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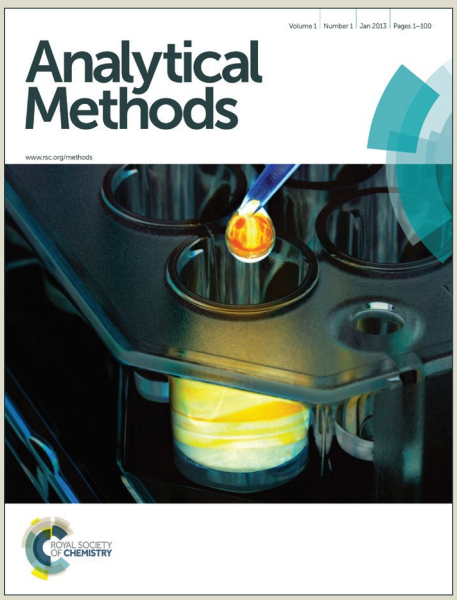

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A novel ultrasensitive electrochemical immunosensor based on carboxy-endcapped conductive polypyrrole for the detection of gypican-3 in human serum

*Guolin Yuan, Junlin He, Yuan Li, Wailan Xu, Liuliu Gao, Chao Yu**

Institute of Life Science and School of Public Health, Chongqing Medical University, Chongqing 400016, P. R. China

Junlin He and Guolin Yuan contributed equally to this work.

*Corresponding author: Prof. Chao Yu,

Email: yuchaom@163.com

Tel: (86) 23-68485589

Fax: (86) 23-68486294

Full address: Box 174#, Institute of Life Sciences, Chongqing Medical University, No.1 Yixueyuan Road, Yuzhong District, Chongqing 400016, P. R. China.

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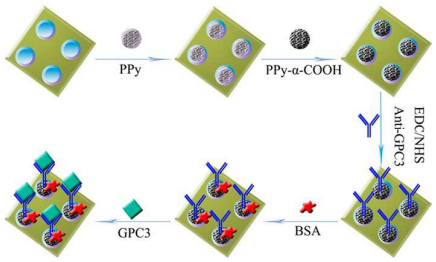
Pyrrole-α-carboxylic acid (Py-α-COOH) coated disposable indium tin oxide (ITO) glass array was used as detection platform for detection GPC3



Abstract

In this work, a novel ultrasensitive electrochemical immunosensor has been fabricated for the determination of the gypican-3 (GPC3) in human serum for the first time. A disposable indium tin oxide (ITO) glass array was used as the working electrode on which pyrrole-α-carboxylic acid (Py-α-COOH) was electropolymerized. This approach provided a high content of surface confined carboxyl groups suitable for direct covalent bind of GPC3 antibody. In the immunosensor system, the "Fluorescence microscopy" technique was first used for characterization of carboxyl groups. Meanwhile, the electrode was characterized by scanning electron microscopy (SEM) and electrochemical impedance spectroscopy (EIS). Using GPC3 as a model analyte, the immunoassay exhibited high sensitivity, acceptable stability and reproducibility with a wide linear range from 0.9 pg mL^{-1} to 9 ng mL^{-1}, and an ultra-low detection limit of 0.3 pg mL^{-1} (S/N = 3). The results for real sample analysis in human serum demonstrated that the newly constructed immunosensor array provided a rapid, simple immunoassay with high throughput, cost-effective and sufficiently low detection limits for clinical applications.

Key Words: Immunosensor; Conducting polymer; Polypyrrole; Gypican-3; ITO array electrode

1. Introduction

Hepatocellular carcinoma (HCC) is one of malignant tumor. It is usually asymptomatic in the early stages and tends to be intravascularly and intrabiliary invasive[1]. Therefore, the availability of a suitable serological marker to distinguish between HCC and benign liver lesions would be very useful for early diagnosis [2-4]. Glypican-3 (GPC3), a member of the heparan sulfate proteoglycan family, is an oncofetal protein and as a potential tumor marker for hepatocellular carcinoma (HCC) was first suggested in 1997 by Hsu et al [5].Several studies showed increased GPC3 expression at the protein level in HCC, when compared with healthy livers and benign hepatic lesions [6-8]. In addition, GPC3 can be detected in the serum as a secreted protein in a subset of patients with HCC but is undetectable in healthy individuals or patients with hepatitis or cirrhosis [9 8-11 9]. So far, one of the challenges in diagnosis of HCC using the available immunomarkers in clinical practice, such as α-fetoprotein, polyclonal carcinoembryonic antigen, and CD34, have significant diagnostic limitations[12-15]. Thus, GPC3 has shown promise as a useful diagnostic immunomarker. Serum GPC3 levels were measured by using a sandwich enzyme-linked immunosorbent assay method[8, 16] and chemiluminesence immunoassay[17]. However, these protocols are complicated, expensive, time-consuming, and a poor detection limit. So it is desirable for diagnostic applications to develop a simple and rapid method for the determination of serum GPC3 with high selectivity and sensitivity. For this reason, electrochemical immunosensor have aroused very great interest with expectations of providing fast and highly sensitive immunological response[18]. Several assay methods with electrochemical immunosensor have been found widespread applications in clinical diagnostics. Hence, it is important to design electrodes that are specific, selective and sensitive towards GPC3.

Polypyrrole (PPy), a type of organic polymer formed by polymerization of pyrrole, presented some important characteristics like dopant-mediated tunable physical properties and good stability in air and aqueous media[19, 20]. PPy is an insulator, but its oxidized derivatives are good electrical conductors. The conductivity of the material depends on the conditions and reagents used in the oxidation [21].

In the current study, a carboxy-endcapped polymeric structure was prepared through electropolymerization of α-carboxypyrrole onto a thin film of electroactive polymers[20]. Disposable indium tin oxide (ITO) glass array was employed as detection platform. The four-electrode array on ITO glass was developed to fabricate with the carboxy-endcapped polymeric film for direct covalent binding of probe antibodies. In addition, an antibody of GPC3 was immobilized onto the prepared thin film and its electrochemical behavior towards GPC3 was investigated. The results clearly revealed that the immobilized antibody onto the carboxy-endcapped polymer can be used as an immunosensor for the determination of the GPC3 concentration in human serum. The proposed method exhibited good analytical performance. Such strategy shortened the immunoassay time and provided a promising platform for low-cost, rapid, and automated immunoassay for the first time.

2. Experimental

2.1 . Materials and apparatus

Pyrrole (98%), Pyrrole-α-carboxylic acid (99%, Py-α-COOH), 1-ethyl-3-(3-dimethylaminopropyle)-carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS) were purchased from Sigma-Aldrich. 5 - amino-fluorescein, Sodium perchlorate ($NaLiO_4$) were supported by Aladdin Co.. Acetonitrile (ACN) was provided by Tedia, United States of American. Bovine Serum Albumin (BSA) was bought from Beijing Chemical Reagents Company (Beijing, China). GPC3 antibody was from Abcam Co., GPC3 stand solution was purchased from Zhenjiang Hope Biotechnology Co., Ltd (Jiangsu, China). All other chemicals were of analytical grade and used without further purification. Human serum samples were purchased from a local hospital and stored at 4 ºC. Five millimolar $[Fe(CN)_6]^{4-/3-}$ was used as electrolyte for all electrochemistry measurement. 0.1 M phosphate buffered saline (PBS, PH 7.0). De-ionized water (18.2 MΩ cm) was obtained from a Millipore Mill-Q purification system.

The electrochemical experiments were performed utilizing an electrochemical workstation (CHI660D) (Shanghai Chenhua Apparatus Corporation, China). The fabricated ITO glass was used as the working electrode (6 mm in diameter, 5 mm edge-to-edge separation), an Ag/AgCl electrode (saturated KCl) as the reference electrode, and a platinum wire electrode as the counter electrode, respectively. Scanning electron microscopy (SEM) was investigated using Hitachi-7500158 (Hitachi Limited, Japan).

2.2. Fabrication of immunosensor array

Before use, the customized ITO glass (3.0 cm×3.0 cm) must be carefully cleaned in acetone, ethanol, NaOH (1 mol mL^{-1}) and distilled water in an ultrasonic bath for 20 min in order, then, the slice was dried in an oven at 50 ℃. Rubber O-rings with a diameter of 8 mm and Eppendorf tubes whose bottom were removed containing circular openings were placed onto the ITO glass to make four separate reaction wells. The fabrication procedure of the proposed immunosenors was illustrated in Scheme 1.

2.3. Preparation of PPy-α-COOH /ITO –glass array electrodes

The electropolymerization was carried out in two steps according to a previously reported method[20]. The PPy polymer film was electrochemically prepared on ITO-glass plates from a degassed (N_2 purged) aqueous solution containing 0.1 M pyrrole in de-ionized water, 0.1 mol L^{-1} of $NaLiO_4$ as supporting electrolyte by running 5 CV cycles in a potential window of 0.7 V to 0 V vs Ag/AgCl, at a scan rate of 20 mV s^{-1}. After a through rinse with de-ionized water, the polymer electrode, PPy/ITO-glass, was transferred into a cell to create carboxy-endcapped PPy (PPy-α-COOH), 0.1M Py-α-COOH in acetonitrile containing 0.1 mol L^{-1} of $NaClO_4$, and an offset voltage of 2 V was applied for 15 min to ensure the α-terminus C-C coupling between PPy and Py-α-COOH. Then, the PPy-α-COOH/ITO-glass array electrode was thorough rinsed

with de-ionized water to eliminate the Py-α-COOH monomer from the hybrid nonmaterial.

2.4 . Fluorescent microscopy of polymer covered ITO-glass

The prepared PPy and PPy-α-COOH polymer were used as model (control and test sample). Then, 20 µL of the freshly prepared dye was added to the surface of the polymer film and incubated for 4 h under dark atmosphere. Before the experiment, PPy/ITO-glass and PPy-α-COOH/ITO-glass were carefully washed with de-ionized water to remove unbounded dye. Florescence microscopy was performed with 490 nm primary filter and 520 nm secondary filter or blue filter. The mechanism of –COOH modification using florescence technique was that if the polymer qualified carboxyl group it could combined with 5 - amino-fluorescein through esterification reaction, which showed green under fluorescent microscopy, while the polymer lacked carboxyl group it could not combined with 5 - amino-fluorescein, which kept black under fluorescent microscopy. That is, we could judge whether the -COOH has been successfully electropolymerized on the PPy surface through the florescence technique.

2.5 . Preparation of biofunctionalized PPy-α-COOH/ITO-glass array electrode with Anti-GPC3 antibody

To covalently immobilize anti-GPC3 antibody, the well-known EDC/NHS cross-linkers were used. Firstly, 100 µL solution containing 173 µM EDC and 150µM NHS was added to the surface of the film to activate the carboxyl terminal groups of the PPy-α-COOH for 3 h at ambient temperature, then, it was rinsed three times with PBS. After that, 20 µL of anti-GPC3 antibody solution was dropped onto the polymer-coated surface, it was incubated for an overnight period at 4 ºC to immobilize antibody. The yielded anti-GPC3/PPy-α-COOH /ITO-glass array electrode was rinsed with 0.1M PBS to remove any physically absorbed antibody. Subsequently, the

immunosensor was incubated in 1 wt% bovine serum albumin (BSA) solution for 1 h to avoid non-specific adsorption. Finally, the array was thoroughly rinsed with 0.1 M PBS (pH 7.0) to wash away the excess BSA. These BSA/anti-GPC3/PPy-α-COOH /ITO-glass array electrodes, as prepared, were stored at 4 ºC unless used.

2.6 . Experimental measurements

The Electrochemical impedance spectroscopic were scanned in 5 mM $[Fe(CN)_6]^{4-/3-}$ containing 0.1 M KCl solution in the ac frequency range of 100 kHz to 1 Hz. To carry out the immunoassay of tumor marker, different concentrations of GPC3 protein were dropped into the well to react for 1 h. After that, the modified electrode was washed with PBS (0.1 M, pH 7.0). Subsequently, 5 mM $[Fe(CN)_6]^{4-/3-}$ was added into the cell and differential pulse voltammetry (DPV) curves were recorded. All measurements were done at room temperature.

3 . Results and discussion

3.1 . Surface morphology studies

The surface morphology of an immunosensor is a vital factor affecting the performance of the sensor. Whereby, the morphologies of PPy, PPy-α-COOH and anti-GPC3/PPy-α-COOH modified ITO-glass array electrode was characterized by SEM. As seen in Fig. 1A, morphology of the PPy polymer film shows a three uniform intercalated rope like structure and PPy-α-COOH film is also of rope like dimensional structure, but with more uniform porous, where pores are more evident within the polymer matrix at high magnification (showed in Fig. 1B). However, after Human Glypican-3 antibody grafted onto the polymer film, the pores disappeared in the SEM image (Fig. 1C), indicates a good adherence of protein molecule spreading well all over the surface of the PPy-α-COOH matrix. The insets are the corresponding

magnified SEM images of the modified surface. Therefore, these results indicate the presence of polymer film coverage by the GPC3 antibody.

In order to confirm the presence of carboxylic acid functional groups in the PPy-α-COOH film, Natural light microscopic images (Fig. 2A, 2C) and fluorescent micrographic images (Fig. 2B, 2D) were taken under pure PPy (Fig. 2A, 2B) and PPy-α-COOH film (Fig. 2C, 2D). As shown in the picture, in the Fluorescent micrographic images, PPy film was dark, however, the significant strong green fluorescence was observed under the Fluorescence microscopy of PPy-α-COOH film, which was attributed to the presence of carboxylic acid functional groups in the PPy-α-COOH film. The presence of carboxylic acid functional groups in the PPy-α-COOH film show great potential for the strong covalent linkage of antibody molecules for the biofunctionalization of the PPy-α-COOH /ITO-glass array electrode.

3.2 . Electrochemical impedance spectroscopic characterization of the anti-GPC3/ PPy-α-COOH /ITO-glass array electrodes

Electrochemical impedance spectroscopy (EIS) is one of the most informative tools for probing the features of the surface modified electrodes [22]. Nyquist diagrams of electrochemical impedance spectra for our electrodes were shown in Fig. 3. The PPy modified ITO showed a small semicircle which was characteristic of infinitesimal electric resistance. After Py-α-COOH grafted on the surface of PPy film, the modified electrode exhibited a remarkable increase in diameter over that of PPy/ITO electrode, indicating much higher Ret values after modification. The reason may be that the presence of negatively charged carboxyl functional groups of PPy-α-COOH, leading to a repulsive interaction with negatively charged redox probe on the electrode surface. After NHS activated, the Ret value of PPy-α-COOH/ITO-glass array electrode reduced sharply, indicating that the mutual sealing between the negatively charged terminal carboxyl anion of Py-α-COOH and the positively charges of NHS ester [23] .When antibodies were captured onto the modified electrode, the Ret increases sharply, owing to the insulating layer of the protein at the PPy-α-

COOH/ITO-glass array electrode surface. These results demonstrated that the proposed electrode was successfully modified, which agree with the SEM result.

3.3 Optimization of the experimental parameters and electrochemical performance of PPy-α-COOH /ITO-glass array electrode.

Initially, to achieve an optimal electrochemical immunosensor response (ΔI), the electropolymerization cycles of the PPy film, the concentration of anti-GPC3, the binding time of anti-GPC3 to Ppy-α-COOH, and incubation time were optimized.

As seen in Fig. 4A, with the increment of electropolymerization cycles, the current increased gradually, indicating that the formation of PPy film on the ITO electrode. What's notably, the current increased slightly after five cycles. As shown in Fig. 4B, the impedance decreased sharply after 2 cycles, it changed negligible with the increasement of polymerization cycles. Hence, 5 cycles was selected for the electropolymerization.

As shown in Fig. 5A, the current response increased with increasing concentrations of anti-GPC3 from 1 to 10 μg mL^{-1}, and then, the current response tended to level off upon further increases in concentration. As a result, the electrode exhibited the highest current response at the concentration of 10 μg mL^{-1}, expounding that anti-GPC3 bonded to the modified electrodes had been saturated. Therefore, 10 μg mL^{-1} was selected as the incubation time for the immunoassay. Furthermore, the binding time of anti-GPC3 to Ppy-α-COOH was also a crucial factor for the appliance of the immunosensor.

As shown in Fig. 5B, the current response increased rapidly with increasing the binding time from 1 h to 4 h. A further increase in the binding time of anti-GPC3 seemed to do not obviously increase the current change, indicating that all of the available recognition sites of Ppy-α-COOH were matched with the anti-GPC3. Thus, 4 h was used for the binding time of anti-GPC3.

As shown in Fig. 5C, with the incubation time increased and tended to a steady value after 60 min, indicating a saturated formation of the immunocomplex. Longer incubation time did not enhance the peak current. Therefore, an incubation time of 60 min was chosen as the optimal incubation time. These conditions were used in the subsequent experiments.

The quantitative analysis of GPC3 was investigated by DPV in 0.1 M KCl, containing 5mM $[Fe(CN)_6]^{4-/3-}$. It is clear from Fig. 6 that with increasing GPC3 concentration the current decreased linearly in the range 0.9 pg mL^{-1} - 9 ng mL^{-1}, obeyed the linear equation ΔI (μA) = 0.6441+0.4959 log[GPC3] (n = 3) with the correlation coefficient of 0.994. The limit of detection was 0.3pg mL^{-1} (S/N = 3). Therefore, the proposed immunosensor showed high sensitivity. The serum GPC3 concentration for HCC patient (the median values[24] was 924.8 pg mL^{-1}) fell in the linear range of this immunosensor. That is to say, this proposed method had great potential to be in clinical trials.

3.4 . Selectivity, stability and reproducibility

In order to evaluate the specificity of the immunosensors for the target GPC3 analyze, some possible interferents such as BSA (0.1 mg mL^{-1}), CRP (6.4 μg mL^{-1}) and ST6-Gal1 (250 ng mL^{-1}), L-ascorbic acid (AA) (100 ng mL^{-1}), Uric Acid (UA) (100 ng mL^{-1}) and glucose (100 ng mL^{-1}) were used. Fig. 7 shows the current change due to the binding of the non-specific proteins and GPC3 (9 ng mL^{-1}) to the GPC3 immunosensors. Compared with the current response obtained from 9 ng mL^{-1} GPC3, the responses of current caused by the interferences were weak and negligible, which indicated that the specificity of the immunosensor based on the specific antigen-antibody immunoreaction was acceptable. Namely, the possible interferences and matrix effects in real samples was negligible.

Stability of immunosensor is also a very important feature in their application, whereby the stability of the immunosensor array was conducted. When the immunosensor was not in use, the newly constructed immunosensor array was stored

at 4 ºC. After a storage period of 20 days, the immunosensor retained 95.16 % of its initial response, indicating the stability of the immunosensors was good.

The reproducibility of the immunosensor was estimated by assaying 1ng ml^{-1} GPC3 for four replicate measurements, the relative standard deviation (RSD) was 3.7 % for four electrodes, suggesting the precision and reproducibility of the immunosensor was acceptable.

3.5 . Application in human serum samples

To evaluate the application potential of this immunosensor array, serum sample were assayed by the proposed method. 0.9 pg mL^{-1}, 0.45 ng mL^{-1} and 9 ng mL^{-1} GPC3 standard solutions were added in Human Blood Serum and the recoveries of the three GPC3 concentrations were determined. The results listed in Table 1, the ranges of the recovery and RSD were 88.89 - 109.96 % and 4.72 - 6.17 %, respectively. Therefore, the proposed immunosensor provided promising potential in clinical analysis to detect the concentration of GPC3.

4 . Conclusions

In this paper, an ultrasensitive electrochemical immunosensor based on carboxy-endcapped conductive polypyrrole film was successfully developed for the detection of GPC3. The carboxyl groups of the film were exploited for covalent immobilization GPC3 antibody and the immunosensor probe on electropolymerized film provided an efficient method for the reagentless and label free detection of GPC3. Highlights of this work could be summarized as follows: (1) The GPC3 was detected by the electrochemical immunosensor for the first time; (2) To the best of our knowledge, in an immunosensor system, the “Fluorescence microscopy” technique was first used for characterization of carboxyl groups; (3) Such fabricated immunosensor showed an excellent performance with an ultralow detection limit of 0.3 pg mL^{-1}, a wide linear range from 0.9 pg mL^{-1} to 9 ng mL^{-1} and good accuracy towards the real samples. In

addition, the high-density micro-array could be constructed to realize high-throughput assay, which showed great potential for simple and efficient diagnostic for varieties of biomarkers, not limited to GPC3. This could facilitate the application of multi-analytics and point of care testing.

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References

1.A. Nassar, C. Cohen and M. T. Siddiqui, *Diagnostic cytopathology*, 2009, 37, 629-635.

2.C. S. Wang, C. L. Lin, H. C. Lee, K. Y. Chen, M. F. Chiang, H. S. Chen, T. J. Lin and L. Y. Liao, *World journal of gastroenterology : WJG*, 2005, 11, 6115-6119.

3.J. Yu, Q. Ma, B. Zhang, R. Ma, X. Xu, M. Li, W. Xu and M. Li, *Science China. Life sciences*, 2013, 56, 234-239.

4.C. Xu, Z. Yan, L. Zhou and Y. Wang, *Journal of cancer research and clinical oncology*, 2013, 139, 1417-1424.

5.H.-C. Hsu, W. Cheng and P.-L. Lai, *Cancer research*, 1997, 57, 5179-5184.

6.T. Nakatsura, T. Kageshita, S. Ito, K. Wakamatsu, M. Monji, Y. Ikuta, S. Senju, T. Ono and Y. Nishimura, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 2004, 10, 6612-6621.

7.T. Nakatsura, Y. Yoshitake, S. Senju, M. Monji, H. Komori, Y. Motomura, S. Hosaka, T. Beppu, T. Ishiko, H. Kamohara, H. Ashihara, T. Katagiri, Y. Furukawa, S. Fujiyama, M. Ogawa, Y. Nakamura and Y. Nishimura, *Biochemical and biophysical research communications*, 2003, 306, 16-25.

8.M. Capurro, I. R. Wanless, M. Sherman, G. Deboer, W. Shi, E. Miyoshi and J. Filmus, *Gastroenterology*, 2003, 125, 89-97.

9.Y. Hippo, k. Watanabe and A. Watanabe, *Cancer research*, 2004, 64, 2418-2423.

10.M. Hanlin L.Wang, PhD, M. Florencia Anatelli, M. Qihui "jim" Zhai, M. Brian Adley, D. Shang-Tian Chung and M. Ximing J. Yang, PhD, *Arch Pathol Lab Med*, 2008, 132, 1723-1728.

11.M. Capurro and J. Filmus, *Cancer research*, 2005, 65, 372-372.

12.B. Li, H. Liu, H. W. Shang, P. Li, N. Li and H. G. Ding, *African health sciences*, 2013, 13, 703-709.

13.R. S. Saad, T. M. Luckasevic, C. M. Noga, D. R. Johnson, J. F. Silverman and Y. L. Liu, *Diagnostic cytopathology*, 2004, 30, 1-6.

14.A. Wee, *Applied Immunohistochemistry & Molecular Morphology*, 2006, 14, 266-272.

15.T. S. Huang, Y. C. Shyu, R. Turner, H. Y. Chen and P. J. Chen, *Systematic reviews*, 2013, 2, 37.

16.P. Tangkijvanich, T. Chanmee, S. Komtong, V. Mahachai, N. Wisedopas, P. Pothacharoen and P. Kongtawelert, *Journal of gastroenterology and hepatology*, 2010, 25, 129-137.

17.b. Qian-Yun Zhanga, Hui Chena, Zhen Lina, Jin-Ming Lina,n, *Journal of Pharmaceutical Analysis*, 2011, 1.

18.Y. Xiao, C. M. Li and Y. Liu, *Biosensors & bioelectronics*, 2007, 22, 3161-3166.

19.M. Shamsipur, S. H. Kazemi and M. F. Mousavi, *Biosensors & bioelectronics*, 2008, 24, 104-110.

20.J. W. Lee, F. Serna and C. E. Schmidt, *Langmuir : the ACS journal of surfaces and colloids*, 2006, 22, 9816-9819.

21.N. Onnela, V. Savolainen, M. Hiltunen, M. Kellomaki and J. Hyttinen, *Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference*, 2013, 2013, 539-542.

22.G. S. Popkirov, E. Barsoukov and R. N. Schindler, *Journal of Electroanalytical Chemistry*, 1997, 425, 209-216.

23.J.-E. Im, J.-A. Han, B. K. Kim, J. H. Han, T. S. Park, S. Hwang, S. In Cho, W.-Y. Lee and Y.-R. Kim, 2010, - 205, Supplement 1, - S278.

24.E. Yasuda, T. Kumada, H. Toyoda, Y. Kaneoka, A. Maeda, S. Okuda, N. Yoshimi and O. Kozawa, *Hepatology Research*, 2010, 40, 477-485.

Figure captions:

Scheme 1: Schematic diagram of step-wise fabrication of the ultrasensitive GPC3 immunosensor.

Fig. 1: SEM images of Ppy/ITO (A); PPy-α-COOH/ITO (B); anti-GPC3/PPy-α-COOH/ITO- glass electrode (C); inset showed the corresponding magnified SEM image of the modified surface.

Fig. 2: Natural light microscopic images (A, C) and fluorescent micrographic images (B, D) of polymer before carboxyl group grafted (A, B) and after (C, D) carboxyl group grafted.

Fig. 3: EIS of different modified electrodes in 0.1 M KCl containing 5mM $[Fe(CN)_6]^{4-/3-}$. The frequency range was 1-100000 Hz with the signal amplitude of 5mV .

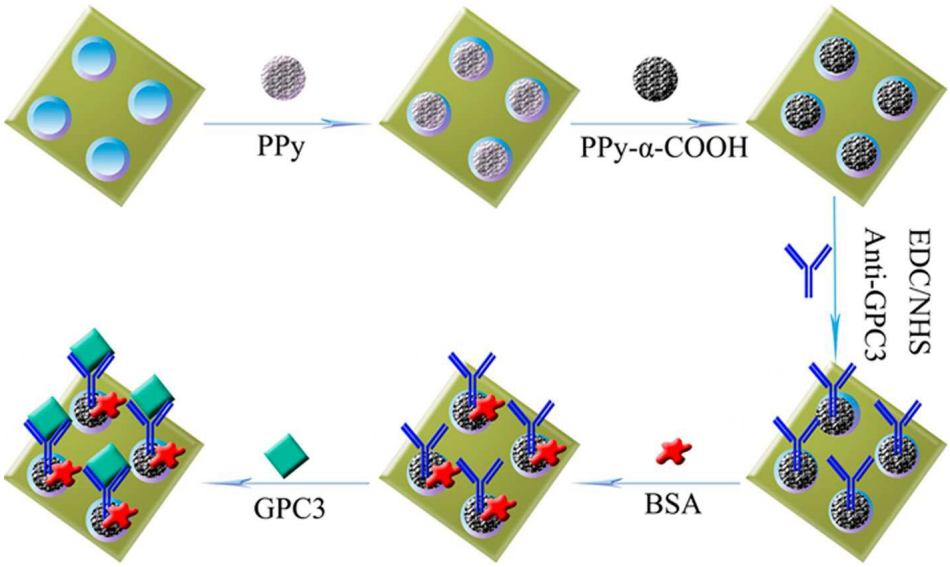
Fig. 4: (A) the electropolymerization cycles of the PPy film; (B) Bode plot for polymerization of Ppy film on ITO-glass in 0.1 M Ppy with 0.1 M $NaLiO_4$, scan rate 0.02 V s^{-1}.

Fig. 5: (A) the amount of anti-GPC3, binding time of anti-GPC3 (B), and effects of immune-binding time (C) on the immunosensor.

Fig. 6: (A) DPV responses of the proposed immunosensor after incubation with different concentrations of GPC3; and (B) calibration curves of the proposed immunosensor toward GPC3 in 0.1 M KCl containing 5mM $[Fe(CN)_6]^{4-/3-}$.

Fig. 7: Specificity of the proposed immunosensor at the concentration of 9 ng mL^{-1} GPC3 with 0.1mg ml^{-1} BSA, 6.4 μg mL^{-1} CRP and 25 ng mL^{-1} ST6-Gal1, 100 ng mL^{-1} L-ascorbic acid (AA), 100 ng mL^{-1} Uric Acid (UA) and 100 ng mL^{-1} glucose.

Table 1: Spike and recovery results for real sample analysis.



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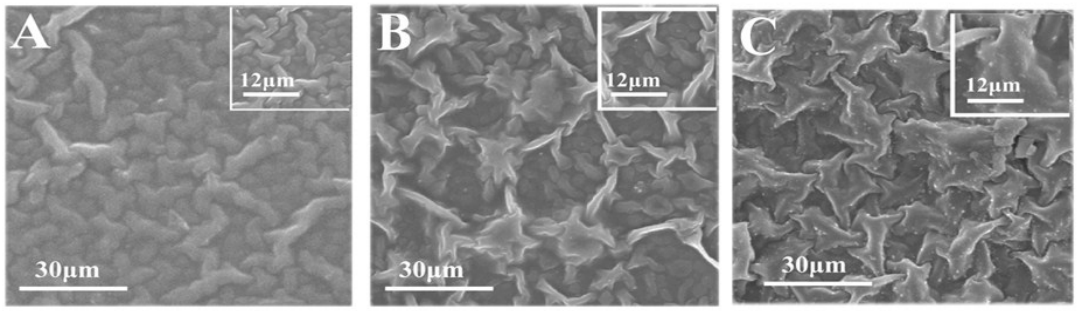

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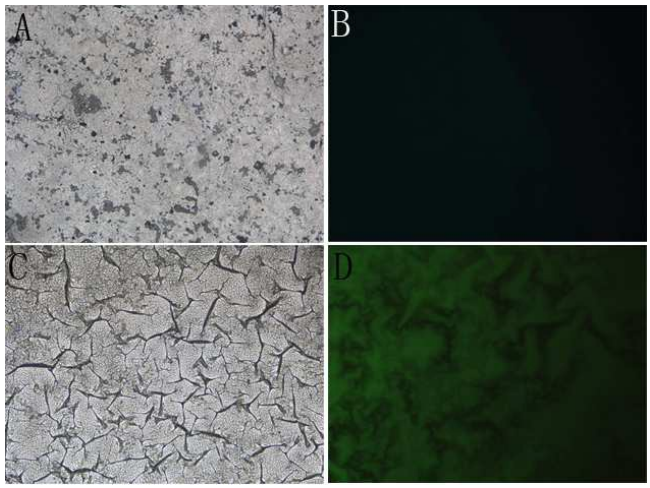

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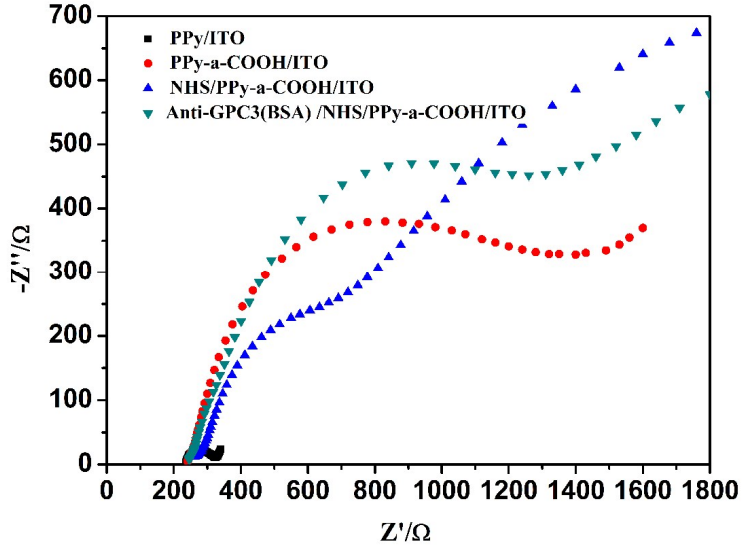

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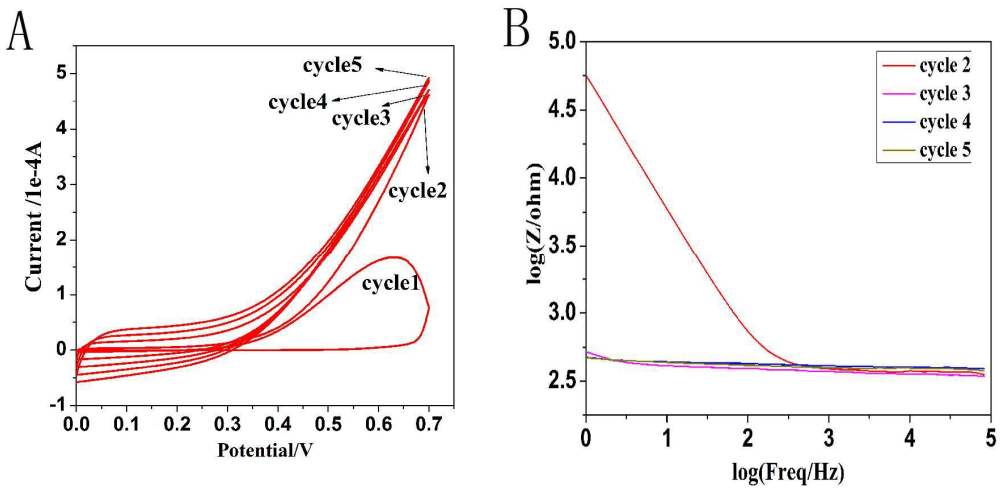

Fig. 4: (A) the electropolymerization cycles of the PPy film; (B) Bode plot for polymerization of Ppy film on ITO-glass in 0.1 M Ppy with 0.1 M $NaLiO_4$, scan rate 0.02 V s^{-1}.

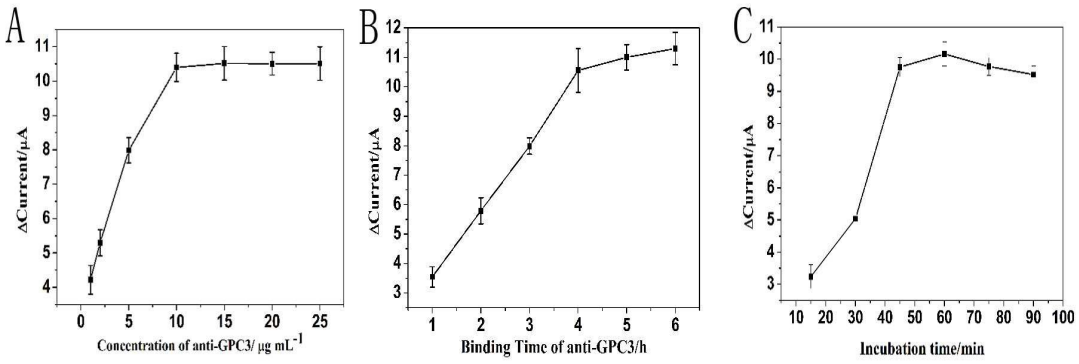

Fig. 5: (A) the amount of anti-GPC3, binding time of anti-GPC3 (B), and effects of immune-binding time (C) on the immunosensor.

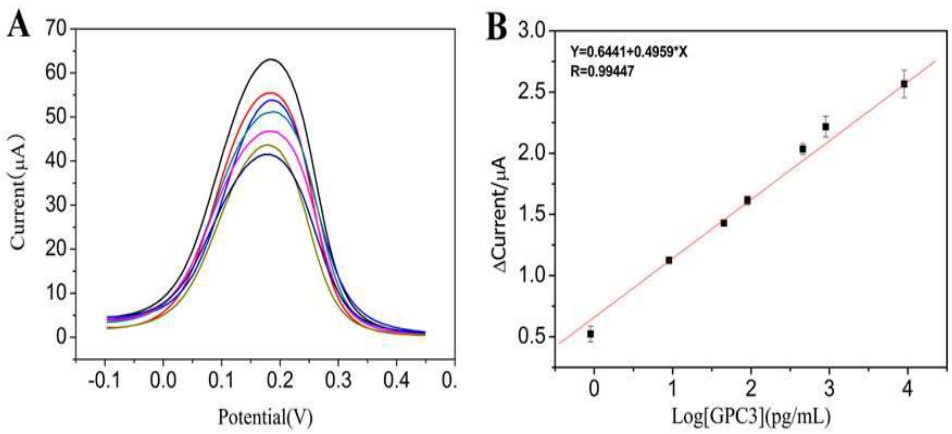

Fig. 6: (A) DPV responses of the proposed immunosensor after incubation with different concentrations of GPC3; and (B) calibration curves of the proposed immunosensor toward GPC3 in 0.1 M KCl containing 5mM $[Fe(CN)_6]^{4-/3-}$

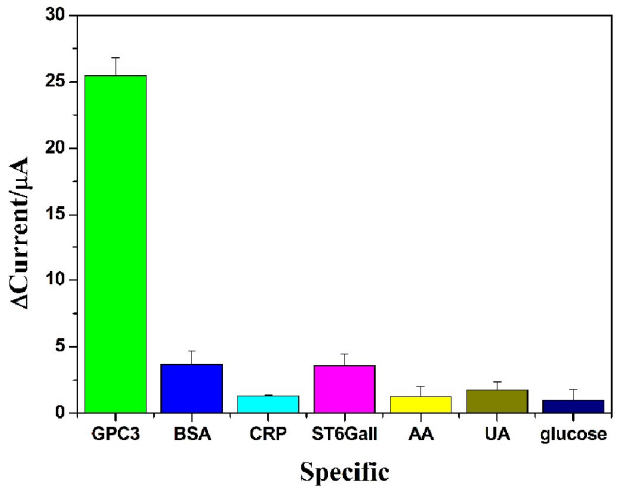

Fig. 7: Specificity of the proposed immunosensor at the concentration of 9 ng mL^{-1} GPC3 with 0.1mg ml^{-1} BSA, 6.4 μg mL^{-1} CRP and 25 ng mL^{-1} ST6-Gal1, 100 ng mL^{-1} L-ascorbic acid (AA), 100 ng mL^{-1} Uric Acid (UA) and 100 ng mL^{-1} glucose.

Table 1: Spike and recovery results for real sample analysis.

Added (ng ml^{-1})	Found (ng ml^{-1})	Recovery (%)	RSD (%)
0.009	0.008	88.89	4.72
0.45	0.447	99.42	6.17
9	9.986	109.96	5.60