

2018-04-28

A Label-free Immunosensor Based on $K_3[Fe(CN)_6]$ and Aldehyde-Functionalized Pyridinium

Xia HU

You-ming SHEN

Guang-yu SHEN

School of Materials Engineering, Shanghai University of Engineering Science, Shanghai 201620,;
sgyrab@163.com

Recommended Citation

Xia HU, You-ming SHEN, Guang-yu SHEN. A Label-free Immunosensor Based on $K_3[Fe(CN)_6]$ and Aldehyde-Functionalized Pyridinium[J]. *Journal of Electrochemistry*, 2018 , 24(2): 154-159.

DOI: 10.13208/j.electrochem.170406

Available at: <https://jelectrochem.xmu.edu.cn/journal/vol24/iss2/7>

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DOI: 10.13208/j.electrochem.170406

Artical ID:1006-3471(2018)02-0154-06

Cite this: *J. Electrochem.* 2018, 24(2): 154-159

Http://electrochem.xmu.edu.cn

基于铁氰化钾电子媒介体及醛基吡啶盐的电化学免疫传感器

胡 霞,申有名,沈广宇*

(湖南文理学院化学与材料工程学院, 湖南 常德 415000)

摘要: 采用聚二烯丙基二甲基氯化铵(PDDA)将铁氰化钾电子媒介体固定在电极表面,构建无标记的电化学免疫传感器. 醛基吡啶盐不仅作为基底物质直接固定抗体, 还可以很好地增强电极表面的导电性能. 将构建的传感器用于肿瘤标志物甲胎蛋白的检测,其线性范围为 $0.01 \sim 20 \text{ ng} \cdot \text{mL}^{-1}$, 检测下限为 $0.004 \text{ ng} \cdot \text{mL}^{-1}$ (3S/N). 此传感器的构建简单方便、无标记、特异性好,为甲胎蛋白及其他肿瘤标志物提供了新的检测方法.

关键词: 电化学免疫传感器; $\text{K}_3[\text{Fe}(\text{CN})_6]$; 醛基吡啶盐; 甲胎蛋白

中图分类号: O646;O657.1

文献标识码: A

电化学免疫传感器因其灵敏度高、简单易行、检测快速、微型化和价格低廉深受欢迎^[1-2]. 通常,大多数免疫传感器是基于标记抗体或抗原,或将可逆氧化还原物质溶解于缓冲溶液中实现对分析物检测^[3-5]. 然而,标记过程较费时,步骤繁琐,成本较高,并容易引起生物分子的变性^[6-7],溶解在缓冲溶液中的氧化还原物质可能污染生物分子并降低其生物活性. 为了克服这些不足,人们将电子媒介体如二茂铁、硫堇、铁氰化钾固定于电极表面构建无标记的电化学免疫传感器^[8-11]. 其中,铁氰化钾具有很好的氧化还原性能,且价格便宜,更为广泛地应用于构建无标记的电化学传感器. 铁氰化钾固定于电极表面一般是通过静电作用,如 Weng 等^[11]用壳聚糖将铁氰化钾固定于电极表面,再用传统的交联试剂戊二醛固定抗体,实现抗原的检测. 壳聚糖溶液粘度较高,成膜厚度不易控制;而且不能直接固定抗体,需要戊二醛“搭桥”. 但戊二醛容易相互交联,降低了抗体的固定效果,因此寻找一种新的固定电子媒介体和抗体的方法十分必要.

聚二烯丙基二甲基氯化铵(PDDA)是极好的阳离子有机高分子导电共聚物,其电荷密度范围大,阳离子单体占共聚物的摩尔分数范围 10%~58%,能很好地吸附 $\text{K}_3[\text{Fe}(\text{CN})_6]$,且其成膜性能

好,便于修饰电极表面,已有很多构建电化学免疫传感器的报道^[12-14]. 醛基功能化的吡啶盐(PyCHO)具有季铵盐结构,带有正电荷,可以通过静电作用与 Nafion 结合,其混合物可以滴加在电极表面形成一层修饰膜,其醛基可以直接固定抗体,不需任何交联试剂,简化了抗体固定过程.

本文以 $\text{K}_3[\text{Fe}(\text{CN})_6]$ 为电子媒介、醛基吡啶盐为基底物固定抗体,构建了无标记的电化学免疫传感器. 首先在电极表面修饰 PDDA- $\text{K}_3[\text{Fe}(\text{CN})_6]$ 膜,然后再修饰 PyCHO/Nafion 膜,通过层层组装的方法将 $\text{K}_3[\text{Fe}(\text{CN})_6]$ 和 PyCHO 固定在电极表面. 将构建的电化学免疫传感器用于肿瘤标志物甲胎蛋白的检测,结果满意. 此传感器的制备及检测过程简单、方便,选择性好,为甲胎蛋白及其他肿瘤标志物的检测及研究提供了新平台.

1 实 验

1.1 试剂与仪器

甲胎蛋白抗原(AFP)、anti-AFP 抗体购自上海领潮生物科技有限公司;人免疫球蛋白(IgG)、牛血清蛋白(BSA)购自北京鼎国生物技术有限责任公司;4-醛基吡啶、二氯甲烷、碘甲烷、全氟磺酸(Nafion, 5%, v/v)、聚二烯丙基二甲基氯化铵(PDDA, $M_w = 200000 \sim 350000$)购自 Sigma 公司. 磷酸缓冲

溶液(PBS, pH 7.0)由 $0.1 \text{ mol} \cdot \text{L}^{-1}$ 的 Na_2HPO_4 和 $0.1 \text{ mol} \cdot \text{L}^{-1}$ 的 KH_2PO_4 溶液按一定比例配制。

CHI 660D 电化学工作站 (上海辰华仪器有限公司)。

1.2 醛基吡啶盐的制备

根据文献^[15]制备醛基吡啶盐。5 mL (56.9 mmol) 的醛基吡啶加入到 20 mL 的二氯甲烷中, 再向此混合溶液中加入 7 mL (112.4 mmol) 的碘甲烷, 搅拌 2 天后, 过滤, 得到黄色的醛基吡啶盐(PyCHO)。

1.3 膜溶液的准备

将 $50 \mu\text{L}$ ($10 \text{ mmol} \cdot \text{L}^{-1}$) 的 $\text{K}_3[\text{Fe}(\text{CN})_6]$ 加入到 $100 \mu\text{L}$ (0.25wt%) PDPA 溶液中, 超声 5 min, 即得 PDPA- $\text{K}_3[\text{Fe}(\text{CN})_6]$ 溶液 (PDPA-KFC solution)。1 mg 醛基吡啶盐加入到 0.5 mL Nafion 溶液 (0.25%, v/v) 中, 超声 5 min, 即得 Nafion-PyCHO 溶液 (Nafion-PyCHO solution)。

1.4 电化学免疫传感器的制备

玻碳电极 GCE (直径为 3 mm) 依次在直径为 $0.3 \mu\text{m}$ 和 $0.05 \mu\text{m}$ 的 Al_2O_3 抛光粉上抛光成镜面, 在蒸馏水中超声清洗 5 min, 室温条件下自然晾干。取 $10 \mu\text{L}$ PDPA-KFC 溶液滴加在干净的电极表面, 晾干, 形成 PDPA-KFC 膜。取 $10 \mu\text{L}$ Nafion-PyCHO 溶液滴加在 PDPA-KFC 膜上, 晾干, 形成 PDPA-KFC/Nafion-PyCHO 膜。 $10 \mu\text{L}$ ($80 \mu\text{g} \cdot \text{mL}^{-1}$) 的抗体溶液滴加在 PDPA-KFC/Nafion-PyCHO 膜上, 37°C 下温育 30 min 后, 用蒸馏水洗涤除去未结合的抗体; $10 \mu\text{L}$ 的 BSA (2.0wt%) 滴加到被抗体修饰的电极上, 37°C 下温育 30 min, 除去非特异性活性位点。最后, 将 $10 \mu\text{L}$ 不同浓度的 AFP 滴加到电极表面, 37°C 下温育 40 min 后, 采用差示脉冲伏安法 (DPV) 检测电信号。传感器的构建过程如图 1 所示。

1.5 电化学测试

电化学实验采用三电极系统, 工作电极为修饰的玻碳电极, 参比电极为甘汞电极, 辅助电极为铂电极。循环伏安法 (CV) 和差示脉冲伏安法 (DPV) 测量均在 PBS 缓冲溶液 (pH 7.0) 中进行。循环伏安法电位扫描范围为 $-0.2 \sim 0.6 \text{ V}$, 扫速为 $100 \text{ mV} \cdot \text{s}^{-1}$, 差分脉冲伏安法电位扫描范围在 $-0.1 \sim 0.4 \text{ V}$, 脉冲幅度为 50 mV , 脉冲宽度为 50 ms , 并在优化实验条件下运用该法进行线性范围测定、样品测定。阻抗测量在 $5 \text{ mmol} \cdot \text{L}^{-1} [\text{Fe}(\text{CN})_6]^{3/4-}$ 溶液中进行, 其频率范围为 $10^{-1} \sim 10^5 \text{ Hz}$ 。

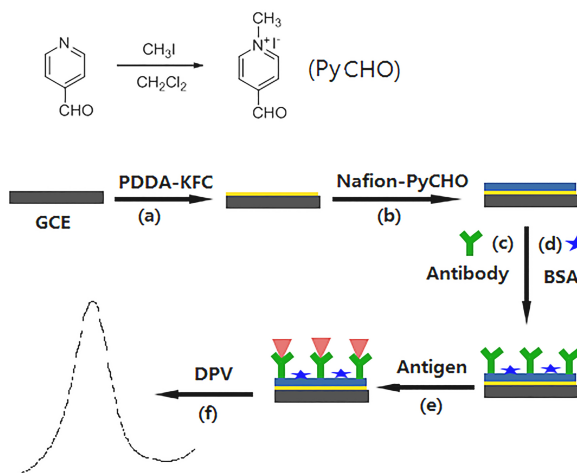


图 1 电化学免疫传感器的构建机理图

Fig. 1 Schematic diagrams showing preparation of the electrochemical immunosensor

2 结果与讨论

2.1 电极修饰过程表征

为表征电极修饰过程, 将电极插入 PBS (pH 7.0) 缓冲溶液中测量循环伏安曲线 (图 2)。电极表面修饰 PDPA 膜后, 曲线 a 的峰电流很小, 没有氧化还原峰。裸电极修饰 PDPA-KFC 膜后, 曲线 b 的峰电流值增加很多, 而且出现了氧化还原峰, 说明 $\text{K}_3[\text{Fe}(\text{CN})_6]$ 被固定到电极表面, 是一种很好的电子媒介体。在 PDPA-KFC 膜上修饰 Nafion-PyCHO 后, 曲线 c 的峰电流继续增加, 这是由于 PyCHO 具有季铵盐结构, 提高了电极表面的导电性。随后逐步固定抗体 (曲线 d)、BSA (曲线 e)、抗原后, 峰电流依次下降, 这是由于抗体、BSA 和抗原作为非导电性物质阻碍了电子的传递。

2.2 实验条件的优化

PBS 缓冲液的 pH 值是影响检测信号的重要因素, 碱性或强酸性都会导致抗体或抗原的生物活性降低。通过考察 pH 在 $5.0 \sim 8.0$ 范围内对差示脉冲伏安曲线 (DPV) 峰电流值的影响, 图 3A 表明峰电流值在 $5.0 \sim 7.0$ 范围随 pH 值的增加而降低, pH 值等于 7.0 时, 峰电流最小; pH 值大于 7 后, 峰电流反而增加。因此, PBS 溶液的 pH 值选择 7.0。抗体浓度是对检测信号产生重要因素。结果发现 (图 3B), 当抗体的浓度从 $20 \mu\text{g} \cdot \text{mL}^{-1}$ 逐步增加到 $80 \mu\text{g} \cdot \text{mL}^{-1}$ 时, 峰电流依次下降至最低, 高于 $80 \mu\text{g} \cdot \text{mL}^{-1}$ 时, 峰电流基本不变。因此, 抗体的浓度选择 $80 \mu\text{g} \cdot \text{mL}^{-1}$ 。在抗体浓度为 $80 \mu\text{g} \cdot \text{mL}^{-1}$

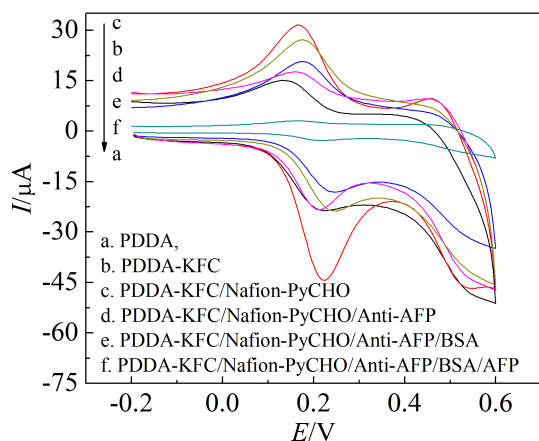


图 2 修饰电极的循环伏安曲线图 (AFP 的浓度是 $10 \text{ ng} \cdot \text{mL}^{-1}$)

Fig. 2 CV profiles of the different modified electrodes (The concentration of AFP was $10 \text{ ng} \cdot \text{mL}^{-1}$)

条件下,作者考查了抗体的固定时间.随着固定时

间的增加,氧化峰电流值逐渐下降,说明抗原与抗体的反应不断加强,反应时间超过 30 min ,曲线趋于平缓,抗原-抗体反应已基本达到平衡,故实验过程中选择抗体固定时间为 30 min (图 3C).

2.3 AFP 的检测

在优化的实验条件下,将不同浓度的 AFP 溶液滴加到被抗体修饰的电极上,反应 40 min 后,在 $\text{PBS}(0.1 \text{ mol} \cdot \text{L}^{-1}, \text{pH} = 7.0)$ 中测定 DPV 响应电流,图 4 表明电流响应随 AFP 浓度的增加而不断降低,在 $0.01 \sim 20 \text{ ng} \cdot \text{mL}^{-1}$ 范围内与电流响应呈良好的线性关系,其线性方程为 $y = 22.42 - 3.23x$,相关系数为 0.9943 ,检测下限为 $0.004 \text{ ng} \cdot \text{mL}^{-1}$ ($3S/N$). 此检测下限比文献报道的 $3 \text{ ng} \cdot \text{mL}^{-1}$ ^[16]、 $2.4 \text{ ng} \cdot \text{mL}^{-1}$ ^[17]、 $1 \text{ ng} \cdot \text{mL}^{-1}$ ^[18]、 $0.45 \text{ ng} \cdot \text{mL}^{-1}$ ^[19] 和 $0.06 \text{ ng} \cdot \text{mL}^{-1}$ ^[20] 低.

2.4 免疫传感器的特异性、重现性和稳定性

将非特异性蛋白质人免疫球蛋白 (IgG, 100

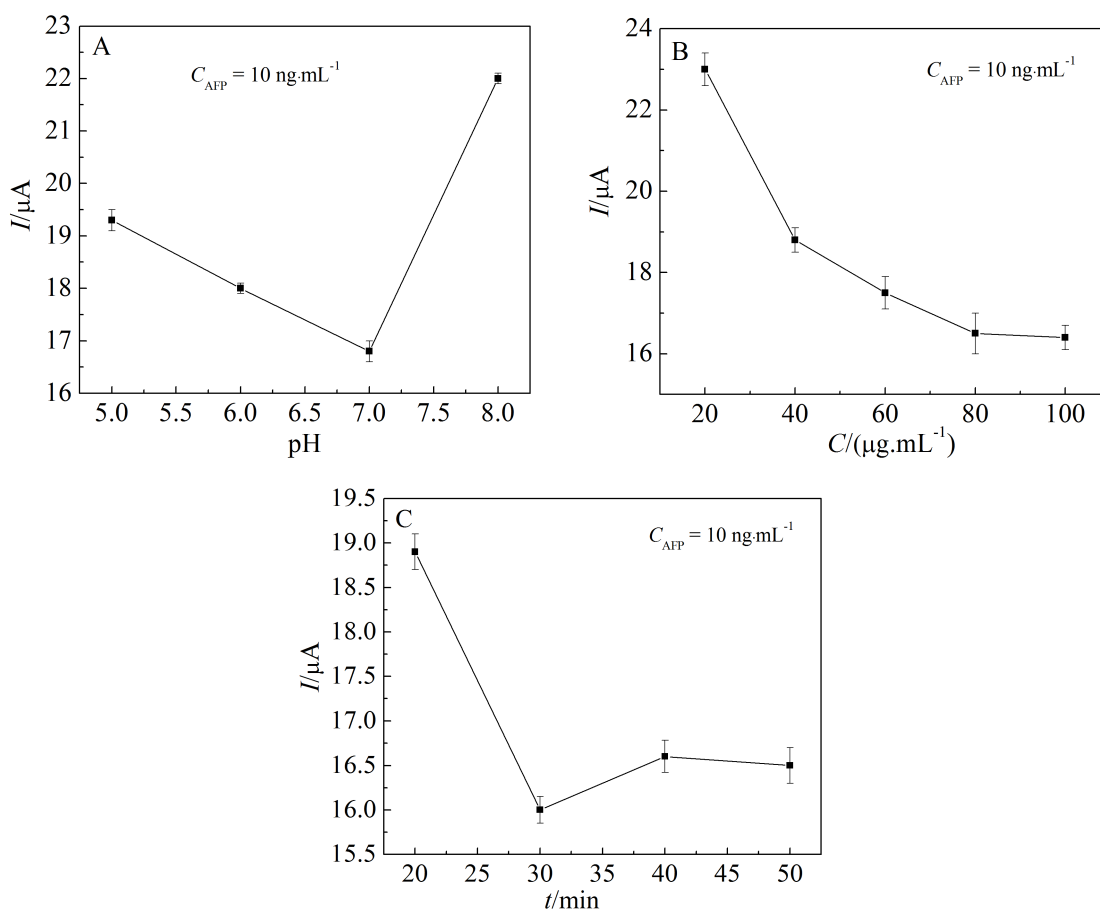


图 3 A. pH 值对峰电流的影响;B. 抗体浓度对峰电流的影响;C. 抗体固定时间对峰电流的影响

Fig. 3 Effects of pH (A), antibody concentration (B) and reaction time of antibody with PyCHO (C) on the peak current of immunosensor

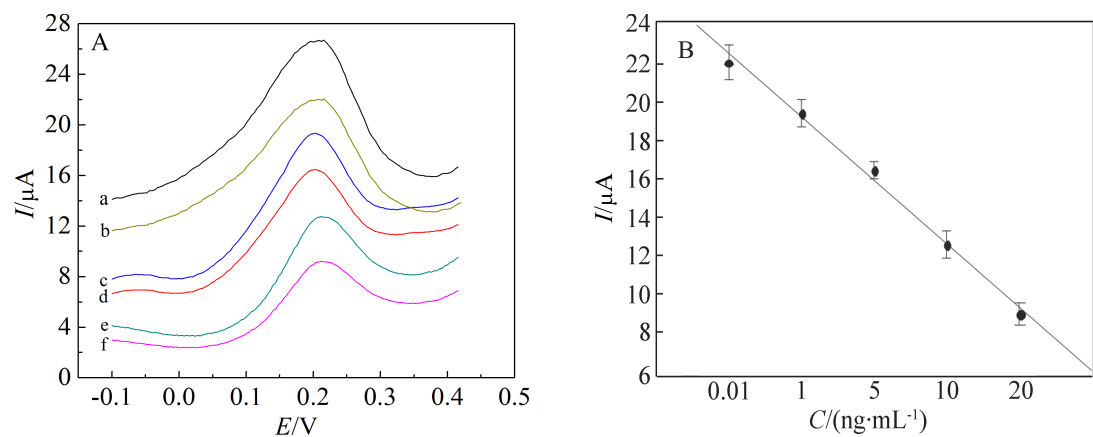


图 4 A. 免疫传感器对不同浓度的 AFP 的电信号响应 (从 a 到 f 的浓度分别为 0, 0.01, 1, 5, 10, 20 $\text{ng}\cdot\text{mL}^{-1}$); B. 校正曲线 ($n = 3$)

Fig. 4 A. DPV responses of the immunosensor to different concentrations of AFP from a to f: 0, 0.01, 1, 5, 10, 20 $\text{ng}\cdot\text{mL}^{-1}$; B. Calibration curve of the immunosensor to different concentrations of AFP ($n = 3$)

$\text{ng}\cdot\text{mL}^{-1}$)、绒毛膜促性腺素 (HCG, $100\text{ ng}\cdot\text{mL}^{-1}$)、前列腺特异性抗原 (PSA, $100\text{ ng}\cdot\text{mL}^{-1}$) $10\text{ }\mu\text{L}$ 分别与构建的免疫传感器作用 40 min 后,检测 DPV 信号. 图 5 表明, 其峰电流的大小与空白溶液比较, 没有显著差异; 3 种非特异性蛋白质与 $20\text{ ng}\cdot\text{mL}^{-1}$ AFP 混合溶液的峰电流与单独 AFP 溶液的峰电流值也没有显著差异. 此结果说明了传感器的选择

性能良好. 采用批间和批内分析方法考察传感器的重现性, 批间和批内的变异系数分别为 4.7% 和 5.3% ($n = 5$), 表明此传感器的重现性良好. 传感器不使用的時候, 应在 $4\text{ }^{\circ}\text{C}$ 温度下保存. 3 个星期后再检测 DPV 信号, 结果发现其峰电流值仍是初始值的 94.1% , 表明此传感器的稳定性良好.

2.5 实际样品的检测

用上述免疫传感器测定人血清样品, 并将测定结果与经典的酶联免疫分析方法 (ELISA) 的测定结果对比, 结果见表 1, 说明本法与 ELISA 方法有很好的 consistency, 用本法测定血清中 AFP 的浓度, 准确可靠.

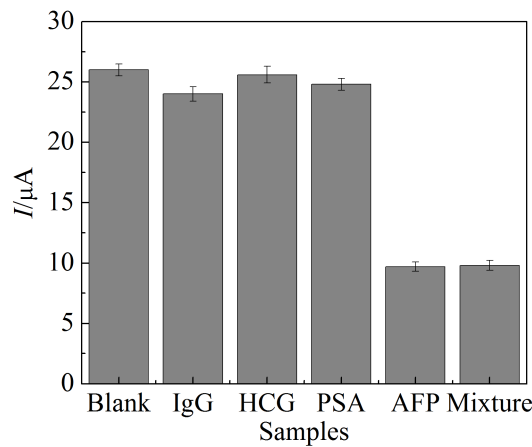


图 5 免疫传感器对空白溶液、IgG、HCG、PSA、AFP 溶液及它们的混合溶液的峰电流响应. IgG、HCG、PSA 的浓度为 $100\text{ ng}\cdot\text{mL}^{-1}$, AFP 的浓度为 $20\text{ ng}\cdot\text{mL}^{-1}$

Fig. 5 A comparison in peak currents of the immunosensor obtained in blank solution, IgG, HCG, PSA, AFP, and solution containing the mixture of IgG, HCG, PSA, and AFP. The concentrations of IgG, HCG, PSA were $100\text{ ng}\cdot\text{mL}^{-1}$, and the concentration of AFP was $20\text{ ng}\cdot\text{mL}^{-1}$

表 1 两种不同方法检测实际样品的结果比较
Tab. 1 Real sample analysis results and comparison with ELISA method

Sample	ELISA/ ($\text{ng}\cdot\text{mL}^{-1}$)	This method/ ($\text{ng}\cdot\text{mL}^{-1}$)	Relative deviation/%
1	1.22	1.16	-4.9
2	6.38	5.97	-6.4
3	11.74	12.03	2.5

3 结 论

采用 PDDA 成功地将 $\text{K}_3[\text{Fe}(\text{CN})_6]$ 固定在电极表面, 构建了性能良好的无标记的免疫传感器. 醛基吡啶盐可直接固定抗体, 不需要其他交联试剂,

为抗体的固定提供了一种更为便捷的方法,而且醛基吡啶盐能增强修饰膜的导电性能,是构建电化学传感器很有前景的材料.该免疫传感器制作简单,使用方便,用于实际样品的检测,结果满意.

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A Label-free Immunosensor Based on $K_3[Fe(CN)_6]$ and Aldehyde-Functionalized Pyridinium

HU Xia, SHEN You-ming, SHEN Guang-yu*

(College of Chemistry and Material Engineering, Hunan University of Arts and Science,
Changde 415000, Hunan, China)

Abstract: A novel aldehyde-functionalized pyridinium was successfully synthesized. Using $K_3[Fe(CN)_6]$ as a redox species modified on the surface of the electrode through Poly(diallyldimethylammonium chloride), we developed a label-free immunosensor based on aldehyde-functionalized pyridinium that was applied to simplify antibody immobilization on the electrode surface, in which no additional chemical cross-linking was required. In addition, the introduction of $K_3[Fe(CN)_6]$ on the surface of electrode avoided the use of other redox species in the electrolyte solution. Under the optimal experimental conditions, differential pulse voltammetry (DPV) signals corresponding to different α -Fetoprotein (AFP) concentrations were measured. After antigens with different concentrations reacted with antibodies immobilized on the electrode surface, the peak current of DPV gradually decreased with increasing concentrations of AFP. The calibration curve showed a good linear relation in the range from 0.01 to 20 $ng \cdot mL^{-1}$ with the linear regression equation of $y = -3.23x + 22.42$ ($R^2 = 0.9943$). The detection limit was 0.004 $ng \cdot mL^{-1}$ based on a signal-to-noise ratio of 3. The constructed immunosensor is simple, label-free and specific, thus, provides a good diagnostic tool for detection of α -Fetoprotein and other cancer markers.

Key words: electrochemical immunosensor; $K_3[Fe(CN)_6]$; aldehyde-functionalized pyridinium; α -Fetoprotein