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# Highly sensitive amperometric immunosensor for the detection of *Escherichia coli*

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ABSTRACT

With the aim of detecting rapidly the presence of *Escherichia coli* (*E. coli*), a disposable amperometric immunosensor was developed based on a double layered configuration at the transducer surface, consisting first of a polypyrrole-$NH_2$-anti-*E. coli* antibody (PAE) inner layer followed by an alginate-polypyrrole (Alg-Ppy) outer packing layer. In the presence of the substrate *p*-aminophenyl β-D-galactopyranoside (PAPG), the bacterial enzyme, β-D-galactosidase produces the *p*-aminophenol (PAP) product, also generating an amperometric signal due to PAP electrooxidation by potentiostating the glassy carbon (GC) electrode at 0.22 V. The operational procedure consists in first adding the test sample containing the bacteria, then coating it with Alg-Ppy to ensure the confinement of the released enzyme and the analyte (being generated by the enzymatic catalysis) to the electrode active surface. This procedure facilitates the diffusion of the substrate within the complex and thus creates a higher oxidation level of the PAP enabling a detection limit of 10 colony forming units (CFU)/ml. The immunosensor setup demonstrates an improved detection limit of more than 10 times less bacteria detected than other immunosensing techniques without the need for multi step pretreatments of the test sample and/or incubation as found in some of the existing methods.



## 1. Introduction

Detection of microorganisms is important in food and water safety, while the presence of *Escherichia coli* (*E. coli*) is used as a potential marker for the presence of pathogens originating from humans and warm-blooded animals (Tryland and Fiksdal, 1998). Estimating the number of coliforms is essential due to enteric disease, such as enterohemorrhagic strains of *E. coli* (Buchanan, 1997; Tokarskyy and Marshall, 2008) contracted from food or polluted coliform water supplies which still constitute public health concerns (Lin et al., 2008; Tokarskyy and Marshall, 2008).

Conventional procedures for *E. coli* detection and monitoring are mainly based on cultures grown on differential agar media followed by counting the number of target organisms in the sample, a procedure that can take 1–3 days (George et al., 2000; Lin et al., 2008). The time-consuming drawback increases the motivation for the development of rapid and sensitive methods aiming at estimating *E. coli* (nucleic acid assays and immunological techniques with chromogenic, fluorogenic, or chemiluminogenic substrates) (Datsenko and Wanner, 2000; Feng and Hartman, 1982; Gehring et al., 2004; Loge et al., 2002), usually showing a good linear correlation with those obtained from traditional methods (George et al., 2000; Tryland and Fiksdal, 1998; Venkateswaran et al., 1996).

Biosensors are also being developed for the detection of pathogenic bacteria including *E. coli* (Ivnitski et al., 1999; Leonard et al., 2003; Tokarskyy and Marshall, 2008). Hence biosensors using electrochemical (Abdel-Hamid et al., 1999a,b; Lin et al., 2008; Mittelmann et al., 2002; Palenzuela et al., 2004), piezoelectric (Su and Li, 2004), acoustic (Howe and Harding, 2000) and optical (Pyle et al., 1995) transducers were applied for *E. coli* detection. The number of electrochemical biosensing platforms (Bakker, 2004; Mehrvar and Abdi, 2004) for the detection of coliform contamination have grown rapidly. For example, endogenous enzymatic activity such as β-D-galactosidase which participates in lactose metabolism was used as a general marker for monitoring coliforms (Mittelmann et al., 2002; Tryland and Fiksdal, 1998), while some studies have used indirect sandwich enzyme-linked immunoassay with amperometric immunosensing (Abdel-Hamid et al., 1999a,b; Lin et al., 2008). In all these methods, *E. coli* bacteria could be

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detected at ranges of concentrations between $10^2$ and $10^8$ CFU/ml without preincubation of the original sample. There is still room to improve on the detection limits of environmental samples.

We report an alternative immunosensing approach for the amperometric detection of *E. coli*. The method is based on electropolymerization and coating of glassy carbon (GC) electrodes with polypyrrole-amine (Ppy-$NH_2$). The immobilization of the anti-*E. coli* antibody is carried out through carbodiimide chemistry (Abu-Rabeah et al., 2005) later enabling the specific attachment of *E. coli*. This step is followed by induced bacterial lysis and the subsequent release of β-D-galactosidase which in the presence of PAPG produces PAP that is then oxidized on the electrode thereby creating a current (Mittelmann et al., 2002). The system is entrapped in an Alg-Ppy matrix (Ionescu et al., 2005) which will confine the enzymatic catalysis to the electrode surface facilitating the diffusion and oxidation of the PAP. Electropolymerized Alg-Ppy fills the porous alginate with polypyrrole filaments reducing possible leaching of bacterial contents that was made available by lysis. Use of a secondary antibody was not necessary because detection was based on the activity of the intrinsic β-D-galactosidase enzyme. This system was tested with a model *E. coli* strain K-12 MG1655, however, introducing the appropriate capture antibodies would allow for the detection of any wild type strains of *E. coli* including O157:H7. An immunosensor that is very sensitive, specific, rapid, simple to fabricate and cost effective was thus developed.

## 2. Experimental

### 2.1. Chemicals and reagents

PAPG (A9545), isopropyl β-D-thiogalactopyranoside (IPTG) (16758), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (E1769), and Tris–HCl (77-861) were purchased from Sigma; pyrrole (py) (13170-9), lithium perchlorate (431567), sodium *p*-toluene sulfonate (NaPTS) (152536) and 1,1′-ferrocenedicarboxylic acid (FeCN) (1293-87-4) were purchased from Aldrich; lysozyme (Amresco 0663), perchloric acid (17931) and hydroxy-2,5-dioxopyrrolidine-3-sulfonicacid sodium salt (sulfo-NHS) (5648) were purchased from Fluka; acetonitrile (75-05-8) was purchased from Bio Lab; Rabbit anti-*E. coli* polyclonal antibody (BO357) was purchased from Dako; Sodium alginate (Alg) Protanal LF 10/60 (Laminaria hyperborea, average molecular weight of 128 kDa, viscosity of 1% (w/v) solution is 39 cP) was provided by FMC Biopolymer (Norway); and Alg-py and py-$NH_2$ were synthesized according to the protocols described in literature (Abu-Rabeah et al., 2005).

### 2.2. Bacterial culture

Bacterial strains of wild type *E. coli* K-12 MG1655, *E. coli* BW25113 with no β-D-galactosidase activity and *Pseudomonas aeruginosa* were grown over night in Luria Bertani (LB) medium at 37 °C with shaking. The experiment was begun by cultures inoculated into Erlenmeyer flasks containing 10 ml of LB medium, supplemented with IPTG to a final concentration of 0.5 mM. These cultures were grown during 2–2.5 h at 37 °C while shaking until reaching logarithmic phase. The cultures were then diluted with phosphate-buffered saline (PBS), pH 7.2, to concentrations ranging from 1 CFU/ml up to $10^7$ CFU/ml and analyzed later by the electrochemical amperometric technique.

### 2.3. Electrochemical instrumentation

The voltametric and the amperometric measurements as well as the electropolymerization process were carried out using a PGSTST30 electrochemical workstation: a conventional electrochemical cell (Metrohm) consisting of GC disk electrodes as working electrode (diameter 3 mm—polished with 1 μm diamond paste MECAPREX Press PM), a saturated Ag–AgCl–KCl electrode (Ag/AgCl) as reference electrode and a platinum wire as counter electrode. The data acquisitions were achieved using GPES and FRA software.

### 2.4. Permeability studies

The permeability of a GC bare electrode, coated with Ppy-$NH_2$, $10^2$ and $10^6$ CFU/ml coatings were examined by a rotating-disk electrode (RDE) equipped with a rotation controller. The experiments were conducted at different rotation speeds using FeCN (2 mM) in 0.1 M Tris–HCl (pH 7) as a redox probe.

The permeability ($P_m$) of the coatings was estimated using RDE experiments at different rotation rates. The results were analyzed and examined by applying Eqs. (1)–(4) (Gough and Leypoldt, 1979). A description of the variation of the steady state limiting current $I_{lim}$ is shown, with the mass transport for a RDE coated with different electro-inactive membranes.

$$\frac{1}{I_{lim}} = \frac{1}{I_s} + \frac{1}{I_m} \tag{1}$$

$$I_s = 0.62nFAC^0 D_S^{2/3} \upsilon^{-1/6} \omega^{1/2} \tag{2}$$

$$I_m = \frac{nFAKC^0 D_m}{\delta} = NFC^0 P_m \tag{3}$$

$$P_m = \frac{KD_m}{d}\,(\mathrm{cm\,s^{-1}}) \tag{4}$$

Terms $D_s$ and $D_m$ are the diffusion coefficients of the substrate in the bulk solution and in the membrane respectively; $\upsilon$, the kinematic viscosity of the solution; $\omega$, the rotation rate of the RDE; $\delta$, the thickness of the membrane; $K$, the partition equilibrium constant of the substrate between solution and membrane; $A$, the electrode surface; $n$, the number of exchanged electrons and $C^0$, the substrate concentration. Eq. (1) is composed of two terms, where the first represents the current flow under the same conditions, in the absence of a membrane, and is therefore characteristic of the diffusion of the substrate in the bulk solution (Levich current, Eq. (2)). The second term accounts for the diffusion of the substrate in the membrane and depends on the product of the partition equilibrium constant $K$, the diffusion constant of the substrate in the membrane and the film thickness. Only the first term of the equation is dependent upon the rotation rate of the RDE. Therefore, a plot of $1/I_{lim}$ versus $1/\omega^{1/2}$ (Fig. 2) presents a linear behavior with the same slope as for a bare electrode with a positive intercept, whose value depends on the permeability $P_m$ of the membranes (Eqs. (3) and (4)). The relative deviation of the values was <5%. AFM micrographs were taken with AFM systems manufactured by Nanonics Imaging Ltd., Jerusalem.

### 2.5. Amperometric detection of β-D-galactosidase activity

β-D-Galactosidase activity was detected using PAPG as a substrate. The enzyme catalyzed PAPG to form PAP which is then oxidized at the electrode surface. The amperometric detection was carried out in 15 ml of an aqueous solution of 0.1 M Tris–HCl buffer electrolyte (pH 7). The modified electrode was potentiostated at 0.22 V versus the reference electrode until reaching equilibrium. To start detection, the substrate PAPG in final concentration of 0.8 mg/ml was introduced and the current resulting from β-D-galactosidase activity was collected and measured by PGSTAT 30 equipped with GPES4 software.