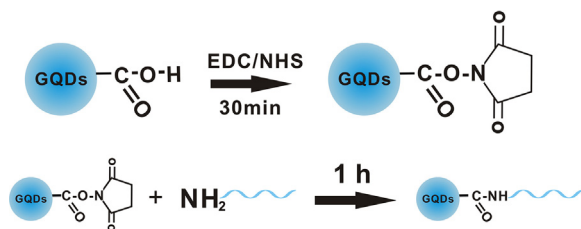


Figure 2. Synthetic scheme of the GQDs-PMO hybrids

- f. Combine 200 μL of PMO solution (1 μM) with 200 μL of the activated GQDs solution in a new microtube.
- g. Shield the mixed solution tube with tin foil and place it on a shaker at 25°C for 60 min with gentle shaking at a slow speed to form the GQDs-PMO hybrids.
6. Characterization of the GQDs-PMO hybrids using TEM, fluorescence, and Zeta potential tests.
 - a. TEM: The carboxylated GQDs have a uniform size with particle diameters of approximately 4–5 nm.
 - b. Fluorescence: Under the excitation wavelength of 365 nm and detection wavelength of 465 nm, the fluorescence signal values of the GQDs-PMO hybrids are lower than those of the GQDs due to the fluorescence quenching effect.
 - c. Zeta potential: We initially measure the zeta potential of GQDs to be -21.22 mV, which reduces to -10.37 mV after combining PMO with GQDs, indicating successful synthesis of the GQDs-PMO hybrids.

Note: To prepare EDC and NHS stock solutions correctly, allow the reagents to equilibrate to room temperature before use. Mix EDC and NHS at the time of use, not beforehand. Advisably, store or handle the GQDs solution in an environment away from light. These steps ensure the protocol's success and must be followed precisely.

Preparation of GQDs-PMO hybrids decorated RGO-FET (GPPR-FET) sensor

⌚ **Timing:** 3 h

7. Deposition of the GQDs-PMO hybrids on the PLL-paved RGO-FET sensor.
 - a. Dispense 10 μL of the GQDs-PMO hybrids solution onto the surface of the PLL-RGO-FET sensing channel.
 - b. Cover the sensor chip with opaque sheets and incubate at 25°C for 8–12 h. The reaction between the carboxyl group on GQDs and the amino group on PLL enables the successful assembly of GQDs-PMO hybrids on the sensor surface.
 - c. Sequentially rinse the sensor 3 times with 1×PBS solution and ultrapure water to eliminate redundant unbinding PMO probes.
 - d. Dry the sensor using nitrogen flow.
 - e. To prevent clumping, gradually sprinkle 1 mg of molecular biology grade BSA powder onto the liquid surface of 1 mL distilled water and stir until fully dissolved, resulting in a BSA solution with a concentration of 1 mg/mL.
 - f. Dispense 10 μL of the BSA solution onto the chip sensing channel and incubate for 1 h to seal non-specific binding sites.
 - g. Rinse the sensor chip with deionized water 3 times.
 - h. Dry the chip with nitrogen flow.
8. Characterization of the GPPR-FET using XPS, fluorescence, atomic force microscope (AFM), and I_d - V_g curve.

$$\Delta V_{\text{CNP}} = V_g - V_{g_0}$$

Figure 3. The formula for ΔV_{CNP} calculation

- a. XPS: We test the P-element of PLL-RGO-FET and GPPR-FET for XPS. The modification of GQDs-PMO leads to the appearance of a clear P-element peak (134.4 eV), carried by the phosphate group of the PMO probes.
- b. Fluorescence microscope: The fluorescence microscope shows a red fluorescent signal of GQDs-PMO on the sensor surface, where the PMO is labeled with Cy5.
- c. AFM: AFM reveals that GQDs-PMO deposits a large number of nanoparticles on the chip surface after fixation, compared to the PLL-RGO sensor planar surface.
- d. I_d - V_g : We observe a shift of the Dirac point of the sensor to the right after PLL deposition for I_d - V_g . This is due to the positive charge of PLL effectively inducing RGO P-doping. Upon immobilization of GQDs-PMO, we notice a shift of the Dirac point to the left, which is caused by the negative charge of GQDs-PMO N-doping the RGO. See [Table 2](#) for further details.

PMO/miRNA hybridization and electrical measurements

⌚ Timing: 1.5 h

This section outlines the procedures for measuring miRNA21 using the GPPR-FET sensor. It includes assessing the sensor's detection capabilities, such as selectivity, sensitivity, stability, and operating curve plots. These steps are essential to ensure accurate quantification of the analyte.

9. Specific hybridization of the PMO probe and analyte miRNA21.
 - a. Apply 10 μL of the synthetic miRNA21 oligonucleotide solution onto the GPPR-FET sensor surface and incubate it for 1 h at 25°C.
 - b. Rinse the sensor sequentially with 1×PBS solution, 0.2% SDS solution, 1×PBS solution, and nuclease-free water to remove any unhybridized molecules.
 - c. Dry the sensor with nitrogen flow in a ventilation cabinet.
10. Setting up the I_d - V_g measurement parameters.
 - a. The semiconductor characterization system (Keithley 4200-SCS) coupled with a probe station (Ever Being BD-6) conducts the electrical measurements of the GPPR-FET sensor.
 - b. Utilize a silver wire as the gate electrode and apply a constant bias of $V_{ds} = 0.1$ V to record the I_{ds} - V_g (I_{ds} being the drain-source current and V_g the gate voltage) curve.
 - c. Measure the I_d - V_g curves at a specified V_g value by applying a liquid-gate bias of 0.1 V in 0.01× PBS.
 - d. Dirac point offset (ΔV_{CNP} , refer to [Figure 3](#)) serves as the electrical signal.^{7–9}
11. Optimizing the GPPR-FET sensor.
 - a. GQDs/PMO ratio optimization.
 - i. Fix the GQDs concentration at 250 $\mu\text{g/mL}$ and dilute the PMO stock solution to different concentrations, ranging from 0.5 μM to 2.5 μM , to establish various ratios of GQDs/PMO complexes.
 - ii. Drop-add GQDs/PMO complexes solutions with different ratios onto the PLL-functionalized RGO-FET biosensor surface to prepare the corresponding GPPR-FET biosensors.
 - iii. Expose the biosensors to 1 nM miRNA21 solutions for hybridization and record the ΔV_{CNP} .
 - iv. Select the sensor that produces the largest signal response for subsequent experiments.
 - b. PLL concentration optimization.
 - i. Add different concentrations of PLL solution (0.2 mg/mL, 0.5 mg/mL, and 1 mg/mL) dropwise onto the RGO film to prepare the corresponding PLL-paved RGO-FET sensors.
 - ii. Expose the biosensors to 1 nM miRNA21 solutions for hybridization and record the corresponding ΔV_{CNP} .

- iii. Select the sensor that produces the largest signal response, this will specify the optimal PLL concentration.
 - c. PMO-RNA hybridization time optimization.
 - i. Record the ΔV_{CNP} at varied miRNA21-PMO hybridization times of 0.5, 1, and 2 h.
 - ii. Select the sensor that produces the largest signal response, as this determines the optimal hybridization time.
 - d. PMO-RNA hybridization temperature optimization.
 - i. Hybridize the GPPR-FET biosensors with 1 nM miRNA21 solutions at 4, 25, and 37°C.
 - ii. Record the corresponding ΔV_{CNP} and select the sensor with the largest response to determine the optimal temperature.
12. Performance evaluation of the GPPR-FET biosensor.
 - a. Specificity.
 - i. Utilize a series of synthesized analyte oligonucleotides, including OM-, TM-mismatch, random sequences (miRNA10b), and miRNA21.
 - ii. Investigate the response of the GPPR-FET sensor to these analytes.
 - iii. Record and analyze the Dirac point shifts ($-\Delta V_{\text{CNP}}$) generated by the hybridization of PMO-RNA.
 - iv. Determine the sensor's ability to differentiate the target RNA from the mismatched RNA.
 - b. Sensitivity.
 - i. Sequentially deposit 10 μL of miRNA21 solutions with varying concentrations (ranging from 100 aM to 1 nM) onto the sensing channel of the GPPR-FET sensor, starting from low to high concentrations.
 - ii. Incubate the sensors at 37°C for 1 h to facilitate the base complementary pairing reaction between the analyte miRNA21 and the PMO probes.
 - iii. Record the $-\Delta V_{\text{CNP}}$ of the sensors.

Note: We anticipate that the protocol steps will result in a gradual increase in the average $-\Delta V_{\text{CNP}}$ values (11, 23, 37, 51, 65, 78, 88, and 104 mV, respectively) with increasing concentrations of the target miRNA21. This trend is due to the accumulation of miRNA21 on the sensing channel surface of the GPPR-FET sensor, resulting in the buildup of negative charges and subsequent N-type doping of graphene.

- c. Stability.
 - i. Add 1×PBS solution dropwise onto the GPPR-FET biosensor surface and then incubate it for 2, 4, 6, and 12 h.
 - ii. Record the ΔV_{CNP} at each time point, and plot histograms in Origin software to visualize the data.
 - iii. Test the stability of the GPPR-FET biosensor over a prolonged period by placing it in a vacuum oven for 7 days and measuring the transfer characteristic curve daily.
 - iv. Analyze the ΔV_{CNP} of the GPPR-FET biosensor over time to assess its prolonged stability.

Note: To evaluate the stability of the GPPR-FET biosensor, we conduct two experiments. First, we add 1×PBS solution dropwise onto the sensor chip and measure the resulting ΔV_{CNP} after incubating for 2, 4, 6, and 12 h. We observe a minimal shift of less than 5% in the sensor's response. In the second experiment, we place the GPPR-FET sensors in a vacuum drying oven and monitor the ΔV_{CNP} continuously for 7 days to assess long-term stability. We find that the Dirac point of the sensor undergoes few shifts, less than 12%, over the 7-day period. These results indicate the excellent stability of the GPPR-FET biosensor (Figure 4).

13. Creating a Work Curve Plot.
 - a. Measure the $I_{\text{d}}-V_{\text{g}}$ curves of the GPPR-FET sensor at varying analyte miRNA21 concentrations.
 - b. Generate a plot of $I_{\text{d}}-V_{\text{g}}$ within the same coordinate axis using Origin software and observe changes in the $I_{\text{d}}-V_{\text{g}}$ curves at different miRNA21 concentrations.

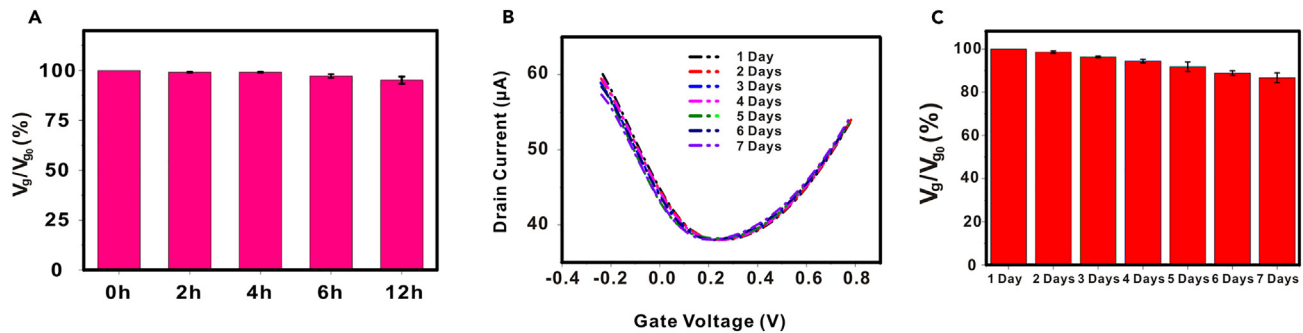


Figure 4. Stability of the GPPR-FET sensor

Error bars represent the standard errors ($n = 3$).

(A) Percentage shift in Dirac point of the GPPR-FET biosensor after incubation in $1 \times$ PBS for 2 h, 4 h, 6 h, and overnight.

(B) Transfer curves of the GPPR-FET biosensor for 7 consecutive days.

(C) Percentage shift in Dirac point of GPPR-FET biosensor for 7 consecutive days.

- c. Record the change in $-\Delta V_{CNP}$ at different miRNA21 concentrations.
- d. Use Origin software to plot miRNA concentration on the X-axis and $-\Delta V_{CNP}$ on the Y-axis to create a linearity curve of the GPPR-FET sensor.
- e. Calculate the correlation between $-\Delta V_{CNP}$ and the logarithm of miRNA concentration.

Note: The protocol observes a strong linear relationship between different concentrations of miRNA21 and $-\Delta V_{CNP}$, yielding a high correlation coefficient (R^2) of 0.99 (Figure 5). The regression equation ($-\Delta V_{CNP} = 13.23 \lg C + 222.64$, where C represents the concentration of the analyte RNA). The graph depicts a dotted line denoting the 3-fold signal-to-noise ratio (10 mV). Utilizing the regression equation and the 3-fold signal-to-noise ratio, the protocol calculates the GPPR-FET biosensor detection limit for miRNA21 at 85 aM.

14. Exosomes isolation from cell-culture supernatant.

△ CRITICAL: To prepare for exosome isolation, pre-treat cells with serum-free or vesicle-free medium for 48 h beforehand. Use the cell culture supernatants (CCS) directly or store them at 2°C – 8°C for up to 6 h. For long-term storage, freeze the CCS aliquots at -80°C . Thaw CCS samples gently by heating them in a 37°C water bath until fully thawed. Avoid prolonged incubation at 37°C to prevent RNA degradation.

- a. Cell culture.
 - i. Culture MCF-10A and MCF-7 cells separately under a humidified atmosphere of 5% CO_2 at 37°C .
 - ii. Use DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin for MCF-7 cells.
 - iii. Use MCF-10A cell culture medium (Procell) for MCF-10A cells.
 - iv. Pre-treat both cells with a serum-free medium for 48 h before exosome isolation.
- b. Exosome isolation.
 - i. CCS collection: Wash the cells three times with ice-cold $1 \times$ PBS when they reach 70% confluence, then culture them in a serum-free medium for 48 h before collecting the CCS.
 - ii. Standard overspeed centrifugation: Centrifuge CCS samples at 300 g using the Beckman Coulter Allegra® X-15R centrifuge for 15 min to remove cell fragments. Then, centrifuge the supernatant at 2 000 g for 20 min to remove cell debris. Filter them with a $0.22 \mu\text{m}$ membrane. Subsequently, centrifuge the filtrates at 1 000 g for 30 min. Precipitate exosomes by centrifuging the supernatant at 110 000 g for 70 min. For purer exosomes, re-centrifuge (110 000 g, 70 min) after resuspension.¹⁰

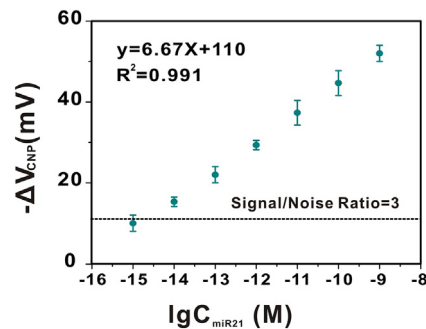


Figure 5. Response of the GPPR-FET sensor to varying concentrations of miRNA21

- iii. Exosome storage: Resuspend the purified exosome pellets in 400 μ L 1 $\times$ PBS solution and store them at -80°C .
15. Exosomes isolation from human plasma.

△ CRITICAL: Time sensitivity and laboratory biosafety are critical considerations for the study.

Time sensitivity: To maintain sample quality and obtain accurate results, it is crucial to centrifuge whole blood samples within 1 h of reception to isolate the plasma exosomes.

Laboratory biosafety: Strict adherence to universal precautions when handling blood samples is essential. This includes wearing appropriate personal protective equipment (masks, gloves, eye protection, and lab coats) to mitigate any potential risks.

- a. Centrifuge the whole blood sample at 300 g at 4°C for 20 min.
- b. Remove 1.5 mL of the plasma, which is the supernatant portion, and place it into a 2 mL Eppendorf tube with a patient ID label.
- c. Immediately subject the plasma samples to an exosome extraction process or freeze them at -80°C for future use.
- d. Use the exoRNeasy Serum/Plasma Midi Kit (Qiagen) protocol to extract the plasma exosomes.
16. Exosomes characterization.
 - a. TEM.
 - i. Load 15 μ L of the exosome suspension in a 400-mesh, carbon-coated copper grid and stabilize with 100 μ L of 2% glutaraldehyde.
 - ii. Stain the grid with 2% phosphotungstic acid for 10 min, then rinse twice with ice-cold 1 $\times$ PBS.
 - iii. Flip the grid so the sample membrane side is facing down, and dry the opposite side for 2 min.
 - iv. Dry the grids before storing them in an appropriate box for observation with the JEM-2100 TEM.
 - b. NTA.
 - i. Dilute 20 μ L of exosome suspension sample 100 times in ice-cold 1 $\times$ PBS and pass through a 0.22 μ M filter.
 - ii. Load the sample into the LM10 unit chamber and capture three 30–60 s videos with a CMOS camera.
 - iii. Use NTA 1.1 and 2.1 software (Zeta view Ltd., Germany) to analyze the sample, and represent results as the average and standard deviation from video recordings.

Note: We used control beads from Duke Scientific (Palo Alto, CA). The NTA analysis demonstrated accuracy within the range of 2×10^8 to $20 \times 10^8/\text{mL}$.

- c. Western blotting.

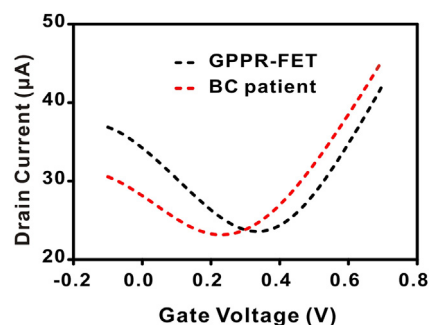


Figure 6. An example of GPPR-FET sensor-based plasma exosomal miRNA21 testing in a breast cancer patient

- i. Lysate exosome samples with protease inhibitors, denature them at 95°C for 10 min, quantify protein levels with a micro-BCA assay, and separate with SDS-PAGE.
- ii. Transfer proteins to PVDF membranes, then incubate with primary antibodies (CD63, EpCAM, and TSG-101) for 8–12 h at 4°C.
- iii. Incubate membranes with HRP-conjugated secondary antibody for 2 h at 25°C, then visualize protein bands with a GeneGnome XQR chemiluminescence imaging system.
17. Exosomal RNA extraction.
 - a. Adhere strictly to Qiagen's exoRNeasy Serum/Plasma Midi Kit protocol to ensure consistency and accuracy when extracting and purifying exosomal RNA from CCS and human plasma.
 - b. Split exosomal RNA into two aliquots for subsequent experiment.
 - c. Quantify miRNA21 level in the first aliquot using reverse transcription-polymerase chain reaction (RT-PCR).
 - d. In the second aliquot, analyze the same analyte with the GPPR-FET biosensor.
18. Exosomal miRNA21 quantification via RT-PCR.
 - a. Convert approximately 2 μg of RNA into cDNA (reverse transcription product) using Super Script III reverse transcriptase (Invitrogen) following the manufacturer's instructions. The resulting cDNA solution will be 20 μL.
 - b. Set the reverse transcription program to run at 42°C for 60 min, followed by 95°C for 3 min. Dilute the cDNA 10-fold before using it as templates for PCR analysis.
 - c. Prepare the amplification reaction by mixing 2 μL of each RT product with 10 μL of 2× miRNA Premix (SYBR&ROX) and 0.4 μL of designed forward and reverse primers. The total volume of the mixture will be 20 μL.
 - d. Set the PCR reaction conditions to 95°C for 15 min, followed by 40 cycles of 94°C for 20 s and 60°C for 34 s. Record the melting curve.
19. Exosomal miRNA21 quantification via GPPR-FET biosensor.
 - a. Extract and purify the total RNA from exosomes using exoRNeasy Serum/Plasma Midi Kit (Qiagen).
 - b. Add 10 μL of the total RNA sample dropwise onto the GPPR-FET sensor surface and incubate it at 37°C for 1 h to ensure complete binding of miRNA21 to the probes.

Table 1. SYBR™ PCR reaction master mix

Reagent	Amount
2 × miRNA Premix (SYBR&ROX)	10 μL
reverse transcription products	2 μL
forward primer	0.4 μL
reverse primer	0.4 μL
ddH ₂ O	7.2 μL
Total	20 μL

Table 2. PCR cycling parameters

Steps	Temperature	Time	Cycles
Incubation	95°C	15 min	40 cycles
Denaturation	94°C	20 s	
Annealing and Extension	60°C	34 s	

- Thoroughly rinse the sensor chip with 1×PBS solution and pure water. Then blow-dry the sensor chip with nitrogen.
- Add 0.01×PBS solution as a liquid-gate to test the I_d - V_g plot of the sensor before and after incubation.
- Calculate the level of miRNA21 in the test sample by using the working curve drawn in step 11 and utilizing the value of ΔV_{CNP} as the signal (Figure 6).

EXPECTED OUTCOMES

In patients diagnosed with breast cancer, a noteworthy rise in plasma exosomal miRNA21 levels is expected, unlike those found in healthy individuals (Figure 7). This elevation can be detected through the GPPR-FET biosensor by tracking a substantial shift in the Dirac point ($-\Delta V_{CNP}$).

LIMITATIONS

The study's limitations include a small number of clinical samples and insufficient data on disease staging and pathological grading. Improving the reproducibility of the biosensor methodology requires a

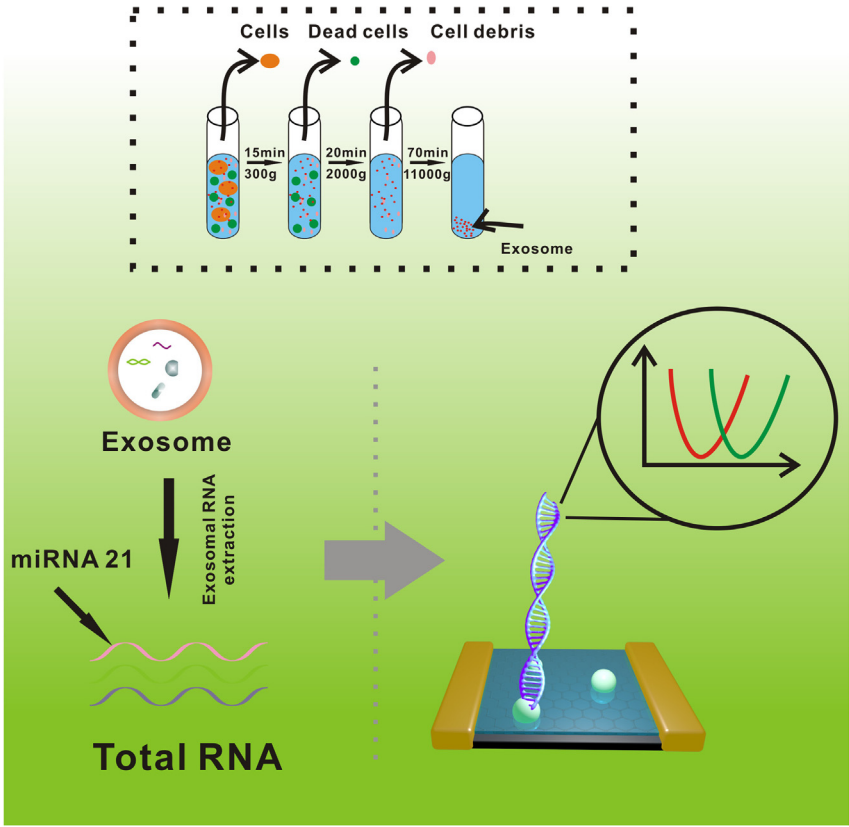


Figure 7. Schematic diagram of exosomal miRNA detection and representative formula of the QGD functionalization

larger clinical sample size and comprehensive data. Prior to measurement, researchers should purify RNA samples using an RNA purification kit following the manufacturer's instructions to prevent background noise interference caused by impurities.

TROUBLESHOOTING

Problem 1

Chip-to-chip variation caused by manual operation (Steps 1 and 2).

In the process of producing sensors for this project, certain critical steps, such as manually drop-casting GQD-PMO hybrids and RGO solution onto the chip surface, are necessary. Unfortunately, human error often occurs due to factors such as unstable personnel skills, inconsistent droplet volumes, irregular coating thickness caused by bubbles, and environmental and operational contamination. As a result, variations in biosensor performance are inevitable among different batches.

Potential solution

- Optimize the manual coating process to achieve consistent droplet size and speed, ensuring the uniformity and accuracy of the coating.
- Perform I_d - V_g measurement on each sensor as part of the standard quality control procedures. This allows us to monitor and maintain control over the sensor current, ensuring that fluctuations are kept within an acceptable range.
- Discard any chip that do not pass our rigorous quality control screening test.

Problem 2

Unstable modification of GQDs-PMO hybrids on the RGO surface (Step 7).

Stable deposition of GQDs on the RGO surface is the key to increasing the density of the probe and enhancing the sensitivity of the biosensor. Traditionally this step can be performed by techniques such as electrochemical deposition and heat-induced self-assembly. However, in this project, we utilized a surface modifier, PLL, to functionalize RGO.

Potential solution

- Optimize PLL concentration.
- Extend the fixation time of the GQD-PMO hybrids.
- Implement a quality control check to assess the stability of the biosensors before use. As can be seen in [Figure 4](#), the GPPR-FET biosensors exhibit excellent stability both in the short term (0–12 h) and the long term (1–7 d).

Problem 3

Unsatisfactory sensitivity and selectivity of miRNA detection using the GPPR-FET biosensor (Step 9).

Potential solution

- Select the right type of sensor probe, such as PMO, a neutral synthetic oligonucleotide used in this study, to facilitate binding with the target RNA.
- Optimize probe sequences at the beginning of the study to ensure the best base complementary pairing reaction, considering that miRNA is short, with low content and high similarity.
- Optimize the temperature and time for probe-RNA hybridization. Before use, it is important to keep the working area RNA enzyme-free with RNaseZAP solution to prevent miRNA degradation.
- Include purification steps during exosome and RNA extraction to overcome interference from impurities such as proteins and lipids in the sample, ensuring accurate detection of miRNA analytes by the sensor.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guo-Jun Zhang (zhanggj@hbtcu.edu.cn).

Materials availability

This study did not generate or use new unique reagents.

Data and code availability

Data reported in this paper will be shared by the [lead contact](#) upon request. This study did not generate/ analyze code. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

C.L. and K.L. authored the manuscript, formulated the biosensor construction protocol, and executed the experiments. Y.P. and L.M. undertook revisions to the manuscript in response to reviewers' feedback. G.-J.Z. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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