Optical pH sensor based on polyelectrolyte complex (PEC) pectin-chitosan/methanol anthocyanin extract of *Catharanthus roseus* for a new optical urea biosensor development

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Abstract. Construction of optical pH sensor as a new platform optical urea biosensor based on polyelectrolyte complex (PEC) pectin-chitosan membrane and total phenolic (anthocyanin) of Tapak Dara flower (*Catharanthus roseus*) has been successfully carried out. The anthocyanin was extracted by a maceration method for 72 hours using methanol, and a total extract yield was 21.56% or 1.803 mg/L. Flavonoid and phenol tests showed positive results indicated by the formation of red and black colours. The anthocyanins showed maximum absorption at 578 nm for phosphate buffer and 575 nm for tris HCl buffer solution. The sensor fabrication was performed using a mixture of pectin and chitosan solutions with a ratio of 3:7. The anthocyanin was mixed into the solution with various concentrations. The sensor has an optimum sensitivity at the anthocyanin concentration of 0.05 mg/L (phosphate buffer) in the pH range of 7.0-9.5 and 0.025 mg/L (Tris HCl buffer) with a narrower pH range of 6.0-7.5. This sensor produced higher sensitivity, a wider linear range, and good linearity when it was exposed in 0.03M PBS. Reproducibility test with a relative standard deviation percentage (% RSD) was 9.20. The sensor showed a stable response after 5 minutes exposed to PBS solution, and it can be used to measure pH within the 20th day. The optimized optical pH sensor has been successfully developed as a urea optical biosensor by immobilizing urease on its surface. The biosensor showed a linear response in a series of 10^-1-10^-10 M urea concentrations and has good linearity.

Keywords: Anthocyanin, *Catharanthus roseus* optical pH sensor, polyelectrolyte pectin-chitosan, urea biosensor

INTRODUCTION

Sensors and biosensors are devices that still grab researchers attention to detect samples in various fields such as health, food, environment, and medicine. The devices have some advantages of being simple equipment and operation, sensitive, and selective. One of the sensors that continue to develop today is the pH sensor. Many sample measurements are based on the detection of pH. The monitoring of pH also is an indirect sample detection that used a concept enzyme and substrate reactions where the reaction produces H+ or OH- ions. Some enzyme reactions with their substrate generate changes in pH, such as urease (1), alcohol oxidase (2), glucose oxidase (3), and peroxidase (4). Therefore, the need for a sensitive pH sensor to be further developed as a biosensor is still needed. A good performance optical pH sensor is promising to be developed as a
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biosensor. The sensing films have shown reversible and reliable responses to pH changes. As previously mentioned, the sensor consists of several important parts, namely the matrix and the active substance. The matrix has a function as a binding agent of the active substance. In addition, it must have good resistance and mechanical properties. The matrix can be obtained from synthetic polymers and biopolymers (5). Synthetic polymers have several advantages in which this substance has good mechanical properties (6,7). Synthetic polymers also have a weakness because they are generally hydrophobic which causes the diffusion process between the active ingredient and analyte to be slow, resulting in a long response time (8,9). Furthermore, natural membranes have also been widely developed as sensor matrices such as cellulose nanofibers chitosan (10,11), pectin (12,13), polyvinyl alcohol, and polyvinyl alcohol glycerol (14).

Pectin and chitosan have been used successfully as a matrix for the development of sensors and biosensors. Pectin and chitosan individually have weaknesses based on their solubility properties (15,16). Pectin is known to be very soluble in water, and chitosan is soluble in acidic solutions (7,17). Pectin has been used as a matrix of optical pH sensor and triglyceride biosensor using microliter samples (12,13). The study reported the hydrogel properties of the pectin membrane were not suitable for measuring samples by immersion. At the same time, several studies have also been developed for chitosan membrane-based sensors, but the sensors also have limitations due to the acid-soluble nature of chitosan. The weakness of pectin and chitosan can be improved by the formation of a polyelectrolyte complex (PEC) which has better mechanical strength. The PEC membrane is formed by electrostatic interaction between the carboxylate group of pectin and the primary amine group of chitosan (18).

In addition, another important component of the biosensor is the active substance. Anthocyanin is one of nature’s active substances which is pH-sensitive properties. Several studies reported on the use of anthocyanins to detect changes in pH. The anthocyanin can be obtained from red cabbage (19), lychee skin [23], black rice, bitter melon, paprika, onion peel, grape skin, mulberry, and beetroot (20).

In this study, the anthocyanin from C. roseus flower has been used as pH-sensitive active substance which was immobilized on the pectin-chitosan PEC membrane. The optimized optical pH sensor was further modified as a urea biosensor by immobilizing the urease enzyme on the surface of the pH sensor. The biosensor works based on the pH change due to the reaction between urease and urea. Thus the pH change sensor environment is marked by a change in the color of the anthocyanins in the PEC membrane matrix.

METHODOLOGY

Chemical used in this research include, potassium dihydrogen phosphate (KH₂PO₄), dipotassium hydrogen phosphate (K₂HPO₄) were acquired from Fluka and pectin and methanol - from Sigma. Chitosan was obtained from Tokyo Chemical Industry Co., Ltd. Japan (obtained from shrimp shell, with deacetylation degree of 75–85%). Catharanthus roseus L was collected from local garden, HCl, NaOH, citrate acid, sodium citrate, pectin, acetic acid- from Merck.

Absorbance measurements associated to the response of the biosensor were performed by spectrophotometer UV-Vis Shimazu 1800 and buffer pH was monitored with a pH-meter (Thermo Scientific Orion Star A211).

Perparation of anthocyanin/pectin-chitosan PEC membrane

Anthocyanin was extracted from Catharanthus roseus using methanol solvent following the previously reported procedure (21). Pectin-chitosan PEC membrane was prepared by mixing each pectin solution and chitosan 1% (w/v) in a ratio of 3:7. Pectin and chitosan were dissolved using distilled water and 4 M CH₃COOH until the 100 mL of total volume. The mixture was stirred until homogeneous. The pectin-chitosan PEC solution was mixed with anthocyanin from the stock extract of 0.05 mg/L so that the PEC solution contained anthocyanin at a concentration of 0.025; 0.0375 and 0.05 mg/L. As much as 30 µL PEC-anthocyanin mixture was dripped onto a circle with a diameter of 6 mm onto a 4 x 0.75 cm mica plastic as illustrated by Figure 1 and dried in a refrigerator at 4°C for 24 hours.
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**Figure 1.** Optical pH sensor design

**Sensitivity optical pH sensor determination in citrate and phosphate buffer solutions**

The sensitivity of optical pH sensor was determined based on measuring absorbance in citrate (pH 4-8), and phosphate (pH 4-9) buffer solutions at each wavelength maximum of absorbance. Thus, volume of 30 µL, taken individually of 0.1M buffer solution (citrate and phosphate) with a certain pH were dropped onto the anthocyanin/pectin-chitosan membrane (optical pH sensor) and after that the measurements were performed taking into account the maximum absorbance values exhibited by anthocyanin. The measuring of absorbance was carried out 10 minutes after dropping 30 µL of buffer solution at various pHs. Then a plot between absorbance and pH is made to obtain a dynamic range. The slope of the curve was determined according to a regression linear, and it indicated the sensor sensitivity value.

**Determination of sensitivity of optical pH sensor at various phosphate buffer concentrations**

The buffer solution which showed the highest sensitivity value was changed in its concentration (0.01, 0.03, 0.05 and 0.1M) to get the maximum sensitivity within a wide pH range.

**Reproducibility, response time and life time studies on the optical pH sensor**

The response time was determined by measuring the response of sensors at 5, 10, 15, 20, 25 and 30 minutes after dropping 30 µL of various pHs of the buffer solutions (with optimum concentration) onto the sensor surface. The reproducibility was assessed based on absorbance measurements of 10 sensors by adding 30 µL of 0.1M of PBS at pH 7. The lifetime was studied by measuring the absorbance of sensor on days (1, 2, 3, 4, 5, 10, 15 and 20).

**RESULT AND DISCUSSION**

**Characterization and optical pH sensor optimization**

The characterization of the optical pH sensor was based on the measurement of sensor absorbance at various pH using a UV-Vis spectrophotometer. The scan measurement results showed that the anthocyanins of methanol extract had two peaks at wavelengths 578 nm and 575 nm for both tris HCl and phosphate buffers. However, the highest absorbance was at a wavelength of 578 nm for anthocyanin in phosphate buffer solution. Hence, the wavelength was used for further absorbance measurement of the optical pH sensor.

**Figure 2.** Maximum wavelength profile of the anthocyanin methanol extract of *C. roseus* in phosphate buffer (578 nm) and tris HCl (575 nm)

Figure 2 shows that the highest absorbance peak was obtained when anthocyanins were in phosphate buffer. Based on visualization observations, anthocyanins in tris buffer have fading color than phosphate buffer at the same pH and concentration. Several previous studies reported that the color intensity sensor based on anthocyanin active substances has a more concentrated color when exposed to phosphate buffer compared to buffers that have a lower pKa value such as citrate buffer solution (8,9,22,23).

**Effect of anthocyanin concentration on sensor sensitivity**

Determination of variations in anthocyanin concentration on the optical pH sensor was carried out on phosphate buffer and tris HCl buffer. Absorbance measurements for variations in anthocyanin concentrations were 0.025; 0.0375; and 0.05 mg/L performed at λmaxs for phosphate buffer (578 nm) and tris HCl (575 nm, respectively). Sensitivity values were obtained from the slope of plot between absorbance and various pHs with acceptable dynamic range. The measurement results are shown in Table 1.

Table 1 shows the optimum anthocyanin concentration for phosphate buffer, which is 0.05 mg/L where these results provide a linear response of the optical pH sensor on the pH 7.0–9.5 with a sensitivity of 0.22 AU/pH unit and an R² value of 0.9891. However, for Tris HCl buffer, the best anthocyanin concentration is at a concentration of 0.025 mg/L linear response.
Table 1. Effect of anthocyanin concentration on sensor performance in phosphate and citrate buffer solution

| Anthocyanin Concentration (mg/L) | Phosphate Buffer | Tris HCl Buffer |
|----------------------------------|------------------|-----------------|
|                                  | Linear Sensitivity (AU/pH unit) | Linear Sensitivity (AU/pH unit) |
| 0.025                            | 7.0 - 13.1 0.969 7.0 - 0.140 0.98 | 7.0 - 0.001 0.969 7.0 - 0.001 0.98 |
| 0.0375                           | 6.5 - 0.201 0.997 5.0 - 0.017 0.93 | 6.5 - 0.101 0.997 5.0 - 0.017 0.93 |
| 0.05                             | 7.0 - 0.220 0.989 5.5 - 0.250 0.93 | 9.5 - 0.0003 0.989 7.5 - 0.004 0.93 |

Effect of phosphate buffer on sensor sensitivity

The concentrations of phosphate buffer used were 0.01 M, 0.03 M, 0.05 M and 0.075 M. The absorbance measurement concentration was carried out at max 578 nm. The sensor sensitivity values with variations in the concentration of phosphate buffer are shown in Table 2.

Table 2. The value of the sensitivity of the pH optical sensor to variations in the concentration of phosphate buffer

| Concentration (M) | Linear range (pH) | Sensitivity (AU/pH Unit) | R² |
|-------------------|-------------------|--------------------------|----|
| 0.01              | 7.0 - 8.5         | 0.030±0.0090             | 0.994 |
| 0.03              | 7.0 - 9.5         | 0.161±0.0003             | 0.925 |
| 0.05              | 7.5 - 9.5         | 0.139±0.0020             | 0.953 |
| 0.075             | 6.5 - 8.0         | 0.367±0.0080             | 0.991 |

Table 2 shows the best sensitivity value at 0.161 for the sensor response measured using 0.03M phosphate buffer compared to the sensor response produced using 0.01M, 0.05M and 0.075M. The concentration of phosphate buffer less than 0.03 M has lower sensitivity and a narrower measurement linear range. Based on observations, the colour of the sensor exposed in 0.03M phosphate concentration was more concentrated than the colour of the sensor exposed in 0.01M phosphate buffer and caused the sensor to be less able to respond at a wide pH range. Employing a phosphate buffer concentration of more than 0.03M showed a decrease in sensitivity and a narrower pH range. The following is a profile of the pH range for the optical pH sensors at various concentrations of phosphate buffer solution.
Reproducibility
The reproducibility measurement of the pH optical sensor was carried out to determine how the pH optical sensor was performed on seven different sensors under the same manufacturing conditions and treated with the same buffer solution. The results obtained are shown in Table 3.

Table 3. Absorbance measurement on reproducibility study of the pH optical sensor

| No | Optical sensor | pH | Absorbance (AU) |
|----|----------------|----|-----------------|
| 1. | A              | 7.0-9.0 | 0.554 |
| 2. | B              | 7.0-9.0 | 0.532 |
| 3. | C              | 7.0-9.0 | 0.496 |
| 4. | D              | 7.0-9.0 | 0.642 |
| 5. | E              | 7.0-9.0 | 0.512 |
| 6. | F              | 7.0-9.0 | 0.505 |
| 7. | G              | 7.0-9.0 | 0.561 |
| Average |  |  | 0.543 |
| Deviation |  |  | 0.05 |
| % RSD |  |  | 9.20 |

Table 3 shows that the reproducibility of the optical pH sensor measurements obtained an average value of 0.543 with a standard deviation of 0.05 and a per cent value of the relative standard deviation of the determination (% RSD) around 9.20%. Measurement of reproducibility in terms of standard deviation and % RSD acceptable values to the sensor performance criteria.

Lifetime
The sensor lifetime is to evaluate how long the sensor show stability in response. The evaluation was performed from the first day to the fifth day. Furthermore, measurements were made at intervals of 5 days, and the sensors were treated with similar conditions. The results of sensitivity determination and linear range for 30 days are as shown in Table 4.

Table 4. Lifetime optical pH sensor

| Day | pH range | Sensitivity (AU/pH Unit) | R² |
|-----|----------|--------------------------|----|
| 1.  | 7.0-9.0  | 0.091±0.0003             | 0.999 |
| 2.  | 6.5-8.5  | 0.154±0.0002             | 0.996 |
| 3.  | 6.5-8.5  | 0.037±0.0004             | 0.974 |
| 4.  | 7.0-8.5  | 0.098±0.0002             | 0.980 |
| 5.  | 6.0-7.5  | 0.082±0.0010             | 0.995 |
| 10. | 5.5-7.5  | 0.026±0.0010             | 0.990 |
| 15. | 6.0-9.0  | 0.036±0.0001             | 0.960 |
| 20. | 5.5-8.5  | 0.055±0.0003             | 0.960 |
| 25. | 7.0-8.5  | 0.051±0.0004             | 0.878 |
| 30. | 6.0-9.0  | 0.035±0.0002             | 0.922 |

Based on Table 4 data, the sensitivity and pH range of the optical pH sensor during 30 days measurements were different. The fluctuation in sensitivity value might be caused by the appropriate linear range adjustment. In addition, the difference was also probably caused by the % RSD value of 9.20%. It was indicated that the sensor response also has a high fluctuating value but is still acceptable. The decrease in sensitivity and changes in pH range during the measurement period was also caused by the stability of anthocyanins which has limited over a certain period (22). Based on the data presented in Table 4, the lifetime of the optical pH sensor using the anthocyanin methanol extract from C. roseus still worked until the 20th day. The sensor showed a narrower in dynamic ranges with poor of linearity after 20 days measurement. It might be due to the unstable properties of anthocyanins in a longer than 20 days.

Response of the pH sensor as a modification of the urea biosensor
The optimized optical pH sensor was aimed as a basic sensor to construct an optical urea biosensor performed by further modifying with immobilization an urease enzyme on the optical pH sensor surface. Urease will react with urea in solution to produce OH- ions which will change the pH environment of the pectin-chitosan PEC/anthocyanin membrane so that the color changes As followed by a reaction below:

\[ \text{NH}_2\text{CONH}_2 + 3\text{H}_2\text{O} \xrightarrow{\text{Urease}} 2\text{NH}_4^+ + \text{HCO}_3^- + \text{OH}^- \]

The response of the urea biosensor is based on measuring the color change of the pH optical sensor as shown in Figure 5.
CONCLUSION

Pectin-chitosan PEC membrane has been successfully employed as a matrix to immobilize anthocyanins from the C. Roseus flower. The system is called an optical pH sensor with good performance. The sensor has a linear range in the alkaline pH and it has been further modified by immobilizing the urease enzyme. The biosensor is able to respond to urea in solution up to $10^{-10}$ M.

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