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RESEARCH ARTICLE

REVISED A simple optical pH sensor based on pectin and *Ruellia tuberosa* L-derived anthocyanin for fish freshness monitoring

[version 2; peer review: 2 approved]

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Abstract

A simple optical pH sensor using the active compound anthocyanin (ACN), derived *Ruellia tuberosa* L. flower immobilized in a pectin membrane matrix, was been fabricated and employed to monitor the freshness of tilapia fish at room temperature and 4°C storage. The quantitative pH values were measured based on the UV-Vis spectroscopy absorbance. The optimum pectin weight and ACN concentrations were 0.1% and 0.025 mg/L. The sensor showed good sensitivity at 0.03 M phosphate buffer solution. The sensor's reproducibility was evaluated using 10 replicate sensors where a standard deviation of 0.045 or relative standard deviation of 9.15 was achieved. The sensor displayed an excellent response after 10 minutes of exposure, possessing a response stability for 10 consecutive days. The decrease in pH value of the Tilapia fish from 7.3 to 5 was observed in a 48 hour test, which can be used as the parameter when monitoring fish freshness. Overall, this reported optical pH sensor has a novelty as it could be used to monitor the rigor mortis phase of fish meat, which is useful in food industry.

Keywords

optical pH sensor, matrix membrane, pectin, anthocyanin, fish freshness

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REVISED Amendments from Version 1

Abstract has been added with the statement of this study's novelty at the end of the paragraph. More elaboration on the introduction. Methods now contains Study Design subsection. Two figures have been added namely, Figure 1 and Figure 7; as a consequence, the order has been changed as well. A paragraph before Table 1, was reordered. Discussion on pH decrease during rigor mortis has been more elaborated with an additional explanation of this study's novelty. Citation has been reordered, where some have been removed.

Any further responses from the reviewers can be found at the end of the article

Introduction

Fish freshness assessment is the main concern for consumers nowadays as people are more cautious about what they put into their body. Eating spoiled products will cause food poisoning symptoms to various degrees. For example, eating spoiled fish may result in an almost immediate onset of diarrhea, nausea and vomiting. According to the United Nations, about 4.5 billion people rely on fish for 15% of their animal protein intake.¹ Therefore, it is imperative to monitor the freshness and quality of fish. Currently, consumers rely on their own experience in determining fish freshness. This is mostly based on the physical condition of the fish like its color and smell. This method is very subjective; hence, there is a need for a more quantitative monitoring method for fish freshness. Heising *et al.* (2012)² has produced a fish freshness monitoring method by detecting total volatile basic nitrogen using an ammonia ion-selective electrode. However, not all of the ammonia produced will dissociate in the aqueous phase, which is a challenge in the conductivity changes-dependent method. Determination of fish freshness can also be performed by measuring trimethylamine (TMA) levels using electrochemical sensing, as reported by Bourigua *et al.* (2011)³ However, determining the freshness of fish via measuring TMA requires a complicated procedure and experts to operate the equipment. Fish freshness can be monitored using an ammonia optical sensor. Wells *et al.* (2019)⁴ reported the determination of fish freshness through ammonia measurement that also used TMA solution standard and a dye indicator for pH measurement. Beside these two methods, a pH sensor can also be employed to monitor fish freshness.^{5–8} There have been several methods proposed to determine pH levels of a fish sample. The most common methods used are optical sensors and ion-selective electrodes (ISEs).⁹ The measurement of pH using an H⁺ ISE is dramatically affected by interferences from samples, especially the presence of alkaline ions.¹⁰ Thus, the determination of pH through optics may be an excellent alternative for samples that contain interfering ions.

Several organic pH-sensitive dyes, immobilized in synthetic membranes, have been utilized in the construction of optical pH sensors. Nonetheless, safer compounds derived from natural products have attracted the attention of researchers in developing pH sensors. An earlier report of optical pH sensors includes the construction of a pH sensor using phenol red as an active molecule.¹¹ The further report had described the development of a pH sensor utilizing polyvinyl chloride as the matrix and the fluorescence compound fluorescein-O-methacrylate as the active molecule.¹² Nevertheless, these aforementioned pH sensors could only be used on solutions with near-neutral pH as more basic or acidic solutions will give an insignificant response time. Pourjavaher *et al.* (2017)¹³ has designed a pH sensor using bacterial cellulose (BC) nanofiber matrix to immobilize anthocyanin (CAN) from red cabbage (*Brassica oleracea*) extract. The sensor has a fairly wide pH range but it needs further characterization to evaluate the sensor performance, especially, for real foodstuff analysis. The use of anthocyanin ACN from blackberries and chitosan membrane in an optical pH sensor has been established.¹⁴ The interaction and mechanical properties of chitosan membrane with entrapped ACN have also been reported.¹⁵ Anthocyanins are flavonoids possessing a number of hydroxyl groups contributing a strong interaction with chitosan via hydrogen bonding.

A more recent study on fish freshness monitoring through optical methods was reported by Moradi *et al.* (2019)¹⁶ using nanofiber bacterial cellulose with ACN. However, this method requires a relatively long analytical time as the pH measurement could not be conducted *in situ*. Chen *et al.* (2020)⁷ has developed a sensitive novel film prepared from starch polyvinyl alcohol and starch polyvinyl alcohol glycerol. The study used curcumin from turmeric and anthocyanin from purple sweet potatoes. The results showed that the mixture of curcumin and ACN improved the stability than that of the individual active substances. As the consequence, the sensor could be employed to detect volatile ammonia as the fish freshness indicator.

Herein, we constructed a new optical pH sensor based on pectin (PC) matrix and ACN extract from the *Ruellia tuberosa* L flower. The ACN derived from the crude extract of *Ruellia tuberosa* L flower has been reported to be pH sensitive.¹⁷ PC is a non-toxic biopolymer that can be crosslinked with the assistance of CaCl₂. PC membrane is transparent, deeming it suitable as a matrix for optical measurements. Moreover, PC is also a hydrogel that will enable easy diffusion of analytes leading to a faster response time compared to another hydrophobic matrices.¹⁸ In addition, PC application as an optical pH sensor for fish freshness monitoring has not been well-explored. ACN is well known to be pH sensitive and will

undergo color changes at different pH.¹⁹ This compound is easily obtained from nature and is relatively cheap compared to other pH sensitive active molecules. In the present work, ACN has been extracted from the flower *Ruellia-tuberosa* L. The ACN was immobilized onto PC membrane to produce CAN/PC composite membrane which can be used for *in situ* detection of fish freshness without requiring a destructive procedure.

Methods

Materials

All chemicals used in this research are analytical grade. Monopotassium phosphate (KH_2PO_4) and dipotassium phosphate (K_2HPO_4) were purchased from Merck (Merck Millipore, Darmstadt, Germany); PC, ethanol, and CaCl_2 – from Sigma-Aldrich (Sigma Aldrich Chemie GmbH, München, Germany); and methanol and acetic acid – from Fluka (Fluka Chemie GmbH, Buchs, Switzerland). As for the plant sample, wild *Ruellia tuberosa* L. was collected from the area near Universitas Syiah Kuala in Banda Aceh, Aceh, Indonesia. To study the application of the optical pH sensor on the real sample, dead tilapia fishes were used and purchased from the traditional market in Banda Aceh, Aceh, Indonesia.

Study design

The first step in sensor fabrication was the extraction of anthocyanin from *Ruellia tuberosa* L. The extracted anthocyanins were then mixed with pectin solution and printed proportionally as an optical pH sensor. The optical pH sensor was then characterized and the optimized and then applied to monitor the freshness of tilapia. The image below is a schematic diagram summarizing research procedures conducted in this work (Figure 1).

Anthocyanin extraction

The procedure follows a previous report.²⁰ Briefly, 200 g fresh *R. tuberosa* L. was macerated in 85 mL methanol for 24 h at room temperature (32–34°C). The residue was then separated from the filtrate by simple filtration. Finally, ACN was obtained after the solvent was removed from the filtrate by means of steaming at 50°C until the volume reached 50 mL.

Construction of optical pH sensor with various ACN concentrations

The optical pH sensor was constructed by dissolving PC powder into a matrix solution (0.1% w/v) in 100 mL CaCl_2 0.1 M solution, heated at 60°C. After the mixture was cooled down, the previously obtained ACN extract (1.503 mg/L) was added to 1.66, 2.49 and 3.33 mL PC matrix solution to produce three different 100 mL ACN/PC solutions with respective ACN concentrations of 0.025, 0.0375 and 0.05 mg/L. A total of 40 μL the ACN/PC solution was dropped onto a polyvinylchloride plastic mold surface with a diameter of 0.8 cm (Figure 2). The sensor was allowed to dry for 24 h at 4°C.

Fourier Transform Infrared (FTIR) Cary 630 Anti Agilent (Penang, Malaysia) was used to identify the structure and functional groups. The membrane morphology was observed under Zeiss Merlin/Merlin Compact/Supra 55VP Field Emission Scanning Electron (FESEM) (Selangor, Malaysia). Thermal stability of the constructed membrane was analyzed using Shimadzu DTG-60 Thermal Gravimetric Analyzer (Kyoto, Japan) and Differential Scanning Calorimetry (DSC) Shimadzu DSC-60 (Kyoto, Japan). Unless otherwise stated, the conditions for these characterizations followed that of reported work for film specimens.^{21,22}

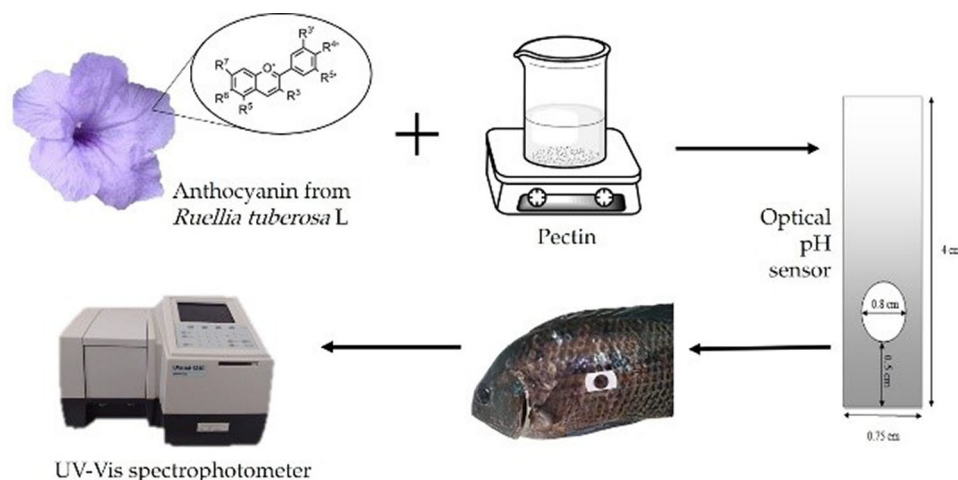


Figure 1. Schematic diagram of optical sensor fabrication and its application for fish freshness monitoring.

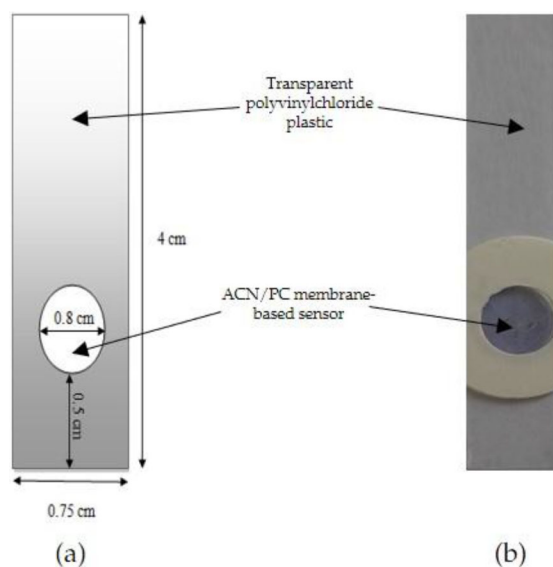


Figure 2. (a) The designed shape and (b) the visual appearance of ACN/PC optical pH sensor.

To test its response and evaluate its analytical performance, each sensor was dripped with 30 μL 0.1 M phosphate buffer solution with a variety of pH values ranging from 5.0 to 8.5 with 0.5 interval –the pH values of each phosphate solution on the sensor were checked by pH-meter Thermo Orion Star A2111 (Selangor, Malaysia). The sensor color changed corresponding to the different pH values of the administered buffer solutions. It consequently resulted in the difference of the absorbance that was then measured nm using UV-VIS Spectrophotometer (Shimadzu Uv-mini-1240, Kyoto, Japan) at $\lambda_{\text{max}} = 635$,¹⁷ until the sensitivity value for pH determination was obtained.

Effect of PC concentration

The effect of PC concentration was tested based on % weight of PC in CaCl_2 0.1 M solution; 0.05, 0.10, and 0.15%. In total, 40 μL of the three different PC solutions containing 0.025 mg/L ACN were casted as previously explained above. Finally, the pH sensor was pipetted with 30 μL phosphate buffer 0.1 M (pH 4-9), and its absorbance was measured.

Selection of the optimum buffer solution and concentration

The optical pH sensor with optimum ACN and PC concentrations was used to test its performance against phosphate and citrate buffers 0.1 M (pH 5.0-8.5) to select which buffer generated the best outcome. To select the optimum buffer concentration (once the best buffer had been chosen; phosphate), the best buffer solution was varied in concentration (0.01, 0.03, and 0.05 M) and used in the optical pH sensor performance with pH ranging from 6-8 following the previously explained procedure. The optimum concentration was selected based on its sensitivity and linearity of the absorbance versus pH plotting curve.

Evaluation of reproducibility, response time and lifetime study of the optical pH sensor

Response time of the optical pH sensor was determined by measuring the optimum absorbance of the pH sensor at a range of 5, 10, 15, 20, 25 and 30 minutes. For reproducibility, the performance was conducted 10 times using ten optical pH sensors. For the determination of the optical pH sensor's lifetime, the absorbance measurement was carried out after 1, 2, 3, 4, 5, 10, 15 and 20 days after the sensor preparation. All of these studies were conducted under optimum buffer conditions.

Optical pH sensor test on fish sample

The pH values of the tilapia fishes were measured by attaching the sensors onto the fishes' surface for 5 minutes before measuring the absorbance, as explained before. The fish were stored at 4°C and ambient temperature (32-34°C). The pH analysis was carried out every 7, 12, 24, and 48 h of the storage time.

Results and discussion

Characteristics: structure, crystallinity, morphology, and thermal behavior

Anthocyanin (ACN) is one of the most important components in the construction of this optical pH sensor other than PC. ACN is obtained from the extract of *R. tuberosa* L. flower that displays different colors at different acidic or basic pH levels.^{23,24} FTIR analysis of the extract showed that the broadening vibrational band with medium intensity at the wavenumber, ranged between 3333 cm^{-1} and 3291 cm^{-1} , indicating the presence of free O-H groups (Figure 3). The presence

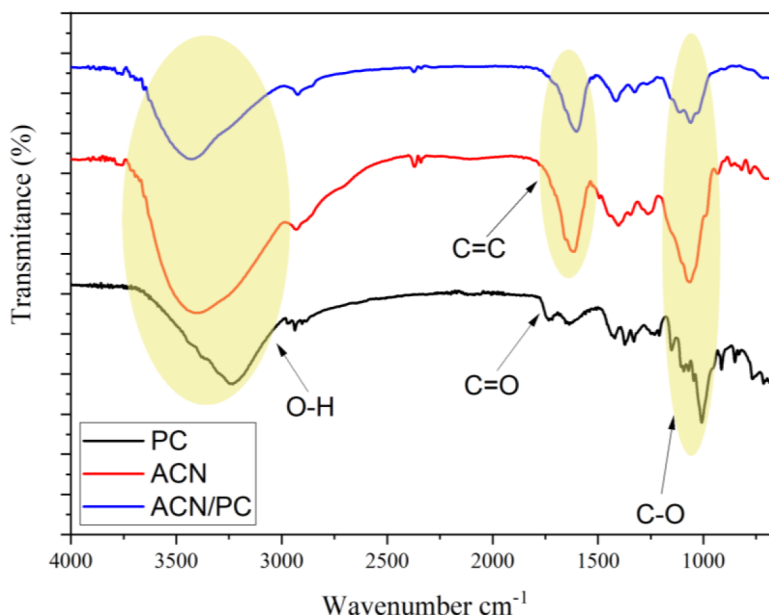


Figure 3. FT-IR spectral profile of PC, ACN, and ACN/PC.

of the aromatic C=C vibrations at wavelength region 1644 cm^{-1} and 1454 cm^{-1} indicates the typical characteristics of an ACN compound.²⁵ The vibrations by group C-O were recognized from wavelength range 1111 and 1015 cm^{-1} . The FT-IR characterization shows that the ACN is in the form of cyanidin-3-glucoside; similar vibration patterns has been reported previously.²⁶

FT-IR characterization on PC displayed typical PC functional groups at wavenumber range of $1000\text{--}2000\text{ cm}^{-1}$. Spectral band at 1717 cm^{-1} and 1624 cm^{-1} are assigned to be vibrations of C=O stretching from ester and carboxylate. The presence of other spectral band at 3370 cm^{-1} is assigned to the vibrational absorbance of O-H functional groups. The ether bonds of C-O-C is observed by the presence of the absorbance peaks at 1219 and 1096 cm^{-1} . In the case of ACN/PC, free O-H groups from the PC molecule were observed from the overlapping band at $3200\text{--}3650\text{ cm}^{-1}$. The other spectral bands at $1630\text{--}1850\text{ cm}^{-1}$ and $1050\text{--}1260\text{ cm}^{-1}$ are assigned to carbonyl groups (C=O) and symmetrical ether groups (C-O-C) from glycoside bonds, respectively.^{27,28}

TGA/DTGA and DSC profiles of PC membrane

Thermal stability is one of preferable characteristics when it comes to a bio-sensor as it may influence its performance. We conducted thermal gravimetry analysis (TGA) and differential scanning calorimetry (DSC) studies to assess whether the PC membrane has ideal thermal stability. The thermograms of TGA and its derivative (DTGA) and DSC have been presented in Figure 4a and b. At around 58°C , the release of solvent (water) was observed on the TGA and DTGA thermograms (Figure 4a). The second peak of DTGA suggests thermal degradation with 30% weight loss.²⁹ A better insight regarding the thermal stability of the PC membrane can be seen in the DSC thermogram.^{21,22}

The first endothermic peak that appears in the DSC thermogram (Figure 4b) agrees with the water content release observed in the TGA. $T_{\text{onset}} = 83^\circ\text{C}$ indicates the first observable thermal transition, in which it is assigned to melting temperature. It is because within the temperature range ($83\text{--}118^\circ\text{C}$), the decrease in weight does not occur in the TGA thermogram. This finding is in line with a previous report investigating PC powder.³⁰ The exothermic peak ($T_{\text{peak}} = \pm 309^\circ\text{C}$) observed afterward indicates the degradation of the PC polymeric chain. From these data, we can conclude that the PC membrane is thermally stable at room temperature range.

SEM images of PC membrane

SEM images of PC (Figure 5a) and ACN/PC (Figure 5b) depict a clear difference of surface morphology between the two.¹⁸ PC surface has a morphology that is uniform and smooth. With the addition of ACN into the membrane, wavy layers are shown as the result of the presence of the liquid that, as the consequence, possibly creates a stress tension or air gap. Other study showing severe cracks on the membrane surface, associated with the presence of water.³¹ This change

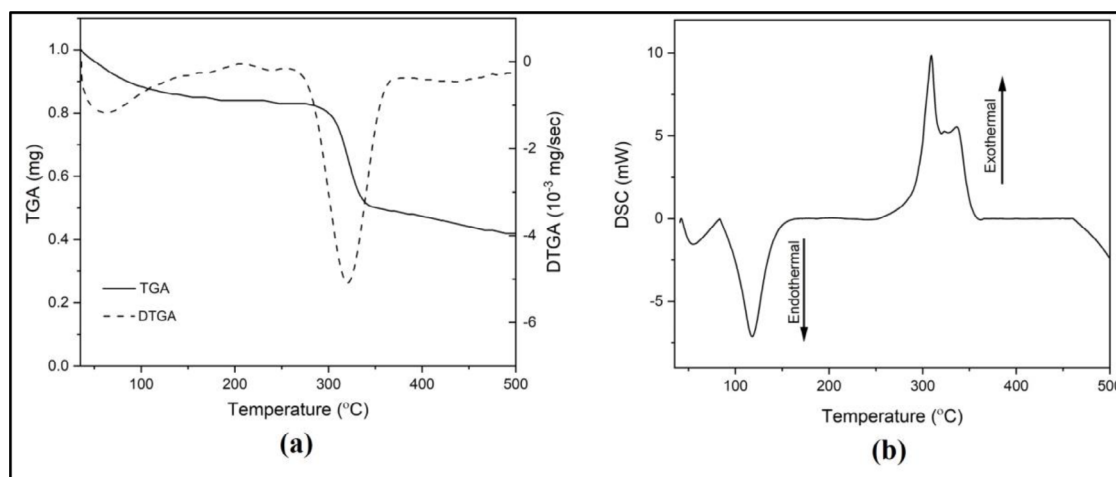


Figure 4. (a) TGA/DTGA and (b) DSC thermograms of thermal analysis on PC membrane.

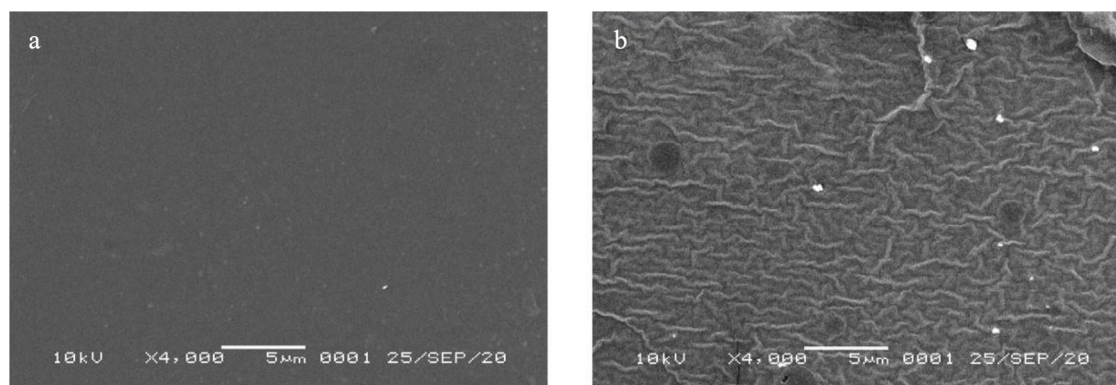


Figure 5. SEM profile of (a) PC and (b) ACN/PC membranes.

may lead to poorer sensor performance as a transparent membrane is preferred for optical sensor to allow the UV light passing through the membrane. Hence, investigation on the sensitivity of the optical pH sensor with respect of ACN or PC loads is important.

Effect of ACN concentration on the sensitivity of the optical pH sensor

The constructed optical pH sensor based on the ACN derived from *R. tuberosa* L flower has hydrogel characteristics. The advantage of a hydrogel membrane in an optical system is the quick interaction between analyte and active membrane which in turn will accelerate the response time.^{18,32} The PC membrane with the immobilized ACN is transparent, where the color change is sensitive against the pH value (Figure 6). This optical pH sensor is optimized by means of ACN variation to achieve the best sensitivity, observed by a wide linear range and good linearity. Further characterization is followed by the determination of sensor performance.

Color change of ACN can be affected by several factors such as temperature, pH, light intensity, sugar moiety and different phenolic derivatives.¹⁸ Due to its solubility in aqueous solution, the color change of ACN is caused by structural transformations of carbon skeleton affected by the levels of H⁺. Four major anthocyanin skeletons have been reported in the literature at different pH values (Figure 7); the red flavylum cation (pH < 3), the blue quinoidal base (pH 6-7), the colorless carbinol pseudo-base (pH 4-5), and the yellowish *cis*-chalcone (pH > 6) (Figure 7).^{33,34}

The effect of ACN concentrations on optical pH sensors response has also been studied and shown (Table 1 and Figure 8). The sensitivity of the sensor toward variations in ACN concentrations showed not significantly different, but the absorbance vs pH plot showed an increase in the value of the intercept. This indicates the intensity of the sensor color increases with increasing ACN concentrations. Furthermore, the ACN concentration of 0.025 mg/L will be used to construct the optical pH sensor for the next characterization.

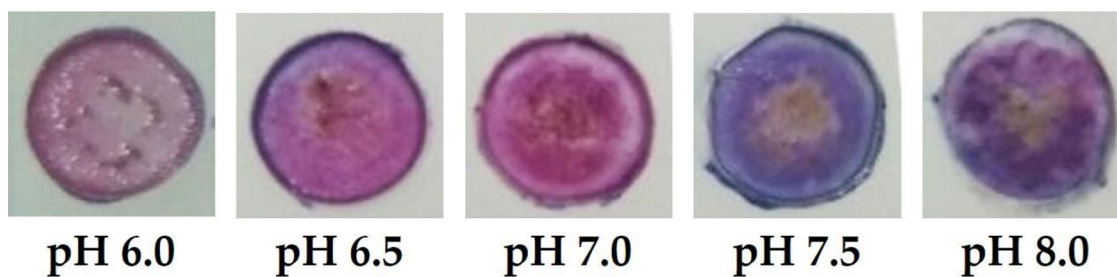


Figure 6. Optical pH sensor color changes at different pH values.

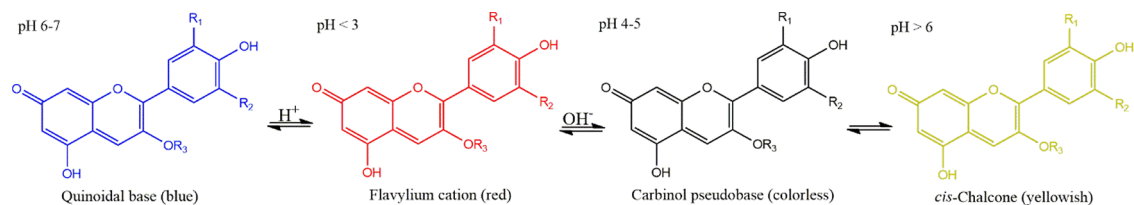


Figure 7. Anthocyanin molecular structures with respect of pH changes.

Table 1. Effect of ACN concentrations on the sensitivity of the optical pH sensors on phosphate buffer.

Concentration (mg/L)	pH range	Sensitivity	R ²
0.025	6-8	0.14 ± 0.03	0.999
0.0375	6-8	0.108 ± 0.05	0.999
0.05	6-8	0.094 ± 0.01	0.995

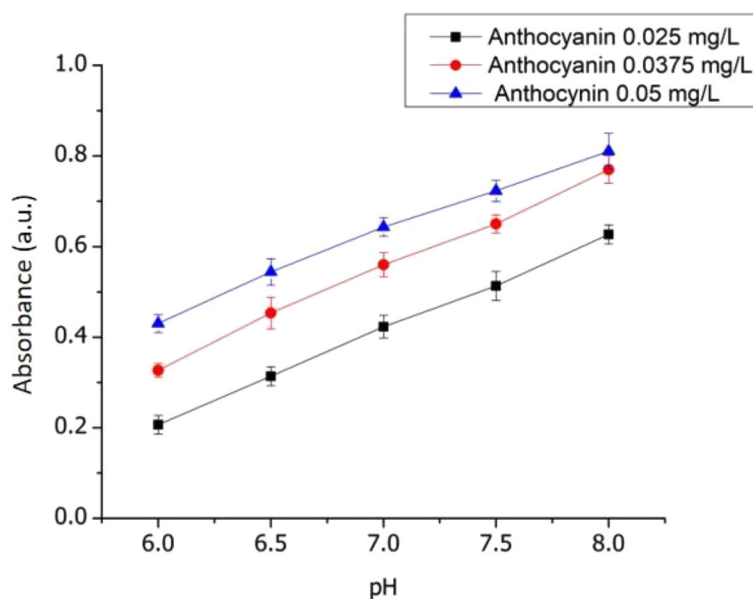


Figure 8. Effect of ACN concentration on sensitivity optical pH sensor.

Effect of PC weight towards sensor sensitivity

The weight variation of PC (0.05, 0.1, and 0.15% w/v) was studied to find the best sensor sensitivity. At varied weights, PC was dissolved using CaCl_2 0.1 M to construct cross linking between Ca^{2+} ion and galacturonate until a pectin solution in the form of gel was produced.³⁵ The effect of PC weight towards the sensitivity of optical pH sensor has been presented (Figure 9). The optimal weight percentage of PC was found at 0.1 % w/v. The membrane with 0.1% w/v pectin has a flatter surface thus making it as the most suitable optical sensor. PC membrane with only 0.05% w/v PC possessed a gel like texture due to the excess of water which causes a longer time to form a solid membrane. This phenomenon is quite similar for membrane preparation using a phase inversion method.^{21,36,37} On the other hand, membrane with 0.15% PC is very dense and has a non-homogenous surface which is not preferred for optical pH membrane application.³⁸

Effect of type and concentration of buffer on the sensor performance

The performance of an optical pH sensor may be affected by the types and concentration of the buffer. Figure 10 shows that the sensitivity of the sensor with phosphate buffer was 0.0877 with an R-square value of 0.993. On the other hand, the

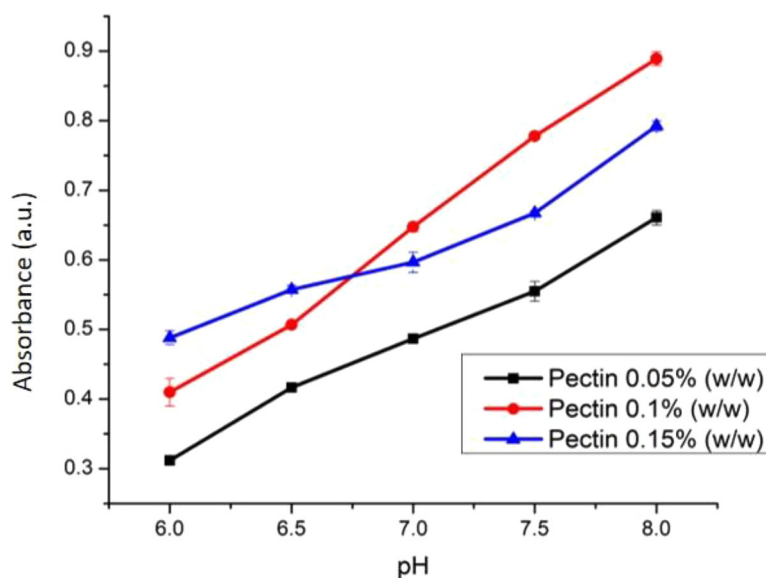


Figure 9. Effect of pectin weight towards the sensitivity of optical pH sensor.

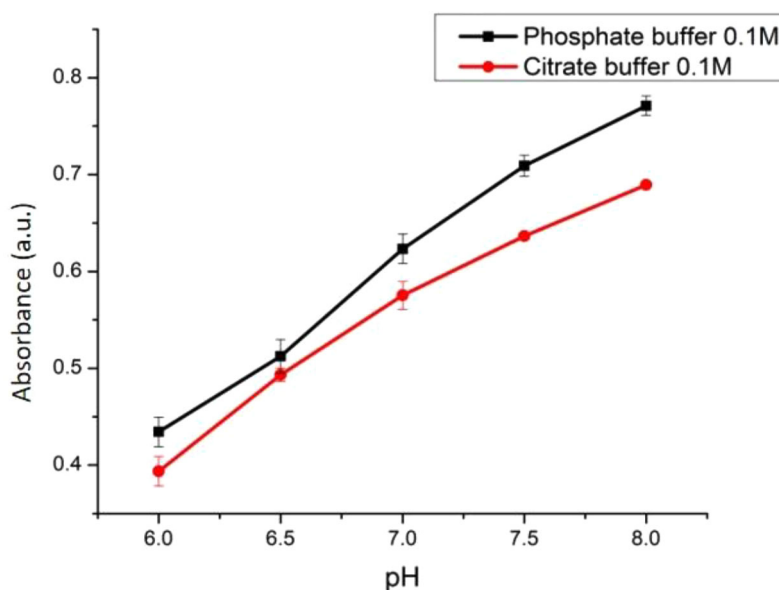


Figure 10. Effect of buffer type towards the sensitivity of optical pH sensor.

ACN/PC sensor with citrate buffer had a sensitivity of 0.074 ($R^2 = 0.981$). Through physical observation, the ANC in the sensor would display a higher color intensity when in phosphate buffer compared to citrate buffer even in the same pH range. This is due to the lower K_a value of phosphate buffer compared to citrate buffer. Altogether, we conclude that the phosphate buffer contributes to better sensitivity of our pH sensor as opposed to citrate buffer. Therefore, the effect of concentration was studied using the phosphate buffer.

The effect of phosphate buffer concentration towards this sensor's sensitivity is shown in Figure 11. This pH sensor produces the best sensitivity of 0.1238 ($R^2 = 0.9989$) when the phosphate buffer 0.03 M was used. Meanwhile, the sensitivities of the pH sensor using phosphate buffer with concentrations of 0.05 M and 0.1 M were found lower at 0.072 ($R^2 = 0.9745$) and 0.084 ($R^2 = 0.9805$), respectively. The pH sensor with phosphate buffer 0.03 M gave a more contrast in the color change at different pH levels, in comparison with that of citrate buffer. In comparison to other earlier studies,^{11,12} our ACN/PC optical pH sensor has a wider working range of pH.

Response time and reproducibility measurement

The response time of this sensor was determined by the required duration (minutes) that the sensor achieves a stable result. Response time was determined at 0, 5, 10, 15, 20, 25, and 30 minutes (Figure 12). The absorbance increased drastically from the first 5 minutes, indicating a good diffusion of the sample onto the membrane. The increase was later observed at minute 10, but no observable significant change afterward. Therefore, the optimum response time of this optical pH sensor is 10 minutes.

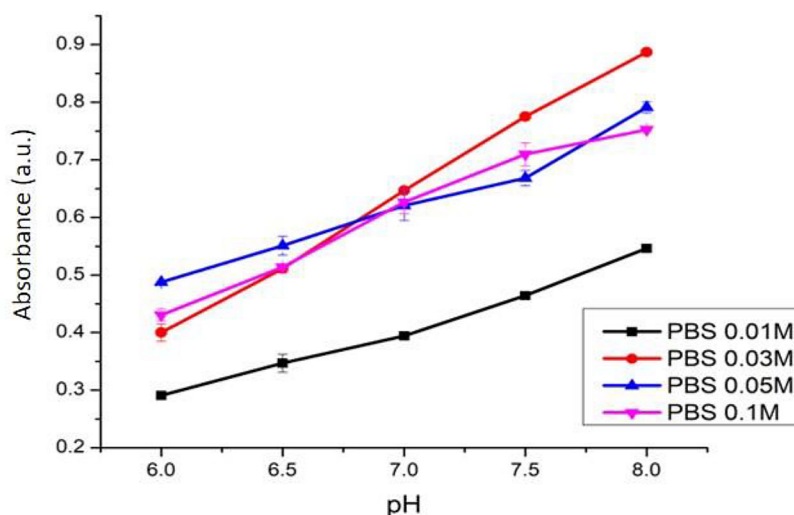


Figure 11. Effect of phosphate buffer concentration towards pH sensor's sensitivity.

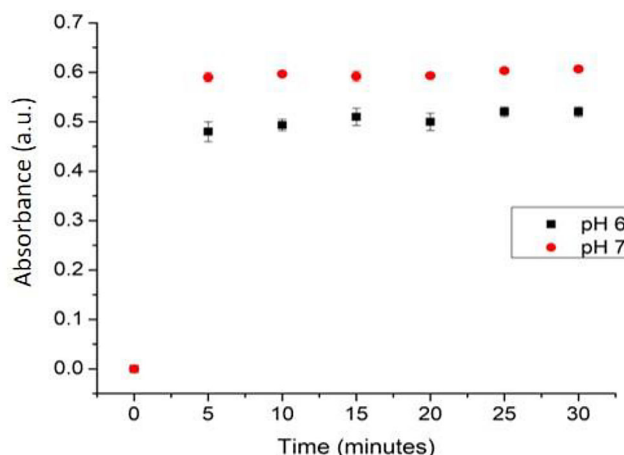


Figure 12. The response time profile of pH sensor.

In addition, the reproducibility measurement was conducted on 10 different sensors with the same condition, where the relative standard deviation (RSD) was 9.15. This shows that there is a small difference in the absorbance values obtained from the repetition using new sensors. However, RSD that is below 10% is still acceptable for qualitative measurement.³⁸

Lifetime of pH sensor

The investigated optical pH sensor had a stable response until the tenth day of storage (Figure 13). Afterward, the sensor response fell as much as 8.3% from the initial response, in which further decline was observed on the 15th day. At the same time, the %RSD also become poor; increasing as much as 36.61% from its initial state. The decrease in sensor performance after particular days of storing depends on the stability of the anthocyanin in maintaining its color. The lifetime of the optical pH sensor in this study is worse in comparison to that of our previous study,¹⁸ in which the performance did not drop until the 15th day. However, previously we used the synthetic chromoionophore ETH 5294 (CI); unlike in this study where we used natural anthocyanin that can be considered more sustainable. Furthermore, in this study, the lifetime is better in comparison to our currently reported sensor using ACN from *Dioscorea alata* L.³⁸

Fish freshness test using real samples

Optical pH sensor with the optimal conditions was used to monitor the freshness of tilapia fish that was kept at 4°C. The pH profile of the fish at two conditions, namely room temperature and 4°C storage temperature, is shown in Figure 14. A living fish has a pH value of around 7.4, but after death the pH decreases.³⁹ The pH of the fish samples was measured

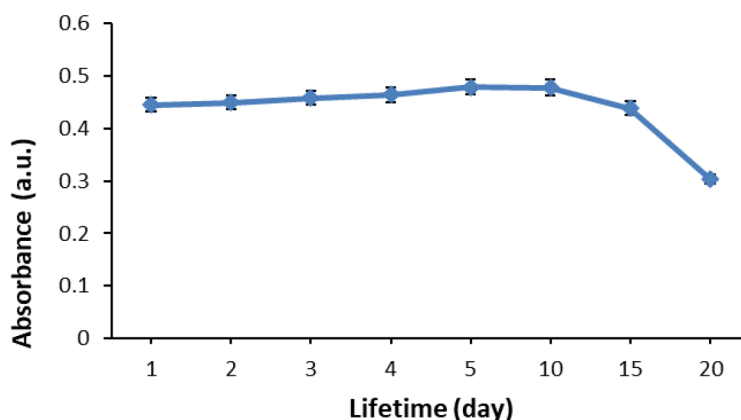


Figure 13. Lifetime of optical pH sensor.

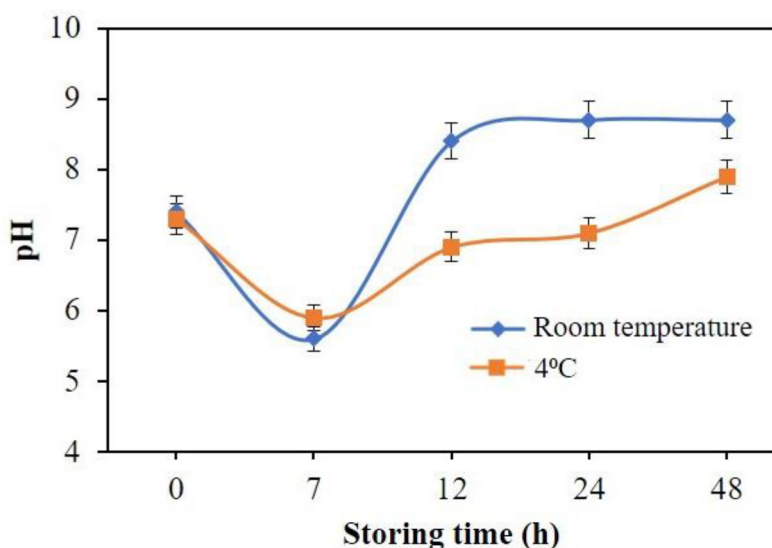


Figure 14. Fish freshness monitoring using optical pH sensor.

after 0, 7, 12, 24 and 48 h storage time at room temperature and 4°C. Fish freshness was measured based on the absorbance value that is converted to pH value based on the constructed calibration curve.

Fish samples kept at room temperature possess a higher pH compared to the fish sample stored at 4°C. Fresh fish that was measured at 0 hours displayed pH of around 7.3-7.4. Following that, the pH decreases to 5.5-5.9, indicating that the fish has reached rigor mortis or postmortem rigidity. The decrease is attributed to the accumulation of lactic acid from post mortem glycolysis. After the rigor mortis phase, the fish will undergo putrefaction due to the microbial activity in the fish sample.⁴⁰ This activity causes the pH to become more basic due to the breakdown of proteins in the fish sample to become ammonia and trimethylamine.²³⁻²⁵ Results achieved from pH measurements at 7, 12, 24 and 48 hours at 4°C using the optical sensor yielded results of pH 5.9, 6.9, 7.1 and 7.9. Based on these results, it can be said that fish that is kept at room temperature will undergo a faster decomposition. This is due to the exposure to sunlight thus a higher temperature that will accelerate the process of decomposition.

Our method of measuring the change of pH is different to the most reported studies using colorimetric response.^{4,5,7,14,16} Indeed, one may argue that colorimetry could give the best practicality of the sensor use. However, it suffers from quantitative information, as it depends on the RGB profiles that requires complex model to convert the response into measured pH value. Moreover, the reported studies rely on the volatile basic compounds released from the meat. Taken altogether, the reported studies were unable to capture the decrease of pH during rigor mortis phase. In food industry, fish meat is best processed by the filleting machine during the pre- or post-rigor mortem. This is the novelty of our optical pH sensor which is useful for the quality control and processing of fish meat in industrial settings.

Conclusion

ACN extracted from *Ruellia tuberosa* L can be immobilized into a PC matrix to produce a sensitive optical pH sensor. The extracted ACN has a similarity over the FT-IR profile of cyanidin-3-glucoside. The amount of ACN and PC in the membrane composite affected the optical pH performance, which was largely indicated by intercept and linearity values. The constructed optical pH sensor works best in phosphate buffer with a long lifetime. Its application in monitoring the freshness of fish has been successfully conducted against the storing time, where the decrease in pH values during rigor mortis period were observed. More studies indeed need carried out to obtain smooth surface morphology to improve the optical sensor performance.

Data availability

Underlying data

Harvard Dataverse: Data Set for Optical pH Sensor Based on Pectin and *Ruellia tuberosa* L-derived Anthocyanin for Fish Freshness Monitoring. <https://doi.org/10.7910/DVN/ZYCXAM>.⁴⁰

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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Sagir Alva

Department of Mechanical Engineering, Faculty of Engineering, Universitas Mercu Buana, Jakarta, Indonesia

After I read the revised results of this article, the authors of this article has added some suggestions that I have conveyed before, so I think the value of this article is better than before.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My specialty is the synthesis and characterization of materials such as polymers for the development of chemical sensors/biosensors.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 11 August 2021

<https://doi.org/10.5256/f1000research.58582.r90078>

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Nur Hamidah Abdul Halim

Institute of Nano Electronic Engineering, Universiti Malaysia Perlis, Kangar, Malaysia

I would suggest the writer to include "non destructive/by *in situ* detection or measurement for fish freshness" in the abstract and title to highlight the novelty of this work.

"The extracted ACN has a similarity over the FT-IR profile of cyanidin-3-glucoside" is suddenly

introduced in the conclusion is hanging. Suggest elaborating how glycoside bonds are important and how it contributed to the pH changes or how it related to freshness. Then it can be concluded how the similarity by having FT-IR profile of cyanidin-3-glucoside in the ACN is desired in this work.

Is having "cyanidin-3-glucoside in ACN" the actual highlight in this work? If yes, the novelty statement in the abstract, introduction and title need to be revised accordingly.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Electrochemical biosensors

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 18 June 2021

<https://doi.org/10.5256/f1000research.56160.r86281>

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Nur Hamidah Abdul Halim

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This paper shows an experimental work on optical sensor using simple optical approach. This paper shows a good work with a potential study on optical pH sensor for fish freshness monitoring. However, it is suggested to elaborate further on discussion how the mechanism and reaction with illustrated figure. The novelty should be explicitly mentioned in the introduction, abstract and findings. A table on reported or published and comparison should also be made available to see the research contribution. It is suggested that the authors may revise based on few comments below:

Abstract

"The sensor displayed an excellent response after 10 minutes of exposure, possessing a response stability for 10 consecutive days. The decrease in pH value of the Tilapia fish from 7.3 to 5 was observed in a 48 hour test, which can be used as the parameter when monitoring fish freshness."

Comment: The statement of decrease in pH value need to be elaborated to highlight the novelty of this research. The authors may add comparison in terms of performance and mechanism that differentiate this works and other reported work. E.g How pH value decrease mechanism is evaluated and correlated to observe the fish freshness.

Introduction

Para 2: "Nevertheless, these aforementioned pH sensors could only be used on solutions with near-neutral pH as more basic or acidic solutions will give an insignificant response time. Pourjavaher et al.¹¹ has designed an optical pH sensor based on cellulose nanofibers with red cabbage (*Brassica oleracea*) extract, while Rajan et al. (2018)¹² has produced an optical pH sensor using peonidin pigment. However, this study did not report the working pH range of peonidin. The use of anthocyanin (ACN) from blackberries and chitosan membrane in an optical pH sensor has been established.¹³ The interaction and mechanical properties of chitosan membrane with entrapped ACN have also been reported."

Comment: This paragraph should elaborate more on fish freshness and its correlation to pH based on previous study. The use of ACN should be illustrated for reader to understand more as the sentences is hanging (Referring to "The interaction and mechanical properties of chitosan membrane with entrapped ACN have also been reported."). It is more helpful if a table or illustrated mechanism is shown to support this study and having a good flow of this paper.

Para 3: "A more recent study on fish freshness monitoring through optical methods was reported by Moradi et al.¹⁵ using nanofiber bacterial cellulose with ACN. However, this method requires a relatively long analytical time as the pH measurement could not be conducted in situ. Chen et al. (2020)⁶ has developed a sensitive novel film prepared from starch polyvinyl alcohol and starch polyvinyl alcohol glycerol."

Comment: Again, this paragraph does not add the value on published work with this work. The mechanism on optical pH to monitor fish freshness is still not addressed. No comparison on the electrochemical performance (LOD, Linear range, selectivity) was mentioned here. How long analytical time is related to pH measurement by having different material like nanofiber and optical properties coming from ACN dye. The ACN sensitivities towards pH correlation to ACN optical properties may need to be added here as well.

Research and Methodology

Comment: The methodology shows a sufficient description a to give reader a good understanding on how this study is conducted. It is suggested that the authors may add process flow/illustration to complete the overall picture on steps and its mechanism.

Results and Discussion

Figure 4. SEM profile of (a) PC and (b) ACN/PC membranes.

Comments: The morphology of ACN/PC membrane does not seem like a crack. It seems to have a wavy layer of membrane that might be the contributed to the adhesion/stress tension or air gap of the ACN/PC compared to PC alone. Is there any study on different ration of CAN added to this PC, or is it already optimized? The caption should be more detailed.

"Color change of ACN can be affected by several factors such as temperature, pH, light intensity, sugar moiety and different phenolic derivatives. Due to its solubility in aqueous solution, the color change of ACN is caused by structural transformations of carbon skeleton affected by the levels of H⁺."

Comments: The color change mechanism is important to be introduced earlier in the introduction section and can be help with illustration. How different phenolic derivatives change this CAN, and which phenolic derivatives took place in this reaction? The authors may put or add this point to

support the color change mechanism towards fish freshness from the finding.

Effect of PC weight towards sensor sensitivity

Comments: The pectin is a membrane that hold the ACN dye to improve the sensitivities. From Fig 4, the importance of having optimum load/weight of pectin is important the membrane with less surface tension, and this is the reason of having crack or wavy like membrane. It is very important optimum ratio of CAN/PC to have smooth ACN/PC membrane in this study.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Electrochemical biosensors

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 09 Jul 2021

Nazaruddin .

Reviewer Nurhamidah

Thank you very much for valuable comments

Comment: The statement of decrease in pH value need to be elaborated to highlight the novelty of this research. The authors may add comparison in terms of performance and mechanism that differentiate this works and other reported work. E.g How pH value decrease mechanism is evaluated and correlated to observe the fish freshness.

Response: The pH of fresh tilapia was 7.3, and the pH decreased to 5 after 7 hours of

storage in two storage conditions (room temperature and 4°C). Changes in pH from 7.3 to 8.7 are the condition of fish monitoring within 48 hours. The post mortem glycolysis-derived lactic acid accumulation is also responsible for the pH decrease.

Additionally, we have amended the manuscript with the following text:

"Our method of measuring the change of pH is different to the most reported studies using colorimetric response. Indeed, one may argue that colorimetry could give the best practicality of the sensor use. However, it suffers from quantitative information, as it depends on the RGB profiles that requires complex model to convert the response into measured pH value. Moreover, the reported studies rely on the volatile basic compounds released from the meat. Taken altogether, the reported studies were unable to capture the decrease of pH during rigor mortis phase. In food industry, fish meat is best processed by the filleting machine during the pre- or post-rigor mortem. This is the novelty of our optical pH sensor which is useful for the quality control and processing of fish meat in industrial settings."

Comment: This paragraph should elaborate more on fish freshness and its correlation to pH based on previous study. The use of ACN should be illustrated for reader to understand more as the sentences is hanging (Referring to "The interaction and mechanical properties of chitosan membrane with entrapped ACN have also been reported."). It is more helpful if a table or illustrated mechanism is shown to support this study and having a good flow of this paper.

Response: The paragraph 2 has been elaborated:

*Nevertheless, these aforementioned pH sensors could only be used on solutions with near-neutral pH as more basic or acidic solutions will give an insignificant response time. Pourjavaher et al.¹¹ has designed a pH sensor using bacterial cellulose (BC) nanofiber matrix to immobilize anthocyanin (CAN) from red cabbage (*Brassica oleracea*) extract. The sensor has a fairly wide pH range but it needs further characterization to evaluate the sensor performance, especially, for real foodstuff analysis. The use of ACN from blackberries and chitosan membrane in an optical pH sensor has been established.¹³ The interaction and mechanical properties of chitosan membrane with entrapped ACN have also been reported.¹⁴ Anthocyanins are flavonoids possessing a number of hydroxyl groups contributing a strong interaction with chitosan via hydrogen bonding.*

Comment: Again, this paragraph does not add the value on published work with this work. The mechanism on optical pH to monitor fish freshness is still not addressed. No comparison on the electrochemical performance (LOD, Linear range, selectivity) was mentioned here. How long analytical time is related to pH measurement by having different material like nanofiber and optical properties coming from ACN dye. The ACN sensitivities towards pH correlation to ACN optical properties may need to be added here as well.

Response: The paragraph 3 has been elaborated:

A more recent study on fish freshness monitoring through optical methods was reported by Moradi et al.¹⁵ using nanofiber bacterial cellulose with ACN. However, this method requires a relatively long analytical time as the pH measurement could not be conducted in situ. Chen et al. (2020)⁶ has developed a sensitive novel film prepared from starch polyvinyl alcohol and starch polyvinyl alcohol glycerol. The study used curcumin from turmeric and anthocyanin from purple

sweet potatoes. The results showed that the mixture of curcumin and ACN improved the stability than that of the individual active substances. As the consequence, the sensor could be employed to detect volatile ammonia as the fish freshness indicator.

Comment: The methodology shows a sufficient description a to give reader a good understanding on how this study is conducted. It is suggested that the authors may add process flow/illustration to complete the overall picture on steps and its mechanism.

Response: The steps have been added Methods:

Study Design

The first step in sensor fabrication was the extraction of anthocyanin from Ruellia tuberosa L. The extracted anthocyanins were then mixed with pectin solution and printed proportionally as an optical pH sensor. The optical pH sensor was then characterized and the optimized and then applied to monitor the freshness of tilapia. The image below is a schematic diagram summarizing research procedures conducted in this work.

[Figure]

Comment: The morphology of ACN/PC membrane does not seem like a crack. It seems to have a wavy layer of membrane that might be the contributed to the adhesion/stress tension or air gap of the ACN/PC compared to PC alone. Is there any study on different ration of CAN added to this PC, or is it already optimized? The caption should be more detailed.

Response: The ratio of ACN has been optimized based on the sensitivity and R^2 , see Table 1 and Figure 8. The description for SEM images analysis has been revised per suggestion.

Comment: The color change mechanism is important to be introduced earlier in the introduction section and can be help with illustration. How different phenolic derivatives change this CAN, and which phenolic derivatives took place in this reaction? The authors may put or add this point to support the color change mechanism towards fish freshness from the finding.

Response: The anthocyanin structure under different pHs has been added in the manuscript as suggested (see Figure 7).

[Figure]

Comment: The pectin is a membrane that hold the ACN dye to improve the sensitivities. From Fig 4, the importance of having optimum load/weight of pectin is important the membrane with less surface tension, and this is the reason of having crack or wavy like membrane. It is very important optimum ratio of CAN/PC to have smooth ACN/PC membrane in this study.

Response: We have optimized the PC weight and the optimum was reached for 0.1% PC to find optimum sensitivity. The membrane with 0.1% w/v pectin has a flatter surface thus making it as the most suitable optical sensor. SEM characterization was carried out on the optimum pectin weight. The wavy like surface structure was probably due to the addition of anthocyanin.

Added as a recommendation in conclusion:

More studies indeed need carried out to obtain smooth surface morphology to improve the optical sensor performance.

Due to the limited features in this comment column, we have uploaded our full response through this [link](#).

Competing Interests: None

Reviewer Report 04 June 2021

<https://doi.org/10.5256/f1000research.56160.r86206>

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Sagir Alva

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After I read and reviewed this article, I found the theme of this article quite interesting. However, unfortunately, there are shortcomings in this article which make it unfit for indexing. Therefore, I suggest a Not Approval status for this article. However, it can be improved in the revision with the following comments:

1. It is true, when fish begin to undergo a process of decomposition, in addition to producing H^+ , ammonia is also produced. In the first paragraph, the authors only compared it with ammonia-ISE, where basically, the concept of measuring ammonia-ISE is an indirect measurement of ammonia based on the dissociation of ammonia in solution to form NH_4^+ . So naturally not all ammonia will be detected. However, there are actually a lot of research on ammonia optical sensors. In other words, the ammonia optical sensor is nothing new. So there needs to be an explanation added to the introduction why choosing an optical pH sensor in detecting the freshness of fish compared to an ammonia optical sensor. What are the advantages of an optical pH sensor compared to an optical ammonia sensor?
2. At the end of the first paragraph, you stated that measuring pH using an optical sensor might be good for samples that have interfering ions. With fish, what ions are supposed to be can interfere with the pH-ISE sensor, so you end up choosing the optical pH sensor over the pH-ISE sensor? An explanation of this needs to be added in the introduction section.
3. Basically, a lot of plants and fruits also have ACN, and here you have also given examples such

as blackberries. But why in this study have you focused on the *Ruellia* flower? Instead, you can also use ACN from blackberries immobilized using Pectin. What are the advantages of ACN from *Ruellia* compared to other plants? It is worth mentioning in the introduction the reasons for this.

4. The use of a hydrogel membrane will indeed facilitate the diffusion of the analyte. However, the hydrogel membrane has serious problems such as easy to swell and break, so that the dye used can be leached and the sensor life time is decreased. There needs to be some clarification on this. In addition, there needs to be additional experimental data on the % swelling index of the pectin membrane used.

5. In optical sensor, leaching study is an important thing to do. However, in this article there are no leaching study data, so it is necessary to add experimental data for leaching study testing.

6. There are several natural hydrogel polymers. It is necessary to add reasons why choose Pectin over other natural hydrogel polymers. What are the advantages of pectin over other natural hydrogel polymers?

7. On page 7 and the beginning of the first paragraph, there is the sentence: "The constructed optical pH biosensor based on the ACN derived from *R. tuberosa* L flower has hydrogel characteristics." - It need clarification, is this really an optical biosensor? Because here I don't see any use of enzymes, peptides, micro-organisms etc.

8. On page 7 it is stated that the colour change is caused by a structural transformation of the ACN. It is necessary to add pictures of the changes in the chemical structure of ACN at various pH variations, such as acidic, neutral and basic.

9. Still from page 7, you stated that one of the factors that caused the change in ACN colour was caused by light. You need to clarify, how do you control the light intensity during the test period, so that the colour of the ACN remains stable and how long can the light change the colour of the ACN?

10. In the ACN variation data, the resulting absorbance will also decrease with the lower ACN concentration, and in the end you use a concentration of 0.025 mg/L as the optimum concentration of ACN. What if the concentration of the ACN is less than 0.025 mg/L? Is ACN still able to respond to changes in pH or not able to respond to changes in pH? Additional data are needed for testing less than 0.025 mg/L.

11. In sensor development, validation testing is very important to ensure that the fabricated sensor performs at least the same as standard test equipment. Here, I don't see that. There needs to be additional validation data with standard methods to test the freshness of fish based on pH changes.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My specialty is the synthesis and characterization of materials such as polymers for the development of chemical sensors/biosensors.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 09 Jul 2021

Nazaruddin .

Comment: It is true, when fish begin to undergo a process of decomposition, in addition to producing H^+ , ammonia is also produced. In the first paragraph, the authors only compared it with ammonia-ISE, where basically, the concept of measuring ammonia-ISE is an indirect measurement of ammonia based on the dissociation of ammonia in solution to form NH_4^+ . So naturally not all ammonia will be detected. However, there are actually a lot of research on ammonia optical sensors. In other words, the ammonia optical sensor is nothing new. So there needs to be an explanation added to the introduction why choosing an optical pH sensor in detecting the freshness of fish compared to an ammonia optical sensor. What are the advantages of an optical pH sensor compared to an optical ammonia sensor?

Response:

- The literature on the development of the NH_3 optical biosensor was developed by Dan-Feng Lu and Zhi-mei Qi in 2019 using bromothymol blue and a porous glass membrane. This sensor can only work at low concentrations of ammonia.
- Another ammonia sensor has also been developed by Maximilian Maierhofer *et al.* (2020), who fabricated the sensor using fluorescence properties of aza-BODIPY dyes with a response time of 390 seconds.
- Detection of ammonia as a total volatile basic nitrogen (TVB-N) to determine fish spoilage requires a sample destruction process (Nathan Wells *et al.* (2019), Talanta 194: 830–836). Then, the standard curve was obtained from measuring the

absorbance of the trimethylamine (TMA) compound that produces ammonia through a complicated procedure. On the other hand, this method was also based on pH measurements. So it can be concluded that ammonia is also correlated with changes in pH to determine the freshness of fish. The same concept has also been previously reported by T. Werner *et al.* (1995) Analyst 120 1627–1631 where the determination of ammonia was based on measuring pH using an ion-pair indicator. Therefore, the detection of fish freshness through pH measurements is more representative of the actual condition of *in-situ* tests.

- An explanation of the ammonia optical sensor and its drawbacks for determining fish freshness has been described in the introduction.

Comment: At the end of the first paragraph, you stated that measuring pH using an optical sensor might be good for samples that have interfering ions. With fish, what ions are supposed to be can interfere with the pH-ISE sensor, so you end up choosing the optical pH sensor over the pH-ISE sensor? An explanation of this needs to be added in the introduction section.

Response: Literature reported ISE H⁺ response is strongly affected by alkaline ions has been added in the Introduction.

Comment: Basically, a lot of plants and fruits also have ACN, and here you have also given examples such as blackberries. But why in this study have you focused on the Ruellia flower? Instead, you can also use ACN from blackberries immobilized using Pectin. What are the advantages of ACN from Ruellia compared to other plants? It is worth mentioning in the introduction the reasons for this.

Response: Mostly, coloured plants contain anthocyanins, including blackberries. Anthocyanins from blackberries can also be used as pH-sensitive active ingredients to develop optical pH sensors. On the other hand, sources of anthocyanins from blackberries are difficult to obtain in our area. In this study, *Ruellia* anthocyanins were used as a sensitive pH compound for optical pH sensor development because the flowers are easy to obtain. In addition, based on a preliminary study on the sensitivity of the anthocyanin at various pHs, we found that the anthocyanin has a great potential to be further applied in developing optical pH sensor.

Comment: The use of a hydrogel membrane will indeed facilitate the diffusion of the analyte. However, the hydrogel membrane has serious problems such as easy to swell and break, so that the dye used can be leached and the sensor life time is decreased. There needs to be some clarification on this. In addition, there needs to be additional experimental data on the % swelling index of the pectin membrane used.

Response: Firstly, the membrane use as optical pH sensor is not applied by immersion into aqueous samples therefore swelling index is not relevant. Secondly, there have been extensive research pertaining to the swelling profile of pectin, of which are Fong H. WEH *et al.* (2014) Lat. Am. J. Pharm. 33(3): 420-31 and Naziha Chirani *et al.* 2015. Journal of Biomedical Sciences. Vol. 4 No. 2:13. P 1-23.

Below is swelling index of pectin in different media based on the reported study.

[Figure]

Comment: In optical sensor, leaching study is an important thing to do. However, in this article there are no leaching study data, so it is necessary to add experimental data for leaching study testing.

Response: In our opinion, not all leaching tests need to be carried out in sensor or biosensor manufacturing studies. It depends on the sensor application. In this study, we did not immerse the sensor in the sample. The sensor is placed directly on the surface of the fish, and then the colour changes are measured. For a liquid sample, only a small amount of sample dropped onto the sensor surface. The sensor produced is a disposal sensor.

Comment: There are several natural hydrogel polymers. It is necessary to add reasons why choose Pectin over other natural hydrogel polymers. What are the advantages of pectin over other natural hydrogel polymers?

Response: Pectin was chosen because of:

1. Its non-toxicity; because the application is for a foodstuff, the sensor should not be toxic.
2. Its ability in forming membrane structure.
3. Transparent and homogenous.
4. In the case of optical pH sensor for fish freshness monitoring, other studies have reported chitosan, starch, and cellulosic materials; while pectin is scarcely reported. Hence, the use of pectine is a novelty.

Those characteristics have been added in the last paragraph of introduction.

Comment: On page 7 and the beginning of the first paragraph, there is the sentence: "The constructed optical pH biosensor based on the ACN derived from *R. tuberosa* L flower has hydrogel characteristics." - It need clarification, is this really an optical biosensor? Because here I don't see any use of enzymes, peptides, micro-organisms etc.

Response: It is a sensor not as a biosensor. Has been modified: "biosensor" to "sensor"

Comment: On page 7 it is stated that the colour change is caused by a structural transformation of the ACN. It is necessary to add pictures of the changes in the chemical structure of ACN at various pH variations, such as acidic, neutral and basic.

Response: Has been added, see figure 7 [Figure].

Comment: Still from page 7, you stated that one of the factors that caused the change in ACN colour was caused by light. You need to clarify, how do you control the light intensity during the test period, so that the colour of the ACN remains stable and how long can the light change the colour of the ACN?

Response: The sensor has been made through a storage process in a dark condition and a temperature of 4°C. At the time of measurement, the sensor is also kept in the dark and needs a short time of exposure to light during the measurement process. We predict no significant colour change. In addition from our preliminary experiment, immobilized anthocyanins on the pectin matrix have good stability.

Comment: In the ACN variation data, the resulting absorbance will also decrease with the lower ACN concentration, and in the end you use a concentration of 0.025 mg/L as the optimum concentration of ACN. What if the concentration of the ACN is less than 0.025 mg/L? Is ACN still able to respond to changes in pH or not able to respond to changes in pH? Additional data are needed for testing less than 0.025 mg/L.

Response: The effect of anthocyanin concentration is not significantly different on sensor sensitivity and linear range. Anthocyanin concentrations less than 0.025 mg/L are predicted still to respond to pH changes. Due to the intensity of the colour decreases, the sensitivity will also decrease. Thus, the determination of sensitivity for anthocyanin concentrations lower than 0.025 mg/L was not determined.

Comment: In sensor development, validation testing is very important to ensure that the fabricated sensor performs at least the same as standard test equipment. Here, I don't see that. There needs to be additional validation data with standard methods to test the freshness of fish based on pH changes.

Response: We have validated the optical sensor method using H⁺ ion-selective electrodes. However, we do not report it. In this paper, we focus more on how the pH changes in fish stored at room temperature and 4°C. The following are the results of the validation of measurements carried out on fish measured using an optical pH sensor with H⁺ ISE.

[Table]

From the results obtained that the ISE measurement is influenced by temperature (as also suggested by other reported studies) so that the results obtained are different from the optical pH sensor.

Due to the limited features available in this comment column, we choose to upload our full response in an accessible link. Please find it through this [link](#).

Competing Interests: None

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