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OPEN Impact of environmental storage conditions on properties and stability of a smart bilayer film

Orapan Romruen¹, Pimonpan Kaewprachu², Samart Sai-Ut³, Passakorn Kingwascharapong⁴, Thomas Karbowski⁵, Wanli Zhang⁶ & Saroat Rawdkuen^{1,7}✉

This study aimed to investigate the behavior of smart bilayer films under various temperature and relative humidity (RH). Smart bilayer films were fabricated using sodium alginate with incorporated butterfly pea anthocyanin and agar containing catechin–lysozyme. Cellulose nanospheres were added at concentrations of 0% and 10% w/w of the film and subjected to test at 4 °C and 25 °C, considering different RHs (0%, 50%, and 80%). The results showed that RH had a greater impact on the mechanical properties than temperature, leading to a decrease in tensile strength and an increase in elongation at break with higher RH. The films displayed increased strength but reduced flexibility at low temperatures. Oxygen permeability was negatively affected by increasing RH, while water vapor barrier properties were better at 25 °C than at 4 °C. In terms of color stability, the temperature played a more important role, with both types of smart bilayer films retaining their color stability throughout 14-day storage at 4 °C, even maintaining their ability to change color with pH. However, the films stored at 25 °C exhibited lower color stability and showed potential for color change with varying pH levels, but with lower intensity. The findings of this study demonstrate the significant impact of temperature and RH on the functional properties of smart bilayer films, with and without the addition of cellulose nanospheres. Such smart bilayer films have great potential for various applications, particularly in food packaging, where maintaining color, mechanical, and barrier properties under varying environmental conditions is crucial.

Keywords Anthocyanin, Cellulose nanosphere, Relative humidity, Temperature, Smart packaging, Storage conditions, Stability

The development of smart packaging materials represents a significant advancement in the food industry, with the potential to enhance food safety, quality, and shelf life¹. Among these innovations, smart bilayer films have emerged as promising candidates, particularly for their ability to act as pH indicators in fresh food products. Smart bilayer films, a notable development in active and intelligent packaging, have received considerable attention due to their ability to interact with the surrounding environment and react in real-time to changing conditions².

In our previous study Romruen et al.³, we successfully developed a smart bilayer film designed to enhance both its functional and protective properties. The top layer, consisting of alginate, butterfly pea anthocyanin extract, and varying concentrations of cellulose nanospheres (CNs), improves mechanical strength and color sensitivity. The bottom layer, composed of an agar matrix fortified with catechin and lysozyme, decreases water vapor permeability (WVP) and prevents microbial growth. Remarkably, the addition of 10% (w/w) CNs resulted in superior mechanical strength, barrier properties, and pH sensitivity, making these films highly suitable for use as pH indicators in fresh food packaging.

While prior studies have explored various smart packaging solutions, our research uniquely addresses the integration of cellulose nanospheres to enhance the film's mechanical and sensory properties. Furthermore, our

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work introduces an innovative approach to mitigating water vapor permeability and microbial contamination, addressing critical gaps in existing smart packaging technologies. This advancement not only improves the films' effectiveness as pH indicators but also extends their application potential across different food products and storage conditions.

The primary objective of developing this film was to utilize it as a pH indicator for monitoring the freshness of protein-rich foods. Films containing anthocyanins have garnered considerable attention for their potential as pH or freshness indicators across diverse applications, such as food packaging and quality monitoring⁴. Anthocyanins are natural pigments present in a wide range of fruits, vegetables, and flowers. They are renowned for their pH-dependent color-changing properties⁵. This unique characteristic enables them to function as natural pH indicators, with the color of the film shifting in response to changes in acidity or alkalinity. In particular, for protein-rich food products like meat or fish, the anthocyanin-infused film is capable of changing color when exposed to volatile compounds produced during the spoilage process⁶. This color change serves as an early warning system, alerting consumers or retailers to potential quality degradation or spoilage, thereby enhancing food safety and reducing waste.

Despite these advancements, the real-world application of smart bilayer films requires a comprehensive understanding of how environmental storage conditions affect their properties and stability. These conditions can cause physical, chemical, and biological changes in the films. To address this issue, natural compounds with suitable properties may be added to enhance the mechanical, barrier, and antimicrobial properties of the films⁷. Exposure of biopolymer films to specific environmental conditions can lead to physical and chemical changes during storage^{8,9}. These alterations impact the mechanical properties and color of the films, influencing their suitability and acceptability for various applications. Consequently, it is crucial to ensure high stability of film properties over extended storage periods¹⁰. Maintaining this stability is crucial for preserving film integrity and functionality and meeting the demands of industrial and commercial uses. For example, changes in mechanical properties can reduce the film's structural integrity and its effectiveness as a barrier, which may compromise the protection of the packaged product. Additionally, color variations can affect not only the aesthetic appeal of the film but also its functionality as a pH or freshness indicator. Temperature and relative humidity are critical factors that significantly influence film properties¹¹. Moreover, the presence of anthocyanin, which imparts pH-sensitivity and color-change properties to the film, can be affected by fluctuations in temperature and humidity¹². Food products are stored under varying temperatures and humidity levels, which can significantly impact the performance of packaging materials. Therefore, it is crucial to investigate the environmental stability of smart bilayer films to ensure their reliability and functionality in diverse storage conditions.

This study aims to fill this gap by systematically investigating the impact of different storage conditions—specifically, varying temperatures and relative humidity levels—on the properties and stability of smart bilayer films. Experiments were conducted at both refrigerated (4 °C) and ambient (25 °C) temperatures, with relative humidity levels set at 0%, 50%, and 80%. The focus was on evaluating the mechanical properties, oxygen and water vapor barrier characteristics, and color stability of the films. This research provides a detailed analysis of how environmental factors influence the performance of smart bilayer films, contributing valuable insights into optimizing their design and application. The findings aim to enhance the practical utility of smart bilayer films in maintaining food quality and safety across diverse storage environments, representing a significant advancement in smart packaging technology.

Materials and methods

Materials

Sodium alginate was purchased from Union Science Co., Ltd. (Chaing Mai, Thailand). Agar was obtained from Krungthepchemi Co., Ltd. (Bangkok, Thailand). Catechin hydrate (C1251) and chicken egg white lysozyme (62971) were obtained from Sigma – Aldrich Chimie SARL (St. Quentin Fallavier, France). All other chemicals used in this experiment were of analytical grade.

Smart bilayer films preparation

The smart bilayer films were prepared following our previous study Romruen et al. (2022a). In brief, the film-forming solution (FFS) for the active (bottom) layer was made by combining 2% (w/v) agar, 50% (w/w polymer) glycerol, and 0.5% (w/v) of catechin–lysozyme in a 1:1 ratio. For the indicator (top) layer, the FFS was composed of 1.5% (w/v) sodium alginate, 50% (w/w polymer) glycerol, and 0.25% (w/v) butterfly pea anthocyanin extract. Corn cob cellulose nanospheres (CNS) obtained from Romruen et al.¹³ were added at 0 and 10% (w/w polymers) into the active FFS. The active FFS, with a total weight of 25 g, was spread evenly on a 12 × 12 cm² plastic plate and left to dry naturally at room temperature for 24 h. Next, the indicator FFS, weighing 30 g, was carefully poured onto the active layer, and allowed to dry for a further 24 h at room temperature. Before any analysis, the dried bilayer film was subjected to a conditioning process inside an environmental chamber at a controlled temperature of 25 ± 0.5 °C and a relative humidity of 50 ± 5% for a period of 72 h.

Characterization of smart bilayer film

Mechanical properties

Mechanical properties of the films, including tensile strength and elongation at break, were evaluated using a universal tension–compression machine (TA-HD Plus+, Stable Micro-Systems, Godalming SU, UK). Rectangular film samples measuring 2.5 × 8 cm² were precisely cut using a standardized double-blade precision cutter (JDC precision cutter, Thwing-Albert Instrument Company, West Berlin, NJ, USA). Before analysis, the smart bilayer film was stored under constant temperature conditions at 4 and 25 °C in a humidity chamber with relative humidity levels of 0%, 50%, and 80% for 72 h. Tests were conducted using a 300 N load cell, and 9 samples, each with a 5 cm grip length, were measured with a cross-head speed of 50 mm/min¹⁴.

Water vapor permeability (WVP)

The water vapor permeability of the film was investigated using a modified method of ASTM¹⁵. The test involved creating a 50% relative humidity gradient by placing the film between a humid environment (water setting 100% relative humidity) and a semi-dry environment (silica gel setting 0% relative humidity) at 4 and 25 °C. The film was positioned on a permeation cell containing silica gel or pure water and then stored at 25 or 4 °C in a 50% relative humidity chamber. The permeation cell was weighted at 24 h intervals, and the WVPs of the films were calculated using Eq. 1 and expressed in $\text{g m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$:

$$\text{WVP} = \frac{WX}{At(P_2 - P_1)} \quad (1)$$

where W is the weight gain or weight loss of the permeation cell (g); X is the thickness of the film (m); A is the surface area of the film (m^2); t is the time (s); and $(P_2 - P_1)$ is the vapor pressure differential (Pa).

Oxygen permeability

The oxygen permeability of smart bilayer films was measured using the manometric method following the manufacturer's protocol with two different instruments: a GTT permeameter (Feinmechanik GmbH, Munich, Germany) at 25 °C and a GDP-C (Brugger Feinmechanik GmbH, Munich, Germany) at 4 °C. The measurement at 25 °C was performed at three different relative humidity levels (0%, 50% and 80%). The test at 4 °C was conducted under dry conditions (0% RH). Before oxygen permeability testing, the system was outgassed under a primary vacuum to remove residual gas. Throughout the testing process, one side of the smart bilayer film was exposed to oxygen, and the resulting pressure increase on the opposite side of the film was monitored and recorded over time. The experiments were conducted in triplicate to ensure accurate and reliable results. Oxygen permeability was calculated using Eq. (2) based on steady-state transfer. Oxygen permeability was expressed in $\text{mol m}^{-1} \text{s}^{-1} \text{Pa}^{-1}$ units.

$$P = \frac{\Delta n \cdot T}{A \cdot \Delta t \cdot \Delta p} \quad (2)$$

where P is the permeability; Δn is the average molar quantity associated with the gas transfer (mol); T is the film thickness; A is the surface area of the film (m^2); Δt is the time (s); Δp is the pressure difference between the two sides of the film (Pa).

Water contact angle

The water contact angle of the smart bilayer film was determined using a goniometer (DSA30, Kruss GmbH, Villebon, France) by using the modified method of Kertész et al.¹⁷. A small drop of water, around 2 μL , was deposited on the film surface. The contact angle of the water droplet on the film surface was then measured over 60 s. The measurement was conducted on both sides of the film. The test was conducted in a controlled room with a temperature of 25 ± 2 °C and a relative humidity of $50\% \pm 2\%$.

Stability of smart bilayer films

The film was subjected to various humidity levels (0, 50, and 80%) and stored at 4 and 25 °C for 14 days. At two-day intervals, the color of the film, represented by L^* , a^* , and b^* values, was measured using a chromameter (CR-400, Konica Minolta Sensing Inc., Osaka, Japan) and the color difference (ΔE) was calculated using Eq. 3¹⁸.

To evaluate the pH sensitivity of the smart bilayer film was assessed using a modified method of Pereira Jr et al.¹⁹ Pieces of the film measuring $1.5 \times 1.5 \text{ cm}^2$ were immersed in buffer solutions with different pH levels ranging from 2 to 12. After a 5 min, the films were taken out of the solutions, and the visible changes or color reactions were recorded using a digital camera (Sony $\alpha 6000$, Sony Thai Co. Ltd., Bangkok, Thailand).

$$\Delta E = \sqrt{(L - L^*)^2 + (a - a^*)^2 + (b - b^*)^2} \quad (3)$$

Statistical analysis

Statistical analysis was conducted using SPSS software (SPSS for Windows version 26.0, SPSS Inc., Chicago, IL, USA) to evaluate the analysis of variance (ANOVA). Duncan's multiple range tests were performed at a 95% confidence level to assess the significance of differences between samples.

Results and discussion

Mechanical properties

The mechanical properties of the films, such as tensile strength and elongation at break, are crucial in determining their performance in food packaging applications. These properties are significantly influenced by storage conditions, including relative humidity (RH) and temperature. Understanding these effects is essential to optimize the use of these films in various food systems. The influence of temperature and relative humidity on the mechanical properties of smart bilayer films is displayed in Table 1. The films were stored at different relative humidity (0, 50, and 80%) and temperatures (4 and 25 °C) for 72 h before mechanical analysis. The results demonstrate that variations in temperature and relative humidity significantly affect the tensile strength and elongation at break of films containing 0% and 10% CNs.

Film	RH (%)	TS (MPa)		EAB (%)	
		4 °C	25 °C	4 °C	25 °C
Without CNS	0	57.69 ± 3.15 ^a	52.15 ± 3.84 ^{bb}	23.71 ± 1.66 ^{db}	33.23 ± 1.24 ^{da}
	50	27.84 ± 1.91 ^{da}	25.53 ± 0.82 ^{db}	38.58 ± 1.82 ^{bb}	44.76 ± 2.49 ^{ba}
	80	15.14 ± 0.76 ^a	12.58 ± 0.78 ^{cb}	41.95 ± 1.70 ^{db}	51.27 ± 1.97 ^{ba}
10% CNS	0	65.37 ± 2.38 ^a	57.74 ± 3.65 ^{ab}	22.38 ± 0.82 ^{db}	30.54 ± 2.73 ^a
	50	34.41 ± 2.30 ^a	28.02 ± 1.03 ^{cb}	33.06 ± 1.96 ^{db}	41.23 ± 1.24 ^a
	80	16.07 ± 0.56 ^a	14.18 ± 1.46 ^{cb}	37.13 ± 1.65 ^{bb}	46.02 ± 2.12 ^{ba}

Table 1. Effect of temperature and relative humidity on mechanical properties of smart bilayer films. Values are given as mean ± SD, n = 9. Different letters within the same column indicate significant differences (p < 0.05). Different capital letters within the same row indicate significant differences between temperatures (p < 0.05). TS: tensile strength, EAB: elongation at break.

An increase in relative humidity led to a decrease in tensile strength and an increase in elongation at break of smart bilayer film at the same storage temperature ($p < 0.05$). This decrease in tensile strength with increasing RH is due to higher moisture equilibrium at high RH levels, leading to increased water absorption by the films, causing a plasticizing effect in the film matrix²⁰. These findings are consistent with Kruk et al.²¹ who found that a multilayer chitosan/furcellaran film enriched with carp protein hydrolysate had the highest tensile strength under dry conditions (0% RH) and the lowest under humid conditions (> 80% RH). At 4 °C, the tensile strength of the film without CNs dropped from 57.69 MPa at 0% RH to 15.14 MPa at 80% RH, while the film with CNs showed a decrease from 65.37 to 16.07 MPa under the same conditions. This trend was also observed at 25 °C, where the tensile strength of the film without CNs decreased from 52.15 MPa at 0% RH to 12.58 MPa at 80% RH, and the film with CNs showed a decrease from 57.74 to 14.18 MPa. In contrast, elongation at break increased with higher RH for both types of films at both temperatures. At 4 °C and 80% RH, the elongation at break for the film without CNs was 41.95%, whereas it was 37.13% for the film with 10% CNs. At 25 °C and 80% RH, the elongation at break for the film without CNs was 51.27%, and 46.02% for the film with CNs. The increase in elongation at break in humid environments can be attributed to the plasticizing effect of moisture absorption, which enhances film flexibility but reduces tensile strength, making the film more resistant to deformation or breaking^{20,22}. Dry conditions, characterized by low RH, lead to film dehydration, resulting in a more rigid and brittle structure with increased TS. This duality underscores the significant impact of RH on the mechanical properties of the smart bilayer film, highlighting the trade-off between tensile strength and flexibility influenced by environmental conditions. The addition of CNs significantly improves the TS of the films stored at 0% and 50% RH. However, this improvement was not observed in films stored at 80% RH, where the TS values for films with and without CNs were not significantly different ($p > 0.05$) (Table 1). This pattern was consistent across both temperature conditions, suggesting that the beneficial effects of CNs on TS are diminished under high humidity. The lack of improvement in TS might be due to the hydrophilic nature of cellulose nanospheres (CNs), which can cause moisture absorption and plasticization of the film matrix, weakening the material²³. Additionally, poor dispersion of CNs, weakened interfacial adhesion in humid conditions, or hydrolytic degradation of the matrix or CNs could further limit the expected reinforcement, preventing the anticipated TS enhancement. This reduction in flexibility could be due to the stiffening effect of CNs, which restricts the movement of the polymer chains within the film matrix. However, the films remain relatively flexible, as indicated by the increased elongation at break with rising humidity levels, due to the plasticizing effect of water.

Temperature variations had a notable impact on the mechanical characteristics of the smart bilayer film. Films stored at lower temperatures exhibited significantly higher tensile strength compared to those stored at ambient temperature while elongation at break decreased significantly ($p < 0.05$). This behavior can be attributed to plasticizer migration and water loss, which influence molecular mobility and cross-linking within the films²⁴. Dry, low-temperature environments enhance the film's mechanical strength due to increased intermolecular cross-linking replacing weaker plasticizer-polymer bonds with stronger ones²⁵. However, the increased molecular rigidity at lower temperatures results in reduced elasticity, contrasting with the increased elongation at break observed at higher temperatures. At 25 °C, the highest elongation at break was noted in CNs-free films stored at 80% RH, while the lowest EAB was observed in 10% CNs films at 0% RH and 4 °C. These results highlight the trade-off between tensile strength and elongation at break, influenced by temperature and RH conditions. Enhanced molecular mobility at higher temperatures leads to greater flexibility and higher elongation at break but reduced tensile strength, whereas lower temperatures restrict molecular movement, increasing tensile strength but decreasing elongation at break. This duality underscores the importance of controlling environmental conditions for optimizing the mechanical properties of smart bilayer films.

The mechanical performance of smart bilayer films, as influenced by storage conditions, directly impacts their suitability for various applications, particularly in food packaging. The tensile strength and elongation at break of these films vary with temperature and relative humidity, which affects their functionality and durability. In food packaging applications, these mechanical property variations are crucial. Higher elongation at break with increased relative humidity and temperature could be beneficial for packaging materials that need to adapt to irregular food shapes or dynamic storage conditions. However, reduced tensile strength at higher relative humidity and temperatures may compromise the film's ability to maintain structural integrity, potentially affecting the protection of food products. Therefore, optimizing storage conditions to balance tensile strength and flexibility is essential for ensuring the smart bilayer films' effectiveness in extending shelf life and maintaining food quality. Understanding these dynamics helps in designing packaging solutions that are both functional and resilient under varying environmental conditions.

Water vapor permeability

Water vapor permeability (WVP) describes a film's ability to allow water vapor to pass through, which is crucial for designing effective barriers for moisture-sensitive products²⁶. The water vapor permeability measurements were conducted under two different conditions: 0–50% relative humidity (dry conditions) and 50–100% RH (semi-dry conditions). The impact of storage conditions on the WVP properties of smart bilayer film is displayed in Table 2. At the same temperature, films exposed to dry conditions exhibited significantly lower WVP compared to those tested under semi-dry conditions ($p < 0.05$). Specifically, at 25 °C, the WVP of the film without CNs in dry conditions was three times lower than in semi-dry conditions, and for the film with 10% CNs, it was four times lower. The same phenomena occurred at 4 °C, where the film's moisture barrier properties were superior under dry conditions. This difference is due to higher humidity creating a greater concentration gradient for water vapor diffusion, increasing permeation rates²⁷. Moreover, high humidity can lead to moisture absorption, swelling, and increased WVP, while low humidity decreases WVP by reducing the concentration gradient and limiting water vapor movement²⁸. According to the results, temperatures also affected WVP significantly. The increase in temperature increased WVP values for both types of films ($p < 0.05$). The increase in WVP

Film	RH _(in) –RH _(out)	WVP ($\times 10^{-11}$ g m ⁻¹ s ⁻¹ Pa ⁻¹)	
		4 °C	25 °C
Without CNs	0–50	2.06 \pm 0.19 ^{cB}	4.25 \pm 0.11 ^{cA}
	50–100	4.06 \pm 0.29 ^{aB}	12.67 \pm 0.28 ^{aA}
10% CNs	0–50	1.39 \pm 0.11 ^{dB}	2.34 \pm 0.11 ^{dA}
	50–00	2.98 \pm 0.11 ^{bB}	10.40 \pm 0.29 ^{bA}

Table 2. Effect of temperature and relative humidity on water vapor permeability (WVP) of smart bilayer films. Values are given as mean \pm SD, n = 3. Different letters within the same column indicate significant differences ($p < 0.05$). Different capital letters within the same row indicate significant differences between temperatures ($p < 0.05$).

may be due to enhanced water vapor diffusion driven by increased energy levels of the molecules and greater motion within the polymer matrix^{29,30}. This observation is consistent with Zhang et al.²⁵ who reported increased WVP with rising storage temperatures in soy protein isolate films. These results underscore the importance of considering temperature and humidity when designing films for moisture-sensitive applications, such as food packaging, where effective moisture control is crucial for maintaining product quality and extending shelf life.

The addition of 10% CNs significantly improved the moisture barrier properties of the smart bilayer film across various environments ($p < 0.05$). Films reinforced with CNs exhibited marked reductions in WVP compared to CN-free films. Under dry conditions (0–50% RH), the CNs-reinforced films exhibited notably lower water vapor permeability values compared to the control films without cellulose nanospheres. This reduction in WVP with CNs was also evident under semi-dry conditions (50–100% RH), highlighting the enhanced moisture barrier properties provided by CNs. The addition of CNs contributed to a notable reduction in WVP, highlighting the enhanced moisture barrier properties conferred by cellulose nanospheres across various moisture levels and temperatures³¹. These findings align with Jiang et al.³² who observed a significant decrease in WVP by incorporating 8% cellulose nanocrystals into Zein, Catechin, and β -Cyclodextrin films. The effectiveness of CNs in augmenting the water vapor barrier can be attributed to their nanoscale nature, contributing to a denser and more compact film structure. This structural enhancement impedes the movement of water vapor molecules, resulting in reduced WVP³³. Such findings resonate with prior research supporting the beneficial influence of CNs on improving water vapor barrier properties, underscoring the potential of smart bilayer films as highly efficient moisture barriers. Optimizing film performance in moisture-sensitive applications, such as food packaging, where maintaining proper moisture levels is imperative for preserving product quality and extending shelf life. Understanding the influence of temperature and humidity on WVP is crucial for designing and utilizing films effectively as practical barriers for moisture-sensitive products.

The water vapor permeability of packaging films is a critical factor influencing their effectiveness in real food systems, as it directly impacts moisture control, shelf life, and product quality³⁴. Films with low water vapor permeability are highly beneficial for maintaining the desired moisture levels of food products, preventing both moisture loss and gain. This is essential for preserving the freshness of dry goods such as snacks and cereals, where excessive moisture loss can lead to staleness, while moisture gain can encourage microbial growth and spoilage³⁰. Additionally, packaging films with low WVP contribute to extending the shelf life of perishable items by reducing moisture transfer, which helps maintain the integrity of products like fresh produce, meats, and dairy³⁵. The performance of these films, however, is influenced by environmental factors such as temperature and relative humidity. High humidity can increase the WVP, potentially compromising the barrier properties of the film and leading to moisture absorption and decreased effectiveness. Conversely, films that maintain their low WVP across varying temperature and humidity conditions ensure consistent protection for food products, safeguarding their quality and extending their usability. The incorporation of cellulose nanospheres (CNs) into films has shown promising improvements in moisture barrier properties, making these enhanced films particularly effective for packaging high-moisture or sensitive foods. Thus, understanding and optimizing WVP is crucial for developing packaging solutions that reliably preserve the freshness, texture, and overall quality of food products in diverse storage environments.

Oxygen permeability

Oxygen permeability refers to a film's ability to allow oxygen molecules to pass through, a crucial factor in selecting suitable packaging materials to preserve product quality and extend shelf life³⁶. Table 3 highlights how temperature and relative humidity impact the oxygen barrier properties of smart bilayer films, both with and without cellulose nanospheres (CNs). At 25 °C, RH significantly affects oxygen permeability, with higher humidity levels resulting in increased permeability ($p < 0.05$). Particularly, at 80% RH, the oxygen permeability was highest, with values of 293.3 and 179.0 ($\times 10^{-19}$ mol m⁻¹ s⁻¹ Pa⁻¹) for films without CNs and with 10% CNs, respectively. This increase is attributed to moisture absorption, which can cause swelling or plasticization of the film, leading to a larger free volume and consequently higher oxygen permeability³⁷. Conversely, low humidity can lead to film desiccation and embrittlement, potentially reducing barrier properties.

The effect of temperature on the oxygen permeability of the film was investigated at 4 and 25 °C in conditions with 0% RH (Table 1). Temperature also affects oxygen permeability, though to a lesser extent than relative humidity. At 4 °C, the oxygen permeability of films was generally higher than at 25 °C, although this difference was not statistically significant ($p > 0.05$). As temperature rises, the kinetic energy of gas molecules increases.

Film	Relative humidity (%)	Oxygen permeability ($\times 10^{-19} \text{ mol m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}$)	
		4 °C	25 °C
Without CNs	0	2.76 \pm 0.27 ^a	6.89 \pm 0.01 ^d
	50	ND	51.97 \pm 2.56 ^c
	80	ND	293.33 \pm 31.77 ^a
10% CNs	0	1.33 \pm 0.32 ^b	6.61 \pm 0.21 ^d
	50	ND	27.20 \pm 1.47 ^d
	80	ND	179.00 \pm 13.11 ^b

Table 3. Effect of temperature and relative humidity on the oxygen permeability of smart bilayer film. Values are given as mean \pm SD, n = 3. Different letters within the same column indicate significant differences ($p < 0.05$). ND = not determined.

This leads to enhanced molecular movement and diffusion rates, resulting in higher oxygen permeability. The film matrix may also soften or become more flexible at higher temperatures, further increasing the free volume available for oxygen transport³⁸. In contrast, lower temperatures reduce the kinetic energy of gas molecules, leading to slower diffusion rates and decreased oxygen permeability. Additionally, the film may become more rigid or brittle at lower temperatures, which can further restrict the movement of oxygen molecules through the film. In conclusion, temperature variations affect the rate of oxygen diffusion through the film. However, relative humidity often has a more pronounced effect on oxygen permeability compared to temperature.

The incorporation of CNs significantly enhances the oxygen barrier properties of the film ($p < 0.05$). The oxygen permeability of films without CNs ranges from 2.76 to 293.33 ($\times 10^{-19} \text{ mol m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}$), whereas films containing 10% CNs exhibit a lower range from 1.33 to 179 ($\times 10^{-19} \text{ mol m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}$). The incorporation of CNs into films has been shown to significantly reduce oxygen permeability, thereby enhancing the film's barrier properties. Cellulose nanospheres contribute to this improvement by creating tortuous pathways within the film matrix, which increases the diffusion distance for oxygen molecules and impedes their movement³⁹. Additionally, cellulose nanospheres improve the packing density of the film, resulting in reduced free volume and further limiting oxygen diffusion⁴⁰. Moreover, cellulose nanospheres mitigate the plasticizing effects of absorbed moisture, which can otherwise soften or swell the film and increase permeability. Collectively, the incorporation of cellulose nanospheres effectively enhances the film's ability to resist oxygen transmission, making it more suitable for applications requiring robust barrier performance.

Films with varying oxygen permeability values are suitable for packaging different types of food products, depending on their sensitivity to oxygen exposure. Films without cellulose nanospheres exhibit higher oxygen permeability, ranging from 6.89 to $293.33 \times 10^{-19} \text{ mol m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}$, and are generally better suited for foods that are less sensitive to oxygen, such as packaged snacks, dried foods, and some frozen items⁴¹. In contrast, films containing 10% CNs have a lower oxygen permeability range, from 6.61 to $179.00 \times 10^{-19} \text{ mol m}^{-1} \text{ s}^{-1} \text{ Pa}^{-1}$, making them more effective at reducing oxygen exposure and thus better suited for more sensitive food products³¹. Specifically, these films with cellulose nanospheres are ideal for packaging fresh meat, poultry, seafood, and fresh fruits and vegetables, which require lower oxygen permeability to extend shelf life and maintain freshness⁴². The choice of film should align with the oxygen sensitivity of the food product and its specific preservation needs.

Water contact angle

The water contact angle is a crucial parameter for evaluating the wetting behavior of polymers and coatings used in food product preservation, protection, or enhancement. This angle represents the degree of tilt between a water droplet and the surface of a material, providing insights into its wettability⁴³. Surfaces with low contact angle values ($< 20^\circ$) are considered highly wettable, while those with high contact angle values ($> 90^\circ$) are considered hydrophobic⁴⁴. The water contact angle values of smart bilayer films, both without CNs and with 10% CNs, are presented in Table 4. The water contact angle measurements were performed on both sides of the film to assess water absorption after 60 s. On the sodium alginate side, the water contact angle was recorded as 51.6° and 53.2° for films without and with 10% CNs, respectively. A decrease in water contact angle after 60 s on the sodium alginate side indicated water absorption. Remarkably, films with CNs exhibited superior water resistance, evidenced by significantly higher water contact angle after 60 s ($p < 0.05$). This finding highlights the effectiveness of CNs in enhancing the water resistance of the film. The result aligns with Ma et al.⁴⁵ who reported increased water contact angles with higher cellulose content in the polyvinylidene fluoride (PVDF) membrane, indicating decreased hydrophilicity. Despite cellulose's inherent hydrophilicity, the close electrostatic association and hydrogen bonding between nanocellulose and polymer molecules contribute to overall hydrophobicity⁴⁶. The agar or bottom side of the bilayer film demonstrated superior water resistance, with a significantly higher water contact angle ($p < 0.05$). Water contact angle values of the agar side ranged from 92.0° to 95.2° for films without and with 10% CNs, indicating hydrophobic surfaces. Although a slight decrease in water contact angle occurred after 60 s, the values remained close to 90° . Hydrophobic properties are highly desirable in packaging applications, offering versatile use⁴⁷. For smart bilayer film applications, utilizing the AG side with a high-water contact angle on the inner layer of food packaging can impart exceptional moisture resistance, leading to extended shelf life, enhanced freshness, and superior protection for packaged food products.





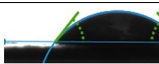
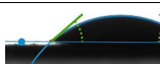


Film	Film side	Contact angle (°)	
		0 s	60 s
Without CNs	SA	 51.6±1.5 ^{Cc}	 39.4±1.6 ^{Cd}
	AG	 92.0±1.6 ^{Ba}	 83.9±2.5 ^{Ab}
10% CNs	SA	 53.2±0.5 ^{Cc}	 43.5±2.8 ^{Bd}
	AG	 95.2±2.3 ^{Aa}	 86.0±2.6 ^{Ab}

Table 4. Water contact angle of smart bilayer films containing 0 and 10% CNs at 0 and 60 s. Values are given as mean ± SD from n = 5. Different upper-case letters indicate a significant difference ($p < 0.05$) between different films at the same time. Different lower-case letters indicate a significant difference ($p < 0.05$) between different times for the same film. SA: sodium alginate; AG: agar.

Color stability and response to pH change of the film

The anthocyanin-enriched smart bilayer film plays a crucial role in monitoring food freshness through its color-changing properties. This characteristic is highly valuable across various sectors, including the food industry, pharmaceuticals, and environmental monitoring, where color stability is essential⁴⁸. pH indicator films that shift color in response to acidity or alkalinity changes offer a straightforward and visual method for pH detection and measurement. Ensuring the color stability of these films is crucial for maintaining the accuracy and reliability of pH measurements. Figure 1 displays the relative color change (ΔE) of the smart bilayer film over a 14-day storage period at 4 °C and 25 °C, under various relative humidity (RH) conditions. The ΔE values increased over time, indicating a decline in color stability, due to the oxidative decomposition of anthocyanin. Films stored at 4 °C (Fig. 1A) exhibited lower ΔE values compared to those stored at 25 °C (Fig. 1B), indicating superior color stability at the lower temperature. Additionally, the smart bilayer film with 10% CNs showed greater color stability than the film without CNs, regardless of storage temperature or relative humidity levels. The results suggest that CNs effectively slow color changes in the smart bilayer film. CNs provide stability by acting as a physical barrier against oxidative factors like oxygen and light, thus protecting anthocyanin from degradation. They also function as antioxidants, reducing anthocyanin's oxidative decomposition, and their hydrophobic nature reduces moisture absorption, which is crucial under high humidity conditions that adversely affect color stability. Additionally, CNs' controlled release properties help sustain anthocyanin within the film, ensuring a more uniform and gradual color expression⁴⁹.

At 4 °C, films without CNs showed ΔE values of 3.03, 3.15, and 3.90 at 0%, 50%, and 80% RH, respectively. Films with CNs had lower ΔE values of 2.62, 2.92, and 3.31 under the same conditions. At 25 °C, films without CNs exhibited higher ΔE values of 4.66, 5.53, and 6.50 at 0%, 50%, and 80% RH, respectively. In contrast, films with 10% CNs showed better color stability with ΔE values of 3.02, 4.48, and 4.99 under the same conditions. Throughout the storage period, the ΔE values of films stored at 4 °C under all relative humidity levels remained below 5, indicating high color stability. Generally, when the ΔE value of an indicator is below 5, the color change is difficult to notice with the naked eye⁵⁰. The results align with Zhu et al.⁵¹ who reported that the ΔE of a cassava starch/polyvinyl alcohol film incorporating anthocyanin, stored at 4 °C with 60% RH, remained below 5. The high stability of the indicator film indicates strong interaction among its components, with good physical properties creating a stable environment that protects the indicator from damage and enhances color stability⁵¹. The high stability of the indicator film indicates strong interaction among its components. Additionally, good physical properties contribute to creating a relatively stable environment for the film, protecting the indicator from damage and enhancing color stability⁵¹. The ΔE value of the smart bilayer film with 10% CNs, stored at 25 °C under various relative humidity levels, was higher than that of the sample tested at 4 °C, but still below 5. However, for the film with 0% CNs, the ΔE value exceeded 5 at 50% and 80% RH at 25 °C. The comparatively lower stability of the indicator pigment in the latter case was attributed to the accelerated oxidation reaction rate at higher temperatures⁵². Higher temperatures accelerate the degradation rate, which can lead to a loss of color stability in the indicator film¹². Therefore, storing anthocyanin indicator films at controlled temperatures is essential to minimize the risk of color degradation. The relationship between relative humidity and the film color change has been observed, as increasing humidity can influence the color change of films, with and without the addition of CNs. Humidity level also plays a role in the color stability of anthocyanin indicator films. Highly humid environments introduce moisture, which can trigger hydrolysis or oxidation of anthocyanins, leading to color changes or fading. Additionally, moisture can facilitate the growth of microorganisms that could degrade

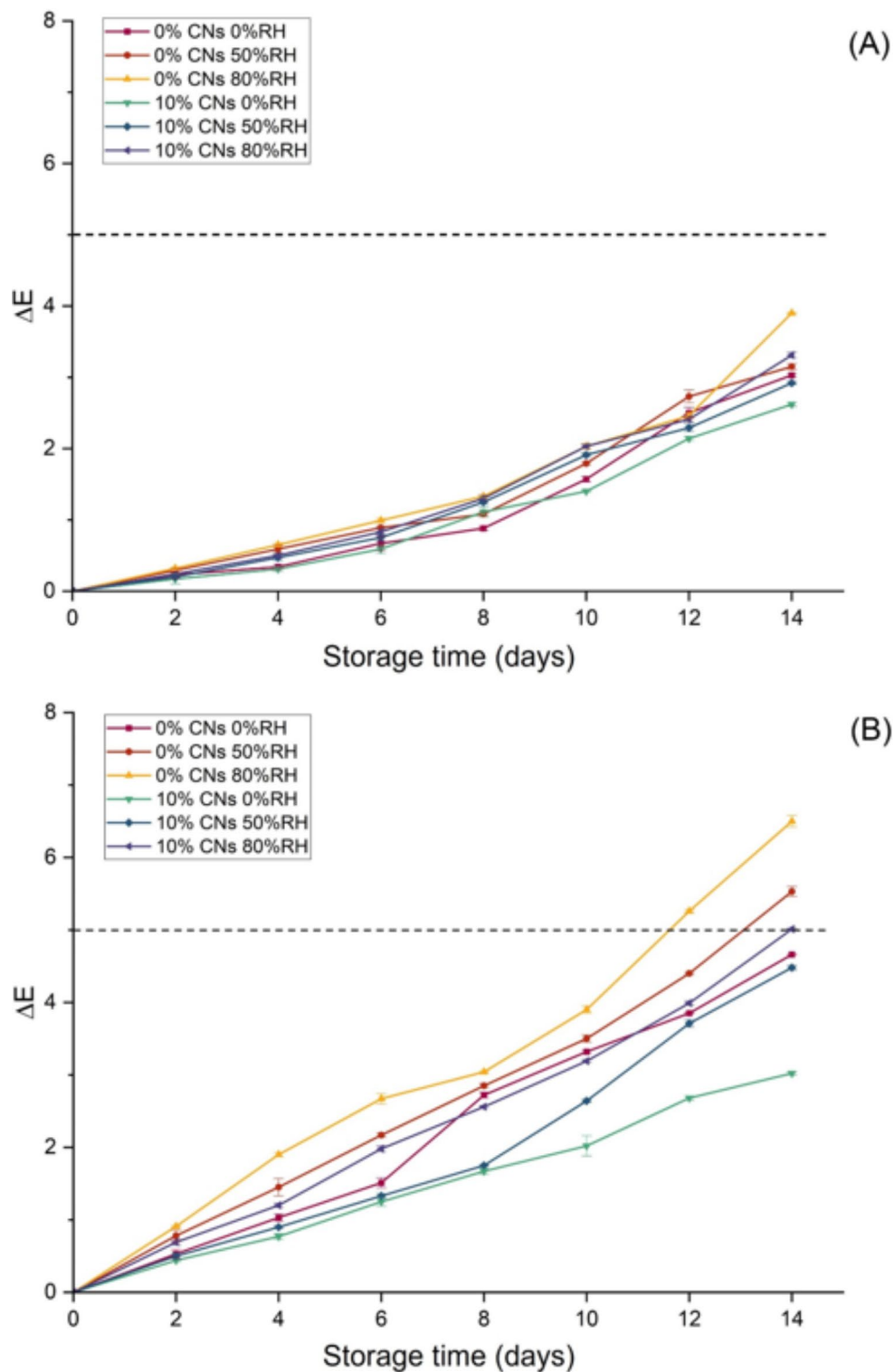


Fig. 1. The relative color change (ΔE values) of the smart bilayer films stored at 4 °C (A) and 25 °C (B) with different relative humidity for 14 days. Color difference distinguished by a naked eye ($\Delta E > 5$), dashed line.

the color stability of the indicator film. Proper humidity control during storage or application of the film is crucial to preserve color stability and ensure accuracy pH response.

The response of the smart bilayer film to different pH buffers was evaluated both before and after a 14-day storage period. Regardless of storage conditions (temperature and relative humidity), film samples demonstrated their ability to change color in response to different pH levels. The films were tested under different temperatures (4 and 25 °C) and exposed to different levels of relative humidity (0, 50, and 80%) (data not shown). The response

to pH changes of the smart bilayer film, stored at 4 and 25 °C with 80% RH for 14 days, is shown in Fig. 2. After 14 days of storage, the film displayed color changes corresponding to the pH levels, albeit with reduced color intensity. These results were noticed under all storage conditions (data not shown). The reduced color intensity observed in the films after the 14 days of storage is related to the color changes presented in Fig. 1. In conclusion, temperature and humidity significantly affect the color stability of anthocyanin indicator films, influencing the degradation and stability of anthocyanin pigments. Understanding and controlling these environmental factors are crucial for preserving anthocyanin indicator films' accuracy and color stability and ensuring their reliability and efficacy in pH sensing.

Conclusion

This study investigated the impact of different storage conditions on the properties and stability of smart bilayer films to ensure their resistance to various environments and retention of color stability for future applications as pH indicators in real food products. The results demonstrate that relative humidity had a more significant impact on the mechanical strength and barrier properties of the films compared to temperature variations. High humidity conditions led to a notable reduction in these attributes, underscoring the importance of controlling moisture exposure during storage and handling. Interestingly, temperature emerged as the more dominant factor influencing color stability, with both types of smart bilayer films, with and without cellulose nanospheres, maintaining stable colors over the 14-day storage period at 4 °C. The incorporation of cellulose nanospheres in the film formulation provided a significant advantage, significantly improving the mechanical strength, oxygen and water vapor barrier properties, and color stability compared to films without CNs. These findings have important implications for the development and application of smart bilayer films as pH indicators in real food products. The ability to withstand various environmental conditions while retaining their color-changing capabilities is critical for ensuring the reliability and effectiveness of these films as monitoring tools for food quality and safety. The insights gained from this study can inform the design and optimization of smart packaging solutions that can accurately track pH changes and provide valuable information to consumers and producers. In conclusion, this research has demonstrated the significant impact of storage conditions on the performance and stability of smart bilayer films for pH indicator applications. By understanding the role of factors such as humidity and temperature, researchers and manufacturers can develop more robust and reliable smart packaging solutions that can effectively monitor food quality and freshness in a wide range of real-world scenarios.

Given these findings, future research should explore the long-term stability of these films under extended storage conditions to assess their durability. Additionally, evaluating their environmental sustainability, particularly their biodegradability and recyclability, will be essential for aligning with sustainable packaging goals. Further optimization of the film composition, including the integration of other functional materials or alternative biopolymers, could enhance their performance. Real-world testing in food packaging scenarios will also be necessary to validate their practical application in monitoring pH changes. By addressing these

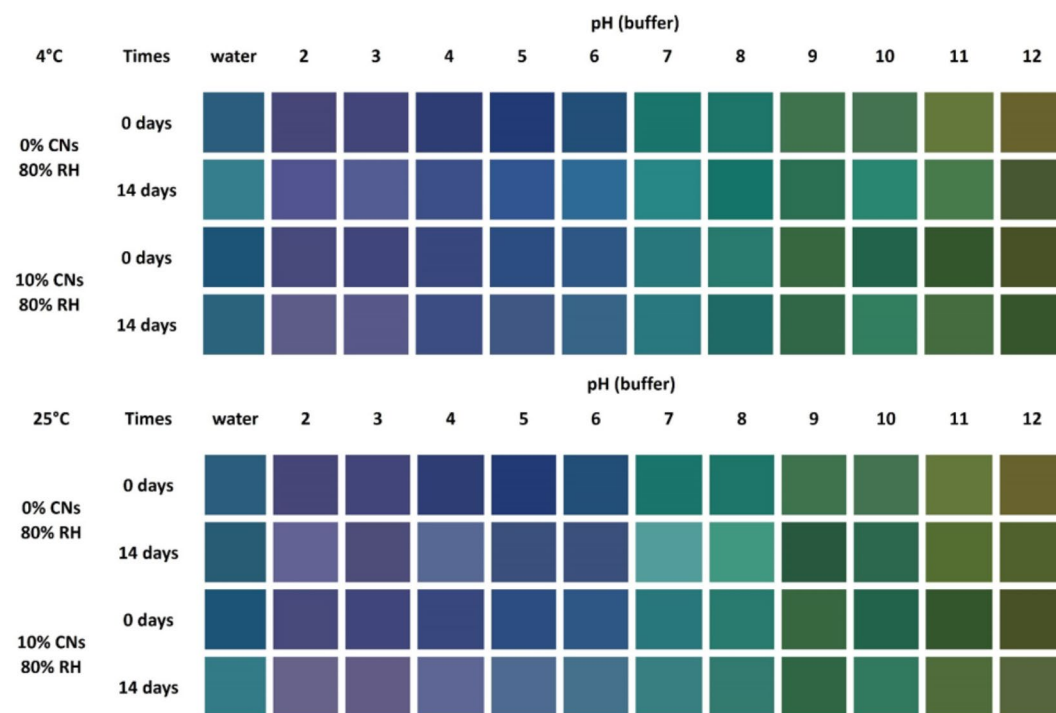


Fig. 2. pH sensitivity of smart bilayer film before and after storage at 4 °C and 25 °C with 80% relative humidity for 14 days.

future research directions, the development of smart bilayer films can progress toward more robust, reliable, and sustainable solutions for monitoring food quality and safety.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

Orapan Romruen: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. Pimonpan Kaewprachu: Writing – review & editing, Visualization. Samart Sai-Ut: Writing – review & editing, Visualization. Passakorn Kingwascharapong: Writing – review & editing, Visualization. Thomas Karbowski: Conceptualization, Methodology, Supervision, Writing – review & editing, Visualization. Wanli Zhang: Visualization. Saroat Rawdkuen: Supervision, Writing – review & editing, Visualization, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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