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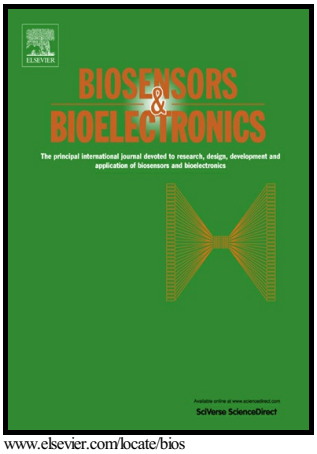

www.elsevier.com/locate/bios

PII: S0956-5663(16)30828-4
DOI: http://dx.doi.org/10.1016/j.bios.2016.08.071
Reference: BIOS9068

To appear in: *Biosensors and Bioelectronic*

Received date: 18 July 2016
Revised date: 19 August 2016
Accepted date: 20 August 2016

Cite this article as: Jinlong Li, Tao Gao, Shiyu Gu, Jun Zhi, Jie Yang and Genx li, An electrochemical biosensor for the assay of alpha-fetoprotein-L3 wit practical applications, *Biosensors and Bioelectronic* http://dx.doi.org/10.1016/j.bios.2016.08.071

An electrochemical biosensor for the assay of alpha-fetoprotein-L3 with practical applications

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Abstract

The detection of alpha-fetoprotein-L3 (AFP-L3) is of great importance for hepatocellular carcinoma (HCC) diagnosis, but remains unsatisfactory owing to poor sensitivity and complex operating steps. In this work, a simple and sensitive method has been proposed for the detection of AFP-L3. Firstly, biotinylated lens culinaris agglutinin-integrated silver nanoparticles (B-LCA-AgNPs) is fabricated. Owing to the specific interaction between lens culinaris agglutinin and AFP-L3, AFP-L3 can be detected directly through the electrochemical signal readout of AgNPs, avoiding separated steps used in clinical practice. Furthermore, after the recognition between B-LCA-AgNPs and AFP-L3, large amount of AgNPs can be gathered at the binding site through avidin-biotin interactions, which can amplify the signal. Therefore, detection of AFP-L3 can be sensitively achieved. Moreover, compared with the other approaches, this new method has a better linear correlation (25 pg/mL- 15000 pg/mL) and a lower detection limit (12 pg/mL). Also, the new method developed in this work has been demonstrated to have good stability and reproducibility for the assay of AFP-L3 in human serum samples, so, it may hold a great application prospect in clinical diagnostics.

Keywords: Electrochemical biosensor; AFP-L3; Lens culinaris agglutinin.

1. Introduction

[1]Authors contributed equally to this work.

Alpha-fetoprotein (AFP) is an important tumor marker that has been extensively employed for diagnosis of HCC. But the low specificity of AFP has been a cause of concern for use as a HCC marker. Studies have shown that AFPs are clarified into three subtypes, including LCA nonreactive AFP (AFP-L1), LCA-weakly reactive AFP (AFP-L2) and LCA-reactive AFP (AFP-L3) based on different affinities to lens culinaris agglutinin (LCA) (Taketa, 1991; Kumada et al., 2014). Among these categories, AFP-L3 is specific to malignant cancer cells, which makes it a potential biomarker for cancer diagnoses (Malaguarnera et al., 2010). In recent years, clinical studies have revealed that the serum level of AFP-L3 is significantly associated with clinicopathological features of HCC, such as tumor size, vascular invasion, proliferation, and metastasis (Kusaba, 1998; Xu et al., 2014; Yamashiki et al., 1999). Therefore, detetion of AFP-L3 is highly required in clinical diagnosis.

To date, the most commonly used method in clinical diagnosis is the affinity adsorption assay, which has shown many shortcomings in clinical practice. First, it contains complex operating steps for AFP-L3 separation (Gao et al., 2010). Second, the sensitivity of this method cannot be good enough, which make it difficult to perform when serum AFP-L3 level is low in the early development of HCC. Last, this method requires sophisticated instruments and trained personnel (Wang et al., 2008). Therefore, other techniques, including immune electrophoresis (Huang et al., 2014), affinity imprinting (Qian et al., 2001), affinity chromatography (Liu et al., 1994) and enzyme-linked immunoassay (Zhang et al., 1992), have been proposed; however they also have shown many disadvantages. Thus, the exploration of a simple, sensitive and costly-effective method is being actively pursued by investigators.

Electrochemical techniques with superb advantages of time-effectiveness, simplified operation steps and high sensitivity, have been widely used in DNA and protein analysis (Gao et al., 2015; Li et al., 2015; Zhao et al., 2014; Miao et al., 2011; Li et al., 2013). So, in this work, an electrochemical method has been proposed for direct and sensitive detection of AFP-L3. Specifically, GO and CS modified glassy carbon electrode (GCE) is used to fabricate a layer of glutaraldehyde (GA) to capture anti-AFP. Biotinylated LCA modified silver nanoparticles (AgNPs) have been

prepared as the recognition probe because of the specific affinity of LCA to the core fucosylation of AFP (AFP-L3). Thus, a biosensor has been fabricated based on the specific interaction between the biotinylated LCA and AFP-L3 at the electrode surface. In the presence of the avidin, large amount of AgNPs can be aggregated on the GCE through avidin-biotin interactions, resulting in enhanced electrochemical signals. Based on the design, this method may have highly improved performance over the present methods, showing great promise for application in clinical diagnosis.

2. Materials and Methods

2.1 Materials and regents

Mouse monoclonal anti-AFP was purchased from Abcam (Cambridge, MA, USA). AFP-L3 standard solutions and carcinoembryonic antigen (CEA) were supplied by Zhengzhou Antu Biological Engineering Co., Ltd. Recombinant Human golgiprotein73 (GP73) was purchased from Beijing Hotgen Biological Technology Co., Ltd. Trisodium citrate, $NaBH_4$, avidin, chitosan, silver nitrate, glutaraldehyde (25%), 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were all received from Sigma-Aldrich. Biotinylated Lens Culinaris Agglutinin (B-LCA) was purchased from Vector Laboratories. Graphene oxide (GO) was bought from Nanjing XF Nano Material Tech Co., Ltd. Serum samples were collected from consenting patients with HCC and healthy individuals. UV–vis absorption spectra were acquired on a TU-1901 UV-vis spectrometer (Beijing Purkinje General Instrument Co. Ltd). Electrochemical measurements were carried out on a CHI660D electrochemical workstation (CH Instruments, Austin, USA).

2.2. Preparation of B-LCA modified silver nanoparticle

AgNPs were prepared according to previous literature with slight modification (Gao et al., 2014). Briefly, 10 μL glutathione (10^{-6} mol/L) was added dropwise to the 1 mL silver colloidal solution and left to stir about 2 h in dark to ensure the self-assembly of the glutathione onto the surface of AgNPs. The mixture was

centrifuged at 14000 rpm for 20 min at room temperature and the mixture was redispersed in PBS for three times. Fresh prepared 20 μL EDC (20 mmol/L) and 20 μL NHS (50 mmol/L) were added into 1 mL above solution. After 15 min, 10 μL B-LCA solution (0.5 mg/mL) was added to the above solution and stored at 4°C overnight. The mixture was centrifuged at 14000 rpm for 20 min at room temperature for three times, and the resulting B-LCA-GSH-AgNPs conjugates were dispersed in PBS and stored at 4 °C before use.

2.3. Biosensor fabrication

The glassy carbon electrode (GCE, 3 mm in diameter) was polished to a mirror sheen using 1.0, 0.3, and 0.05 μm alumina slurry on a polishing pad followed by rinsing thoroughly with deionized water (Wang et al., 2014). After successive sonication in 1:1 nitric acid, ethanol, and deionized water, the electrode was dried under high purity nitrogen. 0.25 mg/mL GO and 0.05% chitosan were dropped onto the GCE surface in sequence (Lin et al., 2014; Yu et al., 2014). After electrochemical reduction of GO at -1.1 V in PBS (pH 8.0) for 400 s, the modified electrode was treated successively with 2.5% glutaraldehyde for 2 h, and with 0.1 mg/mL anti-AFP for 1 h and at 4 °C overnight in a total moisture-saturated environment. Rinse the fabricated biosensor with sufficient PBS (pH 7.4) before use.

2.4. Detection of AFP-L3

Samples containing AFP-L3 were incubated with the modified electrode at 37 °C for 30 min. After washing with 50 mM PBS (pH 7.4), the electrode was incubated with the above-prepared B-LCA-modified AgNPs in a total moisture-saturated environment for 1 h. B-LCA-modified AgNPs were removed by rinsing with PBS (pH 7.4) containing 0.05% (w/v) Tween-20. Then, 10 μL avidin (2.5μg/mL) was dropped onto the GCE surface at 37°C for half an hour. Then, the electrode was incubated with the above-prepared B-LCA-modified silver nanoparticles in a total moisture-saturated environment for 1 h. The modified electrode was finally ready for measurement.

2.5. Electrochemical measurement

All data was collected using a traditional three-electrode system. The modified

GCE, a platinum wire and a saturated calomel electrode (SCE) were employed as the working electrode, counter electrode and reference electrode, respectively. Electrochemical impedance spectra (EIS) was recorded in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 100 mM KCl. Square wave voltammograms (SWVs) were recorded in a mixture of 6 mL 10 mM Tris-HNO_3 buffer (pH7.4) and 100 mM KCl. The detailed experimental parameters were as follows. EIS: bias potential, 0.224 V vs. SCE; amplitude, 5 mV; frequency range, 0.1 Hz-10 kHz. SWV: scan range, -0.2 to 0.3 V; step potential, 5 mV; frequency, 15 Hz; amplitude, 25 mV. The data was obtained at least three independent repetitions of the experiment. Error bars are shown in the figures.

3. Results and discussion

3.1. Method design

Scheme 1 illustrates the principle of this method for electrochemical detection of AFP-L3. The anti-AFP is employed as the capture probe, while B-LCA-AgNPs is used as the recognition nanoprobe to specifically bind oligosaccharide moieties on the captured AFP-L3. For the preparation, the anti-AFP is first immobilized on a chitosan-doped GO substrate which is previously modified on the electrode surface to form a sensing layer with large activated surface area (Pumera, 2013; Lin et al., 2012). Then this prepared electrode is incubated with the test solution to capture AFP-L3 targets. After that, B-LCA-AgNPs are made to bind with the captured AFP-L3 molecules on the electrode surface via the specific lectin–oligosaccharide interaction. With the introduction of avidin, the nanoprobes are assembled through avidin-biotin interactions (Zhu et al., 2015; Iniya et al., 2014). These surface-bound $AgNP_S$ can then generate amplified signal readout through anodic strip (Huang et al., 2016; Tang et al., 2016; Geagea et al., 2015), and it is found that the obtained signal response is positively correlated to AFP-L3 abundance.

3.2. Characterization of B-LCA-GSH-AgNPs

UV-vis spectrophotometry has been used to validate the modification of B-LCA to AgNPs (Zhang et al., 2015). As shown in Fig. 1, compared with the UV-vis spectra of AgNPs (curve a) and GSH-AgNPs (curve b), a strong absorbance peak at 260 nm can

be detected for B-LCA-GSH-AgNPs, which shows an obvious change in the shape, position and symmetry of the absorption peak. The result demonstrates that B-LCA-GSH-AgNPs complexes have been successfully synthesized.

3.3. Characterization of the electrode modification

The stepwise modification of the electrode has been verified by EIS (Fig. 2) (Zhao et al., 2012; Xie et al., 2014). EIS of the bare electrode is nearly a straight line (curve a). After the modification of GO, an evident semicircle appears (curve b), indicating augmented resistance to interface electron transfer. Subsequent absorption of chitosan on GO further repelled $[Fe(CN)_6]^{3-/4-}$ in the solution, leading to a larger impedance (curve c). After assembly with anti-AFP, which is attached through glutaraldehyde linking, the diameter of the semicircle increases remarkably (curve d), because the association of glutaraldehyde with a protein may form an electron transfer barrier. Finally, a larger semicircle diameter (curve e) is observed, confirming the high resistance of the electrode interface as the prepared biosensor is blocked with AFP-L3, which indicates the successful fabrication of the biosensor.

3.4. Characterization of the biosensor

In Fig. 3A, compared the GCE modified with B-LCA-AgNPs/AFP-L3/anti-AFP (curve b) and the GCE modified with B-LCA-AgNPs/avidin/B-LCA-AgNPs/AFP-L3/ anti-AFP (curve c), it can be known that the latter one may exhibit significant increase in the reduction current. It can also be observed that the current response to the GCE electrode modified with B-LCA-AgNPs/avidin/B-LCA-AgNPs/AFP-L3/anti-AFP is about three times as large as that to the response for the electrode modified with B-LCA-AgNPs/AFP-L3/GCE.

Raman spectroscopy has also been used to characterize the modified electrodes prepared in this work (Xu et al., 2015). As is shown in Figure 3B, the B-LCA-AgNPs/avidin/B-LCA-AgNPs/AFP-L3/anti-AFP (Figure 3B-a) shows strong SERS signal, while the B-LCA-AgNPs/AFP-L3/anti-AFP has weak SERS response (Figure 3B-b). One reason is that a large amount of AgNPs have been introduced on the electrode surface through avidin-biotin interaction. Another is that large surface area of the sensing layer can immobilize more anti-AFP.

3.5. Condition optimization

Two important aspects involved in the assay have been examined. Firstly, incubation time is optimized for anti-AFP/AFP-L3 interaction. The amount of AFP-L3 captured from the solution depends on the incubation time before it reaches thermodynamic equilibrium. At 37°C, the SWV response is found to increase with elongation of incubation time and then reaches stabilization, and 40 min has been proved to be sufficient for completely binding (Fig. 4A). Secondly, incubation time is optimized for the amplification strategy in this assay. As shown by Fig. 4B, the DPV response increases with the elongation of incubation time, and the signal tends to be constant after 60 min (Fig. 4B). So, an incubation time of 60 min has been selected for the signal amplification step.

3.6. Electrochemical assay for AFP-L3 determination

Under the optimized conditions, the stripping peak current of AgNPs is observed to increase with increasing concentration of AFP-L3. A linear range can be established between 25 to 15000 pg/ml, with a limit of detection as low as 12 pg/ml. (Fig. 5), defined at a signal-to-noise ratio of 3:1 (S/N=3). The linear regression equation is $I=-2.262+1.82\lg c$ (pg/mL, $R^2=0.999$). By the way, it should be mentioned that the reason for the low detection limit is also due to the following two reasons, in addition to the above described signal amplification strategy. One is the excellent electrical conductivity of rGO, which may enhance the charge transfer. The other is the formation of CS film, which may increase the immobilization amount of anti-AFP by the GA cross-linker.

3.7. Specificity, reproducibility and stability of the biosensor

To further explore the specificity of the fabricated biosensor, we have challenged the biosensor with other interfere proteins, including AFP-L1, AFP-L2, CEA and GP73 proteins. As is shown in Fig. 6, high signal can be obtained only for the samples containing the target AFP-L3. The presence of interfering proteins does not cause significant change in the reduction current. So, the experimental results indicate that the biosensor can exhibit a good specificity for the determination of AFP-L3.

To evaluate the reproducibility of the biosensors, five electrodes have been

fabricated for the detection of the same sample containing 5 ng/mL AFP-L3. The relative standard deviations (R.S.D.) of both the intra- and inter-assay are all not more than 5%, so the reproducibility of the biosensor can be acceptable.

The stability of the developed biosensor has also been investigated in this study. When the biosensor is not in use, it is stored in pH 7.4 PBS at 4°C. After 5 and 10 days, the current response to the same AFP-L3 sample remains 90% and 86% of its initial value respectively, indicating that the as-proposed biosensor may display a satisfactory stability for the determination of AFP-L3.

3.8. Serum sample analysis

To further investigate the application potential of the biosensor in clinical practice, the amount of AFP-L3 in different serum samples have been measured. Firstly, AFP-L3 contents are measured 3 times for each serum sample to obtain the recovery. After 0.1, 0.5, 1, 5, 10 ng/mL AFP-L3 is separately added into the serum sample, the average recovery of the biosensor is calculated to be 96.4-102.6%, as is illustrated by Table 1. So, the recovery can be satisfactorily good. Secondly, the reliability of the proposed biosensor is studied by assaying six clinical serum samples containing AFP-L3, and the obtained results have been compared with those obtained by chemo-luminescence as a reference method. As shown in Table 2, the relative errors between the two methods are ranged from -3.20% to 3.34%, suggesting sufficient precision and high accuracy. So, the developed immunoassay methodology can be satisfactorily applied to the determination of AFP-L3 level in practical clinical samples.

4. Conclusions

In summary, we have successfully developed an immunoassay method for sensitive detection of AFP-L3 with electrochemical technique. This method is based on the assembly of B-LCA modified AgNPs on the surface of the prepared immune-electrode. Taking advantages of the prominent electrochemical activity of AgNPs and the amplification strategy via avidin-biotin interactions, this method has shown significant improvement in sensitivity, stability, and reproducibility. The detection limit for AFP-L3 can be pushed to 12 pg/mL, which is remarkably lower

than the currently used methods and the previous reports. So, this method may open a new way for the determination of AFP-L3, which enables low cost, effective and sensitive determination. Moreover, human serum sample analyses reveal the feasibility of the assay in real clinical samples, so it may have great potential application in the future.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (Grant No. 21235003), and the Science Foundation of Jiangsu Province, China (Grant No. BM2015023).

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Figures and Captions

Scheme 1. Schematic illustration shows the process for biosensor fabrication. (A) Preparation of B-LCA-AgNPs and (B) the fabricated biosensor for electrochemical assay.

Fig.1. UV-vis spectra of AgNPs (a), GSH-AgNPs (b), and B-LCA-GSH-AgNPs (c).

Fig.2. Electrochemical impedance spectra (EIS) of GCE (a), GO/GCE (b), chitosan/GO/GCE (c), anti-AFP/chitosan/GO/GCE (d) and AFP-L3/anti-AFP/chitosan/GO/GCE (e) in 100 mM KCl containing 5 mM $[Fe(CN)_6]^{3-/4-}$.

Fig.3 (A) SWV obtained at the modified electrodes of B-LCA-AgNPs/AFP-L3/ anti-AFP/GCE (a) and B-LCA-AgNPs/avidin/B-LCA-AgNPs/AFP-L3/ anti-AFP/GCE (b) in 100 mM KCl containing 10 mM Tris-HNO_3. AFP-L3 concentration is 5 ng/ml (B) Raman spectra of B-LCA-AgNPs/AFP-L3/anti-AFP /GCE (a) and B-LCA-AgNPs/avidin/ B-LCA-AgNPs/ AFP-L3/anti-AFP /GCE (b), excitation wavelength, $\lambda_{exc} = 514.5$ nm.

Fig.4. Effects of the incubation time for AFP-L3 (A) and B-LCA-AgNPs (B).

Fig.5. SWV obtained at the modified electrodes to quantify AFP-L3. Curves a-j corresponding to AFP-L3 concentrations of 25, 50, 100, 200, 500, 1000, 2000, 5000,

10000, 15000 pg/mL respectively. The inset is the calibration curve. Error bars indicate standard deviation of three independent experiments.

Fig.6. Specificity of the proposed electrochemical biosensor. 10 ng/mL AFP-L3 and 20 ng/mL other interfering proteins were introduced. Error bars indicate standard deviations of three independent experiments.

Table 1 Recovery experiment results of AFP-L3 in serum sample

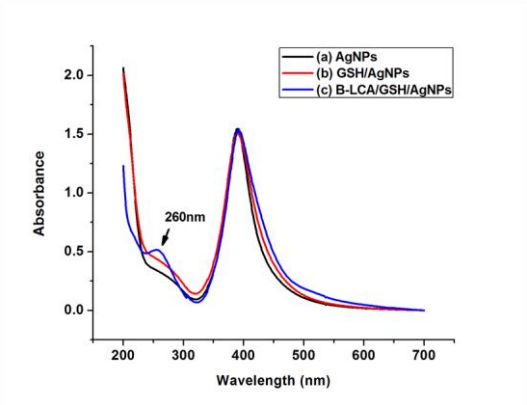
Samples	Added (ng / mL)	Found (ng / mL)	Recovery (%)
1	0.1	0.099	99
2	0.5	0.482	96.4
3	1	1.026	102.6
4	5	4.945	98.9
5	10	9.835	98.4

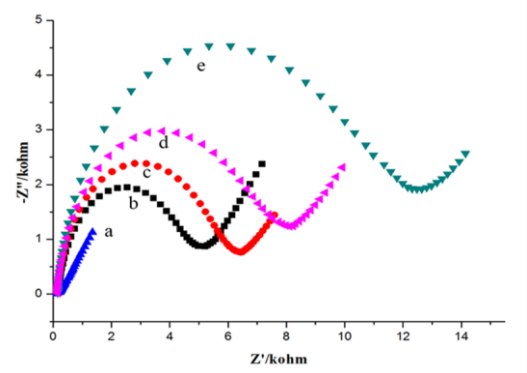
Table 2 Comparison of assay results in clinical serum samples using the proposed and reference methods.

Serum samples	Proposed biosensor	Affinity adsorption assay	Relative eviation
	(ng / ml, n=6)	(ng / ml)	(%)
1	2. 147	2. 096	2. 43
2	0. 929	0. 899	3. 34
3	7. 794	7. 819	−3. 2
4	0. 189	0. 183	3. 28
5	13. 72	14. 11	−2. 76
6	5. 292	5. 181	2. 14

Highlights

- A novel electrochemical method for AFP-L3detection is developed.
- Multifunctional silver nanoprobes have been synthesized.
- Simple and sensitive detection of AFP-L3 can be achieved.
- AFP-L3 is detected effectively in complex serum samples.
- This method may have potential applications in clinical practice.


(a) AgNPs
(b) GSH/AgNPs
(c) B-LCA/GSH/AgNPs
260nm
Absorbance
Wavelength (nm)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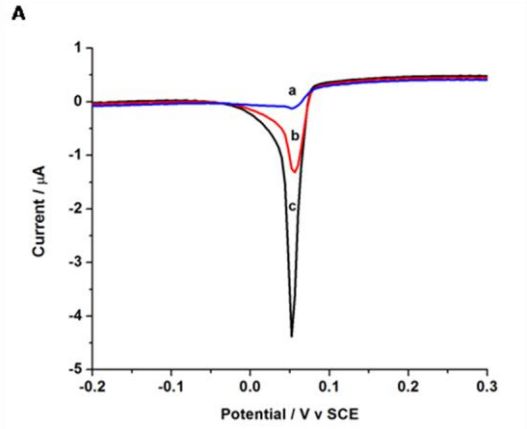
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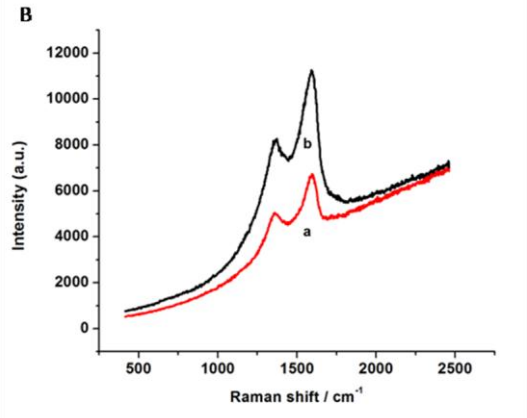
Potential / V v SCE
a
b
c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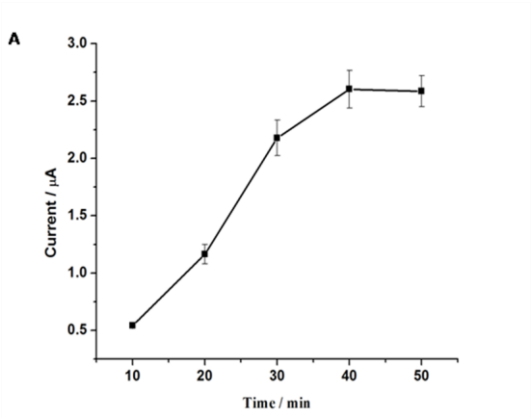
Intensity (a.u.)
Raman shift / cm-1
a
b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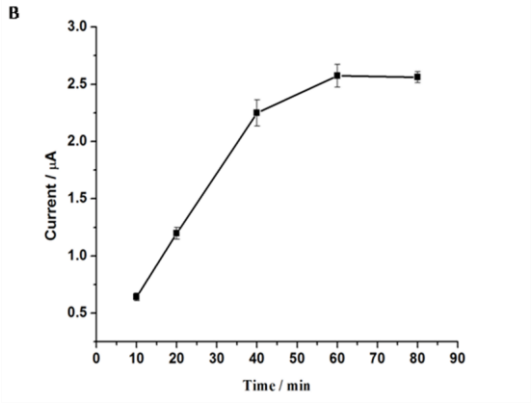
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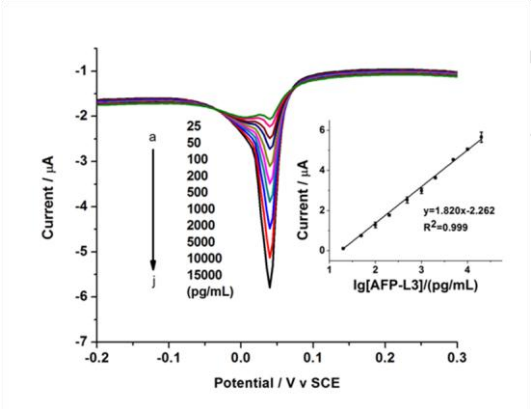
Current / μA
Time / min


a
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25
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100
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500
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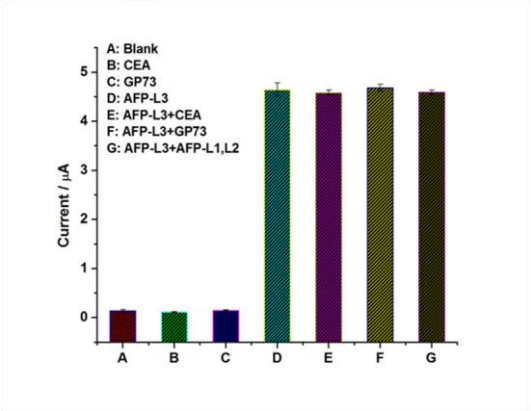
2000
5000
10000
15000
(pg/mL)
Current / μA
y=1.820x-2.262
R²=0.999
lg[AFP-L3]/(pg/mL)
Potential / V v SCE


A: Blank
B: CEA
C: GP73
D: AFP-L3
E: AFP-L3+CEA
F: AFP-L3+GP73
G: AFP-L3+AFP-L1,L2
Current / μA

Scheme