Polyacrylamide/Phytic Acid/Polydopamine Hydrogel as an Efficient Substrate for Electrochemical Enrichment of Circulating Cell-Free DNA from Blood Plasma

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ABSTRACT: A facile method has been developed for the rapid and efficient enrichment of DNAs from different media including synthetic single-strand DNAs (ssDNAs) from buffer solutions and cell-free DNAs (cfDNAs) from blood plasma through electric field-driven adsorption and desorption of DNAs by a polyacrylamide/phytic acid/polydopamine (PAAM/PA/PDA) hydrogel. The as-prepared PAAM/PA/PDA hydrogel possesses regular porosity with a large surface area, strong electric field responsiveness/good conductivity, and a rich aromatic structure, which can be used as an ideal adsorbent for DNA enrichment under a positive electric field. The enriched DNAs can be released efficiently when the positive electric field is converted to a negative electric field. The PAAM/PA/PDA hydrogel-based electrochemical method enables the completion of the process of DNA adsorption and release within 5 min and exhibits reasonable enrichment efficiencies and recovery rates of various DNAs. For instance, the high enrichment sensitivity (0.1 pmol L$^{-1}$) together with the excellent recovery (>75%) of an ssDNA with 78 nucleotides is obtained. Combined with the PCR amplification technique, the practicality of the as-proposed method is demonstrated by the screening of circulating tumor DNAs (ctDNAs) with a BRAFV600E mutation in cfDNAs from the blood plasma samples of patients with papillary thyroid cancer or thyroid nodule and random patients from a clinical laboratory.

INTRODUCTION

The ability to analyze and explore circulating tumor biomarkers in peripheral blood is highly desirable for early cancer diagnosis, progression prediction, therapeutic strategy establishment, and prognosis evaluation.1−13 Among them, circulating tumor DNAs (ctDNAs) are tumor-derived cell-free DNAs (cfDNAs) in blood plasma, which possess the hallmark genomic characterics of a tumor.1−5,7−11 Therefore, the ctDNA-based liquid biopsy in the blood draw form has attracted great attention because the method offers the possibility of minimizing invasiveness to characterize the genetic information on the tumor and monitor the progression of cancer through real-time dynamic monitoring. Although ctDNA has great potential to become a liquid biopsy substitute for tissue biopsies to monitor genetic and epigenetic alterations of carcinogenesis, it is a challenge to directly detect the mutated ctDNAs in the blood sample of cancer patients because the concentration of ctDNA is too low to be extracted and purified by conventional methods for separation and enrichment of genomic DNA.1,3,8,9,13 Due to the extremely trace amount and highly fragmented nature (<200 bp) of ctDNA in blood plasma, there is an urgent demand for the development of a new technique that can enrich short DNA fragments in different media to satisfy the requirement of further clinical applications. Recently, a few strategies including nanoparticle-coupling approach, electrochemical sensing chip, and counter electrophoresis-based assay have been established for isolation and analysis of ctDNA.8,9,13−28 In particular, electrochemical methods exhibit high attraction for ctDNA detection/enrichment because they are rapid, convenient, low-cost, and highly sensitive.21−28

Conductive polymers have been extensively adopted in electrochemical platforms for bioanalytical and biomedical applications.21,29−34 For instance, Cho and colleagues have constructed a polypyrrole (Ppy) electrochemical chip for electric field-mediated extraction of cfDNAs from the blood plasma samples of lung cancer patients.21 Polycrylamide (PAAM) is a kind of material used in gel electrophoresis to separate biomolecules including nucleic acids. Polydopamine (PDA) has been demonstrated as a good DNA carrier under physiological conditions, which can efficiently adsorb DNA on
its surface and inhibit nuclease digestion of the absorbed DNA.35–40 Using phytic acid (PA) as a cross-linker, we have synthesized a kind of PAAM/PA/PDA hydrogel under mild ambient conditions, which has a three-dimensional (3D) network nanostructure, excellent physicochemical stability, and good conductivity.41,42 Especially, the highly 3D porous nanostructure of the PAAM/PA/PDA hydrogel provides a large active surface area for adsorption of ions and molecules.

As a member of the rapidly accelerated fibrosarcoma (RAF) family of kinases, BRAF is the most potent activator of the MAP/ERK kinase (MEK), which regulates the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway.35–51 Various mutations of the BRAF gene (>40) have been identified in approximately 7% of all human cancers. More than 90% of the oncogenic mutation in the BRAF gene involves a thymidine (T) to adenosine (A) transversion at nucleotide 1799 of exon 15. The mutation is known as BRAFV600E due to the substitution of valine (V) with glutamic acid (E) at amino acid residue 600. The missense BRAFV600E mutation leads to the upregulation of the MAPK/ERK activities in tumor cells significantly. Three prevalent cancer types, such as melanoma, papillary thyroid cancer (PTC), and colorectal cancer (CRC), have been shown to have a high incidence of BRAFV600E mutation.44–51 Therefore, the BRAFV600E mutation is an attractive target for the diagnosis and therapy of cancers.

Herein, we introduce a PAAM/PA/PDA hydrogel-based electrochemical method for efficient DNA enrichment through electric field-mediated adsorption/desorption of DNA on a PAAM/PA/PDA hydrogel-modified glassy carbon electrode (GCE). The integration of the 3D porous PAAM/PA/PDA hydrogel and the electric potential regulation endows the method with great enhanced enrichment efficiencies and recovery rates of various DNAs. The mutational status of BRAFV600E in PTC patients was successfully addressed when the enriched cDNAs from as low as 20 μL of blood plasma were used as a template for PCR amplification, which demonstrated the clinical relevance of the PAAM/PA/PDA hydrogel-based electrochemical method.

## RESULTS AND DISCUSSION

### Experimental Condition Optimization of DNA Enrichment

As displayed in Scheme 1, the GCE-PAAM/PA/PDA was fabricated by the in situ gelation reaction of AAM, PA, and DA on the GCE surface using KPS as the initiator and TEMED as the accelerator. The DNAs in different media were adsorbed on the GCE-PAAM/PA/PDA through electrostatic attraction while a positive electrode potential (e.g., 1.0 V) was applied. The adsorbed DNAs were then released into the solution through electrostatic exclusion while a negative electrode potential (e.g., −1.0 V) was applied. The DNA adsorption–releasing process can be measured by the electron transfer resistance change of the GCE-PAAM/PA/PDA (i.e., ΔR_{et} and/or ΔR_{et}^{s}) because DNAs can obstruct electron exchange on the electrode surface.

In order to obtain optimized DNA enrichment conditions, the effect of the PDA amount in the PAAM/PA/PDA on the DNA enrichment was first investigated because the PDA can not only affect the 3D porous nanostructure of the PAAM/PA/PDA but also interact with the DNAs through π–π interactions.35–40 In this case, a synthetic ssDNA (T_M) with 78 NTs from the known oncogene, BRAFV600E, was used as the DNA target. As shown in Figure S1a, the ΔR_{et} was increased by increasing the concentration of dopamine (DA) in the reaction mixture from 3.5 to 6.5 mmol/L and began to saturate above 6.5 mmol/L, while the concentration of T_M was kept constant. Therefore, 6.5 mmol/L was used as the optimized concentration of DA for fabricating GCE-PAAM/PA/PDA. Under this condition, the surface of the GCE-PAAM/PA/PDA has a porous structure, which can facilitate electron transport and enhance the DNA adsorption capability (as shown in Figure S2). In addition, the ΔR_{et} also increased with the increasing incubation time of the GCE-PAAM/PA/PDA with the DNA-containing solution, electrode potential, and standing time of the electrode potential, respectively, while the concentration of T_M remained unchanged (as shown in Figure S1b–d). The saturation values of ΔR_{et} were obtained at 45 min (incubation time), 1.0 V (electrode potential), and 2 min (standing time). In the following study, the DNA adsorption was carried out with the optimized condition, that is, the GCE-PAAM/PA/PDA was firstly incubated in the DNA-containing solution for 45 min and kept at 1.0 V for 2 min. During the DNA desorption, the ΔR_{et} decreased with the decreasing electrode potential and the increasing standing time of the electrode potential (as shown in Figure S3). Considering the potential window of the GCE and the redox of water, the DNAs were released from the GCE-PAAM/PA/PDA/DNA at −1.0 V for 2 min.

Under the optimized experimental conditions, the DNA recovery efficiency was evaluated using T_M and/or T_W (wild type of T_M)-spiked solutions. The recovered ssDNAs were measured by a well-established DNA microarray-based RLS assay.52,53 The T_M and/or T_W standard solutions were employed to calibrate the RLS assay response to the corresponding ssDNAs concentrations. The recovery yield of the ssDNA (Y_{ssDNA}) is calculated by the following equation: Y_{ssDNA} = (C_{I}V_{I}) / (C_{0}V_{0}) × 100%; herein, C_{0} and V_{0} are the concentration and volume of the original ssDNA solution, while C_{I} and V_{I} are the concentration and volume of the recovered ssDNA solution, respectively. The C_{I} was evaluated from the calibration curve of the DNA microarray-based RLS assay (as shown in Figures S4 and S5). As shown in Figure 1, the Y_{ssDNA} slightly increased by increasing the concentration of the ssDNA from 0.1 to 50 pmol/L. In particular, the GCE-PAAM/PA/PDA was sensitive enough to extract 0.1 pmol/L of ssDNA with a high Y_{ssDNA} (>80% of the ssDNA in Tris buffer and > 75% of the ssDNA in Tris buffer plus 10% (v/v) blood plasma), suggesting that the GCE-PAAM/PA/PDA exhibits excellent recovery efficiencies of the ssDNA in different matrices. In addition, we investigated the effect of

Scheme 1. Schematic Representation of the Electric Field-Driven Strategy for Enriching DNAs from Different Media by the GCE-PAAM/PA/PDA

“The illustration is not drawn to scale.
the ssDNA strand length on the recovery efficiency of the ssDNA. As shown in Figure 2, the Y_{ssDNA} was gradually reduced by changing the ssDNA strand length from 78 to 28 NTs. Nonetheless, the data suggested that the GCE-PAAM/PA/PDA can be used to enrich the small fragmented DNA (i.e., 28 NTs) with a relatively high Y_{ssDNA} (>55% of the ssDNA in Tris buffer and >50% of the ssDNA in Tris buffer plus 10% (v/v) blood plasma). Efficient enrichment of the small fragmented DNA from blood plasma may offer an enhanced opportunity for identifying tumor type-related DNA mutations in bloodstreams because tumor-derived cfDNAs (i.e., ctDNAs) are normally presented as highly fragmented forms. Taken together, the experimental results demonstrate that the GCE-PAAM/PA/PDA has great potential for extraction of ctDNA in the clinical blood samples.

To evaluate the batch variation, GCEs modified with the PAAM/PA/PDA in three batches were used to enrich 1 nmol/L ssDNA-spiked Tris buffer (Tris) or Tris buffer plus 10% (v/v) blood plasma. The CV value of the GCE-PAAM/PA/PDA is calculated as 7.74%, which shows that the GCE-PAAM/PA/PDA has good reproducibility. After 7 days of storage at room temperature, the stability was investigated by the same assay, as shown in Figure S7, and the result indicated that the as-prepared GCE-PAAM/PA/PDA has good stability. Furthermore, the actual electrode area of the GCE-PAAM/PA/PDA has been calculated by the peak area of it before and after gel modifying. The actual electrode area can be calculated as 3.822 mm² (Figure S8).

**Enrichment of ctDNA from Plasma Samples of Clinical Patients.** For demonstrating its clinical utility, the GCE-PAAM/PA/PDA was employed to enrich the cfDNAs from blood plasma samples of PTC and TN patients. A 73 bp-amplicon flanking the nucleotide 1799 of exon 15 (BRAFV600 site) fragments was produced by the PCR amplification, while the enriched cfDNAs were used as templates. As shown in Figure 3a, the PCR products of enriched cfDNAs showed gel bands located in parallel alignment with a DNA ladder under 100 bp. Although cfDNAs were enriched from the same volume of blood plasma, the amounts of PCR products showed strongly individual dependence. The result indicates that the amount of cfDNA in blood plasma varies from patient to patient. A set of experiments were designed to determine the sensitivity of the GCE-PAAM/PA/PDA. As a typical example, the PCR products of enriched cfDNAs from various volumes of blood plasma were analyzed by gel electrophoresis (as shown in Figure 3b). The yield of the PCR gradually increased as the blood plasma concentration decreased, reached a maximum at 20% (v/v) blood plasma, and then decreased with the decreasing blood plasma concentration.

The phenomenon suggests that other components of blood plasma (e.g., negative-charged proteins) may affect the enrichment of cfDNA. In addition, the DNA fragments containing the BRAFV600 site could be clearly observed at as low as 20 μL of blood plasma in 980 μL of Tris buffer. This means that the detection of tumor-related genetic aberrations can be achieved by using 100 μL of peripheral blood, increasing its attractiveness for dynamic monitoring at different time points throughout the treatment regimen.
The levels of BRAFV600E mutation in cfDNAs of patients were analyzed by the Sanger sequencing method, which were further compared with the BRAFV600E mutation statuses in genomic DNAs of the correspondent tumor tissues. According to the facts that the short BRAFV6000E amplicon (73 bp) and the high accuracy for the back in the middle of the Sanger sequencing method, reverse sequencing was chosen to obtain accurate results (as shown in Figure 4). Currently, the analysis of genetic aberrations in tumor tissues is considered the golden standard for addressing tumor-related genetic mutations. In this case, the identification of the BRAFV600E mutation was assessed using blood plasma samples and the correspondent tumor tissues from five PTC patients and five TN patients (as shown in the Figure 5). As expected, the PTC patients showed a higher incidence of the BRAFV600E mutation than those of the TN patients (4/5 vs 1/5 of the genomic DNA). The genetic aberration analysis of PCR products from the GCE-PAAM/PA/PDA-enriched ctDNA identified a BRAFV600E mutation in 6 out of 10 cases, while the genetic aberration analysis of PCR products from genomic DNAs of PTC tissues identified a BRAFV600E mutation in 5 out of 10 cases.

The ctDNAs (cfDNAs with a BRAFV600E mutation) were matched well with the BRAFV600E mutation statuses of genomic DNA, except one TN sample. The result indicates that the GCE-PAAM/PA/PDA-enriched ctDNA test exhibits a slightly higher sensitivity than those of the genomic DNA test extracted from PTC tissues (6/10 vs 5/10). Furthermore, we evaluated the capability of the PAAM/PA/PDA-based electrochemical enrichment strategy by detecting BRAFV600E mutants in 10 blood plasma samples from randomly selected patients. Three of the 10 tested samples (30%) were detected positive of the BRAFV600E mutation (as shown in Table S3 and Figure S6). The phenomenon also suggests that the PAAM/PA/PDA-based electrochemical enrichment of the ctDNA strategy has great promising potential for the noninvasive and cost-effective detection of oncogenic mutations. In comparison with genomic DNAs, an additional mutation was detected in cfDNAs with a very high apparent frequency (approximately 75%, as shown in Figure 5 and Figure S6). This mutation was manifested by a T1799C (shows a G (guanine) mutation in the template strand), which caused the substitution of valine (V) with alanine (A) at amino acid residue 600. This BRAFV600A mutation was found in the cfDNAs from both cancer patients and noncancer patients. Because the available data is limited, we still could not figure out the correlation of the specific disease with the BRAFV600A mutation; further studies are expected for answering the important question.

**CONCLUSIONS**

In summary, the PAAM/PA/PDA hydrogel-modified GCE electrode, that is, the GCE-PAAM/PA/PDA has been successfully developed for the enrichment of ctDNA from blood plasma through electric field-mediated transition of the surface charges of the PAAM/PA/PDA hydrogel. Under optimal conditions, the GCE-PAAM/PA/PDA shows excellent enrichment efficiencies and recovery rates of various DNAs and can isolate and enrich ctDNA from as low as 20 μL of blood plasma. Although we focus on the BRAFV600E mutations in PTC at present, the GCE-PAAM/PA/PDA could be applied to detect different oncogenes in other types of malignant tumors, which is expected to accelerate the applications of ctDNA in clinical practices such as early diagnosis of cancer, guidance of patients’ treatment plans through routing analysis, and tumor prognosis.

**EXPERIMENTAL SECTION**

Fabrication of the PAAM/PA/PDA Hydrogel-Modified Glassy Carbon Electrode. The PAAM/PA/PDA hydrogel-modified GCE (GCE-PAAM/PA/PDA) was fabricated by our previously reported strategy (see the Supporting Information for details). Generally, the GCE (3 mm in diameter) with a mirror-like surface was activated by glutaraldehyde (GA, 0.5% wt/v). Five microliters of the mixture of acrylamide (AAM), phytic acid (PA), dopamine (DA), N,N′-methylenebis-acrylamide (MBA), potassium peroxodisulfate (KPS), and N,N,N′,N′-tetramethyldiamine (TEMED) were then pipetted onto the GA-modified electrode, placed at room temperature for 45 min, and gently rinsed by 5 mL of water (3 times). The surface of the as-prepared GCE-PAAM/PA/PDA was characterized by an XL30 ESEM-FEG scanning electron microscope (SEM) at an acceleration voltage of 20.0 kV (FEI Co., USA). The effects of PAAM/PA/PDA components, the incubation time of the GCE-PAAM/PA/PDA with the DNA-containing solution, and the electrode potential on the DNA enrichment were optimized through measuring the electron transfer resistance change of the GCE-PAAM/PA/PDA (ΔRet...
\[ R_{etDNA\text{adsorption}} - R_{etDNA\text{adsorption}} = \Delta R_{et} \text{, herein, } R_{etDNA\text{adsorption}}, R_{etDNA\text{adsorption}} \text{ are the electron transfer resistances of the GCE-PAAM/PA/PDA, GCE-PAAM/PA/PDA after DNA adsorption, and GCE-PAAM/PA/PDA/DNA after DNA desorption, respectively by electrochemical impedance spectroscopy (EIS, see the Supporting Information for details).}

**Patient Characteristics.** Ten blood plasma and 10 thyroid tissue samples were obtained from 10 patients who underwent thyroid surgery in the Department of Thyroid in the First Hospital of Jilin University, and patients’ informed consents were obtained before the surgery. Five patients underwent routine surgery for PTC, while five patients underwent subtotal thyroidectomy for benign thyroid nodules (TN). The tumors were diagnosed based on the latest classification of endocrine tumors of the World Health Organization (WHO). Other 10 blood plasma samples of random patients were obtained from the clinical laboratory of the First Hospital of Jilin University, and patients’ informed consents were obtained.

**Patient Blood Plasma and Tissue Sample Collection.** Patients’ blood samples (4 mL in volume) were collected in EDTA-K2 tubes and were processed within 30 min. The blood samples were treated by two-step centrifugation for removing all blood cells. After centrifugation (4000 rpm) at 4 °C for 10 min, the supernatants of the blood samples were collected and then centrifuged (12,000 rpm) at 4 °C for 10 min. The final supernatants were transferred into 1.5 mL Eppendorf tubes and treated by 50 μL of proteinase K (20 mg/mL), respectively. After incubation at 55 °C for 2 h, the mixtures were centrifuged (12,000 rpm) at 4 °C for 10 min. The supernatants were carefully collected and stored at −80 °C until further processing. The thyroid tissue samples were collected from surgical resection. After rinsing thoroughly by a physiological saline (0.9 wt% NaCl solution), the thyroid tissue specimens were cut into small pieces (approximately 1 mm3). Subsequently, the genomic DNA of the thyroid tissue specimens was extracted by commercial tissue genomic DNA extraction kits (Biotek Inc., Beijing, China) according to the manufacturer’s instruction.

**Enrichment of DNA by the GCE-PAAM/PA/PDA.** For enriching the DNA, the GCE-PAAM/PA/PDA was immersed in 1 mL of Tris buffer (10 mmol/L, pH 7.5) containing different amounts of synthetic ssDNAs or blood plasma samples of patients under stirring for 45 min, exposed to 1.0 V for 2 min, washed by 1 mL of Tris buffer (10 mmol/L, pH 7.5), transferred into 100 μL of a hybridization buffer (0.67 × SSC, 0.1% (w/v) SDS) or Tris buffer (10 mmol/L, pH 7.5), and exposed to −1.0 V for 2 min. For the mimic samples \( (T_{30}, T_{50}, 50 \text{ NT, and } 28 \text{ NT in the buffer or blood plasma, see Table S1 for details}), \) the recovered DNAs were quantified by our previously established DNA microarray-based resonance light scattering (RLS) assay (see the Supporting Information for details). The recovered DNAs from low concentrations (0.1 and 0.5 pmol/mL) of mimic samples were concentrated to 20 μL before quantitative measurement. For the blood plasma samples of patients, the enriched cfDNAs were used as templates for the PCR experiments (see the Supporting Information for details). The raw PCR products were directly analyzed by gel electrophoresis and sequenced by the traditional Sanger sequencing method in Sangon Ltd. Co. (Shanghai, China).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04397.

Additional experimental section including materials and reagents, GCE-PAAM/PA/PDA fabrication, ssDNAs@GNPs synthesis, microarray fabrication and DNA detection, and DNA sequences used in the experiment; figures including experimental condition optimization, SEM micrograph of the GCE-PAAM/PA/PDA surface, calibration curves, recovered ssDNA detection, and sequencing image details of 10 random cfDNA samples; tables of sequences of DNAs used in the experiment; and detailed information of patients (PDF)

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**Notes**

The authors declare no competing financial interest.

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