Sensors and Actuators B xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Research Paper

A whole cell bio-optode based on immobilized nitrite-degrading microorganism on the acrylic microspheres for visual quantitation of nitrite ion

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ARTICLE INFO

Article history:
Received 8 June 2017
Received in revised form 20 August 2017
Accepted 15 September 2017
Available online xxx

Keywords:
Nitrite
Raoutella planticola
Edible bird's nest
Optode
Reflectance

ABSTRACT

A microspheres-based microbial optosensor for NO_2^- ion quantitation was constructed by using immobilized *Raoutella planticola* (*R. planticola*), the bacterium expressing NAD(P)H nitrite reductase (NiR) enzyme, which was isolated from local edible bird's nest (EBN) *via* microbial technique. The whole cells and the lipophilic Nile Blue chromoionophore (NBC) were physically adsorbed on the self-adhesive photocurable poly(*n*-butyl acrylate-co-*N*-acryloxysuccinimide) [poly(nBA-NAS)] microspheres, whilst the reduced co-enzyme NAD(P)H was covalently immobilized on the succinimide-functionalized acrylic microspheres *via* peptide link to produce a reagentless nitrite biosensing system. As the microbial bio-optode responded to nitrite through colour change from blue to pink, a facile reflectometric approach was adopted to measure reflectance intensity at 639 nm, before and after reaction with nitrite at optimum pH 8. The optosensor could quantify NO_2^- ion concentration within a dynamic linear response range of 0.5–400 mg L^{-1} with a limit of detection (LOD) of 0.2 mg L^{-1}. The large surface area to volume ratio of the acrylic microspheres allowed solid-state diffusional mass transfer of the substrate to occur at micro-bio-optode surface, and an equilibrium response was achieved within 5 min. The practical feasibility of using the bio-optode for nitrite assay in food matrix sample showed good agreement with standard ion chromatography method.

1. Introduction

According to Malaysian Food Regulation (1985), the maximum permitted limit for nitrite in food is about 200 mg L^{-1}, and the national legal limit for NO_2^- ion in EBN is about 30 mg L^{-1}. The permissible level of NO_2^- ion in both drinking and natural waters is <10 mg L^{-1} as per Malaysian Ministry of Health limit, and <0.1 mg L^{-1} NO_2^- ion is advocated by World Health Organization for drinking water [1–3].

Various analytical techniques with different detection principles for the determination of nitrite have been developed during the past 15 years. Analytical parameters such as matrix, detection limits and detection range were determined for different practical purposes [4]. Spectrophotometric Griess test is the simplest established standard method for NO_2^- ion detection [5,6]. The Griess diazotization method for nitrite assay has high sensitivity and can be used in measuring as low as 0.5 μM NO_2^- ion [5]. Nevertheless, this method requires careful control of the pH of each reaction step and susceptible to strong oxidants and dyeing interferences. Other traditional methods rely on the use of instruments such as gas chromatography–mass spectrophotometer (GC–MS) [7,8], high performance liquid chromatography (HPLC) [9–21] and ion chromatography (IC) [13–15], which cannot give real-time response, expensive, time-consuming, subject to interferences and not likely for on-site detection purposes. Therefore, it is of significant need for devices there are capable of measuring nitrite concentration *in-situ*, rapidly and without reagents, sample pretreatment or extraction step.

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http://dx.doi.org/10.1016/j.snb.2017.09.102
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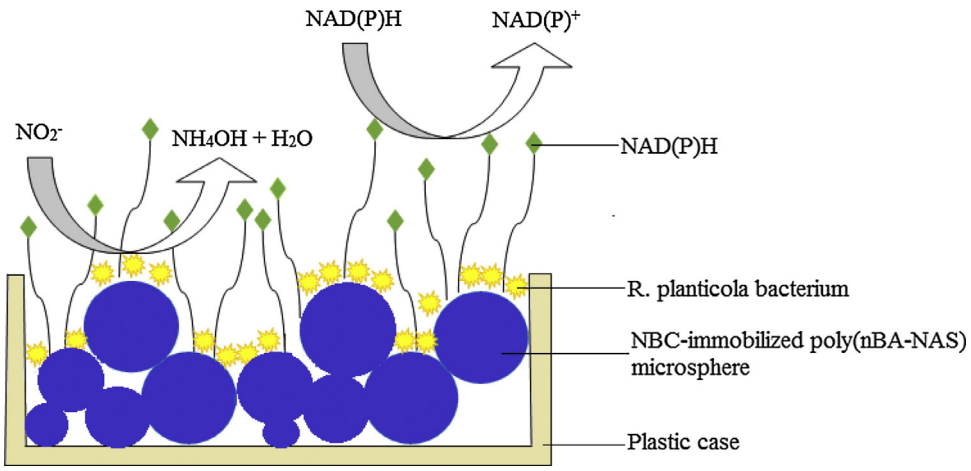

Fig. 1. The schematic design of the reflectance nitrite bio-optode based on nitrite-degrading microorganism, NAD(P)H co-enzyme and NBC pH indicator co-immobilized on the surface of poly(nBA-NAS) microspheres.

Biosensor techniques have been extensively explored alongside with the increasing demand for fast response on-site analysis in order to replace the conventional instrumental methods. Generally, biosensor consists of unique biological sensing elements, which allow it to specifically distinguish the analyte of interest with minimal interferences and optimum sensitivity [16,17]. Various biosensor approaches had been developed with the ability to detect NO_2^- ion in food or environmental samples such as voltammetric [18–22], amperometric [23–26], potentiometric [27], conducometric [28,29] and optical methods [2,16]. Among these techniques, it turns out that optical biosensors could offer better capability compared to electrochemical biosensors because of no reference element is needed during the signal measurement, freedom from electrical interference and baseline variation can be avoided [2], other than its simple instrumentation setting.

Optical NO_2^- ion biosensor based on cytochrome cd_1 nitrite reductase immobilized in isothiocyanato controlled pore glass beads has been previously reported because of its high specificity towards NO_2^- ion and no cross-reactivity with other anions present in the water. The diffuse reflectance optical enzyme bio-optode showed high sensitivity to NO_2^- ion down to micromolar levels, and suitable to be adopted in the surveillance of potable water quality [2]. UV–vis spectrophotometric bio-optode has also been constructed for optical NO_2^- ion determination by immobilizing the cytochrome cd_1 nitrite reductase enzyme in sol-gel matrix on a glass substrate *via* dipping approach. The inert and biocompatible sol-gel immobilization support prevented partial or total loss of enzyme physiological activity, and that the bio-optode remained stable for several months [16].

The use of cell-free enzymes is generally preferred over intracellular enzyme systems because they can directly access to specific substrate in the solution, hence tends to be much more sensitive. Anyhow, the cell-free enzymes often exhibit reaction-rate limited kinetics when they are being extracted out from the natural cellular metabolic environment. In contrast, the intracellular enzyme expression system has a tightly regulated intracellular network that is able to protect the enzyme from toxic and potential interfering substances. Moreover, the whole cell systems are less expensive to prepare compared to purified cell-free enzymes [30]. The usage of whole cell bacterial biosensor based on *Nitrobacter vulgaris* adsorbed on the Whatman chromatographic paper and an amperometric system for oxygen content measurement of cells' aerobic metabolism has showed high selectivity and low nitrite detection limit at micromolar levels [31]. The bacterium-based nitrite biosensor using *Stenotrophomonas nitritireducens* denitrifying microorganism *via* bacterial reduction of nitrite to nitrous oxide (N_2O) was constructed with an built-in Clark-type electrochemical N_2O sensor by [32], and the microbial biosensor was successfully applied for long term on-line assessment of wastewater.

In this study, an optical whole cell micro-bio-optode based on Nile Blue chromoionophore (NBC) pH optode was fabricated in tandem with immobilized nitrite-degrading bacterium, *Raoutella planticola* (*R. planticola*) expressing NAD(P)H nitrite reductase (NiR) enzyme on the copolymer of *N*-acryloxysuccinimide-co-acrylate microspheres for NO_2^- ion detection. The self-adhesive succinimide-modified polyacrylate microspheres were used as the carrier matrix for NAD(P)H co-enzyme *via* amide covalent bond, and the bacterial cells as well as the proton selective chromoionophore were immobilized at the poly(*n*-butyl acrylate-co-*N*-acryloxysuccinimide) [poly(nBA-NAS)] microspheres surface *via* physical adsorption. As the NiR enzyme catalyzed the reduction of NO_2^- ion to ammonium hydroxide (NH_4OH), the NAD(P)H cofactor was immediately oxidized to $NAD(P)^+$ (Eq. (1)). The resulting hydroxide (OH^-) ions led to a large pH change of the optode microspheres film, and a colour change from blue to pink can be perceived as the immobilized NBC underwent deprotonation process (Eq. (2)), which could be quantified by reflectometric technique.

$$NO_2^- + 3NAD(P)H + 3H^+ \rightleftharpoons NH_4OH + 3NAD(P)^+ + H_2O \quad (1)$$

$$\underset{\text{(blue)}}{NBCH^+} \rightleftharpoons \underset{\text{(pink)}}{NBC + H^+} \quad (2)$$

The bio-optode design and the enzymatic reaction involved in the nitrite detection is illustrated in Fig. 1. The bacterial cells were attached at the surface of the copolymer microspheres in order to avoid loss of the enzyme physiological activity, and the use of three-dimensional microspheres immobilization matrix facilitated the small analytes to diffuse before reaching the biological phase. Therefore, the overall analytical performance of the proposed microbial optosensor was tremendously enhanced compared to previously reported optical nitrite biosensor based on two-dimensional substrate.

2. Material and methods

2.1. Chemicals

2,2-Dimethoxy-2-phenylacetophenone (DMPP), 1,6-hexanediol diacrylate (HDDA) and *n*-butyl acrylate (nBA) were supplied by Aldrich. Potassium dihydrogen phosphate (KH_2PO_4)

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and dipotassium hydrogen phosphate (K_2HPO_4) were obtained from R&M Malaysia. *N*-acryloxysuccinimide (NAS) and sodium dodecyl sulfate (SDS) were purchased from Acros Organics and Scharlau Chemie S. A., respectively. Sodium nitrite was procured from Friendemann Schmidt and was dissolved in 0.1 M potassium phosphate (K-phosphate) buffer at pH 7.4. Nile blue chromoionophores ETH 5294 (Fluka) was prepared in 95% ethanol (Dulab Lab Chemical). Nicotinamide adenine dinucleotide phosphate sodium (NADPH, Merck) at 40 mg/L was prepared in 0.1 M K-phosphate buffer (pH 7.4). All the chemicals were of analytical grade and used without further purification. Milli-Q water (18 mΩ) was used to prepare all the chemical and biological solutions.

2.2. Instrumentations

Fiber optic reflectance spectrophotometer (Ocean Optics, USB4000-UV–vis) was used to measure the reflectance intensity of the solid-state bacterium-based bio-optode within the reflectance wavelength range of 300–1000 nm. The optical fiber lamp was placed at a fixed distance on the measurand surface to get the maximum reflectance signal. All the reflectance measurements were done in triplicate. A new bio-optode was used for every measurement. Schott pH meter was used to measure and adjust the pH of the K-phosphate buffer. Sonication of the precursor for copolymer of NAS and nBA was made with Branson ultrasonic cleaner. An UV exposure unit, which built from four UV light tubes with maximum output around 350 nm wavelength was employed in the photolithography process during the synthesis of poly(nBA-NAS) microspheres. IKA hotplate stirrer (C-MAG HS7) was used to prepare homogeneous solutions. The size and surface morphology of the acrylic microspheres were viewed under JEOL field emission scanning electron microscope (FESEM) working at 3 kV acceleration voltage and 2 k × magnification.

2.3. Isolation and identification of nitrite-degrading microorganisms

R. planticola bacterial cells, the nitrite-degrading microorganisms were isolated from edible bird's nest (EBN) sample. In brief, some raw edible bird's nest (EBN) samples were collected from local birdhouse. Feathers and other impurities were manually removed using tweezer and forceps before the EBN was ground up in powder form using mortar and pestle. The powdered EBN was then incubated overnight in saline water (0.9% NaCl) prior to grow the bacterial cells using nutrient agar medium at 37 °C for 24 h. The total viable count of bacteria was 297 colonies based on dilution factor of 10^{-3}. Aliquots of this microbial suspension has been dispatched for polymerase chain reaction (PCR) analysis and agarose gel electrophoresis. *R. planticola* strains NBRC 14939 of 16S ribosomal ribonucleic acid (rRNA) gene with partial sequence was identified as the source of nitrite-degrading microorganism. BLAST (Basic Local Alignment Search Tool) analysis of the 16S rDNA sequence confirmed that the strain NBRC 14939 was belonged to the genus *Raoutella*. The isolates were then grown in an optimum enrichment medium, which consists of sugar compound, K-phosphate buffer (0.1 M, pH 7.4), $MgSO_4{\cdot}7H_2O$, yeast extract, nitrite powder and some trace elements, and stored at −20 °C before use. The nucleotide sequence of *R. planticola* is shown in Appendix A in the Supplementary material.

2.4. Fabrication of microbial bio-optode based on acrylic microspheres carrier matrix

Poly(nBA-NAS) microspheres were synthesized by mixing 4 mL of nBA monomer with 0.09 g DMPP photo-initiator, 400 μL HDDA cross-linker, 0.1 g SDS surfactant, 10 mg NAS and 10 mL of Milli-Q water in a scintillation vial. The mixture was sonicated for 10–15 min to obtain a milky emulsion and photocured with UV radiation for 600 s under continuous nitrogen gas flow. The microspheres were then isolated by centrifugation at 6000 rpm for 15 min, and the pellet obtained was dried at room temperature (25 °C). The dried acrylic microspheres were suspended in 0.1 mg/mL NBC and stirred until a homogenous blue-coloured slurry obtained, and left overnight to allow physical immobilization of NBC onto the poly(nBA-NAS) microspheres. Then, the blue-coloured microspheres suspension was strained using Whatman no. 1 filter paper and washed several times with ethanol to remove the unbound NBC molecules. About 29.7×10^4 bacterial cells/mL (CFU/mL of microbes) and 2 mM of NAD(P)H were then sequentially added onto the microspheres at a volume ratio of 1:3, and incubated overnight at 4 °C to allow spontaneous covalent bonding formed between the succinimide functional group of poly(nBA-NAS) and the amine-bearing NAD(P)H, and physical adsorption of bacterial cells onto the microspheres surface. Finally, the surface-modified microspheres were washed with abundant 0.1 M K-phosphate buffer (pH 7.4) to remove those loosely bound microbes and NAD(P)H co-factor. Some 100 mg of the bio-functionalized copolymer microspheres were deposited in a round plastic case with 8 mm diameter and sealed with parafilm (with tiny holes) followed by drying at 4 °C in a refrigerator to avoid contamination.

2.5. Optimizing the bacterium-based reflectometric nitrite bio-optode

pH effect on the microbial bio-optode response towards the detection of 100 mg L^{-1} NO_2^- ion was carried out by varying the 0.1 M K-phosphate buffer pH between pH 4 and pH 9. The bio-optode reflectance responses before and after reacted with 100 mg L^{-1} NO_2^- ion at different pHs were measured with the fiber optic reflectance spectrophotometer at the wavelength of 639 nm. Dynamic linear response range of the nitrite bio-optode was determined by using a series of NO_2^- ion concentrations from 0.5–600 mg L^{-1} in 0.1 M K-phosphate buffer at pH 8. The bio-optode response time was examined by using some 18 units of batch-produced microbial bio-optodes. The bio-optode reflectance signal at 30 s was recorded after exposure to 100 mg L^{-1} NO_2^- ion. This step was repeated several times using new bio-optodes with different signal capturing times *i.e.* 1 min, 2 min, 3 min, 5 min and 10 min. For bio-optode shelf life study, about 30 units of bio-optodes were batch-produced and stored in a refrigerator at 4 °C. Some three units of bio-optodes were taken out intermittently over an experimental period of a month, and their reflectance responses after reaction with 100 mg L^{-1} NO_2^- ion were measured by means of fiber optic reflectance spectrophotometer. Interference study was conducted by reacting the optical nitrite biosensor with various interfering ions such as NH_4^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+} and NO_3^- ions at 10 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1} and 500 mg L^{-1}. The reflectance signals obtained were then compared with the reflectance response of the bio-optode towards the detection of NO_2^- ion from 10 to 500 mg L^{-1}.

2.6. Validation study

The unprocessed EBN was used as the food matrix in validating the analytical performance of the proposed bio-optode with ion chromatography reference method. About 1 g of unprocessed EBN sample was soaked in 40 mL of Milli-Q water with vigorous shaking for 1 h at ambient temperature followed by heating at 40 °C in a water bath. The bird's nest sample was then cooled to room temperature and centrifuged at 10 000 rpm for 10 min. The clear supernatant was collected *via* filtration through 0.45 μm membrane filter. Standard NO_2^- ion at 10 mg L^{-1}, 40 mg L^{-1}, 80 mg L^{-1},

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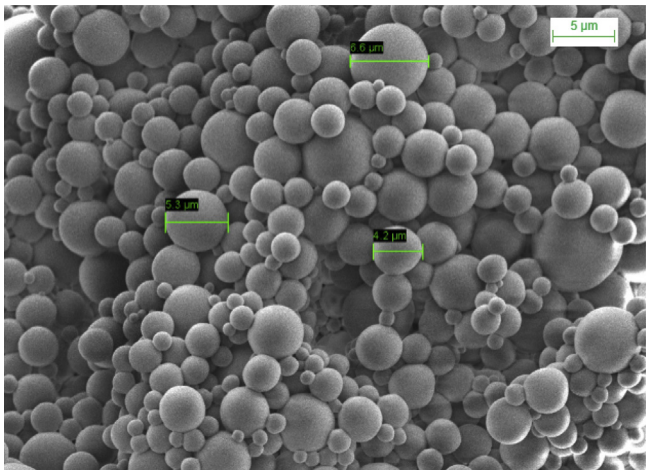

Fig. 2. FESEM image of the poly(nBA-NAS) microspheres captured at an acceleration voltage of 3.0 kV and at 2 k × magnification.

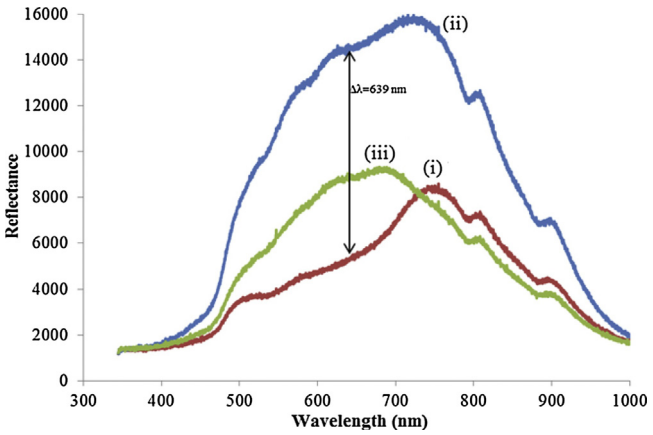

Fig. 3. Reflectance spectra of the microbial bio-optode (i) before and (ii) after reaction with 100 mg L^{-1} NO_2^- ion at pH 8 and (iii) NBC-immobilized poly(nBA-NAS) microspheres without immobilized bacterial cells and co-enzyme in the presence of 100 mg L^{-1} NO_2^- ion (pH 8).

100 mg L^{-1} and 400 mg L^{-1} at pH 8 were then spiked into the EBN aqueous extracts and analyzed with both bio-optode and ion chromatography standard method. An unspiked EBN sample was also examined by both methods for comparison purpose. Statistical analysis using *t*-test was applied to evaluate the results obtained by both methods thereafter.

3. Results and discussion

3.1. Acrylic microsphere and its optical biosensing response with nitrite

The FESEM image in Fig. 2 depicts the spherical acrylic microspheres synthesized by emulsion photopolymerization process. The uniform size distribution of the poly(nBA-NAS) microspheres are in the size range between 1.4 µm and 4.2 µm, and the average diameter of the poly(nBA-NAS) microspheres was determined to be about 2.6 µm. The acrylic microspheres with polymer features such as low glass transition (T_g) value and small molecular weight distributions were the main factors in rendering the resulting immobilization substrates softer and more flexible. The plasticizer-free microspheres composition has been optimized in the previous study [33] to yield the microspheres with sufficient adhesion properties, whereby increasing the amount of HDDA crosslinker could increase the T_g value, and the optimum composition of nBA monomer is important to produce the self-adhesive acrylic microspheres, which are capable to physically bind chemical or biological molecules on the polymeric spheres' surface, and compatible with plastic materials to permit the copolymer microspheres matrix firmly be attached to the plastic surface. Chemical modification of the acrylic microspheres with succinimide functional groups enabled irreversible binding of the co-enzyme to the supporting matrix by covalent bonding. Furthermore, the three-dimensional particle shapes of the acrylic microspheres provided large surface area for high loading capacity of chemical and biological molecules to promote higher sensitivity of the resulting bio-optode. The spherical morphology of the copolymer microspheres also facilitated the diffusion of analyte in a three-dimensional medium.

NBC is a lipophilic pH indicator with long alkyl chains. When 0.1 mg/mL NBC in ethanol was introduced into the hydrophobic acrylic microspheres absorbent, it turned the microspheres matrix surface into blue hue. In the adsorption process, the hydrophobic portions of the NBC molecules attached to the acrylic microspheres surface through non-specific forces i.e hydrophobic interactions. This eliminated the leaching of the components into the aqueous surrounding during sample measurement. Subsequent immobilization of *R. planticola* bacterial cells and NAD(P)H cofactor on the NBC-modified poly(nBA-NAS) microspheres *via* physical and chemical adsorptions did not appear to affect the colour properties of the microspheres. The microbial bio-optode in the protonated state remained blue in colour and absorbed the incident light transmitted from the feed fiber, thus attenuated the reflected light intensity from the measurand surface to the return fiber, and gave a maximum reflectance intensity at 750 nm (Fig. 3, reflectance spectrum i). Upon exposure to 100 mg L^{-1} NO_2^- ion at pH 8, the bio-optode reflected higher light intensity at 720 nm as the immobilized chromoionophore underwent deprotonation reaction and changed to bright pink colour (Fig. 3, reflectance spectrum ii). This was attributed to the dissociation of H^+ ion of the immobilized NBC during enzymatic reduction of nitrite to NH_4OH by the immobilized nitrite-degrading microorganism expressing NiR enzyme [34–36], and that the optical whole cell bio-optode demonstrated higher reflectance response as its colour changed to brighter one. Rather than absorb light, the pink-coloured bio-optode reflected most of the light struck on the substrate surface after partial absorption and multiple scattering within the medium. A control experiment was held by exposing the NBC-immobilized acrylic microspheres without immobilized bacterial cells and co-enzyme to the same NO_2^- ion concentration and pH condition, and a low reflectance intensity, which resembled to the microbial bio-optode before reaction with NO_2^- ion was obtained due to no distinct colour changed was observed (Fig. 3, reflectance spectrum iii). In addition, the reflectance signal of the proton selective optode layer was perceived to move to a shorter wavelength at 680 nm compared to the bio-optode initial response as the reflectance intensity may depend upon factors such as particle size, surface morphology, refractive index, sample absorption and the substrate colour intensity. In view of the maximum reflectance difference occurred at 639 nm between the reflectance spectra of bio-optode before and after reaction with nitrite, this wavelength was used as the working wavelength in the subsequent optimization experiments.

3.2. Effect of different buffer pHs on the microbial optosensor response, response time, shelf life and dynamic linear range of the whole cell nitrite bio-optode

pH is an essential parameter for optimum catalytic activity of enzyme as it plays a major role in protein folding and stability to achieve an optimized biosensor response. The nitrification reaction depends on the pH condition where an optimum pH medium

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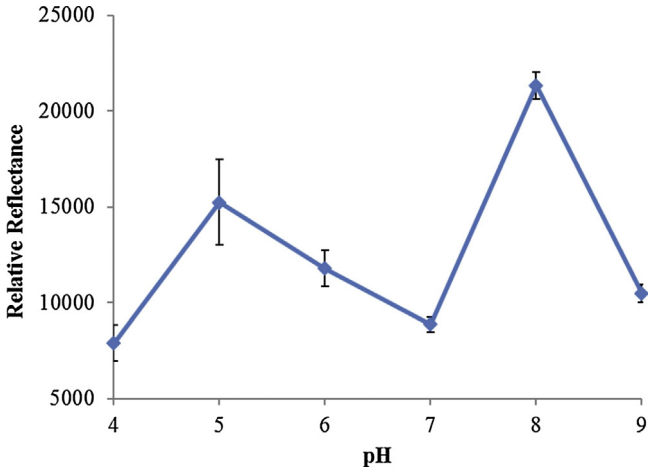

Fig. 4. The reflectance response of the acrylic microspheres-based microbial bio-optode at 639 nm from pH 4–9 in the determination of 100 mg L^{-1} NO_2^- ion (n = 3, RSD = 7.8%).

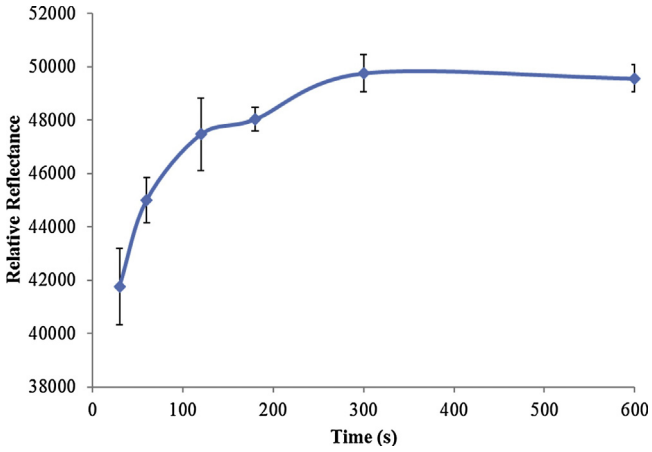

Fig. 5. The response time profile of the bacterium-membrane towards 100 mg L^{-1} of NO_2^- ion solution at pH 8 (n = 3, RSD = 1.9%).

favours the reduction of NO_2^- ion to NH_4OH [37]. Fig. 4 shows the reflectometric bio-optode response as a function of pH. The relative reflectance is defined as the reflectance difference between the bio-optode reflectance response after reaction with nitrite and the bio-optode response before reaction with nitrite at 639 nm. The enzymatic reaction was not favourable in acidic conditions ascribed to the limited nitrification reaction at pH below pH 7 [38], and that the bio-optode did not show significant colour changes upon reaction with 100 mg L^{-1} NO_2^- ion from pH 4 to pH 6, after which the bio-optode exhibited purplish pink coloration with 100 mg L^{-1} NO_2^- ion at pH 7, and the most distinct colour change from blue to pink was observed when the bio-optode was reacted with 100 mg L^{-1} NO_2^- ion in 0.1 M K-phosphate buffer at pH 8. The nitrite bio-optode turned to purplish pink at pH 9, implied that the extreme alkaline condition did not serve to increase the enzymatic reaction rate. This finding is consistent with the previous reported studies by Tarre and Green [39], who suggested pH 8 as the optimum pH for high rate of nitrification reaction.

Biosensor response time is the rate limiting step that determines the *in-situ* assay time. Fig. 5 represents the time dependent bio-optode reflectance response in the presence of 100 mg L^{-1} NO_2^- ion in 0.1 M K-phosphate buffer at pH 8. As the duration of the bio-optode exposed to 100 mg L^{-1} NO_2^- ion increased, the bio-optode response gradually increased from 30 s to 300 s. The characteristic blue-coloured bio-optode surface steadily changed to pink

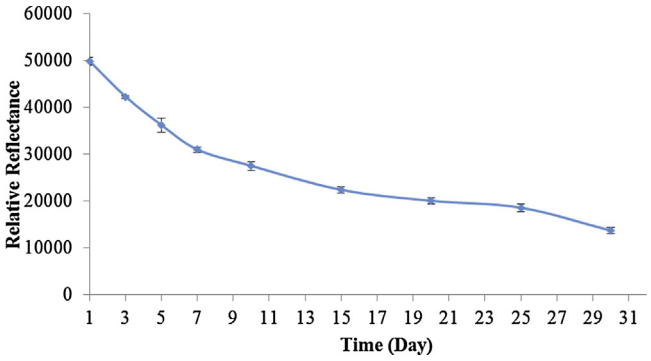

Fig. 6. The lifetime profile of the bacterium-membrane towards 100 mg L^{-1} of NO_2^- ion solution at pH 8 (n = 3, RSD = 3.0%).

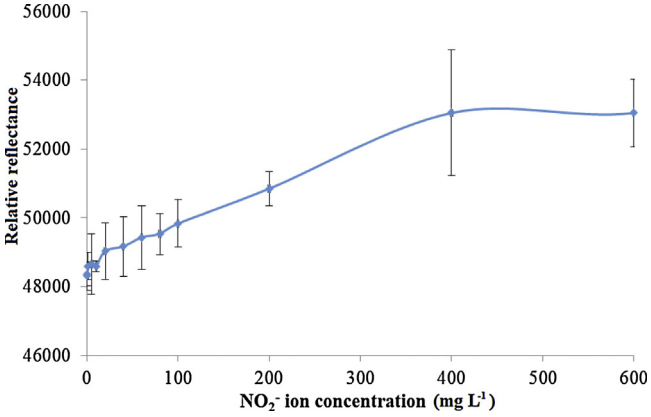

Fig. 7. The response curve of the acrylic microspheres-based microbial bio-optode towards NO_2^- ion determination in the concentration range of 0.5–600 mg L^{-1}.

hue, thereby led to the enhancement of the bio-optode relative reflectance response at 639 nm. This was attributed to the increasing number of immobilized NBC getting protonated as the progress of the enzymatic reaction slowly increased with time. No significant change in the bio-optode reflectance intensity was noticed from 5 min up to 10 min reaction time. This steady-state response suggesting the full colour development of the micro-bio-optode whereby the nitrite degradation by the immobilized *R. planticola* microbes has reached to completion. Therefore, the bio-optode response time was fixed at 5 min in further analyses.

Bio-optode shelf life study was done by intermittently measuring the bio-optode response towards a constant concentration of 100 mg L^{-1} NO_2^- ion for a month. As can be seen in Fig. 6, the bio-optode response decreased to about 84.8% relative to its initial response after kept in the refrigerator at 4 °C for three days. The relative response of the bio-optode remained at 55.1% on day 10, and further declined to 40.2% on the 20th day. The bio-optode response ultimately reduced substantially until reaching some 27.4% relative response on day 30 as the immobilized NAD(P)H cofactors become oxidized and consumed during the enzymatic reduction of NO_2^- ion at the microsensing platform throughout the course of the 30-day experimental period, and that the amount of immobilized NAD(P)H cofactors were becoming increasingly scarce, thereby restricted the lifetime of the bio-optode. Additionally, the survival rate of the immobilized microbes become low and irreversible degradation of both immobilized bacterial cells and co-enzymes may have taken placed after such a long storage time. This can be deduced that the microbial optosensor has an operational stability of 3 days since >80% of its initial response was still be retainable.

The response curve of the bacterium-based bio-optode towards a wide NO_2^- ion concentration detection range from

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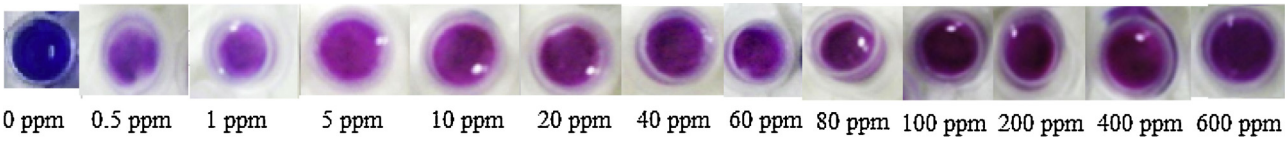

Fig. 8. The visual colour change of the reflectometric microbial bio-optode towards the detection of different NO_2^- ion concentrations.

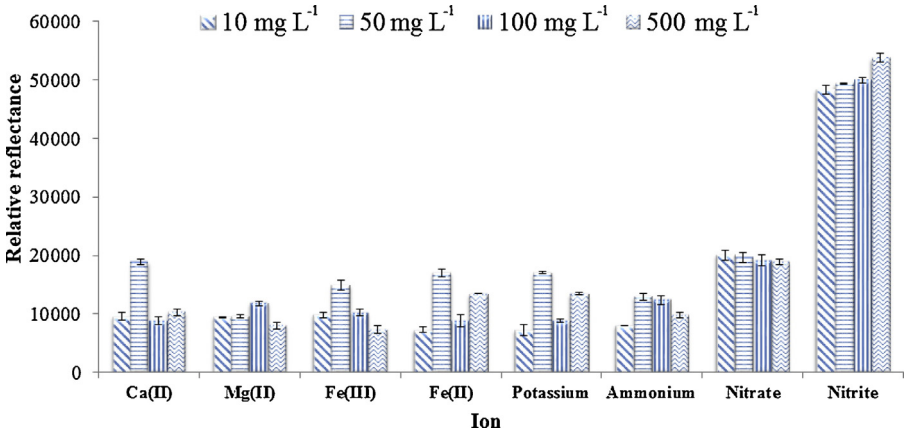

Fig. 9. Interference effect on the bio-optode reflectance response using various potential interfering ions in the concentration range of 10–500 mg L^{-1} at pH 8 (n = 3, RSD = 1.4%).

0.5–600 mg L^{-1} in 0.1 M K-phosphate buffer at pH 8 and 639 nm shows dramatic reflectance response increment with the increasing of the NO_2^- ion concentration between 0.5 mg L^{-1} and 400 mg L^{-1}, and the reaction become constant thereafter until 600 mg L^{-1} NO_2^- ion (Fig. 7). At low NO_2^- ion concentration, the available reactive sites on the bio-optode surface were partially reacted and led to partial protonation of the immobilized NBC (blue colour). In the presence of higher NO_2^- ion concentration, the high enzymatic reaction rate taking part at the bio-optode surface resulted in the formation of the deprotonated bio-optode (bright pink colour). Further increasing the NO_2^- ion concentration up to 600 mg L^{-1} did not show to further enhance the reflectance signal, which indicates that the bio-functionalized microspheres surface has entirely deprotonated as a result of the nitrification reaction by the immobilized *R. planticola* bacteria. Each bio-optode response was taken at 5 min after the reaction between bio-optode and NO_2^- ion commenced. Fig. 8 shows the visual colour change of the proposed microbial optosensor towards different nitrite concentrations.

The bio-optode linear concentration range was determined to be between 0.5 mg L^{-1} and 400 mg L^{-1} NO_2^- ion. The dynamic linear range has a linear regression equation of $y = 12.521x + 48593$ and correlation coefficient (R^2) of 0.994. The limit of detection (LOD) of the bio-optode, which was calculated based on the average of blank signal plus three times the standard deviation of blank [40,41] was obtained at 0.2 mg L^{-1} NO_2^- ion. As the proposed nitrite micro-bio-optode involved visible colour change, and that the NO_2^- ion concentration can be determined by visual colour inspection, hence it can be used as a portable colorimetric test-kit for on-site rapid detection on nitrite levels in food and environmental samples.

The average reproducibility relative standard deviation (RSD) of each calibration point, which obtained from three different individual bio-optodes, and analyzed within the first three fabrication days was estimated at <2.0% (n = 3). The low RSD value implies high reproducibility of the developed microspheres-based bio-optode in the quantitation of nitrite concentration. This can best be explained by the uniform sized of the copolymer microspheres, which allowed consistent loading of the chemical and biological components on their surfaces to enable a uniform colour change on the three-dimensional bio-optode surface.

3.3. Interference study

The effect of potential interfering ions at different concentration levels towards bio-optode reflectance response was investigated using Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, K^+, NH_4^+ and NO_3^- ions. These ions were chosen because they may co-exist with the target NO_2^- ion in food, water and environmental samples. The interfering agent was introduced separately onto the bio-optode surface at 10 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1} and 500 mg L^{-1} under the bio-optode optimum working conditions. The influence of possible interferents on the relative reflectance of the bio-optode is featured in Fig. 9. The reflectometric microbial bio-optode was found to be specific to NO_2^- ion detection, whereby the bio-optode acquired significant reflectance response that is proportional to the increasing of the NO_2^- ion concentration, and showed negligible inconsistent results at low reflectance signal levels upon exposure to some other foreign ions at various concentrations [42]. As the microbial bio-optode gave a three-fold higher reflectance signal towards nitrite compared to those potential interferents in the same concentration range from 10 to 500 mg L^{-1}, this signifies the high selectivity of the developed bio-optode towards NO_2^- ion determination.

3.4. Validation of the microbial bio-optode with standard ion chromatography method

To assess the accuracy and feasibility of the developed microbial bio-optode for NO_2^- ion detection in real sample matrix, several raw EBN samples, which have been spiked with known concentrations of standard nitrite within the bio-optode linear range were examined with both reflectance bio-optode and ion chromatography (IC) reference method. An unspiked swiftlet nest sample was also analyzed by the bio-optode and IC method to quantify the original nitrite level present in the sample. The results obtained by both methods are tabulated in Table 1. The nitrite levels obtained both by IC and the present methods are much higher than the spiked and unspiked sample values. The discrepancies observed are attributed

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Table 1
Validation of the microbial bio-optode performance with ion chromatography standard method for NO_2^- ion concentration determination in raw swiftlet nest samples.

EBN Sample	NO_2^- ion concentration by IC ($mg\,L^{-1}$)	NO_2^- ion concentration by biosensor ($mg\,L^{-1}$)	*t*-value
Unspiked EBN	1.37 ± (0.12)	2.10 ± (1.28)	0.003
Spiked 10 $mg\,L^{-1}$	14.73 ± (0.12)	15.99 ± (1.28)	0.004
Spiked 40 $mg\,L^{-1}$	46.33 ± (0.38)	45.89 ± (0.48)	0.001
Spiked 80 $mg\,L^{-1}$	84.33 ± (0.59)	83.21 ± (0.51)	0.003
Spiked 100 $mg\,L^{-1}$	129.83 ± (0.99)	152.28 ± (1.07)	0.058
Spiked 400 $mg\,L^{-1}$	413.33 ± (0.52)	435.66 ± (0.04)	0.059
RSD (%)	6.5	5.5	

Table 2
Comparisons of developed bio-optode performance with several previously reported optical NO_2^- ion chemical sensors and biosensors.

Sensing elements and immobilization matrix	Analysis method	Linear range ($mg\,L^{-1}$)	Detection limit ($mg\,L^{-1}$)	Response time (min)	Reference
R. planticola-NAD(P)H-NBC-poly(nBA-NAS) microspheres	reflectometric	0.5–400	0.2	5	This work
cd_1 nitrite reductase enzyme-isothiocyanato controlled pore glass beads	reflectometric	0–0.184	0.043	–	[2]
cd_1 nitrite reductase enzyme- sol-gel monoliths	UV–vis spectrophotometric	0.003–0.058	0.003	5	[16]
Dapsone-NED-filter paper	reflectometric	0.29–5	0.09	–	[43]
BCB-triacetyl cellulose membrane	UV–vis spectrophotometric	0.0002–0.03	0.0001	2	[44]

to the presence of nitrite in the raw EBN samples, which could be determined by both methods. The statistical *t*-test data analysis was applied to determine if the two methods show significant difference in the quantification of NO_2^- ion concentration. The calculated *t*-values obtained were lesser than the critical t-value at 2.776 with 4° of freedom suggests that the fabricated bio-optode analytical performance is in good agreement with the standard IC approach. This indicates that the microspheres-based reflectometric bio-optode can be used with best accuracy and precision in NO_2^- ion concentration determination.

3.5. Comparison of proposed bio-optode performance with previous reported optical nitrite chemical sensor and biosensor

Table 2 outlines the performance comparison between the developed microbial bio-optode with some other optical NO_2^- ion chemical sensors and biosensors in terms of dynamic linear range, detection limit and response time. In general, the efficiency of cytochrome cd_1 nitrite reductase immobilized on the isothiocyanato controlled pore glass beads was highly dependent on the pH, being very low enzyme loading capacity at basic pH, and the bio-optode performance was susceptible to oxygen interference. The use of bulk sol-gel monolith as the carrier matrix for cd_1 nitrite reductase enzyme served lower capacity of biological phase, and rendered a narrow dynamic linear range of NO_2^- ion concentration. The reflectometric chemical sensor developed using filter paper as the reaction medium for diazotization of dapsone and (naphthyl)ethylenediamine hydrochloride (NED) with NO_2^- ion in acidic medium was found to be very much dependent on the order of reagent addition, volume of solution added and quality of filter paper used. The chemical sensing system yielded no colour development when the chemical reagents were added separately onto the filter paper. The detection scheme of the optical sensor fabricated from brilliant cresyl blue (BCB)-immobilized triacetyl cellulose membrane was based on the oxidation of immobilized BCB by bromate ion in the presence of NO_2^- ion in an acidic reaction medium that resulted in the decolouration of the membrane. However, the addition of an extra bromate ion during optical nitrite assay obviating the need for reagentless nitrite sensing upon online field measurement is carried.

Since the detection range of the proposed microbial bio-optode is between 0.5 $mg\,L^{-1}$ and 400 $mg\,L^{-1}$ NO_2^- ion, thus it can be used to determine the presence of elevated levels of NO_2^- ion in food samples without further dilution, and this avoids sample treatment step (*e.g.* dilution) as the bio-optode is aimed for on-site rapid analysis of nitrite. This is particularly so for cured meat products such as bacon, sausage and ham as well as EBN products (*e.g.* blood-red cubilose) with an average NO_2^- ion content of 400 $mg\,L^{-1}$. Besides, the high levels of nitrite also extensively applied as a preservative in fishery products in most countries. Although most of the previously reported methods were able to determine NO_2^- ion at lower concentration range compared to the developed bio-optode, however, they cannot perform reagentless nitrite assay and possessed very narrow linear response range, which necessitates dilution to be carried out for real samples with NO_2^- ion concentration of >0.2 $mg\,L^{-1}$.

The proposed acrylic microspheres-based reflectance microbial bio-optode demonstrated the widest linear calibration range compared to previously reported optical nitrite chemical sensors and biosensors. This could be due to the uniform size distribution of the acrylic microspheres. The microspherical shape of the poly(nBA-NAS) microspheres provided large reaction surfaces, hence allowed higher loading capacity of biological and chemical molecules to promote mass transfer reaction rate at the bio-optode surface [45,46]. Therefore, the overall analytical performance of the proposed microbial bio-optode based on three-dimensional spherical shaped microspheres considerably enhanced compared to previously reported optical nitrite sensor/biosensor based on two-dimensional immobilization support materials. As such, the developed acrylic microspheres-based microbial bio-optode showed high potential for *in-situ* nitrite analysis. Response time of the developed bio-optode is however slightly slower than the

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reported sensor/biosensor for nitrite since the kinetics governing the intracellular enzyme biocatalysts are always about 10–100 folds slower than the cell-free enzyme.

4. Conclusions

The poly(nBA-NAS) microspheres-based microbial nitrite bio-optode showed a well-defined reflectance change at the working wavelength of 639 nm with a broad dynamic linear range from 0.5–400 mg L^{-1}, and that high level of dilution may not be required upon *in-situ* nitrite screening. The average reproducibility RSD obtained at <2.0% implied that the preparation of the microbial bio-optodes were highly reproducible with the proposed fabrication method. The poly(nBA-NAS) microspheres-based microbial bio-optode showed facile approach for optical NO_2^- ion determination. The usage of *R. planticola* bacterium, the intracellular enzyme expression system, which can be isolated from EBN allowed the fabrication of economical sensing device. The small size of the developed bio-optode requires only small portions of analyte aliquots to affect the enzymatic reaction. As the nitrite bio-optode based on intracellular enzyme expression system involved a distinct colour change, nitrite assay by visual colour inspection would offer a more user-friendly on-site analysis measure for the surveillance of food and water quality.

Acknowledgements

This work was supported by Universiti Malaysia Pahang *via* Operation Research Grant (UMP-RDU130308, UMP-GRS130389) and Ministry of Higher Education through Fundamental Research Grant Scheme (FRGS/2/2014/SG01/UKM/02/1, FRGS-RDU140131) and Research Acculturation Grant Scheme (RAGS-RDU131406).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2017.09.102.

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