A Practical Approach to Potentiometric Biosensors Based on Consolidated Composites: Construction and Evaluation of a D-Amygdalin Biosensor

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Abstract: A simple procedure to be used in an analytical chemistry laboratory by undergraduate students to prepare a potentiometric biosensor for D-amygdalin is given. The membrane material is prepared by simple compression of a solid sensing mixture (β-glucosidase mixed with Ag_2S and AgI). This new technology has some advantages. It presents a simple way to prepare a biosensor membrane and this methodology adapts well to mass production technology. Simple polishing before each new measurement can renew the membrane. This type of biosensor produced by consolidated biocomposites can serve as a base material for different biosensing schemes.

Using this technique, students can easily envision the functioning of a potentiometric biosensor where the classic detection mechanism as of an I-ISE is combined with the biological recognition of an enzyme.

Different kinds of materials are used to prepare biosensor membranes. Enzymes may be immobilised within an insoluble gel matrix (physical entrapment), to a support matrix via covalent bonds, or by a multifunctional reagent (chemical entrapment) or they can be adsorbed directly onto the surface [1]. Electrochemical composite materials, however, represent an attractive alternative for fabrication of biosensors [2–7]. They are formed by the combination of two or more different phases. As presented in Figure 1, composite electrodes can be classified according to how the phases are distributed within the composite material. For example, a conducting composite electrode surface can be prepared as an ordered array or as a random arrangement (ensemble) of conducting regions separated by an insulator [5]. The random composite mixtures are classified according to the distribution of the conductor within the composite matrix. If the conductor particles are distributed randomly within the composite matrix the composite is said to be of the dispersed type. If the conductor extends throughout the composite in a random fashion with regions of pure insulator and pure conductor that do not intermix, the composite is said to be of the consolidated type. According to this classification we will introduce a practical approach to the design of a D-amygdalin biosensor based on the consolidation of a silver salt mixture (Ag_2S, AgI) and the enzyme β-glucosidase. The first step consists of the preparation of the electroactive salt mixture of Ag_2S and AgI [8]. The second step is the mixing of this salt mixture with the β-glucosidase enzyme and the thorough homogenisation of these dry components using an agate mortar. The third step is the consolidation of all the components at high pressure using a pellet die and a hydraulic press. The fourth step is the biosensor construction.

This type of biosensor offers several advantages. Because they have a single active surface the reaction occurs directly in the liquid phase at the interface in contact with the biocomposite membrane; therefore, it is faster than other types of biosensor using active membranes attached directly to the transducer. Furthermore, this kind of biocomposite membrane can be polished, renewing the enzyme active surface leading to improved reproducibility.

To evaluate the biosensor, D-amygdalin substrate is used as the analyte. Amygdalin is a potentially toxic cyanogenic glycoside. The name amygdalin (*d*-mandelonitrile-β-*d*-glucosido-6-β-*d*-glucoside) is currently used interchangeably with *laetrile*. It occurs in seeds; mainly in bitter almonds, but also in peaches and apricots [9, 10]. These seeds have a high protein content and can be used as a food or feed ingredient; however, they contain approximately 50 nmol of the potentially toxic cyanogenic glycosides amygdalin and prunasin per mg [11]. Cyanoglycosides yield glucose, benzaldehyde, and hydrocyanic acid when hydrolysed in vitro by mineral acids or in vivo by enzymes (see reaction 1). An application of this biosensor in diluted and undiluted samples is possible considering the concentration of amygdalin in these seeds. The most important application would be in food technology wastewater control. The process effluents in food technology that involve amygdalin materials are regarded as highly toxic and cyanide must be destroyed prior to its disposal to aquatic environments. An amygdalin biosensor can substitute for the gas chromatographic detection of amygdalin [12], which is a more costly technique.

Consider a biosensor using the previously mentioned membrane in contact with an aqueous solution of D-amygdalin. The hydrolysis of the substrate is catalysed by the enzyme leading to the liberation of cyanide at the membrane–solution interface as presented in the following equation:

$$\text{D-amygdalin} + H_2O \xrightarrow{\beta\text{-glucosidase}} \text{benzaldehyde} + 2\ \text{glucose} + \text{HCN} \quad (1)$$

The presence of cyanide generates the following concurrent reaction at the membrane-solution interface:

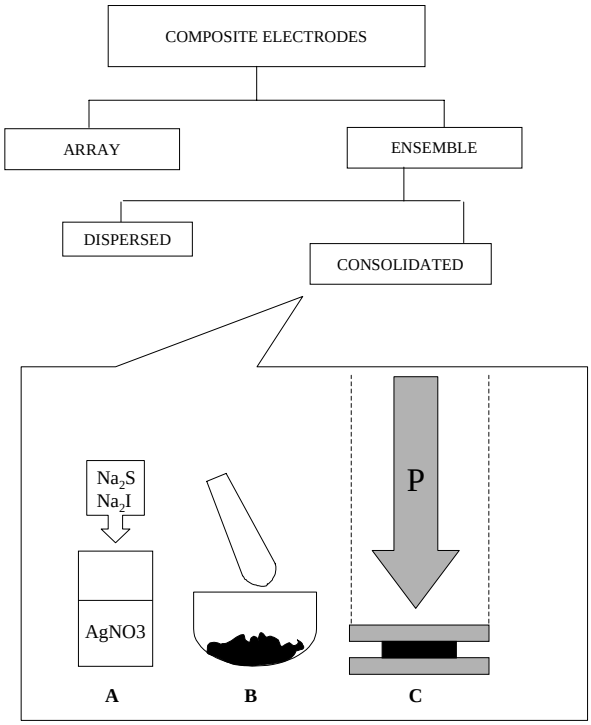

Figure 1. Classification of composite electrodes. For the preparation of consolidated biocomposite the experimental steps were: (A) preparation of the electroactive salts Ag_2S and AgI, (B) homogenisation of the electroactive salts and the enzyme using an agate mortar, (C) consolidation of the mixture of electroactive salts and enzyme at high pressure using a hydraulic press.

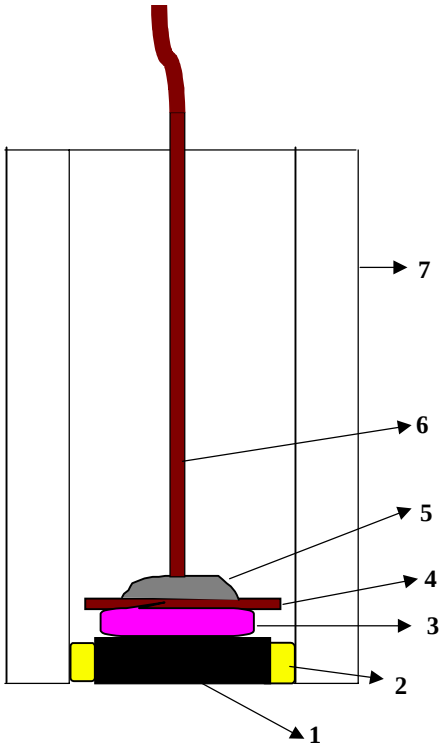

Figure 2. A typical electrode assembly used for the construction of a potentiometric biosensor based on consolidated biocomposites. (1) Consolidated biocomposite membrane (AG_2S+AGI + enzymes). (2) Epoxy resin glue. (3) Graphite epoxy glue. (4) Copper disc. (5) SN stick. (6) Electrical conductor. (7) PVC body.

$$AgI_m + 2CN^- \rightleftharpoons Ag(CN)_2^- + I^- \qquad (2)$$

where iodide from the membrane (m) is released into the solution.

Admitting that there are too many unknown variables embedded in the *y* intercept (potential of the biosensor) a constant term, *S*, results as presented in the following section.

The concentration of D-amygdalin can be monitored through the potentiometric detection of cyanide [9, 14]. A linear relationship is found to exist between the potential measured and log [D-amygdalin].

$$E = \text{constant-}S \log[\text{D-amygdalin}] \qquad (3)$$

The quantity *S* is the slope of the linear plot or, in other words, the sensitivity of the biosensor device.

Reaction 1 is not very fast and it controls the velocity of the overall reaction as well. In this situation it is important to control the time of each measurement after each addition of the amygdalin substrate.

Experimental Procedure

Reagents. All reagents used were of analytical grade unless otherwise specified. Deionised water was used for preparing all solutions.

β-Glucosidase (5.0 units/mg solid) from almonds was obtained from Sigma (EC 3.2.1.21, product number G0395). D-Amygdalin from apricot kernels was also obtained from Sigma. Epoxy resin (Araldit M and Hardener) was obtained from CIBA. Graphite powder (particle size: 50μm) was from BDH England. Solutions were prepared daily in a pH 7 phosphate buffer.

0.1-M phosphate buffer solutions at pH 7 and at pH 11 were prepared using dihydrogen phosphate (NaH_2PO_4). The pH was adjusted with a 1 M sodium hydroxide solution. 0.1-M aqueous solutions of Na_2S, NaI, and $AgNO_3$ were prepared. A 1 M HCl solution was also used.

Materials. The biosensor was constructed using the following materials.

A PVC tube, 6 mm i.d. and 8 mm o.d, cut in 12-cm lengths.

Disks, 5-mm o.d., cut from a 0.5-mm-thick copper plate. Shielded electric wire was used to make contact with a copper disk through a Sn stick. Connectors compatible with the input of the measuring instrument for the indicator electrode were used. Medium-sized abrasive paper (P-400) and alumina polishing strips (aluminum oxide std 3 micron, Orion 301044-001) were used to polish the membranes.

Instrumentation. The equipment necessary to carry out the experiments is not elaborate. A Digilab 517 pH/mV meter (Crison, Barcelona) was used with an Orion 90-02 double junction reference electrode with an outer phosphate buffer solution. The working electrode was built as shown in Figure 2. To produce the membranes a 5-mm pellet die and a hydraulic press (as used in the preparation of samples for IR spectroscopy) were used. A solder iron was used to fix the copper wire to the copper plate.

Exercises

Part I: Construction Of The D-Amygdalin Biosensor. *Section A: Preparation of the Electroactive Salts.* 50 mL of a 0.1 M NaI solution are mixed with 50 mL of a 0.1 M Na_2S solution. This mixture is poured gradually on approximately 160 mL of a 0.1 M $AgNO_3$ solution in slight excess as needed

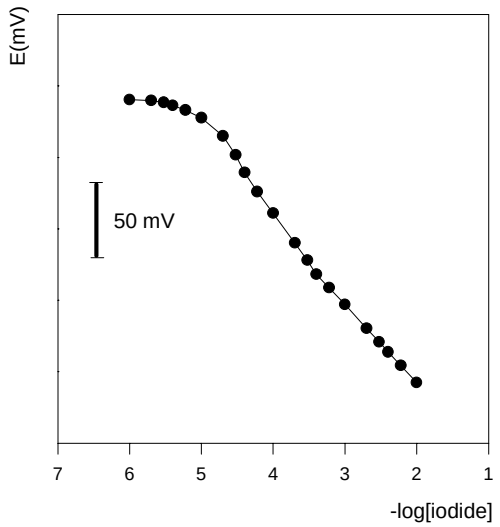

Figure 3. Iodide response for the biosensor using biocomposite membranes consolidated at 2.0×10^4 kg cm^{-2}. Membrane composition: 100 mg silver salts (AG_2S + AGI) and 10 mg (50 U) β-glucosidase. Working media: PH 7 phosphate buffer (0.1 M). Additions of iodide solutions prepared in the same buffer.

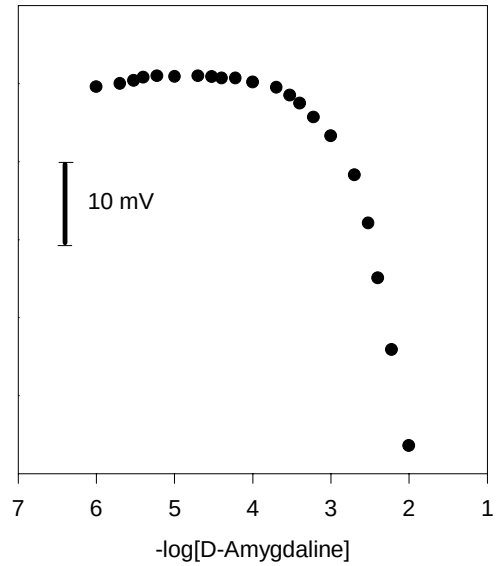

Figure 4. A typical D-amygdalin response for the biosensor using biocomposite membranes consolidated at 2.0×10^4 kg cm^{-2}. Membrane composition and working media are as described in Figure 3. Additions of D-amygdalin solutions prepared in the same buffer.

to precipitate the iodide and the sulphide. The precipitate of sintered glass funnel and dried overnight at 100 °C. This AgI and Ag_2S is washed carefully several times, filtered with a material is than pulverised using an agate mortar and is stored away from the light.

Section B: Preparation of the Biosensor Membrane by Consolidation. A quantity of 100 mg of the above electroactive salts is weighed and mixed thoroughly with 10 mg of β-glucosidase (5 U/mg) using an agate mortar. This mixture is introduced into a 5-mm pellet die. Using an hydraulic press (as used to make IR sample pellets), a pressure of 20,000 kg cm^{-2} is applied to produce a 5-mm diameter membrane with an 0.8–mm thickness.

Section C. Construction of the Biosensor. A dielectric epoxy resin is prepared by mixing 1 g of Araldite M with 0.4 g of its hardener. The graphite–epoxy composite used as a conductive glue is prepared by mixing 0.5 g of the above epoxy mixture with 0.5 g of graphite powder.

A copper disc (5-mm diameter) is soldered to a wire as shown in Figure 2. The biosensor membrane is fixed to the disk using the graphite composite. The dielectric epoxy resin is used to seal the space between the PVC tube and the membrane. The electrode body is left in an oven at a temperature of 40 °C for 12 hours to cure the epoxy. After the glue is cured, the membrane is polished with a medium-sized abrasive paper (P400) and then with 3-μm alumina polishing strips (Orion 301044-001) under wet conditions. The membrane is conditioned by leaving it for at least 2 hours in a 10^{-4} M iodide solution.

While the biosensor is not in use it is kept refrigerated at 4 °C.

Part II Analytical Evaluation of the D-Amygdalin Biosensor. Section A. *Calibration of the Biosensor Using an Iodide Solution.* 25 mL of 0.1 M phosphate buffer at pH 7 is put in a 50-ml beaker with a magnetic stirrer. During potential measurements the biosensor is used as the indicator electrode.

Different volumes of an iodide solution prepared from the stock solution are added to the cell (in the range 10^{-2} to 10^{-6} M iodide concentration). The potential is measured with a pH/mV meter 2 min after the addition.

Figure 3 presents the relationship between the measured potential (*E*, mV) and the negative logarithm of the iodide concentration (–log [iodide]). From this data the iodide sensitivity of the biosensor, *S*, in the linear portion of curve; the standard potential, E^o; and the practical detection limit, the abscissa of the interception of the linear parts of the curve, for iodide can be calculated.

Section B. D-Amygdalin Calibration of the Biosensor. After washing the cell and the electrodes well, 25 mL of the phosphate buffer is placed in a 50-mL beaker and the cell is prepared as in the previous section.

Solutions of D-amygdalin ranging in concentration from 10^{-6} to 10^{-2} M are made and the potential is measured two minutes after each solution has been completed.

The measured potential (*E*, mV) as a function of the negative logarithm of the concentration of the D-amygdalin is recorded as shown in Figure 4. The relative standard deviation of our potential measurements was 5.6% (n = 3). The standard potential, E^o; the sensitivity of the biosensor, *S*; and the practical detection limit, the abscissa of the interception of the linear parts of the curve, can all be calculated from this data.

Caution: Collect the waste D-amygdaline solutions in a high-pH waste disposal container to avoid exposure to hydrogen cyanide.

Section C. Effect of pH on the Biosensor Response. A 25-ml quantity of 10^{-2} M D-amygdalin in 0.1 M phosphate buffer at pH 11 is added to a cell where the D-amygdalin biosensor and a reference electrode (as in Part II) have been introduced. A pH meter featuring a combination pH electrode is introduced to monitor the pH of the solution. After each addition of a small volume of a 1 M HCl solution, the pH is measured with the pH meter and the potential is measured with the biosensor and the associated instrument. Figure 5 shows the relationship between the potential measured by the biosensor setup, *E*, versus the measured pH.

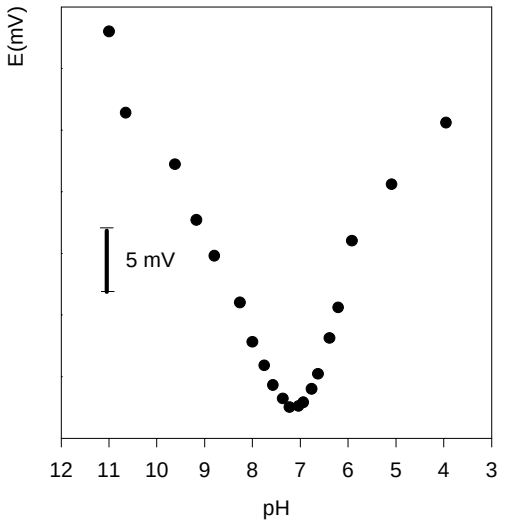

Figure 5. Effect of pH on the potential of D-amygdalin biosensor. The biosensor membrane has the same composition as for the experiments presented in Figure 3. Additions of 1 M HCl were followed upon the cell with 10^{-3} M D-amygdalin in 0.1 M phosphate buffer at a pH of 11.

Discussion

To avoid studying the electrode response to cyanide only the iodide response is studied. By performing this calibration students can check how the biosensor responds to the primary ion, iodide. When calibrating with D-amygdalin, the biosensor responds to variations of iodide as a result of the overall reaction presented previously (reactions 1 and 2). Figure 3 shows the response of the biosensor to the addition of iodide. From the pseudo-Nernstian response [5], the sensitivity and detection limit can be calculated. These calculations are based on the dependence of the measured potential on the concentration of D-amygdalin. These parameters are important for the analytical characterisation of the biosensor even though they do not show Nernstian behaviour.

From the intercept of the linear part of Figure 3, the practical detection limit for iodide is 4×10^{-6} M. As shown in Figure 4, the practical detection limit for D-amygdalin is 10^{-3} M. This high detection limit for D-amygdalin can be explained by the fact that reaction 1 is not very fast. For this reason, the transformation of D-amygdalin into HCN is not stoichiometric during the time in which the potential is measured. It can be concluded from the D-amygdalin calibration curve that the biosensor has a sensitivity of 50 mV/decade (in the range from 10^{-2} M to 10^{-3} M).

Figure 5 shows the effect of pH on the biosensor response. The optimal pH value of 7 reflects a compromise between the cyanide acid–base equilibrium and the enzyme activity. High pH values increase cyanide activity (due to the equilibrium $HCN + H_2O \rightleftharpoons H_3O^+ + CN^-$), but they are detrimental to the enzyme, which functions optimally at pH values between 4.4 and 5.0 [15].

The exercises presented show that using easy technology students can learn how to prepare a simple biosensor. The membrane material is prepared by simple compression of a solid sensing mixture (β-glucosidase mixed with Ag_2S and AgI). This new technology has some advantages. It presents an easy way of preparing a membrane and the methodology adapts well to mass production technology. Simple polishing before each new measurement renews the membrane. This type of biosensor produced by consolidated biocomposites can serve as a base material for different biosensing schemes.

Using this technique students can easily envision the functioning of a potentiometric biosensor, where the classic detection mechanism as of an I-ISE is combined with the biological recognition of an enzyme. The above experiments can also be using linamarin [16] as the substrate instead of D-amygdalin. The possibility of using this biosensor for monitoring linamarin is based on the hydrolysis of linamarin in the presence of β-glucosidase (reaction 6).

$$\text{linamarin} + H_2O \xrightarrow{\beta\text{-glucosidase}} \text{glucose} + \text{acetone} + \text{HCN} \quad (6)$$

Reaction 6 can also be catalysed by linamarase; therefore, students can see the possibility of the construction of a linamarin potentiometric biosensor that contains linamarase in the consolidated membrane.

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