Diagnostic assay of carcinoembryonic antigen tumor markers using a fluorine immobilized biosensor with handmade voltammetric circuit

Suw Young Ly[a], Jung Eun Kim[a] and Woo Yeon Moon[b]

The tumor marker carcinoembryonic antigen (CEA) was investigated using a graphite pencil electrode (PE) and fluorine immobilized onto a graphite pencil carbon electrode (FPE). The optimum diagnostic conditions for square wave (SW) stripping voltammetry and cyclic voltammetry were searched. The voltammograms indicated three other detection ranges of 0.4–1.6 μg/l cyclic voltammetry (PE) seven points, 0.2–1.2 μg/l SW (PE) cathodic six points, and better sensitive ranges of 0.05–0.45 μg/l SW (FPE). These were obtained within a diagnostic accumulation time of 120 s in 0.1 mol/l ammonium phosphate electrolyte solution of pH 5.0. Under optimum SW conditions, the detection limit (S/N) approached 0.08 μg/l CEA, and the relative standard deviation at 10 mg/l CEA was 0.074% in 15 measurements. The proposed method can be applied in tumor assay using cancer patient urine. *European Journal of Cancer Prevention* **20:58–62 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The level of carcinoembryonic antigen (CEA) is elevated in many benign conditions such as collagen disease, chronic lung disease (Niklinski *et al.*, 1992), liver disease (Ldward *et al.*, 1967), pancreatic cancer (Ona *et al.*, 1973) and gastric cancer (Youji *et al.*, 2006), in which CEA tumor markers are widely found in patients' serum at low-concentration ranges. For medical diagnosis, sensitive CEA detection methods are needed. Some of these CEA diagnostic methods depend on DNA separation, polymerase chain reaction (PCR) amplification and other detection methods of photometric diagnostic systems (Shukla *et al.*, 2009), such as PCR (Tsavellas *et al.*, 2001), real-time PCR assay (Andrew *et al.*, 1997; Eva and Michael, 1998; Ronald and Satyajit, 2001), the surface plasmon resonance method (Marc *et al.*, 1998) and fluorescence immunocytology (Marieta *et al.*, 2004). Moreover, PCR-amplified DNA detection methods require electrophoresis gel separation and photometric fluorescence detection. Such methods have been shown to be useless for quantitative analysis. In addition, these methods are time consuming and technically expensive. Recently, however, there is an increasing demand for faster and simple analytical techniques. For these reasons, inexpensive and simple electrochemical methods have been pursued, such as immunoassay (Hao *et al.*, 2007), amperometric immunosensor (Xuelian *et al.*, 2006) and nanoparticle coated gold immunosensor (Yin *et al.*, 2007). However, a more sensitive and fast analytical method is needed. In this study, a sensitive electrochemical sensing technique (Suw, 2005; Suw and Yun, 2006) using a simple process (Suw, 2008) of CEA assay was developed using an FPE sensor. The FPE electrode was prepared by fluorine immobilization on a graphite carbon surface through voltammetry accumulation. The fluorine and carbon structure (-CF-) has specific properties, and the -CF- molecular surface selectively interacts with CEA, and is sensitive and stable. Moreover, the graphite surface can serve as a metal semiconductor, and it can perform electron transfer with CEA molecules, which makes the -CF- structure chemically stable (Weglikowska *et al.*, 2007) and electronegative (Matsumoto *et al.*, 2006). Thus, the fluorine-immobilized surface reacts sufficiently with the CEA molecule by anodic and cathodic accumulation. Here, a handmade sensor was interfaced to the voltammeter in the second version of our system. The circuits are optimized with stripping and cyclic voltammetry. The optimized conditions achieved low-detection limits, had a very short accumulation time and easy detection of the CEA molecule. The developed FPE sensor and electric systems can be used in tumor and other medical diagnosis.

Materials and methods

The voltammetric apparatus was developed using the new Bioelectronics-2 system, which is the second version of a system made at the authors' institution. The developed circuitry is a computerized handheld voltammetric system with a 3.0 V potential range, a 2 mA current range, a 10 pA measuring current and a $3'' \times 2'' \times 1''$

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compact size. It uses a rechargeable battery with a USB power source and has a USB port for data telecommunication with a PC. The instrument's size is similar to that of a typical cellular phone (Fig. 1a, inset) and it can be used for bioassay (Suw *et al.*, 2010), microorganism detection and sensor techniques for individual and laboratory applications. It can also be used with Bluetooth personal systems. The graphite pencil carbon (PE) working electrode was prepared using 0.5×10 mm diameter Hipolymer HB, and H and 3H grade pencil leads were used. The details of the pencil electrode have been described in earlier studies (Suw *et al.*, 2004). The FPE electrode was prepared using PE, where the PE carbon surface was polished using sand paper, then immersed in a standard fluorine solution (50% Fluka fluoroboric acid in water, BF4H; molecular weight 87.81) and dried using a hair dryer. The procedure was repeated three times. The FPE and target electroactive interaction were shown in earlier studies (Suw, 2008), and the FPE and CEA reaction is $-[C_x\text{-}(H\text{-}F)_n]^{n+} \leftrightarrow CEA^{+(-)}$. The FPE–CEA reaction sensitively responded in anodic cyclic voltammogram and cathodic stripping. The hand-made sensor was connected with 0.2 mm copper wire and the Bioelectronics-2 input circuits. An Ag/AgCl/KCl was used as a reference electrode and 0.2×5 mm platinum wire was used as the auxiliary electrode. All experiments were performed at room temperature, without removing oxygen. The electrolyte solution used was 0.1mol/l H_3PO_4 with a pH of 4.0, which was found to be the most suitable electrolyte solution. Hao *et al.* (2007) used N_2-saturated phosphate buffer (pH 7.4 $NH_4H_2PO_4$). Moreover the effect of the concentration of phosphoric acid was studied within the range of 0.01–0.3 mol/l. A concentration of 0.1 mol/l phosphoric acid was found to be the most suitable. First, the FPE surface was stabilized 10 times by cyclic scanning from 2.0 to −2.0 V at a 100 mV/s scan rate, whereas the other stripping voltammetry parameters were maintained at optimal conditions. All the experiments were performed in an open circuit. The CEA and chemicals used were of standard analytical grade.

Results and discussion

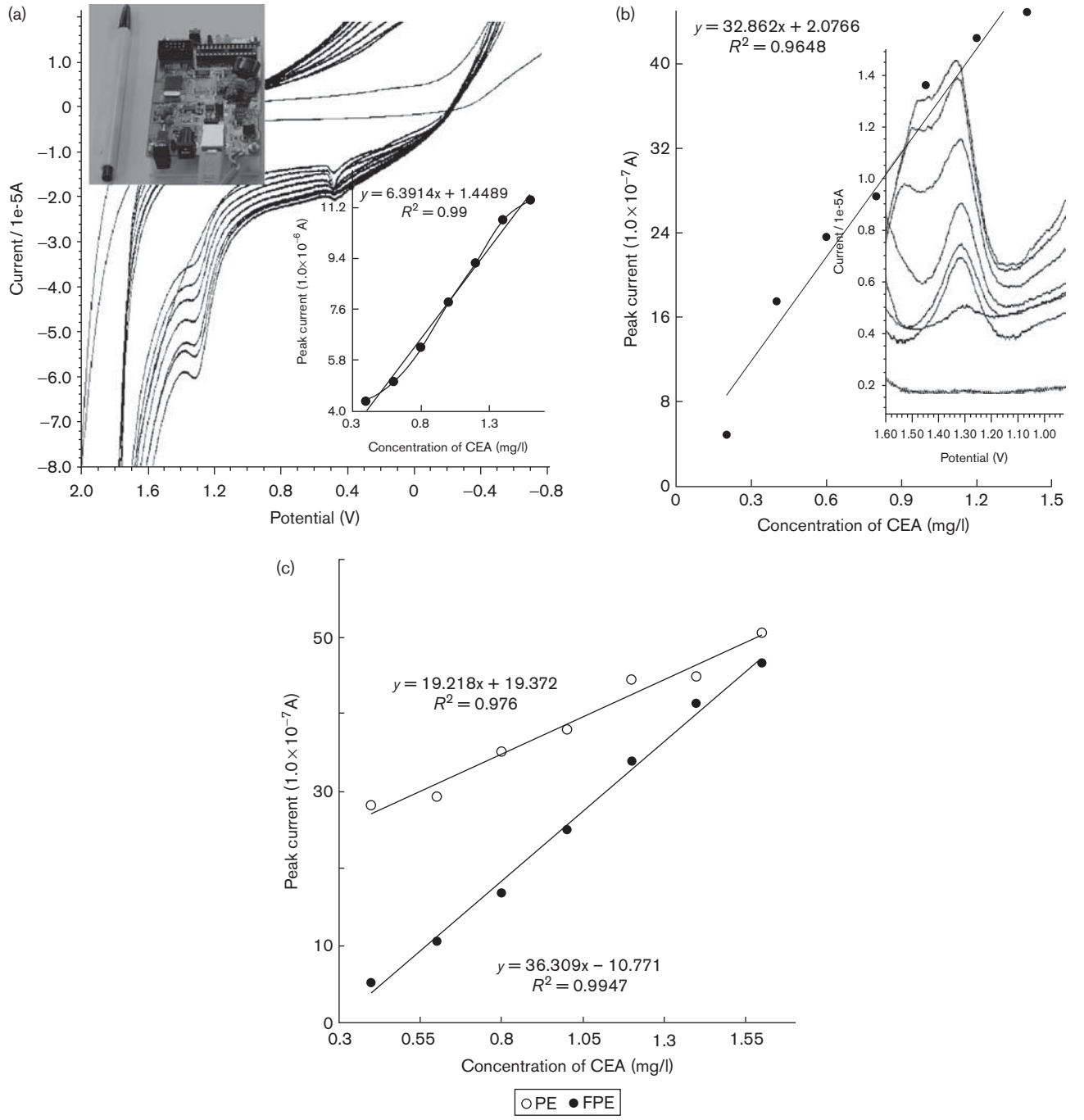
Voltammetry of carcinoembryonic antigen

In electrolyte conditions, the analytical peak potential was searched by PE with CEA variation from 0.4–1.6 μg/l spike using wide ranges for 2.0 and −2.0 V switching potential with a fast scan rate. Figure 1a shows the results, in which the electrolyte blank solution is simple and no peak appeared. Then 0.4 μg/l CEA spike, at the positive scan, 0.4 V (same peak potential with 40 ng/ml CEA containing cyclic voltammetry by Dian *et al.*, 2006; Yin *et al.*, 2007; Xiulan *et al.*, 2008) and 1.2 V peak potential appeared, thus spiking was continued. In these results, a linear equation was calibrated (insert curve) using 1.2 V peak potential, which was found to be sufficiently sensitive, then 0.4 V other potential appeared. Moreover, reverse scan appeared for 1.1 V peak potential, out of which 1.1 V was used with square wave (SW) accumulation techniques. Therefore, a more sensitive stripping method was examined. Figure 1b shows the CEA variation from 0.2 to 1.4 μg/l continuous spiking, PE was used with cathodic stripping, the peak appeared at 1.3 V and peak current increased from 4.87×10^{-7} to 44.83×10^{-7} A; the slope sensitivity was 32.86 and determination coefficient was $R^2 = 0.9648$. In this solution, anodic stripping was performed using the same conditions and only a 0.4 V peak potential was obtained. The 1.2 V peak was not obtained, and so the data are not shown here. In this condition, electrode sensitivity was compared using a common PE and specially prepared FPE sensors. Under the same electrolyte cell systems, both electrodes were placed and the CEA concentration was varied from 0.2 to 1.6 μg/l spike using anodic stripping. The calibration curves are shown in Fig. 1c. The linear peak current was obtained of 4.87×10^{-7}–50.5×10^{-7} A PE q and 5.0×10^{-7}–46.56×10^{-7} A FPE. In these curves, slope sensitivity was of PE = 19.21 and FPE = 36.31. Here, FPE is more sensitive than PE, and the working ranges are wide. Thus, more practical conditions were examined using stripping parameters. Moreover, both sensors were examined by cathodic stripping. However, less sensitive signals were detected when anodic stripping was performed.

Square wave stripping voltammetric optimization with FPE

As shown in Fig. 2a (-○-), the 1.0 μg/l CEA addition and electrolyte hydrogen ionic strength were varied by spiking with 0.1 mol/l HCl and 0.1 mol/l NaOH standard solution for 1.65, 1.79, 2.03, 2.29, 2.42, 4.94, 4.98, 5.59, 5.83, 6.01, 6.13, 6.34, 6.39, 6.77 and 7.12 pH ranges; the peak current was increased from 1.65×10^{-8} to 5.62×10^{-8} A, at pH 5.0. Maximum current appeared with a narrow peak width, then the peak current decreased to 1.12×10^{-8} A. The pH 5.0 was found to be sufficiently sensitive, so the setup was fixed at this pH. At this pH, SW accumulation time was examined within 30, 60, 90, 120, 150, 180, 5.0 210, 210, 240, 270 and 300 s variations. Peak results are shown in Fig. 2a (-●-), peak currents increased slowly from 5.15×10^{-8} to 9.923×10^{-8} A, then continuously decreased. Here, 150 s is very sensitive and a large current appeared so it was fixed at 150 s accumulation time and 5.0 pH. With these parameters, SW accumulation potential was searched by anodic scanning during −2.0, −1.9, −1.8, −1.7, −1.6, −1.5, −1.4, −1.3, −0.8, −0.4, −0.2, 0.0, 0.2, 0.4, 0.6 and 0.8 V variations. The results are shown in Fig. 2b (-●-), the first eight peaks were quickly increased from 5.15×10^{-8} to 8.65×10^{-8} A and slowly decreased to 1.3×10^{-8} A. At the final ranges, the peak current did not obtain any peak signals and disappeared. Here, the −1.3 V accumulation potential was large and a sharp peak width appeared. However, optimization of cathodic stripping was not pursued. At this potential, SW amplitude variation was examined. The results are shown in Fig. 2b (-○-). The variations were 0.001, 0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.04 and 0.05 V, peak current increased continuously from

Fig. 1



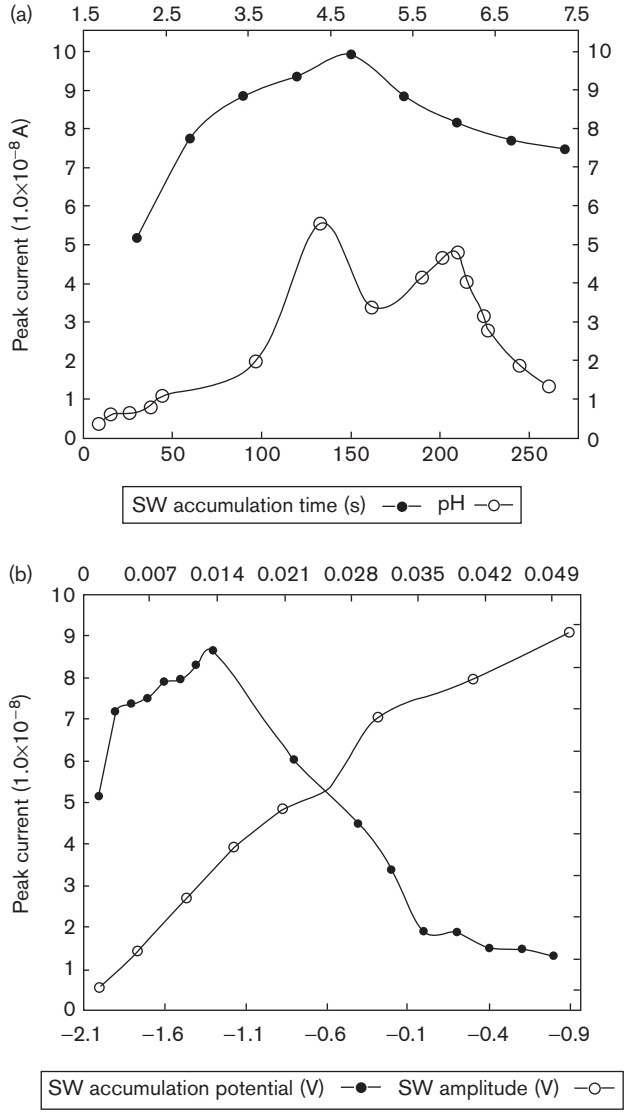
Concentration effects for carcinoembryonic antigen (CEA), (a): CEA variation for 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 μg/l spike by cyclic voltammetry with −2 initial, 2.0 V switching potential, 0.1 V/s scan rate using pencil electrode (PE). (b): CEA variation for 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 μg/l spike in cathodic square wave (SW) for PE. (c) Linear ranges of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 μg/l in anodic SW using the PE and FPE. In the 0.1 mol/l phosphate buffer electrolyte solution, the other parameters in Fig. 3 were held constant. 1e-5A, exponential; y, exponential.

0.45×10^{-8} to 8.75×10^{-8} A. Here, a sharp peak width of 0.025 V was obtained. In these conditions, other parameters of SW frequency and SW increment potential were examined, although the findings are not shown here. The final condition was fixed at 5.0 pH, 150 s accumulation time, −1.3 V anodic and 0.025 V amplitude. In this state, analytical statistics, working curve and analytical application were performed using standard CEA.

Working curves, statistics and application

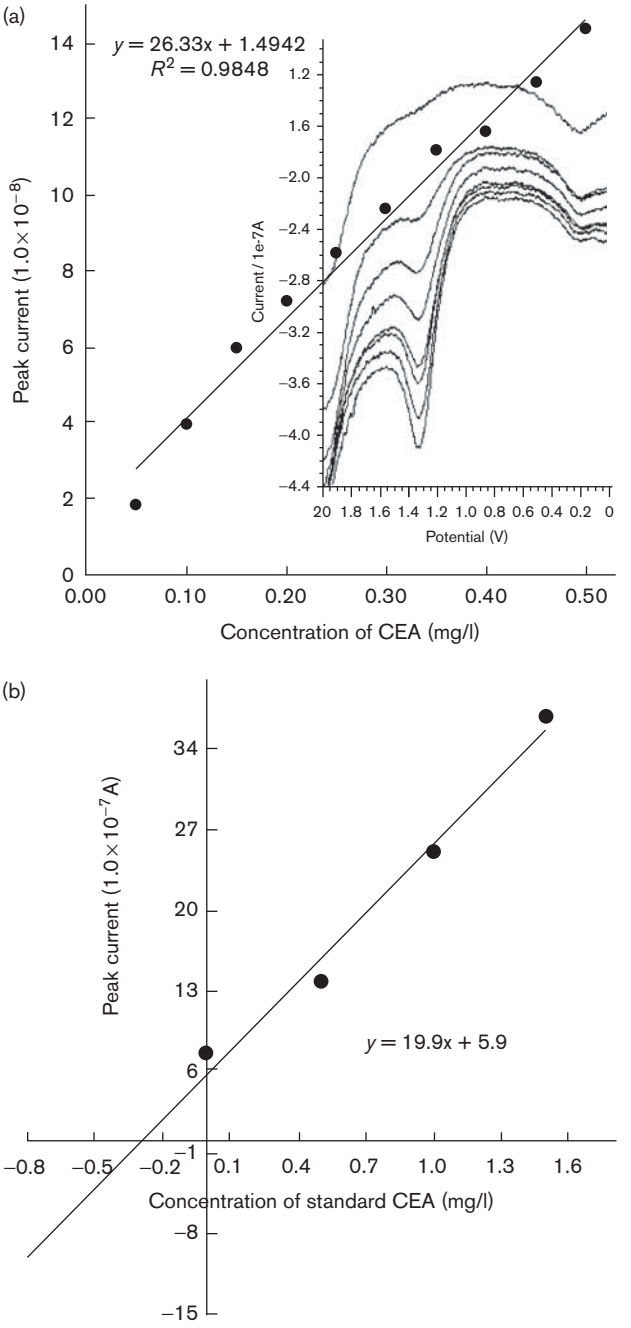
Under optimum SW conditions, the analytical regression equation was examined by anodic stripping; the working

Fig. 2



Square wave (SW) anodic stripping optimization. (a): electrolyte hydrogen ionic strength variation from 1.65 to 7.12 pH (-○-) and accumulation time variation from 30 to 300 s (-●-). (b): accumulation potential variation from −2.0 to 0.8 V anodic (-●-) and SW amplitude variation from 0.001 to 0.005 V (-○-) in 1.0 μg/l CEA, 0.1 mol/l ammonium phosphate buffer; the other experimental conditions were as in Fig. 3.

Fig. 3



Application. (a) Square wave (SW) carcinoembryonic antigen (CEA) variations of 0.0, 0.05, 0.10, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50 μg/l spike by 150 s accumulation with optimum conditions for −1.3 V initial potential, 2.0 V final potential, 0.004 V SW increment potential, 0.025 V amplitude and 15 Hz frequency in 5.0 pH electrolyte strength. (b) Application on cancer patients' urine using standard addition methods, first point is sample spike, and standard spike in optimized conditions of 0.1 mol/l ammonium phosphate buffer under 0.004 V SW increment potential, 0.025 V amplitude and 15 Hz frequency. le-7A, exponential; y, exponential.

ranges were 0.05–0.45 μg/l with eight points (Fig. 3a). The blank electrolyte and the final two points are not shown here. In this equation, sensitive working ranges were obtained using slope = 26.33, intersect of 1.49, and determination coefficients of $R^2 = 0.9848$. These results can be useful in patient diagnosis using any fluid or blood assay. Thus, an unknown patient's urine (a 45-year-old female from Asan Medical Center) was examined using low working curves. Figure 3b shows the standard addition methods. In this curve, the electrolyte blank solution was simple and no peak current appeared. Thus, patient urine (1.0 ml) was spiked and 0.412×10^{-6} A peak current was obtained. In these conditions, 0.5, 1 and 1.5 ml CEA standard was continuously spiked and linear

plots were obtained. In this equation, the slope was 19.9 and a current intersect of 5.9 was obtained. The final result of 0.66 μg/l ± 0.005 mg/l was obtained using the same solution; the fifth repetition ranges were 0.56–0.78 μg/l, which shows that the proposed method can be applicable to tumor assay.

Conclusion

Diagnostic PE and FPE handmade CEA tumor sensors were researched, in which the FPE sensor was found to be more sensitive than the PE electrode. The FPE sensor was used with the Bioelectronics-2 voltammetric system. The system was reconstructed based on an earlier version and can be applied to CEA detection. Analytical optimum conditions were pH 5.0 electrolyte solution, 150 s accumulation time, −1.3 V initial potential and 0.025 V amplitude. Under these conditions, diagnostic detection limits can approach in-vivo or in-vitro detection ranges. This proposed method features fast analysis, an inexpensive simple circuit and is handheld. The developed technique can be used in various tumor assays for biological serum or urine analysis.

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