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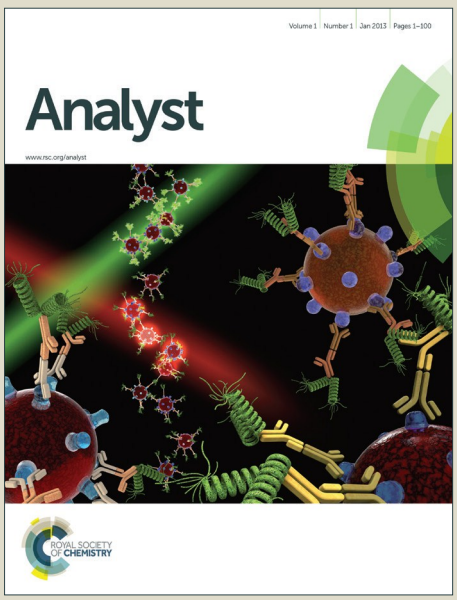

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A new electrochemical substrate for rapid and sensitive *in-vivo* monitoring of *β*-galactosidase gene expressions

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Abstract

4-Methoxyphenyl-*β*-Galactopyranoside (4-MPGal) substrate incorporating 4-methoxy phenol (4-MP) as an electrochemical reporter is described for the monitoring of *β*-Galactosidase (*β*-Gal) gene expressions. *β*-Gal derived from *Escherichia coli (E. coli)* and *Aspergillus oryzae (A. oryzae)* were investigated, while graphene oxide film modified electrode was employed as transducer. The electrochemical signal of 4-MPG within 4-MPGal was masked by protecting their hydroxyl group with galactose. The externally added *β*-Gal triggered the deprotection through specific enzymatic hydrolysis with concomitant release of 4-MP. The apparent K_m and V_{max} values of 4-MPGal are determined to be 0.21 mM and 0.51 µM min^{-1} mg of *β*-Gal^{-1} (*E. coli*) which were consistent with the previous reports. To detect *β*-Gal derived from *E. coli*, cyclic voltammetry (CV) provides linear ranges of 12–1200 ng mL^{-1} and 1.2–12 µg mL^{-1} with limit of detection (LOD) of 5 ng mL^{-1}, while differential pulse voltammetry (DPV) shows linear range of 1.2–120 ng mL^{-1} and LOD of 1 ng mL^{-1}. To detect *β*-Gal derived from *A. oryzae*, CV provides linear ranges of 0.1–100 ng mL^{-1} and 0.1–1 µg mL^{-1} with LOD was 0.06 ng mL^{-1}, while DPV shows linear range of 10–10 ng mL^{-1} with LOD of 8 pg mL^{-1}. Moreover, we set up a platform for the real-time *in-vivo* monitoring of *β*-Gal gene expressions in *E. coli* cultivated through microbiological culture. The developed sensing platform using 4-MPGal as substrate is simple, rapid, sensitive, specific and advantageous over its laborious optical analogues.

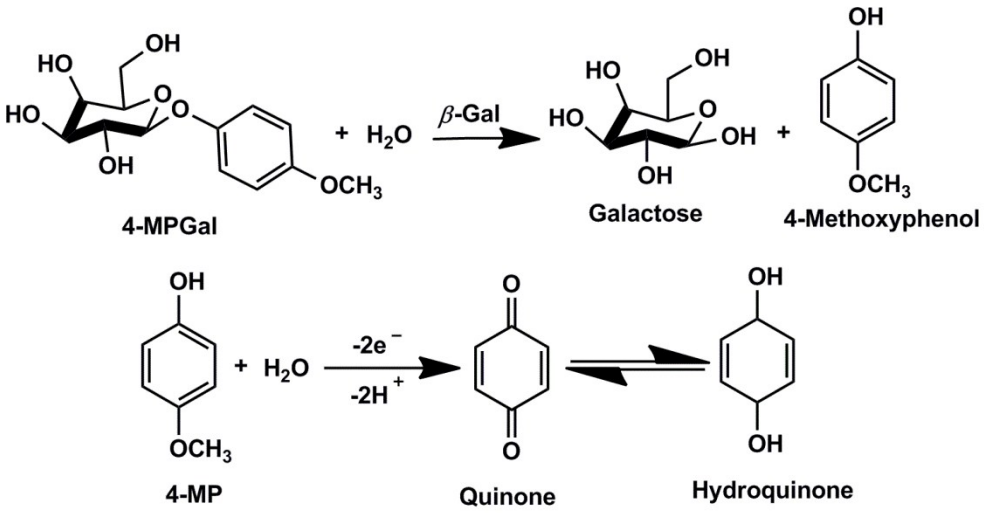
Keywords: *β*-Galactosidase, 4-methoxy phenol, *Escherichia coli*, *Aspergillus oryzae*, electrochemical probe

1. Introduction

In many events, *β*-galactosidase (*β*-Gal) enzyme has a crucial role in genetics, molecular biology, and other life sciences and its commercial interest is originated from its homeland habitat microorganism of bacteria, fungi, animals, and plants[1-3]. *β*-Gal having pervasive occurrence in mammalian organs interrelated to their multiple physiological accessibilities. *β*-Gal is widely used as an antibody-tag for cell-ELISA (cell-enzyme-linked immunosorbent assays (ELISA)[4] and frequently used as a marker to determine transcriptional regulation or transfection efficiency in gene expression of a promoter gene that is nearer to the source of lacZ gene [5, 6], heavy-metal ions determination[7] and faecal coliform detection based on

inhibition of *β*-Gal[8]. On the analytical assay perspective, *β*-Gal provides plenty of advantages in molecular biology field and therefore many substrates have been established for the detection of *β*-Gal. In general, optical substrates (chromogenic and fluorogenic) are the largely available probes to detect *β*-Gal, such as ortho-nitrophenyl- *β*-galactoside (ONPG)[7, 9, 10], chorophenol red-*β*-D-galactopyranoside, 5-bromo-4- chloro-3-indolyl-*β*-D-glucuronide, 4-methylumbelliferyl-*β*-D-galactopyranoside, fluorescein di-*β*-D–galactopyranoside [11-14], and 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-*β*-D-galactopyranoside[15]. However, optical assay approaches are laborious and encounter certain important limitations; (1) the probes itself has absorbance or fluorescence, (2) the reporter emits shorter wavelength and (3) they requires high pH conditions to enhance fluorescence signal of the reporter. In addition, the optical methods are involving multi-step procedures, require high amount of microorganisms, comprises permeabilization of the cells and require additional equipment to convert the analytical signal into electronic signal, However, electrochemical methods directly convert the signal into electronic signal and involve simple operating protocols, fast, direct use in point-of-care assays, portable and highly sensitive. 4-aminophenyl-*β*-D-galactopyranoside (PAPG) is the most widely used electrochemical substrate, where the electrochemical behavior of the reporter 4-aminophenol is monitored to assay the activity of expressed *β*-Gal[16].

Designing latent ratiometric electrochemical probes and exploring their applications are ongoing research interests of our research group[17]. In this present work, we have described a new electrochemical substrate 4-methoxyphenyl-*β*-galactosidopyranosides (4-MPGal) to assay the *β*-Gal expressions. For our studies, we have used *β*-Gal derived from *Escherichia coli (E. coli)* and *Aspergillus oryzae (A. oryzae)* (**scheme 1**). The reporter 4-methoxy phenol (4-MP) featured with obvious redox peaks attributed to the quinone-hydroquinone redox couple as shown in the mechanism[18] (**Fig. S2**). We used graphene oxide film (GO) film modified electrode as the transducer electrode to reveal the electrochemical behaviour of 4-MP. In the past years, GO based electrodes widely acclaimed for their excellent electrocatalytic ability owing to their large surface area, presence of abundant oxygen functionalities and larger amount of edge plane like defects[19-21]. Therefore, GO film electrode is expected to be the excellent candidate to accelerate the electrocatalysis of 4-MP in efficient manner than the bare GCE. Interestingly, the described substrate 4-MPGal can be applicable for the real-time *in-vivo* monitoring of *β*-Gal gene expression in *E. coli* cultivated through microbiological culture.



Scheme 1. Mechanistic pathway for the electrochemical determination of *β*-Gal employing 4-MPGal as a substrate, 4-MP as a electrochemical reporter and GO film modified electrode as the transducer.

2. Experimental

2.1 Reagents and Instrumentation

Graphite (powder, <20 μm), *β*-Gal from *E. coli* (780.4 units/mg, Isoelectric point at pH 7.3), *β*-Gal from *A. oryzae* (8 units/mg, Isoelectric point at pH 4.5) and isopropyl-b-d-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich. 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris) purchased from J.J. Baker chemical Ltd, while 4-MPGal was purchased from Tokyo chemical industry co. Ltd. All the other chemicals were purchased from Sigma-Aldrich and used without purification. All the electrochemical measurements were carried out using CHI 612d work station at room temperature. Electrochemical studies were performed in a conventional three electrode cell using GO film modified GCE as a working electrode (area 0.071 cm^2), saturated Ag|AgCl (KCl) as a reference electrode and Pt wire as a counter electrode. Prior to electrochemical experiments, all the electrolyte solutions were deoxygenated with nitrogen for 5 min unless otherwise specified.

2.2 Preparation of graphene oxide film modified electrode

Graphite oxide was synthesized by modified Hummer's method [22] and it was suspended in water (0.5 mg mL^{-1}) (**Fig. S1**). The graphite oxide dispersion was exfoliated through ultrasonication for 2 h to yield graphene oxide (GO). GO was subjected to centrifugation for 30

min at 4000 rpm to remove any unexfoliated graphite oxide. The yellowish brown GO supernatant was collected and stored. GCE surface was polished with 0.05 µm alumina slurry using a Buehler polishing kit, then washed with water, ultrasonicated for 5 min and dried. Then optimized GO concentration of 5 µl was drop casted onto the pre-cleaned GCE and dried at room temperature for 20 min. The resulting electrode was named as GO/GCE and used as working electrode.

2.3 Measuring conditions

10 mM Tris buffer (pH 7.3) prepared from Tris and HCl was used as supporting electrolyte for *E. coli* studies, while 50 mM acetate buffer (pH 4.5) prepared using acetic acid and sodium acetate was used for *A. oryzae* studies. For the *β*-Gal determination studies, 4-MPGal and *β*-Gal were mixed in the respective buffers and incubated for 1 h at 37°C. Subsequently, the solutions were transferred to an electrochemical cell and electrochemical experiments were carried out using GO/GCE as a transducer. Cyclic voltammetry measuring parameters: potential range of -0.3 V to +1.0 V and scan rate of 0.1 Vs^{-1} (unless otherwise specified); differential pulse voltammetry measuring parameters: amplitude= 0.05 V, sampling width= 0.0167 s, pulse period= 0.5 s. All the experimental parameters were optimized and given as Table S1.

2.4 Cultivation of E. coli bacteria

E. coli (strain DH5α) was grown overnight in lysogeny broth (LB) medium (5 mL). Then, 0.1 mg mL^{-1} of isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to induce the activity of *β*-Gal. Subsequently, the whole bacterial culture system was grown for 1 h at 37°C with vigorous shaking. The solution was distributed into aliquots (1 mL) in a test tube. Then, particular concentration of probe 4-MPGal was added and the whole solution was incubated for 1 h at 37°C. Finally, the solution was transferred to an electrochemical cell and electrochemical experiments were carried out at GO/GCE to monitor the activity of *β*-Gal.

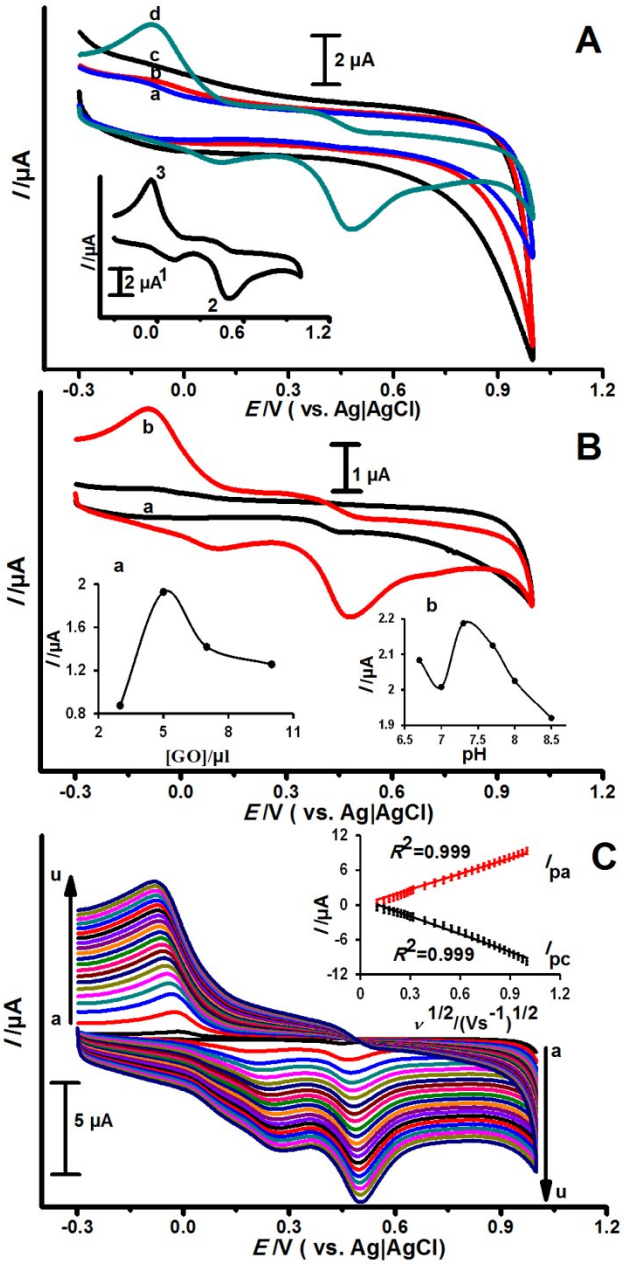


Fig. 1 (A) CVs obtained at GO/GCE in Tris buffer (pH 7.3) (a) containing 1 mM 4-MPGal (b), 0.63 μg *β*-Gal (c), and mixture of 1 mM 4-MPGal and 0.63 μg *β*-Gal (*E. coli*) (d). Inset: CV obtained at GO/GCE in Tris buffer (pH 7.3) containing pristine 4–MP (100 μM). (B) CVs obtained at unmodified GCE (a) and GO/GCE (b) in Tris buffer (pH 7.3) containing 1 mM 4-MPGal and 0.63 μg of *β*-Gal (*E. coli*) at scan rates of 0.1 Vs^{-1}. Inset (a): optimization of GO concentration, [current] (μA)/ [GO]/μL; CVs obtained at GO/GCE in Tris buffer (pH 7.3) containing 1 mM 4-MPGal and 0.63 μg of *β*-Gal. Inset (b): pH optimization: [current] (μA)/pH. CVs obtained at GO/GCE in Tris buffer (different pH) containing 1 mM 4-MPGal and 0.63 μg

of *β*-Gal. (C) CVs obtained at GO/GCE in Tris buffer (pH 7.3) containing 1 mM 4-MPGal and 0.63 µg of *β*-Gal (*E. coli*) at different scan rates from 0.01 Vs^{-1} to 1 Vs^{-1}. Inset: Plot of I_{pa} and I_{pc} versus $v^{1/2}$; $I_{pa}/\mu A = 9.83\ v^{1/2}/(Vs^{-1})^{1/2} - 0.602$; $I_{pa}/\mu A = -10.23\ v^{1/2}/(Vs^{-1})^{1/2} + 1.12$.

3. Results and Discussion

3.1. Determination of β-Gal on graphene oxide film modified GCE

The cyclic voltammogram (CV) acquired at GO/GCE in Tris buffer (pH 7.3) containing only 4-MPGal (1 mM) (**curve b, Fig. 1A**) did not exhibit any characteristic redox peaks in the absence of *β*-Gal which is due to the protection of reporter 4-MP within this probe. Also, the CV obtained at GO/GCE in Tris buffer (pH 7.3) containing only *β*-Gal (0.63 µg) (**curve c, Fig. 1A**) did not present any voltammetric peaks. However, the CV obtained at GO/GCE in the presence of 1 mM 4-MPGal and 0.63 µg *β*-Gal (**curve d, Fig. 1A**) exhibits well defined quasi-reversible redox peaks related to the characteristic redox reaction of 4-MP. The electrochemical behaviour of 4-MP is well-established in the literature and the corresponding mechanism is given as **Fig. S2**. 4-MP is oxidized to benzoquinone; subsequently benzoquinone undergoes reversible conversion to hydroquinone[18, 23]. The redox reaction of benzoquinone-hydroquinone is one of the feasible redox systems for the electrochemical sensing studies. Thus, the CV results are clearly indicating that the addition of *β*-Gal triggered the deprotection and uncloaks the redox active center of 4-MPGal. The concentration of GO required for the maximum performance was optimized. CVs were carried out using GCE modified with different amounts of GO in Tris buffer (pH 7.3) containing 1 mM 4-MPGal and 0.63 µg *β*-Gal (**inset a, Fig. 1B**). The plot between response current and GO concentration is indicating that 5 µl GO has maximum response current and therefore we used this optimized concentration for other studies. Different pH study was carried out in the Tris buffer with different pH ranges. The plot between response current and pH revealing that pH 7.3 is the optimum pH in order to attain maximum response current **(inset b, Fig. 1B)** and therefore, we adopted this pH for further analysis.

The CV obtained for the pristine 4–MP (**Inset to Fig. 1A**) is quite consistent with that of released 4–MP from 4-MPGal (curve d, figure 1A) which adds additional to evidence to the proposed mechanistic pathway. **Fig. 1B** presents the CVs obtained at unmodified GCE (curve a) and GO/GCE (curve b) in Tris buffer (pH 7.3) containing 4-MPGal (1 mM) and *β*-Gal (0.63 µg).

The redox reaction of 4-MP has shown feeble peak currents which indicating sluggish electron transfer at unmodified electrode. Also, unmodified electrodes encounter surface fouling related problems. However, the redox reaction of 4-MP at GO/GCE has shown obvious and highly enhanced peaks currents in comparison with unmodified GCE. GO has very good electrocatalytic activity, large surface area, and abundant oxygen functionalities. Also, GO surface possess more sp^2 like domains and larger amount of edge plane like defects which are greatly accelerate the electrocatalysis of 4-MP at GO/GCE[24, 25]. In addition, the electrochemical active surface areas of bare GCE and GO/GCE have been investigated using $K_3[Fe(CN)_6]$ as a model redox mediator, while Randles–Sevcik equation was adopted to calculate the areas[26]. The electrochemically active surface areas of bare GCE and GO/GCE were calculated to be 0.051 and 0.098 cm^2. Therefore, the electrochemically active surface area of GO/GCE is nearly two-fold enhanced than bare GCE. **Fig. S3** presents the CVs obtained at GO/GCE in tris buffer (pH 7.3) containing different concentrations of 4-MP. As seen from figure, the redox peak currents corresponding to the electrochemical behavior of 4-MP were increased linearly as the concentration of 4-MP increases. The plot between peak currents and concentration of 4-MP is exhibits good linearity. Thus, all the experimental results proved that relatively GO/GCE has appreciably enhanced electrocatalytic ability to catalyze 4-MP than bare GCE and hence, we have employed GO/GCE as a transducer for our studies.

The effect of applied scan rate on the electrochemical behavior of released 4-MP has been examined in Tris buffer (pH 7.3) containing 1 mM 4-MPGal and 0.63 µg of *β*-Gal (*E. coli*) at different scan rates from 0.01 Vs^{-1} to 1 Vs^{-1} (**Fig. 1C**). Both the anodic (I_{pa}) and cathodic peak currents (I_{pc}) increases linearly as the scan rate increases. A plot of peak currents and square root of scan rates ($v^{1/2}$) exhibits good linearity indicating that the redox reaction of 4-MP follows diffusion controlled electron transfer process (**inset to Fig. 1C**)[27]. In order to investigate stability of the GO/GCE to detect *β*-Gal, 100 consecutive CVs were acquired at GO/GCE in Tris buffer (pH 7.3) containing 4-MPGal and *β*-Gal (**curve b, Fig. 1A**). 97.23% of its initial peak currents were retained even after 100 continuous cycles indicating good stability. In addition, the reproducibility of the proposed approach to detect *β*-Gal was investigated. Five individual measurements were carried out at five different GO/GCEs. The relative standard deviation (R.S.D) for these five measurements was 2.57% indicating appreciable reproducibility.

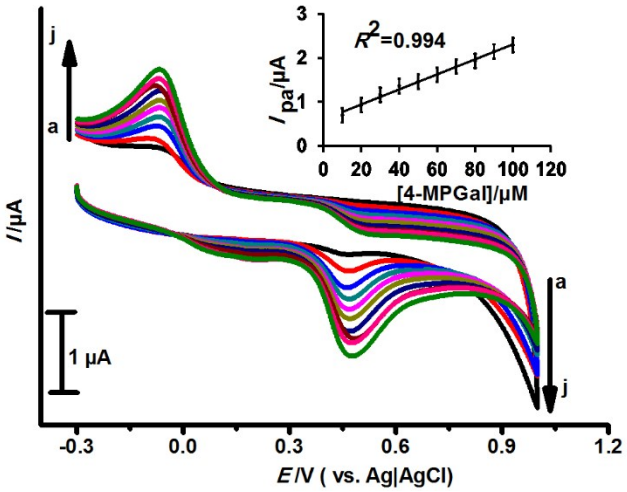


Fig. 2 CVs obtained at GO/GCE in 10 mM Tris buffer (pH 7.3) containing 1.27 µg *β*-Gal (*E. coli*) upon increasing concentration of 4-MPGal (each 10 µM addition; a to j). Scan rate = 0.1 Vs^{-1}. Inset: I_{pa} versus [4-MPGal]. I_{pa}/µA = 0.017 [4-MPGal]/µM + 0.601.

3.2 Apparent Kinetic parameters

The apparent kinetic studies were carried out in the solution containing 4-MPGal and *β*-Gal in Tris buffer (pH 7.3) and the release of 4-MP was monitored. (**Fig. S4**). The incubation of 4-MPGal alone in Tris buffer (pH 7.3) for more than 1 h is unable to release 4-MP (figure not shown), however, incubation of 4-MPGal with *β*-Gal triggered the release of 4-MP within 15 min through enzymatic hydrolysis. A double-reciprocal plot of electrochemical signal appearance rate versus different concentrations of 4-MPGal is shown in **Figure S2**. The apparent K_m and V_{max} values were obtained as 0.21 mM and 0.51 µM min^{-1} mg of *β*-Gal^{-1}. The K_m value determined using 4-MPGal as a substrate is quite comparable to the previously reported substrate o-nitrophenyl-*β*-D-galactoside[28]. **Fig. 2** shows the CVs obtained at GO/GCE in Tris buffer (pH 7.3) containing 1.27 µg *β*-Gal (*E.coli*) with increasing concentration of 4-MPGal (each 10 µM addition; a to j). As can be seen from figure, the peak currents corresponding to 4-MP is linearly increases as the concentration of 4-MPGal increases from 10 µM–100 µM. The plot between peak current and concentration of 4-MPGal is exhibits good linearity (**inset to Fig. 2)**. Therefore, this substrate is well suitable to monitor *β*-Gal enzymatic activity.

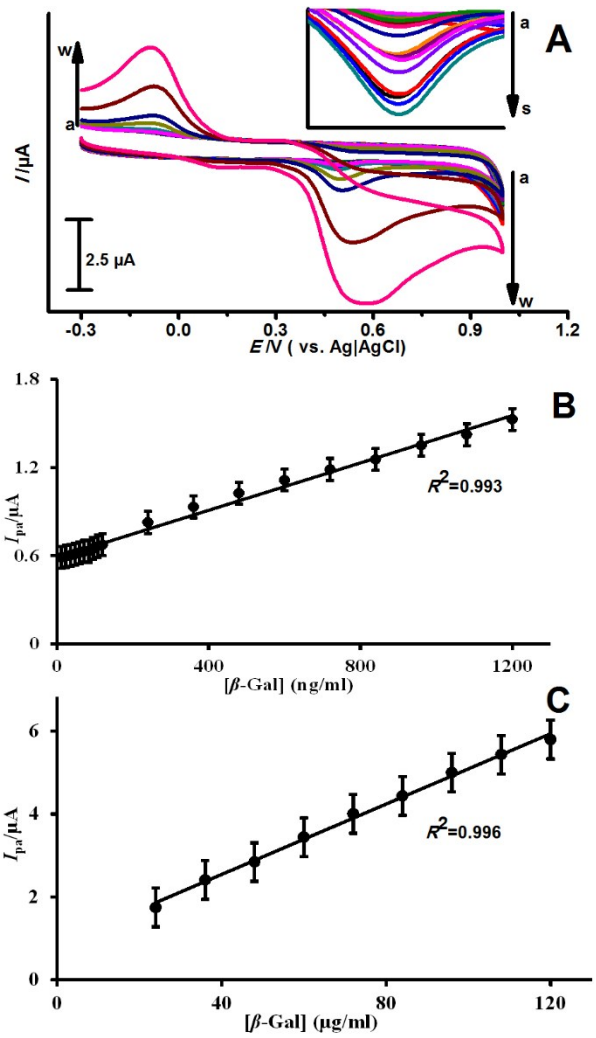


Fig. 3 (A) CVs obtained at GO/GCE in 10 mM Tris buffer (pH 7.3) containing 2.4 mM 4-MPGal with increasing concentration of *β*-Gal *(E.coli)* (a= 12 ng, b= 24 ng, c= 36 ng, d= 48 ng, e= 60 ng, f= 72 ng, g= 84 ng, h= 96 ng, i= 108 ng, j= 120 ng, k= 240 ng, l= 360 ng, m= 480 ng, n= 600 ng, o= 720 ng, p= 840 ng, q=960 ng, r= 1.08 μg, s= 1.2 μg, t= 6 μg, u= 12 μg, v= 60 μg, w= 120 μg). (B) I_{pa} vs. [*β*-Gal]; I_{pa}/μA = 0.8 [*β*-Gal]/(nA/ng) + 0.587. (C) I_{pa} vs. [*β*-Gal]; I_{pa}/μA = 42.5 [4-MPGal]/(nA/μg) +0.587.

3.3 Determination of β-Gal

Fig. 3A presents the CVs obtained at GO/GCE in Tris buffer (pH 7.3) containing 2.4 mM 4-MPGal with different concentration of *β*-Gal (*E.coli*). As evident from the figure, the peak currents corresponding to the electrochemical behavior of 4-MP are linearly increases as the concentration of *β*-Gal increases. A plot between I_{pa} and I_{pc} versus concentration of *β*-Gal

exhibits a linear relationship (**Fig. 3B**). Monitoring I_{pa} of the 4–MP offers more sensitivity than I_{pc} and therefore we choose I_{pa} for the determination of β-Gal. The linear range was found between 12 ng mL^{-1} to 1200 ng mL^{-1} with sensitivity of 11.27 nA ng^{-1} cm^2. The limit of detection (LOD) was calculated as 5 ng mL^{-1}. The LOD was calculated using the formula, LOD= 3 s_b/S where, s_b is the standard deviation of ten blank measurements and S is the sensitivity. A second linear range was observed in higher concentration region between 1.2 µg mL^{-1} to 12 µg mL^{-1} (Fig. 3C) with sensitivity of 0.60 µAµg^{-1} cm^{-2}. In order to develop sensitive determination platform, differential pulse voltammograms (DPV) were carried out. DPVs were performed at GO/GCE in Tris buffer (a) containing 1 mM 4-MPGal with increasing concentration of β-Gal (**Fig. 4**). The response current was increases linearly as the concentration of β-Gal increases. The plot between response current and concentration of β-Gal exhibits good linearity with slope of 9.7 nA ng^{-1}. As evident from the calibration plot, the linear range was 1.2 ng mL^{-1}–120 ng mL^{-1}. The sensitivity and LOD were calculated to be 136.62 nA ng^{-1} cm^{-2} and 1 ng mL^{-1}, respectively. Although, 4-MPGal shows comparatively less performance than the electrochemical substrate PAPG[29], it has advantages over current optical methods. The current optical methods using ONPG as substrate are laborious; they involve multi-step procedures, require high amount of microorganisms and comprises permeabilization of the cells. However, electroanalytical methods are simple, fast, portable and sensitive. Moreover, the described electrochemical approach can be engineered into microbial chip based real-time sensors and these kinds of on-chip electrochemical analysis can be accurately performed with minimal amount of sample, whereas, colorimetry methods does not have these kinds of opportunities.

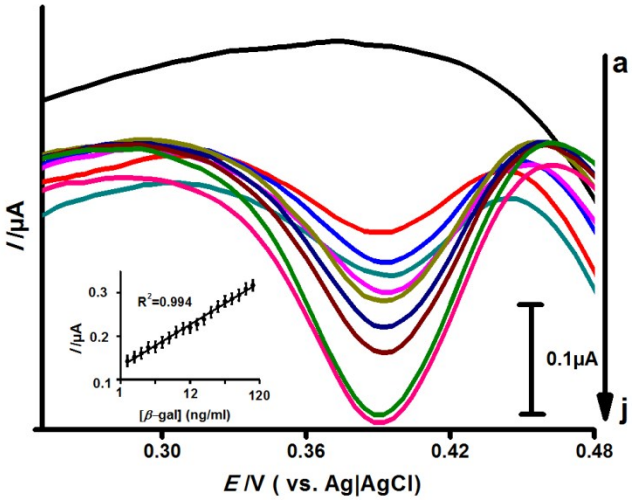


Fig. 4 DPVs obtained at GO/GCE in 10 mM Tris buffer (pH 7.3) (a) containing 1 mM 4-MPGal with increasing concentration of *β*-Gal *(E.coli)* (b= 1.2 ng, c= 2.4 ng, d= 3.6 ng, e= 4.8 ng, f= 6.0 ng, g= 7.2 ng, h= 8.4 ng, i= 9.6 ng, j= 10.8 ng). Inset: Calibration plot, [I_{pa}] vs. [*β*-Gal]; I/μA = 9.7 [*β*-Gal]/(nA/ng) + 0.130.

Similarly, a sensitive electrochemical detection platform has been developed for the determination of *β*-Gal derived from *A. oryzae* employing 4-MPGal as substrate, 4-MP as reporter and GO/GCE as electrode (**Fig. S5**). For the determination of *β*-Gal derived from *A. oryzae*, acetate buffer (pH 4.5) was employed. The mechanistic pathway and experiments are similar as the one explained for *E. coli.* The apparent K_m and V_{max} values were 0.27 mM and 2.62 μM min^{-1} mg of *β*-Gal (**Fig. S6)**. The K_m value determined using 4-MPGal is comparable to the previously reported substrate o-nitrophenyl-*β*-D-galactoside for *β*-Gal in *A. oryzae*[30] and hence the 4-MPGal is a suitable substrate for the electrochemical detection of *β*-Gal **(Fig. S7).** The working concentration ranges: (1) 0.1 ng mL^{-1}–100 ng mL^{-1} with sensitivity of 0.972 μA ng cm^2 and (2) 0.1 μg mL^{-1}–1 μg mL^{-1} with sensitivity of 0. 326 nA μg cm^2. The LOD was 0.06 ng mL^{-1} (**Fig. S8**). DPV based determination platform also developed which can detect low concentration of *β*-Gal with linear range of 10 pg mL^{-1} –10 ng mL^{-1}, LOD of 8 pg mL^{-1} and sensitivity of 173.2 nA μg^{-1} cm^{-2} **(Fig. S9).** All these results clearly revealed that the described electrochemical platform is highly applicable for the determination of *β*-Gal derived from *A. oryzae.*

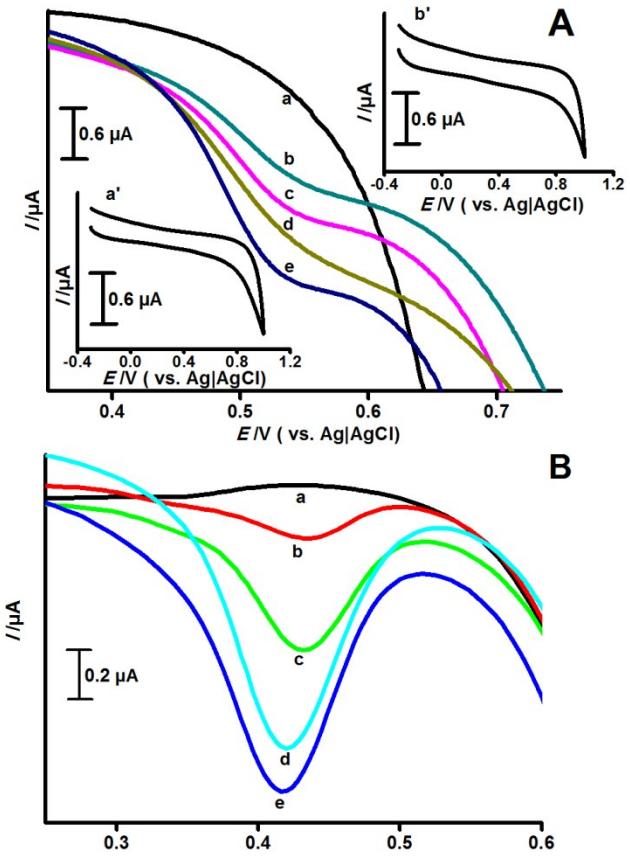


Fig. 5 (A) CVs obtained at GO/GCE in *E. coli* with IPTG induction containing different concentration of 4-MPGal; a= 0 µM, b= 200 µM, c= 300 µM, d= 400 µM, and e= 500 µM. Inset a': CVs obtained at GO/GCE in *E. coli* without IPTG induction containing 500 µM 4-MPGal. Inset b' (negative control): CV obtained at GO/GCE in *E. coli* with IPTG induction containing 5 mM galactose and 500 µM 4-MPGal. (B) DPVs obtained at GO/GCE in *E. coli* with IPTG induction containing different concentration of 4-MPGal. (a = 0 µM, b= 200 µM, C= 300 µM, d= 400 µM, and e= 500 µM).

3.4 Real-time monitoring of β-Gal gene expressions in E. coli

The practical applicability of the described probe, 4-MPGal was verified by real-time monitoring of *β*-Gal gene expressions in IPTG induced *E. coli*. **Fig. 5** displays the CVs obtained at GO/GCE in IPTG induced *E. coli* medium containing different concentration of substrate, 4-MPGal; a= 0 µM, b= 200 µM, c= 300 µM, d= 400 µM, and e= 500 µM. No signal was observed in the absence of substrate (curve a). An enhanced signal corresponding to the electrochemical

behaviour of 4-MP was observed in the presence of substrate revealing the presence of overexpressed *β*-Gal in the IPTG induced *E. coli* (curve b). With IPTG induction, *β*-Gal of IPTG induced *E. coli* undergoes overexpression, and the overexpressed *β*-Gal is sufficient to trigger the release of 4-MP from 4-MPGal. Moreover, the response current was linearly increases as the concentration of 4-MPGal increases (curves c to e).

No electrochemical signal was observed for *β*-Gal without IPTG induction (inset a') which might be due to the absence of enough *β*-Gal to unmask the 4-MPGal. Furthermore, a negative control experiment was also carried out in the presence of galactose. The CV obtained for the IPTG induced *E. coli* containing 4-MPGal (500 µM) and galactose (5 mM) has not shown any characteristic peaks of 4-MP (inset b'). As expected, the large excess concentration of galactose inhibits the electrochemical signal and hence the CV unable to show any peaks. Moreover, experiments carried out in LB medium indicating that the proposed scheme works well in the LB broth medium (supporting information, fig. S10).

Fig. 5 presents the DPVs obtained at GO/GCE in IPTG induced *E. coli* containing different concentration of 4-MPGal. As shown in the figure, highly enhanced sharp signals were observed in the presence of substrate which clearly revealing the presence of overexpressed *β*-Gal in the *E. coli* which validates the practical feasibility of the proposed method. Consequently, the described substrate, 4-MPGal can be used for the real-time monitoring of *β*-Gal gene expressions in bacteria with high sensitivity. Thus, we set up a new detection platform which is applicable for the real-time *in-vivo* monitoring of *β*-Gal gene expressions revealing its potential practical application.

4. Conclusions

In summary, a new electrochemical substrate was established for the sensitive determination of *β*-Gal gene expressions. The product of the enzymatic reaction between *β*-Gal and 4-MPGal is 4-MP which was detected at GO/GCE as a working electrode in a conventional electrochemical cell. The peak currents of 4-MP were linearly dependent with the concentration of *β*-Gal which leading to the ratiometric detection of *β*-Gal. The apparent K_m and V_{max} values of 4-MPGal were 0.21 mM and 0.51 µM min^{-1} per mg of *β*-Gal^{-1} which were consistent with reported values. A sensitive determination platform was developed based on CV and DPV methods which exhibited wide linear ranges and low detection limits. A real-time *in-vivo*

analysis was carried out in *E. coli* cultivated through microbiological culture system. The developed sensing platform using 4-MPGal as substrate is simple, rapid, sensitive, specific and advantageous over its laborious optical analogues.

Acknowledgements

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References

1. D. H. Juers, B. W. Matthews and R. E. Huber, *Protein Science*, 2012, **21**, 1792-1807.
2. Q. Z. Zhou, X. D. Chen and X. Li, *Biotechnology and bioengineering*, 2003, **81**, 127-133.
3. P. Geng, J. Zheng, X. Zhang, Q. Wang, W. Zhang, L. Jin, Z. Feng and Z. Wu, *Electrochemistry Communications*, 2007, **9**, 2157-2162.
4. Z. Liu, T. Gurlo and H. von Grafenstein, *Journal of immunological methods*, 2000, **234**, P153-P167.
5. M. K. Sezgintürk and E. Dinçkaya, *Biosensors and Bioelectronics*, 2008, **23**, 1799-1804.
6. A. Struss, P. Pasini, C. M. Ensor, N. Raut and S. Daunert, *Analytical chemistry*, 2010, **82**, 4457-4463.
7. S. Z. Hossain and J. D. Brennan, *Analytical chemistry*, 2011, **83**, 8772-8778.
8. L. Fiksdal and I. Tryland, *Current opinion in biotechnology*, 2008, **19**, 289-294.
9. J. C. Jokerst, J. A. Adkins, B. Bisha, M. M. Mentele, L. D. Goodridge and C. S. Henry, *Analytical chemistry*, 2012, **84**, 2900-2907.
10. H. Kodaka, M. Ishikawa, M. Iwata, F. Kashitani, S. Mizuochi and K. Yamaguchi, *Journal of clinical microbiology*, 1995, **33**, 199-201.
11. D. R. Henderson, S. Friedman, J. Harris, W. Manning and M. Zoccoli, *Clinical chemistry*, 1986, **32**, 1637-1641.
12. D. Eustice, P. Feldman, A. Colberg-Poley, R. Buckery and R. Neubauer, *Biotechniques*, 1992, 739-740, 742-733.

13. J. D. Berg and L. Fiksdal, *Applied and environmental microbiology*, 1988, **54**, 2118-2122.
14. Z. Huang, *Biochemistry*, 1991, **30**, 8530-8534.
15. H. Gong, B. Zhang, G. Little, J. Kovar, H. Chen, W. Xie, A. Schutz-Geschwender and D. M. Olive, *Analytical biochemistry*, 2009, **386**, 59-64.
16. Y. Cheng, Y. Liu, J. Huang, Y. Xian, W. Zhang, Z. Zhang and L. Jin, *Talanta*, 2008, **75**, 167-171.
17. V. Mani, W.-Y. Li, J.-A. Gu, C.-M. Lin and S.-T. Huang, *Talanta*, 2015, **131**, 121-126.
18. D. Hawley and R. N. Adams, *Journal of Electroanalytical Chemistry (1959)*, 1964, **8**, 163-166.
19. V. Mani, B. Devadas and S.-M. Chen, *Biosensors and Bioelectronics*, 2013, **41**, 309-315.
20. V. Mani, B. Dinesh, S.-M. Chen and R. Saraswathi, *Biosensors and Bioelectronics*, 2014, **53**, 420-427.
21. V. Mani, S.-M. Chen and B.-S. Lou, *Int. J. Electrochem. Sci*, 2013, **8**, 11641-11660.
22. W. S. Hummers Jr and R. E. Offeman, *Journal of the American Chemical Society*, 1958, **80**, 1339-1339.
23. X. Zhu, S. Shi, J. Wei, F. Lv, H. Zhao, J. Kong, Q. He and J. Ni, *Environmental science & technology*, 2007, **41**, 6541-6546.
24. D. R. Dreyer, S. Park, C. W. Bielawski and R. S. Ruoff, *Chemical Society Reviews*, 2010, **39**, 228-240.
25. T. Kuila, S. Bose, A. K. Mishra, P. Khanra, N. H. Kim and J. H. Lee, *Progress in Materials Science*, 2012, **57**, 1061-1105.
26. C. Liu, H. Zhang, Y. Tang and S. Luo, *Journal of Materials Chemistry A*, 2014, **2**, 4580-4587.
27. A. J. Bard and L. R. Faulkner, *Electrochemical methods: fundamentals and applications*, Wiley New York, 1980.
28. K. Wallenfels, *The Enzyme8*.
29. T. Kaya, K. Nagamine, N. Matsui, T. Yasukawa, H. Shiku and T. Matsue, *Chem. Commun.*, 2004, 248-249.
30. Y. Tanaka, A. Kagamiishi, A. Kiuchi and T. Horiuchi, *Journal of biochemistry*, 1975, **77**, 241-247.

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