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Portable potentiostatic sensor integrated with neopterin-imprinted poly(ethylene-*co*-vinyl alcohol)-based electrode

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Abstract: Neopterin is a catabolic product of guanosine triphosphate, a purine nucleotide. Measuring neopterin concentrations in biological fluids such as urine provides information about cellular immune activation in humans under control of T helper cells. A high neopterin concentration in bodily fluids, including serum and urine, indicates cellular immunity activation, which is associated with oxidative stress. In this work, neopterin is the target molecule and imprinted onto poly(ethylene-*co*-vinyl alcohol) via solvent evaporation. The template molecules on the thin film are then removed, and the membrane is used as a sensing element for electrochemical urinalysis. Poly(ethylene-*co*-vinyl alcohol) containing 27 mol% ethylene had high imprinting effectiveness and may be integrated with the proposed portable biosensor. In random urine analysis, the cyclic voltammetry measurements of neopterin with an additional recovery method achieved >95% recovery for the neopterin concentration of 15 ng/mL.

1 Introduction

A high neopterin concentration is associated with increased production of reactive oxygen species [1], and may be the best predictor of adverse outcomes in patients with human immunodeficiency virus (HIV) infection, cardiovascular disease and various cancers [2]. Notably, a high neopterin concentration may be induced by viral infection, bacterial infections, parasites, an autoimmune disease, malignant tumour and allografts.

During the 1980s, the sensing capability of molecularly imprinted polymers (MIPs) in combination with electrodes and electrochemical measurements was first demonstrated [3]. Piletsky and Turner [4] and Blanco-López *et al.* [5] have reviewed electrochemical sensors based on MIPs in 2002 and 2004, respectively. Potentiometric sensors have been reviewed by McCluskey *et al.* [6] and Rao and Kala [7]. Recently, Suryanarayanan *et al.* also reviewed molecularly imprinted electrochemical sensors [8]. The composition of MIPs conventionally include methacrylic acid (MAA)/ethylene glycol dimethacrylate (EGDMA) or 4-vinyl pyridine (4VP)/EGDMA, which is usually used for acidic or basic systems. Divinylbenzene is used as the cross-linking monomer in hydrophobic systems. Other novel functional monomers are synthesised for increased selectivity of target molecules. Prepolymers, such as polyurethane [9, 10], are also utilised to form MIPs to reduce covalent bonding between target molecules and a monomer during polymerisation. Poly(ethylene-*co*-vinyl alcohol) (EVAL) contains hydrophobic and hydrophilic repeat units, accounting for its high biocompatibility. Methods for forming EVAL membranes are precipitation by solvent evaporation and wet-phase inversion [11, 12]. Therefore different EVAL compositions should be suitable for use as artificial antibodies in biosensors [13–15].

In this work, EVAL was utilised to form synthetic receptors on a sensing element and was then integrated with an electrode. The EVALs containing different mole percentages of ethylene were imprinted and their adsorption was compared to identify which had the highest affinity for neopterin. Random urine samples were analysed using the membrane-coated electrode with a proposed homemade potentiostat.

2 Experimental section

2.1 Reagents

Neopterin, 2-amino-6-(1,2,3-trihydroxypropyl)-1H-pteridin-4-one, and poly(ethylene-co-vinyl alcohol), EVAL, with ethylene 27, 32, 38 and 44 mol% were purchased from

Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Panreac (Barcelona, Spain) and used as the solvent to dissolve EVAL particles. Sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used for the removal of template molecules. All chemicals were used as received unless otherwise mentioned.

2.2 Preparation of MIPs-coated electrodes

The synthesis of neopterin- and non-imprinted EVAL thin film briefly included three steps: (i) casting the EVAL solution (EVAL/DMSO = 1.0 wt%) mixed with and without 0.025 wt% of template molecules on a indium tin oxide (ITO) glass electrode (2.5×7 cm^2); (ii) solvent evaporation in an oven at 60°C for 3 h to completely remove DMSO and then (iii) removal of the template molecule by rinsing in 20 mL of 0.1 wt% SDS for 30 min and then deionised water for 10 min, repeated three times. In general, the template molecule for molecular imprinting may be a part or whole molecule of the target molecule.

2.3 Surface characterisation of MIP thin films

The preliminary binding measurements of neopterin to the neopterin- or non-imprinted EVAL polymers were performed with 2 mL of 0.1 mg/mL target molecules for 30 min. A UV–vis spectrophotometer (HaLO DB-20, Dynamica, Australia) was then used to measure the concentration decrease in the stock solutions, determined with wavelength at 346 nm.

Atomic force microscopy (AFM) scanning and electron spectroscopy for chemical analysis (ESCA) measurement of the molecular imprinting polymers coated on the sensor was performed in the steps before and after SDS solution washing of template from EVAL thin film, followed by being freeze-dried under vacuum (FD5030/8530, Panchum, Taiwan). AFM (model: NT-MDT solver P47H-PRO, Moscow, Russia) images were made in air (room temperature (ca. 27°C) and moisture 87%) using the tapping mode with velocity (scan rate) 0.75 Hz. The cantilever was a SiO_2 probe (model: TGS1, NT-MDT, Moscow, Russia) with probe size and resonant frequency are 2 nm and 144 kHz, respectively. ESCA (Axis Ultra DLD, Kratos Analytical Inc., Manchester, UK) was employed to measure the elemental composition of the neopterin- or non-imprinted EVAL polymeric thin film. The energy resolution for O–C = O C 1s of polyethylene terephthalate (PET) is full-width half maximum (FWHM) $<$ 0.68 eV and sensitivity $>$10 KCPS at FWHM = 0.68 eV.

2.4 Portable potentiostat

This work proposes a low-cost three-channel potentiostat to realise the concurrent multiple electrochemical biosensors analysis. The three-channel potentiostat consists of three stand-alone portable potentiostats with a universal serial bus (USB) data interface and USB hub. The USB hub, which is used to expand a single USB port of a computer into several ports, is connected to three stand-alone portable potentiostats to achieve the function of a three-channel potentiostat. Each of the three stand-alone portable potentiostats can be operated in three modes – potentiometry, voltammetry and cyclic voltammetry. Data transmission between different potentiostats and the computer is organised by different time slots to avoid data collision.

Fig. 1 shows the circuit diagram of a stand-alone portable potentiostat, which has a mixed-signal microprocessor (C8051F020), an auto-range current-to-voltage converter, a unity-gain buffer amplifier, an operational amplifier, a direct current power converter and a USB data transfer interface. The DC–DC power converter converts the 5 V provided by the USB port into ±12 V for the potentiostat. The USB data transfer interface with its plug-and-play function provides data communication between the potentiostat and computer, and powers the potentiostat. A three-electrode biosensor in the proposed potentiostat is combined with an operational amplifier and negative feedback loop for potentiostatic control. The digital-to-analogue converters DAC0 and DAC1 in the mixed-signal microprocessor are used to generate reduction potential (V_{set}) of the biosensor and offset potential (1.2 V) of the potentiostat, respectively.

A high-impedance unity-gain buffer amplifier attached to the reference electrode is used to measure the potential of working electrode referred to a reference electrode to ensure that very low current flows through this interface. Furthermore, an auto-range current-to-voltage converter, comprising a transimpedance amplifier, four different value resistors and an analogue multiplexer, can be used to automatically reduce the current range of the sensor from ±12 mA to ±12 μA by adjusting the analogue multiplexer channel using a microprocessor.

2.5 Measurement of random urine sample with the potentiostat and MIP electrodes

The current response of the imprinted polymeric sensing electrodes was measured with the proposed potentiostat, using phosphate-buffered saline (PBS), different concentrations of target molecules in PBS and using random urine samples. The set-up of the electrochemical reaction can be found elsewhere [16]; briefly, the working, counter and Ag/AgCl reference electrodes were placed in a

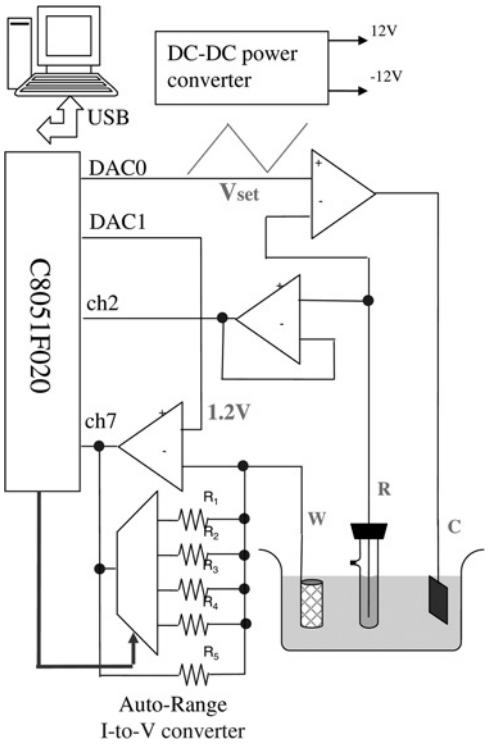

Fig. 1 *Circuit diagram of a stand-alone portable potentiostat*

20 mL 0.5 M KCl, 20 mM $K_4Fe(CN)_6$ and 20 mM $K_3Fe(CN)_6$ solution with the scan rate 0.1 V/s.

3 Results and discussion

Fig. 2 shows the surface analysis of the neopterin-imprinted EVAL thin film, including analyses of its adsorption capacity, surface element and surface morphology. The adsorption of neopterin on the neopterin-imprinted EVAL thin film (Fig. 2*a*) decreased from 2.35 ± 0.10 to 0.90 ± 0.09 μg/cm^2 when the ethylene mole percentage increased from 27 to 44. This analytical result demonstrates that several hydrophilic functional groups on the neopterin, the affinity between neopterin and the EVAL increased when EVAL contained a low percentage of ethylene. Adsorption of neopterin on non-imprinted polymers decreased when the ethylene mole percentage increased. The best imprinting effectiveness for neopterin was 2.05 using 27 mol% ethylene (Table 1). Imprinting effectiveness is defined as the ratio of adsorption for MIPs and to that of non-imprinted polymers. Fig. 2*b* shows the binding energy and the intensity profile of the surface element examined by ESCA. The binding energy of carbon, nitrogen and oxygen was roughly 282–284, 397–400 and 529–531 eV, respectively. Table 2 lists the element ratios of those molecules in the MIP thin film before and after template removal and after rebound with neopterin. Notably, the template neopterin molecule contained five nitrogen molecules. Owing to the high amount of carbon and oxygen in EVALs, the atomic ratios of nitrogen molecules in the EVAL thin film can be easier to compare during imprinting and rebinding. Figs. 2*c*–*e* show the AFM images of the MIP thin film before and after template removal and after target molecules rebounded, respectively. The scanning area of those images was 500 × 500 nm^2, and maximum thickness was 50 nm (Fig. 2*c*) and 40 nm (Figs. 2*d* and *e*). The average roughness of these three images was 5.67, 2.73 and 3.98 nm for each step. Therefore the average thickness of the neopterin rebound on MIPs was about 1.25 nm, which is the approximated monomer layer adsorption of neopterin with the long axis.

Fig. 3*a* shows the typical cyclic voltammetry of the MIP electrode measured by a home-built potentiostat; current peaked when 0.25 V was applied. The current responses for neopterin-imprinted polymer increased monotonically from 130 μA to as high as 200 μA when neopterin concentration

Table 1 Rebinding of neopterin to the neopterin- and non-imprinting EVAL and their imprinting effectiveness on different ethylene mole% of EVALs (bold values indicate the highest imprinting effectiveness achieved)

EVAL (ethylene mole%)	Neopterin adsorption, μg/cm^2		
	MIPs	NIPs	IF
27	**2.35 ± 0.10**	**1.15 ± 0.06**	**2.05**
32	1.93 ± 0.07	1.20 ± 0.07	1.61
38	1.34 ± 0.11	0.95 ± 0.11	1.41
44	0.90 ± 0.09	0.54 ± 0.09	1.66

MIP: molecularly imprinting polymer; NIP: non-imprinting polymer; IF: imprinting effectiveness

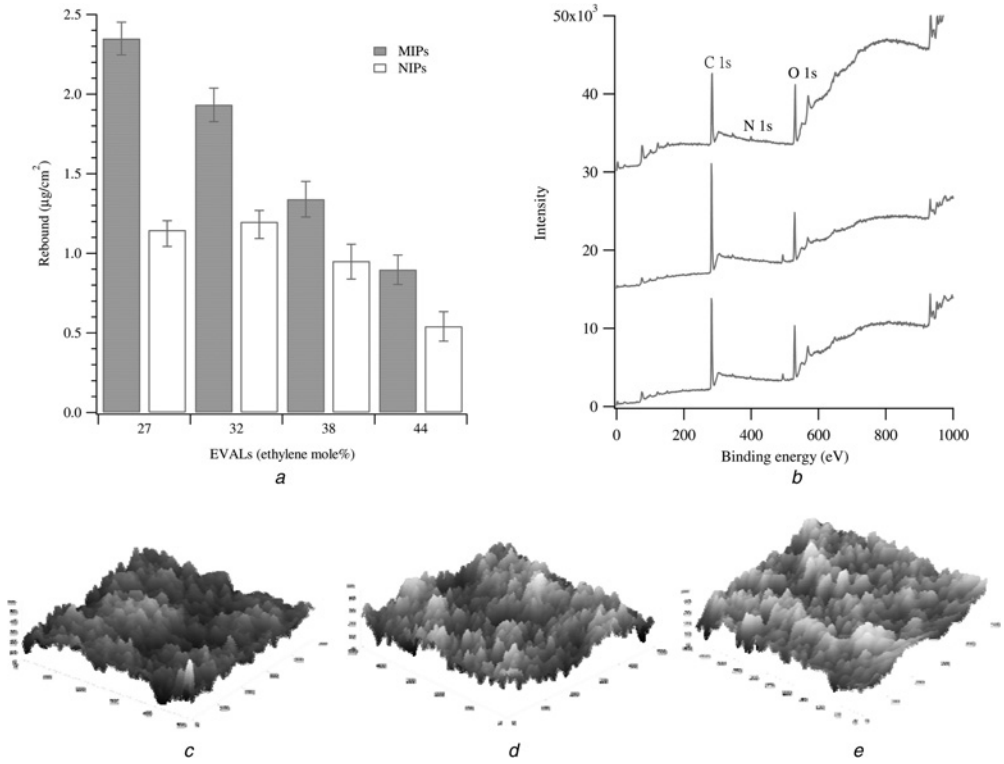

Fig. 2 *Characteristics of neopterin- and non-imprinted EVAL thin film*

a Adsorption of neopterin on neopterin- and non-imprinted EVAL thin film
b X-ray photoelectron spectroscopy spectra of neopterin-imprinted EVAL thin film before, after washing and after rebinding are shown from top to bottom
c AFM image of neopterin-imprinted EVAL thin film before washing
d AFM image of neopterin-imprinted EVAL thin film after washing
e AFM image of neopterin-imprinted EVAL thin film after rebinding
Image size of the AFM is 500 × 500 nm^2 and the maximum height is 50 nm

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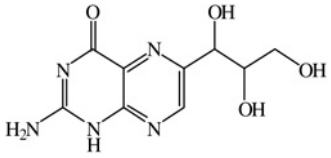
Table 2 Surface element analysis of neopterin-imprinted polymer thin film during the preparation (Grey shading indicates the nitrogen atom % may relate with the amount of neopterin on the EVAL thin film)

Neopterin	Elements	MIPs			NIPs
		Before washing	After washing	After rebinding	
	O 1s	19.74	17.01	13.97	17.30
	N 1s	2.08	0.62	1.07	0.47
	C 1s	77.55	82.13	84.52	81.04

was 50 ng/mL. Fig. 3*b* shows the electrochemical response of the non-imprinted EVAL-coated electrode. In comparison with the electrochemical response of the neopterin-imprinted EVAL-coated electrode, the non-imprinted EVAL-coated electrode was 30–60 μA in the same neopterin concentration range. The peak current changed when the neopterin concentration changes may due to the combination of the reaction between ferricyanide and ferrocyanide and the ketone–ethanol tautomerism [17] of the neopterin. Fig. 3*c* plots the current difference between the buffer and specific neopterin concentration at peak current, and is used as the calibration curve for real sample measurement. The sensitivity of the neopterin- to non-imprinted EVAL-coated electrodes was roughly 4-fold when the neopterin concentration was 10 ng/mL. The selectivity of the proposed electrodes was also examined with high concentrations of urea and creatinine. The electrochemical responses for those molecules were less than ± 3.0 μA as shown in Fig. 3*d*.

Although low-cost highly stable MIPs have significant advantages as sensing materials, sensor reliability must be

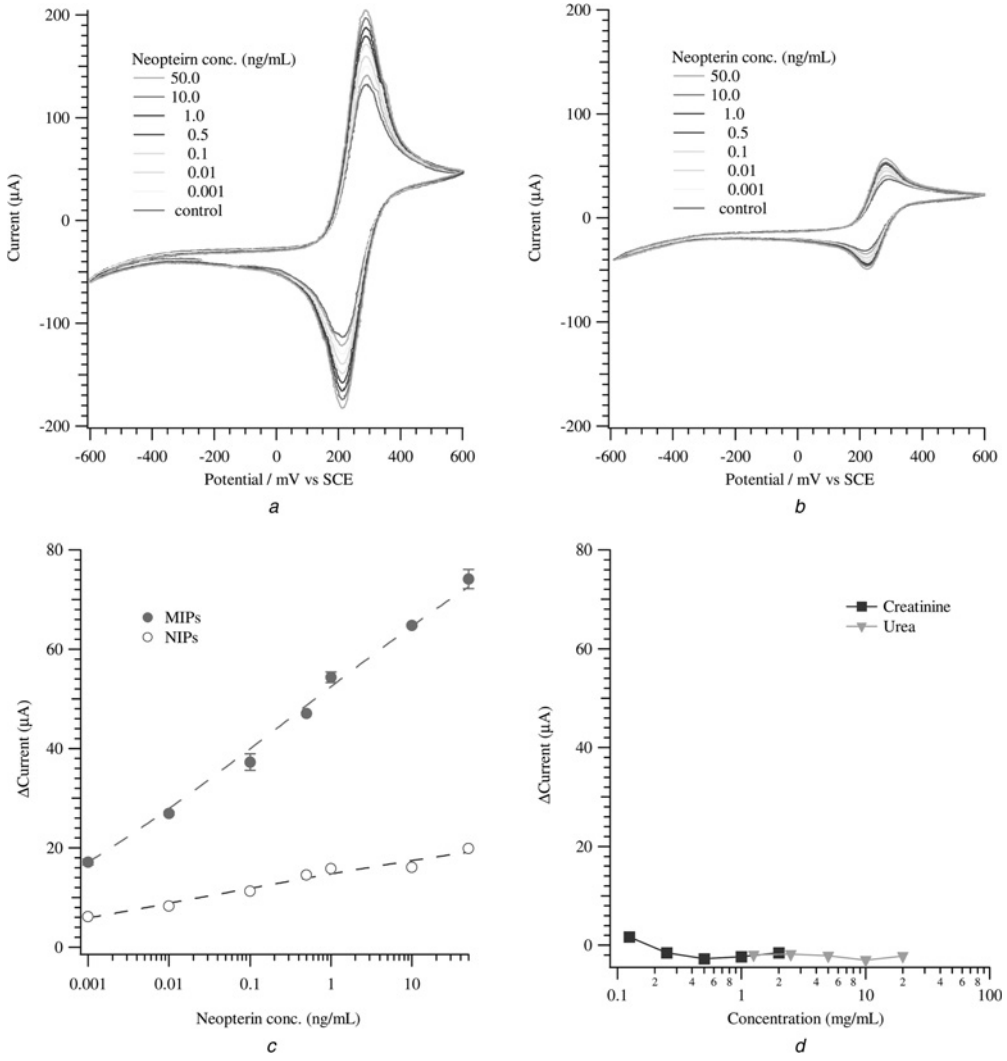


Fig. 3 *Electrochemical responses of EVAL coated ITD electrodes with a home built potentiostat*

a Cyclic voltammetry using neopterin-imprinted polymer coated electrode
b Cyclic voltammetry using non-imprinted polymer coated electrode
c Calibration curve of neopterin to neopterin- and non-imprinted polymer-based sensors
d Current difference when different concentrations of an interfering analyte (creatinine: squares and creatinine: urea) were measured with neopterin-imprinted EVAL polymeric electrode

Table 3 Additional recovery of neopterin in random urine

Sample	Urine, μL	Neopterin (0.1 mg/mL)	Home-built potentiostat sensor				Recovery, %
			ΔCurrent, μA	Converted conc., ng/mL	Recovered, ng	Urinary conc., ng/mL	
I(1)	20		35.27	0.048	0.96	43.96 ± 4.54	
	40		37.76	0.077	1.54		
	40	3 μL	68.25	14.840	297.45		98.64
I(2)	20		36.49	0.049	0.98	44.79 ± 4.13	
	40		39.20	0.081	1.63		
	40	3 μL	68.14	14.888	298.39		98.92
II	20		34.05	0.060	1.21	55.31 ± 5.09	
	40		36.78	0.100	2.01		
	40	3 μL	68.32	14.583	292.28		96.76
III	20		35.38	0.038	0.76	34.92 ± 3.02	
	40		38.07	0.064	1.28		
	40	3 μL	68.27	15.007	300.77		99.83

Recovery of neopterin was defined as the percentage of pure neopterin recovered in the urine containing solution

confirmed before it can be considered for daily homecare use. Additional recovery of neopterin was performed when 20 μL of random urine samples was added to electrochemical sensing repeatedly. Three microlitres of 0.1 mg/mL (i.e. 300 ng) of pure neopterin was then added to determine the amount of neopterin recovered (Table 3). The recovery of neopterin is defined as the percentage of neopterin recovered and added. The recovery of neopterin is based on the calibration curve, and the standard deviation is as less as 2 μA at high neopterin concentration. Random urine samples from the author and colleagues were analysed; the neopterin concentration in urine was 34.92 ± 3.02 − 55.31 ± 5.09 ng/mL. This work demonstrates that EVAL polymeric thin films can be imprinted with neopterin molecules; neopterin is an important biomarker for aging. Moreover, the limit of detection of this electrode for neopterin from the calibration curve was as low as 0.025 pg/mL, which is comparable with some commercial kits like radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Their sensitivities are as low as 0.2 ng/mL [18] and 0.5 ng/mL [19] for RIA and ELISA, respectively. Finally, the homemade potentiostat can be integrated with multiple MIPs sensing system for urinalysis.

4 Conclusions

Although biomarkers in urine for specific diseases are still under investigation, sarcosine in urine was recently strongly correlated with prostate cancer [20]. Non-invasiveness is an important issue for daily monitoring. In this work, a novel low-cost portable potentiostat was integrated with MIPs for the detection of neopterin, an oxidative stress biomarker. The EVALs containing low mole percentages of ethylene had enhanced imprinting effectiveness for recognition of target molecules. The sensitivity of the neopterin-imprinted EVAL-coated electrode was as low as 0.041 pg/mL, compared with the reference concentration of 35–55 ng/mL in urine. This low-cost electrode with high sensitivity and selectivity has potential for use as a homecare system.

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