Int. J. Electrochem. Sci., 12 **(2017)** 10633 – 10641, doi: 10.20964/2017.11.30

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Investigation of Prolactin Based on a Novel Electrochemical Immunosensor

Baosheng Sun[1#], Li Zhou[2#], Fanxu Meng[1], Jian Ou[1], Zhou Wang[1], Jing Du[2], Pingle Wu[2], Jing Li[2] and Jinxia Piao[2,]*

[1] Department of Radiotherapy, Jilin Provincial Cancer Hospital, Chang Chun, Jilin, 130012, P.R.China
[2] Department of Gynecological Oncosurgery, Jilin Provincial Cancer Hospital, Changchun, Jilin, 130012, P.R. China
[*]E-mail: dgojinxiapiao@foxmail.com

Received: 26 July 2017 / *Accepted:* 5 September 2017 / *Published:* 12 October 2017

In this work, a new electrochemical immunosensor to detect the hormone prolactin (PRL) was proposed based on the poly (pyrrolepropionic acid)/multiwalled carbon nanotubes hybrid (transducer) – modified GCE. In the case of the covalent immobilization of the antigen onto the decorated electrode along with the reaction between PRL in the specimen and the alkaline phosphatase (AP) - labelled anti-PRL (certain amount), we performed an indirect competitive measurement. After the residual labelled antibody was attached onto the immobilized PRL, the differential pulse voltammograms (DPVs) of the AP enzyme reaction product was used to monitor the affinity reaction, where the substrate was 1-naphtyl phosphate. The voltammetric assays were confirmed to be desirably reproducible and selective; thus, the proposed immunosensor was successfully used for the detection of urine and human serum of clinically relevant concentration levels.

Keywords: Prolactin; Phosphatase; Carbon nanotube; Electrochemical determination; Voltammetric measurement

1. INTRODUCTION

As a 23 kDa peptide hormone, prolactin is largely released by the pituitary lactotrophs of the anterior pituitary gland [1]. Monomeric prolactin (85–95 %) is the primary form of prolactin, though different molecular sizes of prolactin formed have been detected [2]. With respect to biological actions, the most versatile hormones include the monomeric prolactin [3]. For example, an approximately ten-fold increase in serum prolactin levels during pregnancy leads to the development of breasts, as well as lactation [4]. The serum prolactin level has been proposed to increase in epileptic

seizures, suggesting the possible facilitation of prolactin level to the distinction of psychogenic non-epileptic seizures and epileptic seizures [5]. In addition, serum prolactin exhibits a significant increase in concentration in hypothyroidism, hyper prolactin aemia and Polactinoma [6]. Hence, prolactin determination is of great use to disease diagnostics and pretreatment.

Enzyme immunoassays (EIAs)[7-9], bioluminescent immunoassays [10], and chemiluminescence immunoassays [11] are common immunoassays towards prolactin. Unfortunately, these methods exhibit such disadvantages as poor sensitivity, challenging procedures, and the requirement of lengthy time periods for measurement, as well as false positive results [12-15]. Electrochemical immunoassay, where the antigen-antibody interactions with electrochemical transducers are specific, has gained increased attention recently, since it is portable, inexpensive, and precise in current assays [16, 17].

Carbon nanomaterials have been extensively used as a desirable support for the biosensor preparation [18]. For instance, in material science, increasing focus has been placed on graphene nanomaterials [19]. Graphene has gained extensive use in the fabrication of desirable electrochemical sensors [20, 21] (typical of immunosensors), since it possesses excellent chemical and physical features, flexible two-dimensional (2D) structure, and single-atom thickness [22]. On the other hand, single walled carbon nanotubes (SWCNTs) have also obtained widespread application in the preparation of biosensors [23-25]. The negative charge of SWCNTs was made due to the presence of substantial functional groups left on the edges and surface of SWCNTs, which also contributed to the functionalization of SWCNTs for the preparation of multifunctional nanostructure hybrid materials during the utility of immunosensors [26].

The polymer network incorporated with functional groups has been considered a promising candidate for the covalent immobilization of biomolecules. Polypyrrole functionalization-involved techniques in this work were proven to be excellent. For instance, pPy was functionalized by poly(propionic acid), for the preparation of a label-free SPR immunosensor, where the model protein was goat IgG [27]. Immunosensors were prepared using electropolymerized poly(pyrrole propionic acid) (pPPA), where anti-IgG was covalently immobilized onto the carboxyl-containing film, and IgG was recognized with amperometry [28]or SPR [29]. There have also been studies presenting the preparation of an electrochemical immunosensor towards the detection of the hormone leptin without AuNPs based on the co-electropolymerization of pyrrole propionic and pyrrole acid [30]. In a one-dimensional nanomaterial field effect transistor (FET) biosensor, pPPA could also be applied to encapsulate the anti-rabbit IgG biomolecules, where pyrrole propionic acid was electropolymerized on TiO_2-nanowire (NW)-based FETs [31]. Through the electrochemical polymerization of anti-rabbit IgG and pPPA hybrid films on patterned NWs, a conductance-based immunosensor was fabricated by the same research group [32].

This report proposed the fabrication of the new electrochemical immunosensor for the detection of PRL, where the antigen was immobilized on the pPPA/CNTs composites that were deposited on a GCE, and an indirect competitive measurement was performed based on AP- labelled anti-PRL. This developed immunosensor exhibited great sensitivity and selectivity towards the detection of PRL in human urine and serum due to its low cost and simplicity.

2. EXPERIMENTS

2.1. Reagents and apparatus

Mouse monoclonal antibody towards alkaline phosphatase-labelled PRL (AP-anti-PRL) (Immunometrics) was employed in this study. Dilution (1/100) was conducted using Tris buffer solution (0.1 M, pH 7.2) (Tris) to prepare the test antibody solution. The product, obtained from Sigma, was used for direct preparation of stock PRL solutions (40 mg/L) in the same buffer solution. Multiwalled carbon nanotubes (MWCNTs) with purity of 95% and a diameter of 30 ± 15 nm were obtained from Nanolab, Brighton, MA. Prior to use, MWCNTs were treated with nitric acid (2.6 M) and refluxed for 24 d in order for chemical shortening and carboxylation. We also employed pyrrole propionic acid monomer (PPA) and 99.5% KCl (Sharlau). PPA solutions (0.1 M) were prepared in deionized water + 0.5 M KCl. A terminal concentration of 1% was achieved by dissolving Type VH bovine serum albumin (BSA) into phosphate buffer solution (PBS) (0.1 M, pH 4.5) that contained KCl (0.1 M). A mixture (2 mg/mL) containing *N*-hydroxysuccinimide sulfate (NHSS), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) (both obtained from Acros) was prepared in PBS (0.1 M, pH 5.0). A stock solution of 1-naphthylphosphate (1-NPP) (50 mM) was prepared in 50 mM Tris of pH 9.6 + 10 mM $MgCl_2$ (Trizma) buffer.

All electrochemical experiments were performed on a CHI660D electrochemical workstation (Shanghai Chenhua Instruments) at 25 ± 0.5 °C (ambient temperature), where a traditional triple-electrode configuration was used. The working, auxiliary and reference electrodes were an original or modified glassy carbon electrode (GCE; diameter: 3 mm), a platinum wire and Ag/AgCl/saturated KC.

2.2. Preparation of PRL/pPPA/MWCNTs/GCE immunosensors

Initially, the surface of GCE was polished with alumina slurries (0.3 μm), followed by a thorough rinsing with deionized water, sonication for 0.5 min in water and acetone, and then air drying, to obtain the pPPA/MWCNTs modified GCE (denoted as pPPA/MWCNTs/GCE). 2 μL of a 0.5 mg MWCNTs per mL dispersion in dimethyl formamide was casted to the surface of the electrode to obtain the MWCNTs modified GCEs, which was then dried under IR radiation. With 30 consecutive voltammetric cycles run in a potential range of 0 to 0.85 V at 100 mV/s, pPPA was electrodeposited on the MWCNTs modified GCE in a mixture of KCl (0.5 M) and PPA (0.1 M). This was followed by immersing the as-prepared pPPA/MWCNTs modified GCEs into an EDC/NHSS solution (2 mg/mL) at 25 °C for 15 min. Subsequently, the electrode was washed by 0.1 M Tris pH 7.2 Tween 20, and dropped with a PRL solution (10 μL, 40 μg/mL, denoted as anti-PRL-pPPA/MWCNTs/GCE), followed by incubation at 25 °C for 120 min. Next, the as-prepared bioelectrode was rinsed by the same batch of washing solution, dropped with BSA solution (20 μL, 0.005%), and incubated at 25 °C for 60 min.

2.3. Immunoassay fabrication

The indirect competitive immunoassay was carried out by introducing a mixture (20 μL) of AP-anti-PRL (1/100) + standard PRL solution or the specimen to an Eppendorf tube, and maintained for 0.5 h at 25 °C. This was followed by dropping 10 μL of the above mixture that contained residual non-conjugated AP-anti-PRL to the surface of the electrode, and leaving it incubating at 25 °C for 120 min. For the detection of PRL, the AP-anti-PRL–PRL-pPPA/MWCNTs modified GCE (denoted as AP-anti-PRL–PRL-pPPA/MWCNTs/GCE) was immersed in a 500 μL of a 50 mM Trizma buffer (pH 9.6) + 5 mM 1-NPP for 10 min to maintain the enzyme reaction. Finally, DPVs were obtained.

3. RESULTS AND DISCUSSION

MWCNTs were used for the modification of the GCE followed by the electropolymerization of PPA using cyclic voltammetry (CV) on the as-prepared MWCNTs modified GCE. This finding was followed by the covalent immobilization of PRL onto the pPPA/MWCNTs modified GCE via EDC/NHSS chemistry along with a blocking treatment using BSA. Next, the mixture of AP-anti-PRL and PRL standard solution or the specimen was introduced into an Eppendorf tube (where the reaction took place) to perform an indirect competitive immunoassay. The attachment of the labelled antibody onto the immobilized PRL was achieved by dropping an aliquot of the solution that contained the residual non-conjugated AP-anti-PRL to the PRL-pPPA/MWCNTs modified GCE. This step was followed by immersing the as-prepared immunosensor into a Trizma buffer solution (50 mM, pH 9.6) that contained the AP substrate (1-NPP), along with the detection of PRL via the DPV of the product obtained during the enzyme reaction (1-NP).

As indicated in Fig. 1A, the modified electrode at varying stages was investigated with $[Fe(CN)_6]^{3-/4-}$ (5 mM) + KCl (0.1 M) via CVs, with the behaviour being assessed by the current densities of varying electrodes. It can be seen that the original GCE showed several well-defined oxidation-reduction peaks, while the MWCNTs and pPPA modified GCE showed an obvious increase in the current density, since the charge transfer has been accelerated. Nevertheless, a decrease in the current density was observed, as the captured antibodies blocking solution was immobilized onto the modified surface of the electrode, suggesting the hindrance of charge transfer after the addition of Ab_1. An additional decrease in the current density was observed after the successive immobilization of prolactin onto the surface of the electrode, primarily since the antigen-antibody complex was formed on the modified GCE. When the resulted electrode reacted with the biotinylated again, the current density increased dramatically, indicating that the MWCNTs possessed high conductivity, and favoured electron transmission [13]. A pronounced increase in the current density was observed with the reaction of AP with the final electrode, suggesting the charge transfer between the GCE and biomolecules was enhanced. The results of the above analysis corresponded to that of the electric impedance spectroscopy (EIS) measurement (Fig. 1B).

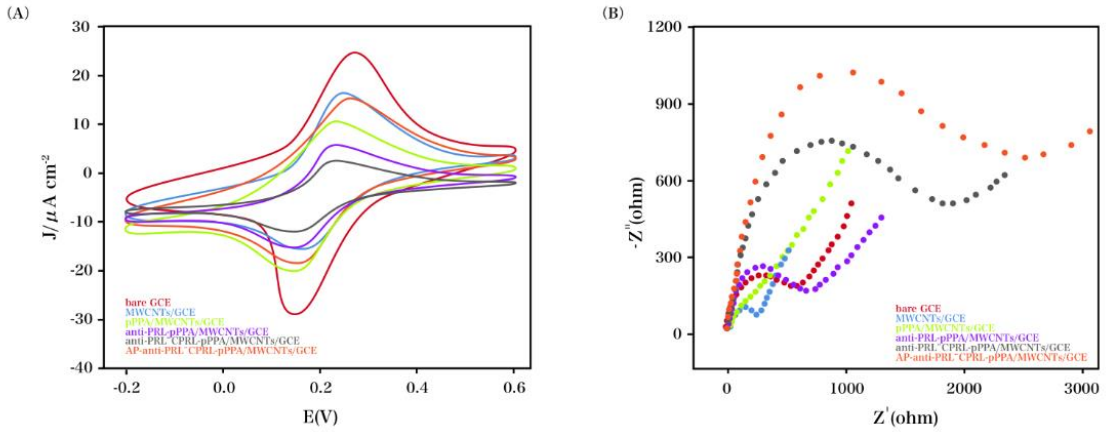

Figure 1. (A) CVs and (B) EIS spectra of each immobilization step in pH 7.4 PBS that contained $[Fe(CN)_6]^{3-/4-}$ (5 mM) and KCl (0.1 M): the original GCE, MWCNTs modified GCE, pPPA/MWCNTs modified GCE, anti-PRL-pPPA/MWCNTs modified GCE, anti-PRL–PRL-pPPA/MWCNTs modified GCE and AP-anti-PRL–PRL-pPPA/MWCNTs modified GCE.

The calibration plot constructed for the determination of PRL by DPV is displayed in Fig. 2A. For a competitive-type assay, a decrease in the peak current value was observed with the increase in the concentration of PRL with a linear range obtained as broad as 10^{-2} to 10^{4} ng/mL (r = 0.99). The highest and lowest analyte concentration in the linear range of the calibration profile exhibited obvious current variation (ca. 10 μA). Varying DPVs were obtained for varying concentrations of PRL (Fig. 2B). Compared with the dynamic ranges obtained for the ELISA kits, the above linear range was obviously broader and was proper for the detection of PRL in urine and serum specimens. This advantage can also be extended to the other immunosensors for PRL described in the literature [2, 33, 34]. This value was remarkably lower than those reported previously using the magneto immunosensor (3.74 ng/mL) [2],the poly(o-phenylenediamine)-carried nanogold (GPPD) particles functionalized with horseradish peroxidase-anti-PRL (0.1 ng/mL) [34], or with the fluorometric multisensor (1.3 ng/mL) [33].

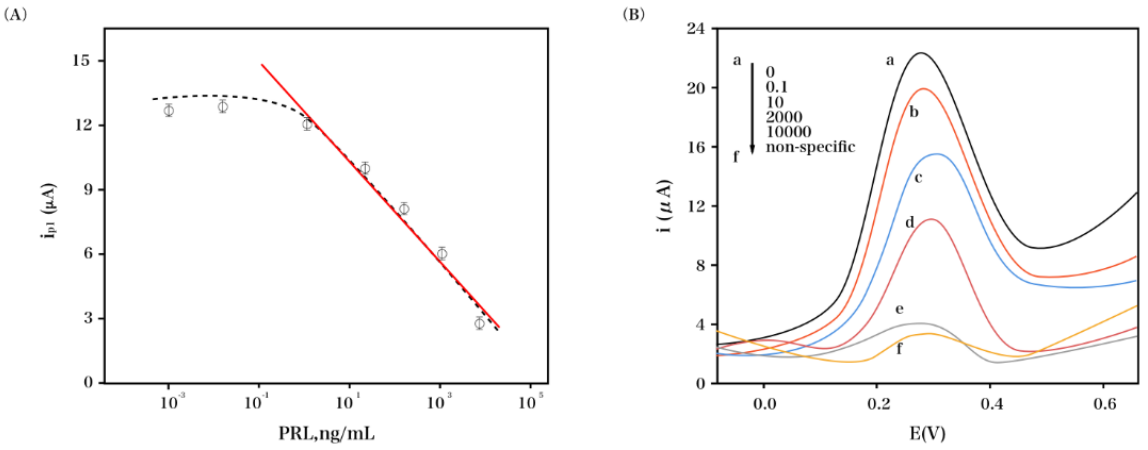

Figure 2. (A) Calibration curve for PRL in semilogarithmic form using the AP-anti-PRL–PRL-pPPA/MWCNTs modified GCE. (B) DPV patterns obtained for varying concentrations of PRL (0 - 10,000 ng/mL).

The calculation of the limit of detection (LOD) was described as the following equation: LOD = i_p (blank) − 3s_b, where i_p represented the protein concentration to produce a voltammetric response (=that from the blank), s_b represented the standard deviation of the blank. The s_b value (±0.07 μA) was calculated from 20 consecutive experiments obtained from solutions in the absence of PRL with an LOD of 4 pg/mL. In addition, compared with this LOD, the LOD for the ELISA kits was considerably higher. Three factors were assumed to contribute to the enhanced sensitivity using the AP-anti-PRL–PRL-pPPA/MWCNTs modified GCE, including the indirect competitive method for immunoassay, the effective covalent immobilization of PRL onto the pPPA/MWCNTs modified GCE, and the strengthened current signals observed via DPV using the nanostructured electrodes.

A series of voltammetric experiments were performed using varying immunosensors for each experiment towards PRL solutions (0.01 ng/mL), both on the same working day and on varying days, to investigate the reproducibility of these measurements. For the measurements performed on the same day, relative standard deviation (RSD) was obtained as 2.0% (n = 5). In addition, for those performed on varying days, the RSD was obtained as 2.6% (n = 5). These results indicated that the preparation process of the immunosensor was highly reproducible. We also studied the storage stability of the PRL-pPPA/MWCNTs/GCE bioconjugates under humid condition at 4 °C. Different bioconjugates fabricated on the same day were stored before use in the preparation of AP-anti-PRL–PRL-pPPA/MWCNTs/GCE immunosensors on different days for the detection of PRL solutions (0.1 ng/mL). Although the storage stability of the conjugate is moderate, it is acceptable to allow the storage of prepared AP-anti-PRL–PRL-pPPA/MWCNTs/GCE conjugates under the abovementioned conditions and their use for the preparation of the immunosensors on request. The DPV peak current values were maintained for five days, the range was ±3 times the standard deviation of the measurements (n = 10) performed on the initial day. The current decreased with prolonged storage time, possibly ascribed to the instable covalent attachment of the protein onto the decorated surface of the electrode. To enable comparison with previous reports, the characteristics of different electrochemical sensors for PRL are summarized in Table 1.

Table 1. Comparison of the major characteristics of electrochemical sensors used for the detection of PRL.

Electrode	Linear detection range	Detection limit	Reference
Carbon nanotube/PEDOTs/SPE	0.1 - 150 ng/mL	0.22 pg/mL	[35]
Streptavidin-functionalized magnetic particles	10-2000 ng/mL	3.74 ng/mL	[2]
Enzymeimmunoassay	10 ng/mL – 5 μg/mL	0.1 ng/mL	[36]
AP-anti-PRL–PRL-pPPA/MWCNTs/GCE	0.1 ng/mL -10 μg/mL	4 pg/mL	This work

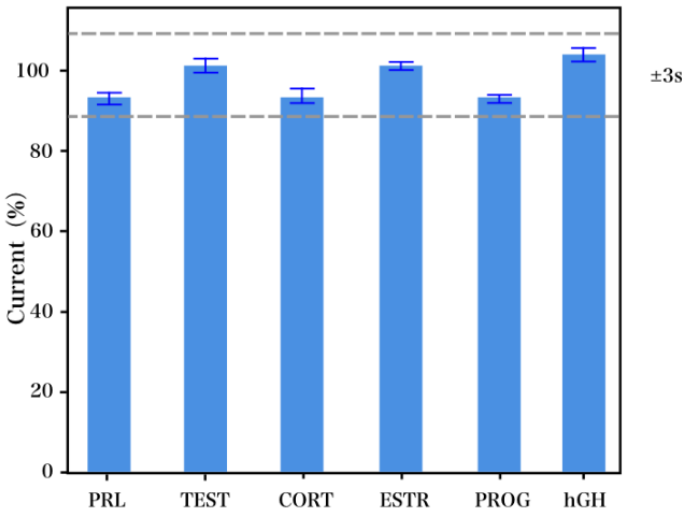

Figure 3. Amperometric currents obtained using the AP-anti-PRL–PRL-pPPA/MWCNTs modified GCE after adding cortisol, testosterone, progesterone, oestradiol and human growth hormone (50 ng/mL).

It has been widely accepted that diverse species might exist in biological fluids besides PRL, thus these species were analysed as possible interference agents based on the DPV response of the AP-anti-PRL–PRL-pPPA/MWCNTs/GCE immunosensor. Next, 50 ng/mL of human growth hormone, progesterone, oestradiol, cortisol, and testosterone were investigated. Conversely, the comparison between the immunosensor responses for the test solutions with and without PRL was presented. Fig. 3 shows that voltammetric currents obtained for the blank solution exhibited no obvious variation to that of the solution that contained each of these agents, suggesting that our proposed immunosensor towards the detection of PRL was excellently selective. Therefore, we further tested the performance of the proposed immunosensor for PRL detection in human serum.

The specimen in the presence of PRL (0.1 to 10 ng/mL) was characterized via calibration plots to assess the potential presence of matrix effects in human serum. The peak current values obtained for the specimens were interpolated to the calibration plot for the PRL standard solutions to perform the detection of PRL. The serum specimens were spiked using PRL of three concentration levels. Recoveries ranging between 95% and 102%, which show the usefulness of the developed immunosensor for the determination of this hormone in human sera with no sample treatment. The measurement of urine specimens showed similarly desirable results. We did not observe obvious intercept and slope value variation with respect to those obtained with PRL standard solutions. In addition, the same protocol for the quantification of PRL could be used. For the test urine specimen spikes with PRL (0.01 - 10 ng/mL), the desirable recovery range of 98% to 104% was obtained.

4. CONCLUSIONS

The electrochemical immunosensor for the detection of the PRL was prepared based on the synergistic effect resulting from two factors. First, biomolecules could be efficiently immobilized due to the poly(pyrrolepropionic acid) conducting polymer. Second, the charge transfer reactions could be

enhanced by the MWCNTs. Compared with other techniques, the proposed fabrication of the test immunosensor was more cost-effective and facile. In addition, our proposed immunosensor could be successfully used for the detection of urine specimens with strong relevance to the consideration of urinary PRL as a biomarker for preeclampsia and related diseases, as well as the absence of immunoassay techniques for the detection of the specimens of this kind. It is possible for the diluted specimens to be analysed using our proposed methodology, which is highly sensitive and could prevent matrix effects.

References

1. M. Freeman, B. Kanyicska, A. Lerant and G. Nagy, *Physiological Reviews*, 80 (2000) 1523.
2. M. Moreno-Guzmán, A. González-Cortés, P. Yáñez-Sedeño and J. Pingarrón, *Analytica Chimica Acta*, 692 (2011) 125.
3. Y. Sinha, *Endocrine Reviews*, 16 (1995) 354.
4. M. Fahie-Wilson and T. Smith, *Best Practice & Research Clinical Endocrinology & Metabolism*, 27 (2013) 725.
5. S. Banerjee, P. Paul and V. Talib, *Indian Pediatrics*, 41 (2004) 827.
6. C. Bole-Feysot, V. Goffin, M. Edery, N. Binart and P. Kelly, *Endocrine Reviews*, 19 (1998) 225.
7. T. Smith, A. Suliman, M. Fahie-Wilson and T. McKenna, *The Journal of Clinical Endocrinology & Metabolism*, 87 (2002) 5410.
8. K. Roy and B. Prakash, *Theriogenology*, 67 (2007) 572.
9. M. Mondal, C. Rajkhowa and B. Prakash, *Animal Reproduction Science*, 99 (2007) 182.
10. A. Kudryavtsev, V. Krasitskaya, A. Petunin, A. Burakov and L. Frank, *Anal. Chem.*, 84 (2012) 3119.
11. A. Rojanasakul, U. Udomsubpayakul and S. Chinsomboon, *International Journal of Gynecology & Obstetrics*, 45 (1994) 141.
12. S. Li, Y. Yan, L. Zhong, P. Liu, Y. Sang, W. Cheng and S. Ding, *Microchimica Acta*, 182 (2015) 1917.
13. Z. Faridli, M. Mahani, M. Torkzadeh-Mahani and J. Fasihi, *Analytical Biochemistry*, 495 (2016) 32.
14. X. Sun, Z. Jiang, H. Wang and H. Zhao, *International of Electrochemical Science*, 10 (2015) 9714.
15. G. Martínez-García, V. Serafín, L. Agüí, P. Yáñez-Sedeño and J. Pingarrón, *Electroanalysis*, 27 (2015) 1119.
16. B. Chikkaveeraiah, A. Bhirde, N. Morgan, H. Eden and X. Chen, *ACS nano*, 6 (2012) 6546.
17. H. Nie, S. Liu, R. Yu and J. Jiang, *Angewandte Chemie International Edition*, 48 (2009) 9862.
18. J. Wang and Y. Lin, *TrAC Trends in Analytical Chemistry*, 27 (2008) 619.
19. A. Chuvilin, E. Bichoutskaia, M. Gimenez-Lopez, T. Chamberlain, G. Rance, N. Kuganathan, J. Biskupek, U. Kaiser and A. Khlobystov, *Nature Materials*, 10 (2011) 687.
20. X. Chen, J. Zhu, Q. Xi and W. Yang, *Sensors and Actuators B: Chemical*, 161 (2012) 648.
21. T. Gan and S. Hu, *Microchim. Acta*, 175 (2011) 1.
22. J. Lu, S. Liu, S. Ge, M. Yan, J. Yu and X. Hu, *Biosensors and Bioelectronics*, 33 (2012) 29.
23. J. Zhu, D.S. Chauhan, D. Shan, X. Wu, G. Zhang and X. Zhang, *Microchim. Acta.*, 181 (2014) 813.
24. L. Zhang, C. Li, D. Zhao, T. Wu and G. Nie, *Microchim. Acta.*, 181 (2014) 1601.
25. F. Khorsand, M. Azizi, A. Naeemy, B. Larijani and K. Omidfar, *Molecular Biology Reports*, 40 (2013) 2327.
26. Y. Liu, Y. Liu, H. Feng, Y. Wu, L. Joshi, X. Zeng and J. Li, *Biosensors and Bioelectronics*, 35 (2012) 63.
27. W. Hu, C.M. Li and H. Dong, *Anal. Chim. Acta*, 630 (2008) 67.

28. H. Dong, C.M. Li, W. Chen, Q. Zhou, Z. Zeng and J. Luong, *Anal. Chem.*, 78 (2006) 7424.
29. H. Dong, X. Cao, C. Li and W. Hu, *Biosensors and Bioelectronics*, 23 (2008) 1055.
30. W. Chen, Y. Lei and C.M. Li, *Electroanalysis*, 22 (2010) 1078.
31. Y. Chu, C. Lin, H. Chang, C. Li and C. Guo, *Biosensors and Bioelectronics*, 26 (2011) 2334.
32. C. Lin, Y. Chu and H. Chang, *Sensors and Actuators B: Chemical*, 187 (2013) 533.
33. P. Petrou, S. Kakabakos, I. Christofidis, P. Argitis and K. Misiakos, *Biosensors and Bioelectronics*, 17 (2002) 261.
34. H. Chen, Y. Cui, B. Zhang, B. Liu, G. Chen and D. Tang, *Analytica Chimica Acta*, 728 (2012) 18.
35. V. Serafín, G. Martínez-García, L. Agüí, P. Yáñez-Sedeño and J. Pingarrón, *Analyst*, 139 (2014) 4556.
36. M. Mondal, C. Rajkhowa and B. Prakash, *Animal Reproduction Science*, 99 (2007) 182.