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Fabrication of an Immobilized Polyelectrolite Complex (PEC) Membrane from Pectin-Chitosan and Chromoionophore ETH 5294 for pH-Based Fish Freshness Monitoring

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Abstract: Considering the significance of its demand around the world, the accurate determination of fish freshness with a simple and rapid procedure has become an interesting issue for the fishing industry. Hence, we aimed to fabricate a new optical pH sensor based on a polyelectrolyte (PEC) membrane of pectin–chitosan and the active material chromoionophore ETH 5294. A trial-and-error investigation of the polymer compositions revealed that the optimum ratio of pectin to chitosan was 3:7. With an optimum wavelength region (λ) at 610 nm, the constructed sensor was capable of stable responses after 5 min exposure to phosphate-buffered solution. Furthermore, the obtained sensor achieved optimum sensitivity when the PBS concentration was 0.1 M, while the relative standard deviation values ranged from 2.07 to 2.34%, suggesting good reproducibility. Further investigation revealed that the sensor experienced decreased absorbance of 16.67–18.68% after 25 days of storage. Employing the optimum conditions stated previously, the sensor was tested to monitor fish freshness in samples that were stored at 4 °C and ambient temperature. The results suggested that the newly fabricated optical sensor could measure pH changes on fish skin after 25 h storage at room temperature (pH 6.37, 8.91 and 11.02, respectively) and 4 °C (pH 6.8, 7.31 and 7.92, respectively).

Keywords: chromoionophore ETH 5294; optical pH sensor; PEC membrane; pectin; UV-Vis spectroscopy

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1. Introduction

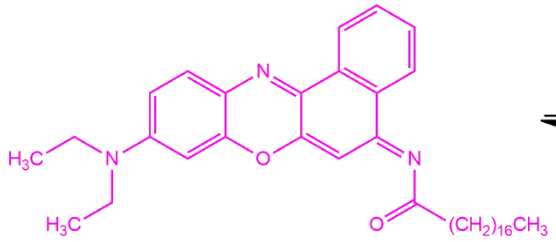
As the global demand for fish consumption is significant, various technologies to measure fish freshness been largely developed. In the post-mortem state, fish can undergo proteolysis, glycolysis and lipolysis, which contribute to fish spoilage [1]. Previous studies have fabricated sensors to determine fish freshness on the basis of concentration increments in total volatile basic nitrogen (TVBN) [2] or trimethylamine (TMA) [3]. Since both TVBN and TMA also play a role in the pH change, as an alternative, fish freshness could also be determined by employing pH sensors [4], which is considered a relatively more straightforward and faster method. Additionally, pH sensors allow real-time analysis, which is not only useful in the fishing industry, but also in other food industries, biomedical applications and environmental monitoring [5].

As of today, the most commonly used and reliable tool to measure pH levels over a wide linear range is the H-selective electrode (H^+ ISE) [6]. Since the H^+ ISE works by measuring the changes in the electrochemical H^+ ion concentration, it has a major drawback of being influenced by interference ions [6]. To overcome this limitation, an optical pH sensor has been proposed as an alternative that operates based on the pH-dependent color changes result from alterations in UV-Vis light absorbance [7]. Hence, active agents that are sensitive to pH changes should be employed in this system, including chromoionophore ETH 5294 [8]. The active agent could be immobilized in a polymeric matrix, for which some researchers have used synthetic polymers such as polyethene [9] and polyvinylchloride (PVC) [7]. During the employment of the foregoing polymers, the narrow pH range and inadequate reaction with the analyte were reported as the main weaknesses of the developed sensors, attributed to the lack of compatibility between the non-polar matrix and the polar analyte [7,9]. To overcome this challenge, several studies have fabricated optical pH sensors using polar polymers, including nanofiber cellulose [10,11].

Since the optical pH sensor prepared in the present study is proposed to be manufactured from good ingredients, biopolymers are considered as good candidates owing to their non-toxic properties [12]. In addition, biopolymers are closely associated with the green properties of a material, which is important in attenuating the carbon footprint of the fishing industry [13]. Pectin, one of the well-known biopolymers used in foods, has been reported for its ability to immobilize active agents, resulting in optical pH sensors with sensitivity (mean ± standard deviation (SD)) reaching 0.14 ± 0.03 [14]. Furthermore, a pectin-based optical pH sensor only required less than 5 min to detect an optimum response due to its hydrogel properties allowing higher analyte diffusion [14]. However, for practical applications, its weak mechanical strength must be resolved [15].

To improve its mechanical strength, modification via polyelectrolyte complex (PEC) could be carried out by exploiting intramolecular ionic interactions between two polymers possessing different charges [16–19]. Pectin has a tendency to form negatively charged molecule surfaces, attributed to the presence of carboxyl moieties ($–COO^-$). Hence, in order to form a PEC interaction, pectin could be mixed with another biopolymer, chitosan, whereby its protonated amine moieties ($–NH_3{}^+$) could be obtained in acidic conditions [1]. In the mixture, pectin would act as the polyanion, while chitosan would act as the polycation. The ease of chitosan modification has been specifically highlighted in a review article [20] and investigative reports [21,22]. Previously, chitosan matrices have been employed for fabricating piezoelectric sensors [23], a H_2S sensor [24] and smart pH-sensing packaging [25]. In our previous report, a pectin–chitosan PEC matrix was used to immobilize natural anthocyanin when developing an optical pH sensor for salivary samples, with satisfying results being obtained [18].

In the present study, we develop and ETH 5294-based optical pH sensor with a similar pectin–chitosan PEC matrix as a tool for fish freshness monitoring. ETH 5294 is a common active agent used for sensors with various immobilizing matrices, such as chitosan [26], pectin [8], κ-carrageenan [27] and polythiophene [28]. The chemical structure of ETH 5294 can be protonated and deprotonated under acidic and alkaline pH ranges, respectively, resulting in changes in color (Scheme 1). The novelty of our current work is two-fold, the first aspect of which is the sample. To measure the pH of a fish sample, only a limited amount of analyte must be present on the surface to diffuse into the matrix. This is perceived as more challenging than salivary samples, which appear in a liquid form, facilitating easier analyte diffusion. Secondly, the use of commercial ETH 5294 in this present study allows the replication of an optical pH sensor, suggesting higher practical value.



Scheme 1. An illustration of deprotonated and protonated ETH 5294.

2. Materials and Methods

2.1. Materials

The chemicals used in this research were chromoionophore ETH 5294, monopotassium dihydrogen phosphate (KH_2PO_4) and dipotassium phosphate (K_2HPO_4) purchased from Fluka (Buchs, Switzerland). Pectin, acetic acid (CH_3COOH) and ethanol (C_2H_5OH) absolute were procured from Sigma (St. Louis, MO, USA). Chitosan was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) obtained from shrimp shells, with a deacetylation degree of 75–85%. Unless mentioned specifically, all chemicals in this research were of analytical grade.

Chromoionophore ETH 5294 reagent was prepared by dissolving 0.4 mg ETH 5294 in 1 mL ethanol and mixed for 10 min until homogenous. Potassium-phosphate-buffered solution (PBS) was prepared by mixing potassium dihydrogen phosphate (KH_2PO_4) with dipotassium hydrogen phosphate (K_2HPO_4). The tilapia fish (*Oreochromis mossambica*) used in this study were obtained in dead condition from the local wet market and directly transported to the laboratory for investigation.

2.2. Fabrication of Optical pH Sensor

Firstly, pectin 1% *w/v* and chitosan 1% *w/v* solutions were obtained by dissolving the biopolymers in distilled water and acetic acid, respectively. The PEC membrane solution was prepared with a composition ratio of 3:7 of previously prepared pectin 1% *w/v* and chitosan 1% *w/v* solutions. The ratio was determined as such because based on our trial and error it produced a membrane with the optimum transparency. The assessment was carried out based on the visible appearance of the membrane. The active substance, ETH 5294 (4 mg), was then dissolved into 10 mL of 96% C_2H_5OH to obtain ETH 5294 solution at 400 mg/L concentration. Thereafter, 1.5 mL of membrane solution was mixed with 500 µL of 5294 ETH 400 mg/L. The casting solution (30 µL) was poured onto the sensor template (4×8 cm^2; radius of 1.5 cm), resulting in a membrane with a thickness of 0.06 mm. The sensors were stored for 18 h at 4 °C upon further use.

2.3. Characterization of the Fabricated Optical pH Sensor

The surface morphology of the prepared PEC membrane was analyzed with a Zeiss Merlin/Merlin Compact/Supra 55VP field emission scanning electron microscope (FESEM) (Berlin, Germany) at an acceleration voltage of 10 kV and 4000 times magnification. Functional groups were observed under Fourier transform infrared (FT-IR) spectrometry with Cary 630 Agilent (Santa Clara, CA, USA).

2.4. Optimization and Sensor Performance

Firstly, the fabricated sensor was evaluated for its response time by using PBS 0.1 M (30 µL), and the contact times ranged between 0 and 20 min. The responses were monitored using a UV-Vis spectrophotometer (Shimadzu 1800, Kyoto, Japan). Afterward, the optimum PBS concentration was determined using variation of 0.1, 0.05 or 0.01 M under a pH range of 5 to 9.5. The PBS concentration yielding the optimum sensitivity was further used to evaluate the sensor's reproducibility. Herein, PBS at pH levels of 6 and 8 was employed, whereby the standard deviation and relative standard deviation were calculated after

10 repetition using different sensors. The sensor lifetime was tested using PBS at pH 6, in which after the exposure, the sample was stored at 4 °C and measured for the absorbance every 5 days for a total of 25 days of investigation.

2.5. Application on Tilapia Fish

Two tilapia fish were prepared, whereby onto each of the fish's skin we attached a prepared sensor (Figure 1). Each sensor was left attached for 5 min for sufficient exposure before removal and then observed with a UV-Vis spectrophotometer (λ_{max} = 620 nm). One fish was stored in a refrigerator at 4 °C, and the other at room temperature (25 ± 1 °C). The pH change monitoring was conducted for 25 h after the first measurement (0 h).

Figure 1. The fabricated sensors were attached onto the skin of the fish to detect the pH changes before the absorbance was measured with a UV-Vis spectrophotometer.

3. Results and Discussion

3.1. Optimum Ratio of Pectin and Chitosan

The PEC membrane in this study was made from pectin and chitosan biopolymers based on electrostatic interactions between chitosan polycations (NH_3^+) and pectin polyanions (COO^-). The ionic bonds formed through the electrostatic interactions of these two charges form stable PEC membranes [1,29]. The formation of pectin–chitosan PEC membranes is influenced by pH solutions ranging from 3 to 6 [30]. However, at lower pH, pectin and chitosan may also form interactions through hydrogen bonds, causing pectin to possess a nearly neutral charge. According to a previous report [31], the interactions between pectin and chitosan to form a PEC membrane can be expressed by Equation (1):

$$\text{P-COOH} + \text{C-NH}_3^+ \leftrightharpoons \text{P-COO}^- + \text{NH}_3\text{-C} + \text{H}^+ \quad (1)$$

The manufacture of PEC membranes involved 1% pectin solution (*w*/*v*) and 1% chitosan solution (*w*/*v*). Each polymer solution was mixed at various pectin/chitosan ratios, including 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Based on our observations, the pectin–chitosan PEC membrane at a ratio of 3:7 was visually transparent and had a homogenous surface. Meanwhile, PEC membranes containing high levels of chitosan were also transparent but rigid. Increasing the ratio of pectin would only result in PEC membranes with less homogeneous surfaces. Therefore, a pectin–chitosan PEC membrane at a ratio of 3:7 was the optimum membrane composition for manufacturing the optical pH sensor matrix.

3.2. FT-IR

FT-IR spectra of chitosan, pectin and PEC pectin–chitosan membranes are presented in Figure 2. The typical infrared absorption spectrum for chitosan is shown in the range of 1645–1597 cm^{-1}, where the absorption peak at 1645 cm^{-1} is assigned for the C=O stretching vibration of the amide I group, while at 1597 cm^{-1} the –NH bending vibration of the amide II group is the cause. Pectin contains esters and C=O from carboxylate functional groups indicated by absorbance bands at 1733 and 1628 cm^{-1}, respectively. Consequently, those functional groups also appeared in the FT-IR spectrum of the PEC pectin–chitosan membrane, which could be seen within the range of 1650–1500 cm^{-1}. In detail, absorption peaks observed at 1656 and 1577 cm^{-1} were assigned to the vibrations of carbonyl from pectin and amine from chitosan, respectively. Shifted wavenumbers in the aforementioned range suggested that the changes in chemical environment of the amine group stemmed from the interaction with pectin [29,32].

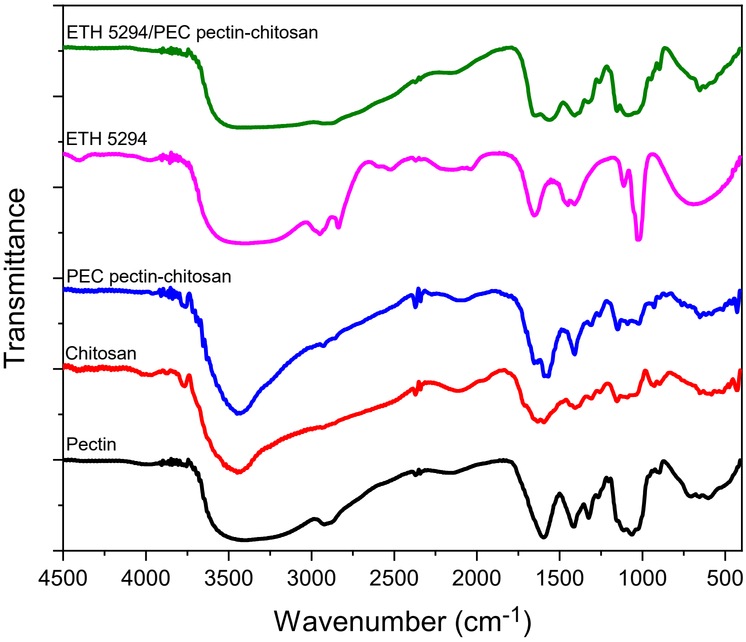


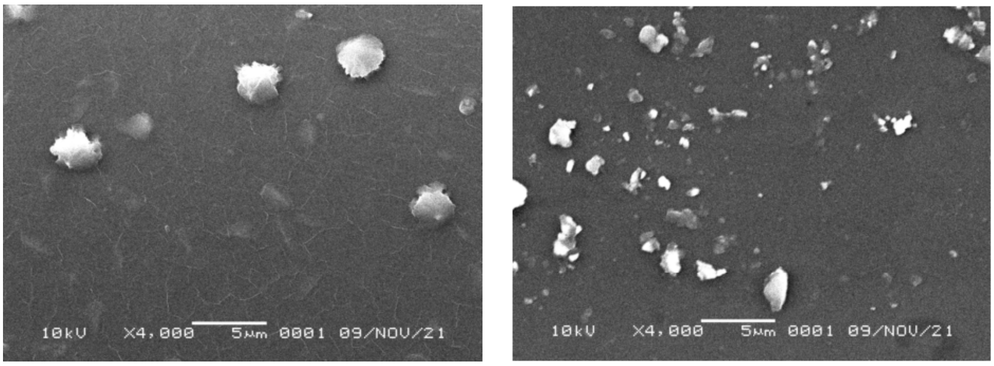
Figure 2. FT-IR profiles of pectin, chitosan, PEC pectin–chitosan, ETH 5294 and ETH 5294/PEC pectin–chitosan.

Infrared spectral profiles obtained from chromoionophore ETH 5294 and the PEC membrane with added chromoionophore (ETH 5294/PEC pectin–chitosan) are also presented in Figure 2. The absorbance peaks observed at 1120 and 1022 cm^{-1} correspond to symmetric C–O–C tensile vibration, suggesting the presence of an oxo cyclic structure in the ETH 5294. Other molecular vibrations that are typical of the chromoionophore ETH 5294 could be observed at 1656, 1463 and 1411 cm^{-1}, corresponding to –C=O stretching vibration, $–CH_3$ stretching vibration in the $–N^+(CH_3)_2$ group and $–CH_2$ bending vibration, respectively [33]. The addition of ETH 5294 into the PEC pectin–chitosan membrane caused a more defined absorbance peak at around 1085 cm^{-1} assigned to C–O–C tensile vibration. This proved that ETH 5294 had been embedded into the PEC pectin–chitosan matrix.

3.3. SEM

The morphological characterization of the membranes was performed using scanning electron microscopy (SEM), with the images presented in Figure 3. Initially, both pectin and chitosan had smooth surfaces, while small cracks and granules were found on the chitosan surface. Upon the formation of polyelectrolyte, the surface was found to be filled with small granules and agglomerations. These may have been contributed from the ionic interaction between the pectin and chitosan. The use of different solvents in the preparation of pectin and chitosan solutions could also be another factor, because the dissolubility of chitosan is sensitive to pH changes. Moreover, thin lines emerged on the membrane surface, which could be attributed to the tension stress from the water molecules owing to the hydrophilicity and hygroscopicity [34]. Although the hydrophilic property results in the rough surface of the membrane, it could also allow better diffusion of the analyte [21,35].

Similar occurrences on membrane surfaces resulting from polymer complex formation have been reported previously [13,14].



(**a**) (**b**)

Figure 3. The morphologies of pectin–chitosan PEC (**a**) and ETH 5294/PEC pectin–chitosan membranes (**b**).

3.4. Effect of PBS Concentration on the Optical pH Response

The concentration of PBS is one of the parameters measured to determine the optimal performance of an optical pH sensor. The PBS concentrations of 0.1 M, 0.05 M and 0.01 M were used to evaluate the optimum sensitivity over a wide dynamic range with acceptable linearity. The obtained measurement results are presented in Figure 4. The overall results suggested that the sensors exposed to the 0.1 M concentration of PBS had greater absorbance ability than the sensors tested with 0.05 M and 0.01 M PBS solutions at both acidic and alkaline pH levels. Hence, the concentration of PBS affects the intensity of the color change produced by the sensor, as similarly witnessed in a previous study [36]. However, sensors with higher color intensity did not always show good sensitivity. Therefore, in this present study, the concentration of 0.05 M PBS resulted in higher sensitivity.

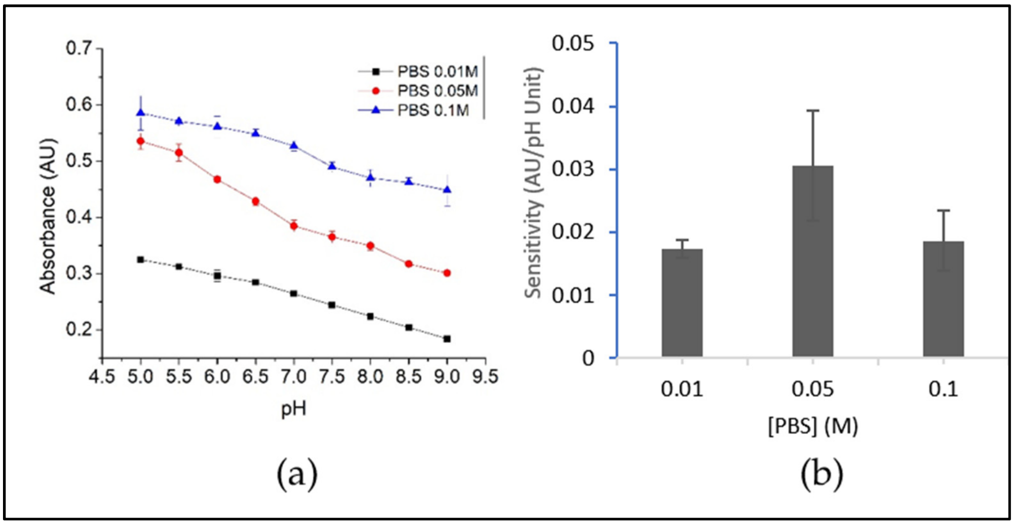


Figure 4. Response profile (**a**) and sensitivity (**b**) values of the manufactured optical pH sensors with various PBS concentrations. The absorbance was measured over a pH range of 4.5 to 9.5.

3.5. Study of the Optical pH Sensor Performance

3.5.1. Optical pH Response

The optical pH sensor based on PEC pectin–chitosan membrane, as shown in Figure 5a, showed color changes when exposed to various pH buffer solutions. The color of the sensor changed from blue to purple as the pH increased (Figure 5a). The color changes stemmed from the protonation and deprotonation of ETH 5294 over acidic and basic pH ranges, respectively. Indeed, insignificant changes in color can be visually observed by the naked eye over certain pH ranges (pH 5–6 and pH 7.5–8.5) (Figure 5a). Hence, UV-Vis spectrophotometry is a requirement for accurate analyses when applying the proposed optical pH sensor. The response of the optical pH sensor based on the UV-Vis absorbance over the range of 400 to 800 nm is presented in Figure 5b. The response was revealed to decrease gradually with increasing pH levels, stemming from the protonation and deprotonation of the ETH 5294. At 610 nm, the sensor's response seemed to form a linear correlation with the pH changes (pH 5–9) at a PBS concentration of 0.05 M. Thus, the wavelength was taken for the construction of the calibration curve. This finding is similar to our previous report employing anthocyanin derived from *Ruellia tuberosa* L. (λ_{optimum} = 635 nm) [14]. The foregoing system yielded a sensitivity value of 0.0306/pH unit.

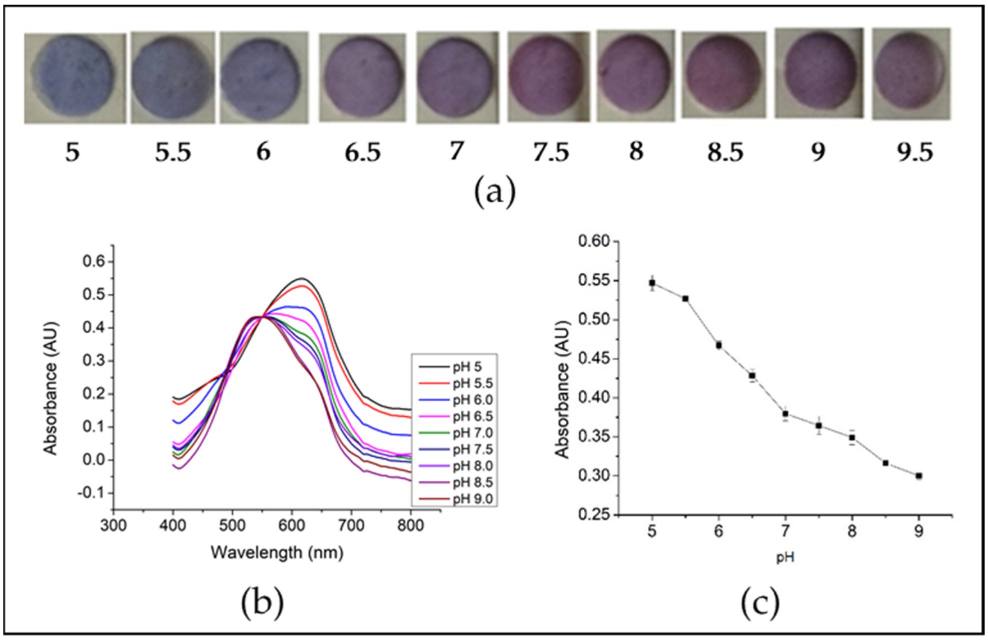


Figure 5. The visual appearance (**a**) and UV-Vis spectral profile (**b**) of the manufactured optical pH sensor. Sensor response with respect to pH changes from pH 4 to 9 (**c**).

3.5.2. Response Time

The determination of the response time was performed using 0.05 M PBS at pH 6, 7 and 8. The absorbance was measured at 0, 5, 10, 15 and 20 min. The measurement results are presented in Figure 6. The responses were recorded after the sensors were exposed to PBS at pH 6 and 8. Changes in color immediately appeared as indicated by the change in absorbance. However, a stable response was obtained after 5 min of exposure. A similar response time profile was obtained from an optical pH sensor prepared using polyurethane hydrogel in a published report [37]. In our previously published report using a similar PEC membrane matrix, a stable response time was obtained after 5 min, suggesting that the response time is not affected by the type of active agent. In fact, the response time of the sensor is influenced by the thickness of the membrane, the activity of the analyte and the measured pH [5].

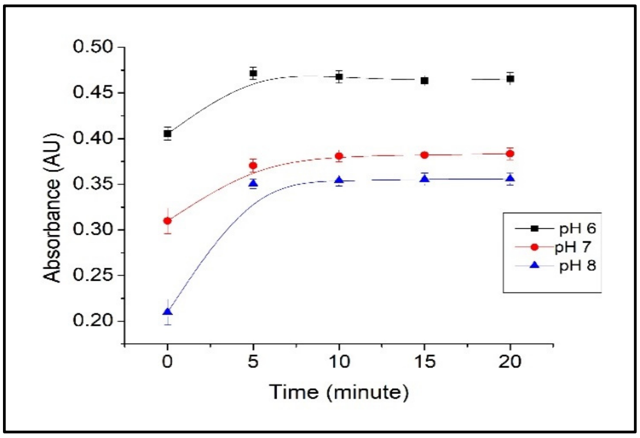


Figure 6. Response times of the prepared sensor measured at pH 6, 7 and 8 at a wavelength of 610 nm.

3.5.3. Reproducibility

To examine the reproducibility of our sensor, we prepared 10 sensors and labeled them from 1 to 10 (Table 1). In this research, reproducibility means the variation in responding absorbance values resulting from measurements of each sensor. The reproducibility evaluation was carried out on ten optical pH sensors whose absorbance was measured using PBS 0.1 M at pH 6 and 8. The data showed that the sensors had good reproducibility values, with a standard deviation of 0.007, while the relative standard deviation was in the range of 2.07 to 2.34% at both acidic and basic pH. According to the previous report, the RSD obtained in the present study was still within the acceptable range [38]. In fact, the RSD of the currently proposed optical pH system with commercial chromoionophore was nearly 4 times lower in comparison with that obtained previously [18].

Table 1. Reproducibility of sensors tested at pH 6 and 8.

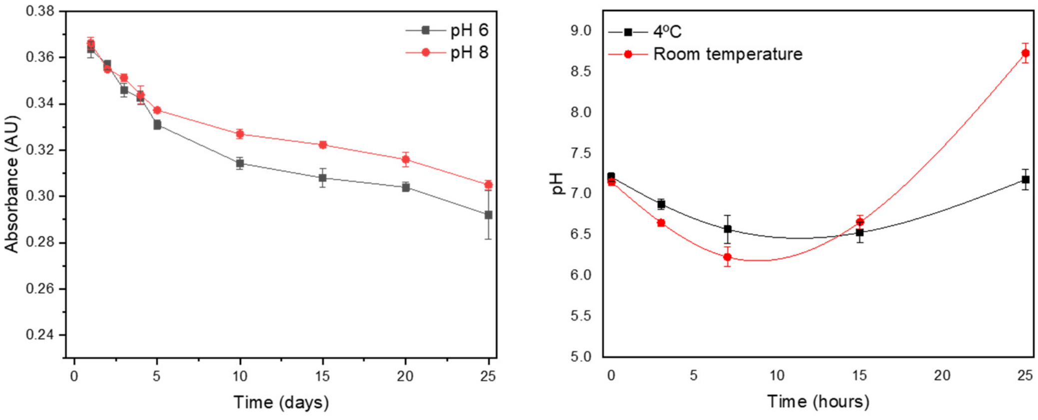
Sensor	**Absorbance (AU)**	
	pH 6	**pH 8**
1	0.325	0.320
2	0.330	0.325
3	0.333	0.327
4	0.335	0.329
5	0.336	0.331
6	0.337	0.332
7	0.339	0.336
8	0.341	0.340
9	0.343	0.342
10	0.350	0.344
Mean ± SD	0.337 ± 0.007	0.333 ± 0.008
RSD (%)	2.073	2.347

SD: standard deviation; RSD: relative standard deviation.

3.5.4. Lifetime Profile

The sensor had a limitation of time in use, so the evaluation of the stability of the sensor to provide an acceptable response value was determined at 1, 2, 3, 4, 5, 10, 15, 20, and 25 days. The evaluation of the lifetime was performed using a 0.1 M PBS solution at pH 6. The obtained results are shown in Figure 7a. The stability of the sensor was affected by time. The decrease of 7.65% in sensor response occurred gradually after five days of storage. However, when the sensor was stored for up to 10 days or 25 days, the decrease in response could reach 10% or 16.67%, respectively. Thus, it can be concluded that the sensor is only stable within five days of storage at 4 °C. The lifetime profile of this fabricated sensor was similar to our previously published study using PEC pectin–chitosan [18].

However, longer lifetimes (up to two weeks) were achieved with the optical pH sensor made solely from the pectin membrane matrix [8,14]. We stipulate that this weakness of PEC is attributed to its hygroscopic properties, allowing the absorbed water molecule to form non-transparent thin lines on the surface, as observed in the SEM images. The high hygroscopicity of chitosan-based PEC was noted earlier in a report preparing PEC carboxymethyl chitosan–alginate [34]. Taken altogether, despite the benefits offered by the PEC, its stability during storage is considered a challenge.



(a) (b)

Figure 7. The lifetime of the prepared optical pH sensor (**a**). Application on tilapia fish stored at room temperature and 4 °C (**b**).

3.5.5. Fish Freshness Monitoring on Tilapia Fish

The optimized sensor was then used to monitor the freshness of tilapia fish (*Oreochromis mossambica*) stored at room temperature and 4 °C. The measurements of the sample absorbance were then converted into pH values, as shown in Figure 7b. The initial pH measurements of fresh fish ranged from 7.1 to 7.2. The initial high and low pH values in fish were influenced by the amount of glycogen present and the strength of the buffer in the flesh. Furthermore, after death (rigor mortis phase), the pH of the fish decreased to the acidic range. However, after the rigor mortis phase ended, the fish entered a post-rigor phase, or the process of decay. The pH in the post-rigor phase increased to a more alkaline pH caused by the breakdown of protein into ammonia, dimethylamine and trimethylamine [39].

The sensor responded to the freshness of the fish with color changes from purple to pink. Based on the data, the pH values of the fish stored at 4 °C were 6.8, 7.31 and 7.92 when measured over three consecutive days. After 25 days storage, the pH was 8.91 for fish stored at room temperature, which was higher than for the fish stored at 4 °C. This finding suggests that the fish left at room temperature decomposed faster than the fish stored at 4 °C, which may have been due to the exposure of the fish to direct sunlight, while the mucus found on the skin, gills and in the digestive tract of the fish has no function and makes it easier for bacteria to attack these parts, causing fish spoilage [40]. Table 2 presents a comparison of optical pH sensor measurements of fish freshness and spoilage that have been reported so far.

Table 2. Comparison of optical pH sensor measurements of fish freshness and spoilage.

Matrices	Active Substance	Linear (pH)	Reference
Polyelectrolite complex (PEC) pectin–chitosan	Chromoionophore ETH 5294	5–9	Present work
Pectin	ETH Nile Blue 5294	5–9	[8]
Pectin	Ruellia Tubelora L	6–8	[14]
Sugarcane wax	Butterfly pea flower	2–6 and 7–12	[41]
Poly(ortho-phenylenediamine-co-aniline)	Anilin	1–13	[42]
Carboxymethyl cellulose (CMC) and cellulose nanofibers (CNF)	Shikonin extracted from Lithospermum erythrorhizon roots	2-12	[43]
Polyvinyl alcohol (PVA)	Acidochromic dye	7–12	[44]
Silicone	Disodium 1-amino-9,10-dioxo-4-[3-(2-sulfonatooxyethylsulfonyl)-anilino] anthracene-2-sulfonate (Remazol Brilliant Blue R, RBBR) and 2-fluoro-4-[4-(2-hydroxyethanesulfonyl)-phenylazo]-6-methoxyphenol (GJM-492)	8	[45]
Sol–gel (ormosil)	Bromophenol blue	1–8.5	[46]

4. Conclusions

The optical pH sensor was successfully fabricated by embedding commercial chromoionophore ETH 5294 (1%) into a PEC pectin–chitosan membrane matrix. The optimum ratio of pectin to chitosan to produce a membrane matrix with suitable properties for the optical pH sensor was 3:7. The optimized optical pH sensor showed good performance and measurements of the pH level over acidic and basic ranges. The response time was stable after 5 min exposure to analyte. The RSD values obtained from the calculation using the optical pH sensor were in the acceptable range for quantitative measurements. The constructed optical pH sensor was capable of monitoring the freshness degradation of tilapia fish stored at room temperature or at 4 °C (refrigerator). The main weakness of this proposed optical pH system is its lifetime, associated with its high hygroscopicity. Future studies need to be carried out to modify the material, which could prevent water molecule absorbance from the air.

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