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1 **Bilayer pH-sensitive colorimetric films with light-blocking ability and**
2 **electrochemical writing property: application in monitoring crucian spoilage in**
3 **smart packaging**

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19

20 **Abstract**

21 Bilayer colorimetric films were developed for monitoring fish spoilage by using gelatin (GN)
22 incorporated with ZnO nanoparticles as the upper layer (GN-ZnO), and gellan gum (GG) incorporated
23 with mulberry anthocyanins (MBA) as the lower layer (GG-MBA). The color stability of the bilayer
24 colorimetric films under visible and ultraviolet light was improved with the increase of ZnO
25 nanoparticles content. Meanwhile, the bilayer films had good NH₃ sensitivity. The limit of detection of
26 the GG-MBA/GN-2.0% ZnO film to NH₃ was 0.01 mM. The electrochemical writing ability of the
27 bilayer films was also identified, indicating the feasibility of inks-free printing on biopolymer films.
28 Finally, the GG-MBA/GN-2.0% ZnO film with an electrochemical writing pattern was used to monitor
29 crucian spoilage. The GG-MBA/GN-2.0% ZnO film with electrochemical writing pattern showed visible
30 color changes with the crucian spoilage. In conclusion, the bilayer colorimetric film was expected to be
31 a good fish spoilage indicator in smart packaging.

32 **Keywords:** Bilayer colorimetric film; Gelatin; Gellan gum; ZnO nanoparticles; Mulberry anthocyanins;
33 Electrochemical writing

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37 Introduction

38 Safety and quality control of foods have attracted significant global attentions (Valdez, Gupta,
39 Lozano, & Mao, 2019). It is well-known that fish is popular with consumers owing to high protein
40 content and nutritional qualities. However, it is perishable due to enzymatic reactions and microbial
41 contamination (X. Zhang, Sun, Xiao, Liu, & Zheng, 2016). Volatile amines, such as trimethylamine,
42 dimethylamine, and ammonia (NH₃), are the degradation products of amine acids and proteins due to
43 microbial growth, and generally known as total volatile basic nitrogen (TVB-N). TVB-N levels have
44 been widely used as the indicator of fish spoilage (Zhai, Shi, Zou, Wang, Jiang, Zhang, et al.,
45 2017). Therefore, the sensitive detection of TVB-N is critical for evaluating the quality and safety of fish
46 products (Erim, 2013). In addition, to provide essential manufacturing information to consumers, most
47 food packaging materials are labeled with inks that mainly consist of petrochemical feedstock, which
48 can potentially contaminate the food and environment, and even endanger human health (Patel, 2015;
49 Wu, Wang, Yan, Ding, Shi, Deng, et al., 2018). Thus, there is a great demand for developing edible labels
50 to minimize food safety risk.

51 Recently, many studies have concentrated on developing smart packaging for tracing food quality.
52 In particular, pH-sensitive colorimetric films have been widely explored, because they can present color
53 changes when exposed to volatile gases, such as biogenic amines generated from foods (Huang, Xiong,
54 Zou, Dong, Ding, Liu, et al., 2019). Generally, a pH-sensitive colorimetric film is composed of solid
55 supports and dyes (Pourjavaher, Almasi, Meshkini, Pirsá, & Parandi, 2017). In recent years,
56 anthocyanins, as edible natural pigments, have been used to develop colorimetric films due to their non-
57 toxic and pH-sensitive properties (Choi, Lee, Lacroix, & Han, 2017; Huang, et al., 2019; Jr, Arruda, &
58 Stefani, 2015; Zhai, et al., 2017). To immobilize the anthocyanins, various polymeric compounds have
59 been utilized as solid supports (Choi, Lee, Lacroix, & Han, 2017; Huang, et al., 2019; Wei, Cheng, Ho,
60 Tsai, & Mi, 2017; Zhai, Li, Zhang, Shi, Zou, Huang, et al., 2018). The color stability of anthocyanins to
61 ultraviolet (UV) light, temperature, and oxygen are important for the colorimetric films in practical
62 application (Bąkowska, Kucharska, & Oszmiański, 2003; Xuran Cai, Du, Cui, Wang, Yang, & Zhu, 2019).
63 Particularly, UV light showed a significant influence on the stability of anthocyanins over other factors
64 (Bąkowska, Kucharska, & Oszmiański, 2003; Tonon, Brabet, & Hubinger, 2010). Therefore, it is
65 essential to improve the stability of anthocyanins embedded in colorimetric films.

66 Nano metal oxides have attracted much attention in the field of smart packaging materials due to
67 their excellent biocompatibility and functional property (Xuechao Cai, Xie, Li, Kassymova, Zang, &
68 Jiang, 2020; Chandra, Kumari, Bontempi, & Yadav, 2020; Varma, 2012). Zinc oxide (ZnO) is an
69 inorganic compound and is currently listed as a generally safe (GRAS) material by the Food and Drug
70 Administration (21CFR182.8991). It has efficient UV absorptivity resulting from its wide bandgap (E_g
71 = 3.37 eV, corresponding to 376 nm UV light). Moreover, gelatin (GN) has been widely used as a film-
72 forming agent because of its out-standing gel formability (Hosseini & Gómez-Guillén, 2018; Quero,
73 Padilla, Campos, Luengo, Caballero, Melo, et al., 2018). Our previous work revealed that multicolor
74 patterns could be marked on the protein/polysaccharide/anthocyanin composite films by electrochemical
75 writing (Zhai, et al., 2018). The electrochemical writing process based on the pH-responsive color change
76 of anthocyanins provides a green printing method for presenting the essential information of the product,
77 such as the storage information and nutritional facts of the food in a safe fashion. However, to the best
78 of our knowledge, there are few studies on printing on biopolymer films (Wu, et al., 2018; Zhai, Li, Shi,
79 Huang, Sun, Zhang, et al., 2019).

80 The aims of this study were: (1) to design a bilayer colorimetric hydrogel film with UV light

81 blocking capability and electrochemical writing properties and (2) to monitor the spoilage of crucian fish
82 in real-time. ZnO nanoparticles were incorporated in gelatin (GN) as the upper layer to improve the light-
83 blocking property of the film. The lower layer of the film consisted of gellan gum (GG) and anthocyanin
84 extracted from mulberry fruits (MBA), which is a traditional anthocyanin-rich fruit (Zeng, Chen, Qin,
85 Zhang, Wang, Wang, et al., 2019). GG is a natural extracellular polysaccharide produced by fermentation
86 of *Sphingomonas paucimobilis*, which has been widely used as an edible food additive and in polymer
87 films (Amin & Panhuis, 2011; Wei, Cheng, Ho, Tsai, & Mi, 2017). The GG-MBA composite with NH₃
88 sensitivity was used as the lower layer to indicate fish freshness.

89 **2. Material and methods**

90 **2.1. Materials and Reagents**

91 Mulberry fruits (*Morus nigra L*), and Crucian (*Carassius carassius*) were obtained from a local
92 supermarket (Zhenjiang, China). Other chemical agents, such as low-acyl gellan gum, gelatin, ZnO
93 nanoparticles, ammonium hydroxide, and ethyl alcohol were purchased from Sinopharm Chemical
94 Reagent Co., Ltd. (Shanghai, China).

95 **2.2. Anthocyanins extraction and its response to pH variation**

96 The anthocyanins in mulberry fruits were extracted according to our previous method (Yang, Zou, Li,
97 Huang, Zhai, Zhang, et al., 2019). Firstly, the dried mulberry fruits were ground with an electric coffee
98 mill. The powder was prepared in 60% ethanol aqueous solution with a solid-liquid ratio of 1:50 and
99 stirred for 30 min by using a magnetic stirrer. Then the extract solution was filtered through a 25- μ m
100 filter paper, concentrated using a vacuum rotary evaporator and freeze-dried to obtain the extract powder.
101 The obtained MBA extract powder was stored at 4 °C in brown air-tight plastic bottle. The spectra at
102 400-800 nm of MBA solutions under pH 2-12 were determined by using the UV-Vis spectrophotometer
103 (GNilent CARY 100, Varian Corporation, USA). The pH of MBA solutions was adjusted by using 0.2 M
104 disodium hydrogen phosphate, 0.2 M citric acid and 0.2 M sodium hydroxide solutions with different
105 proportions.

106 **2.3. Development of bilayer films**

107 The solution of GN was prepared by dissolving 2 g of GN powder in 100 mL distilled water while heating
108 at 80 °C in a water bath under magnetic stirring for 1 h. Thereafter, different amounts of ZnO
109 nanoparticles were added to the GN solution at 0.5 mg ZnO/g GN, 1 mg ZnO/g GN and 2 mg ZnO/g GN,
110 expressed as GN-0.5% ZnO, GN-1.0% ZnO, and GN-2.0% ZnO. Subsequently, the mixture was
111 homogenized (Ultra Turrax IKA T25 digital, Germany) at 8000 rpm for 5 minutes and degassed for 5
112 minutes at 80 °C using a sonicator (Branson CPX 5800H, USA). After degassing, 6 g of the GN-ZnO
113 solution was instantly poured into a plastic Petri dish as the upper layer of the bilayer hydrogel. The
114 solutions formed hydrogels after cooling.

115 The solution of GG was prepared by dissolving 2 g of GG powder in 100 mL distilled water at 95 °C
116 under magnetic stirring for 1 h. 4 mg of MBA powder was then added to the GG solution (65 °C). Under
117 this constant temperature, 40 mg of CaCl₂·2H₂O was completely dissolved into the solution with constant
118 stirring. The GG-MBA mixture was degassed with a sonicator, and then 6 g of GG-MBA solution was
119 instantly poured over the GN-ZnO layer hydrogel. The GG-MBA solution formed to be the lower layer
120 of the bilayer hydrogel after cooling.

121 Meanwhile, the bilayer hydrogels containing GG, GN, and CaCl₂·2H₂O, or containing GG, GN,
122 CaCl₂·2H₂O, and MBA were also prepared, following the same process. The bilayer hydrogels containing
123 MBA were selected for electrochemical writing. The cooled bilayer hydrogels were placed on a platinum
124 (Pt) plate connected with the cathode of an electrochemical analyzer (CHI660E, CH Instruments Co.,

125 Shanghai, China). Then, a 0.5-mm Pt needle connected to the anode of the electrochemical analyzer was
126 contacted the surface of the GG-MBA hydrogel. The electrochemical writing process was performed
127 according to our previous method (Zhai, et al., 2018). All bilayer hydrogels were heated in an oven at
128 40 °C for 4 h to form films, and the films were placed at 4 °C with 75% relative humidity (RH) before
129 use.

130 **2.4. Characterization of the films**

131 The cross-section of the films was observed under a field emission scanning electron microscopy (FE-
132 SEM) (S-4800, Hitachi High Technologies Corporation, Japan) coupled with energy dispersive X-ray
133 (EDX). The films were freeze fractured by liquid nitrogen for cross-section observation. Then, they were
134 attached to double-sided conductive adhesive tape and mounted on the specimen holder. Finally, the films
135 were coated with gold under vacuum. The optical transmittance of the films was measured at 200-800
136 nm using the UV-Vis spectrophotometer.

137 **2.5. Color stability**

138 To simulate the indoor and outdoor light conditions, the films were stored in the incubator at 25 °C with
139 75% RH under fluorescent lights (~ 400-760 nm) and UV light ($\lambda = 365$ nm, 100 W, Spectroline SB-
140 100P, Sylvania, USA), respectively. The photos of the films were captured using an optical scanner
141 (Scanjet G4050, HP) and analyzed using a user program in Matlab R2012a (Matworks Inc., Natick, MA,
142 USA). The stability of the films was defined as the relative color change (S), according to our previous
143 study (Xiao-wei, Xiao-bo, Ji-yong, Zhi-hua, & Jie-wen, 2018).

$$144 \Delta R = |R_0 - R_I| \quad (1)$$

$$145 \Delta G = |G_0 - G_I| \quad (2)$$

$$146 \Delta B = |B_0 - B_I| \quad (3)$$

$$147 S(\%) = \frac{\Delta R + \Delta G + \Delta B}{R_0 + G_0 + B_0} \times 100 \quad (4)$$

148 where R_0 , G_0 , B_0 were the initial gray values of the red, green and blue, R_I , G_I , and B_I were the gray
149 values of the red, green and blue after storage. S was the relative color change of R , G and B values.

150 **2.6. NH₃-sensing ability**

151 The NH₃ was used to determine the color response of the film towards basic gas. Briefly, the films (10×10
152 mm) were placed in an Erlenmeyer flask (500 mL) containing different concentrations of ammonia
153 solution for 30 min at 25 °C. The images of the films were captured using the optical scanner, and the S
154 value of the films was measured as described in Section 2.5. The spectra of the films at 400-800 nm were
155 documented by means of the UV-Vis spectrophotometer.

156 **2.7. Application of films in monitoring fish spoilage**

157 For the fish spoilage test, 200 g of crucian, after removing its tail, innards and scales, was put into a
158 polyethersulfone resin (PES) box with a lid. The hole (15 × 15 mm) on the lid was covered by using a
159 piece of GG-MBA/GN-ZnO film (20 × 20 mm) with a written pattern. The PES box was placed in an
160 incubator at 4 °C with 75% RH. The TVB-N content of the crucian was measured according to our
161 previous method (Zhai, et al., 2018), following the Chinese standard (GB 5009.228-2016).

162 **2.8. Statistical analysis**

163 Duncan's multiple range test was used to compare the means at the 95% confidence level by using SPSS
164 statistics software. The data labeled with different lowercase letters are significantly different.

165 **3. Results and discussion**

166 **3.1. Color and spectral properties of MBA**

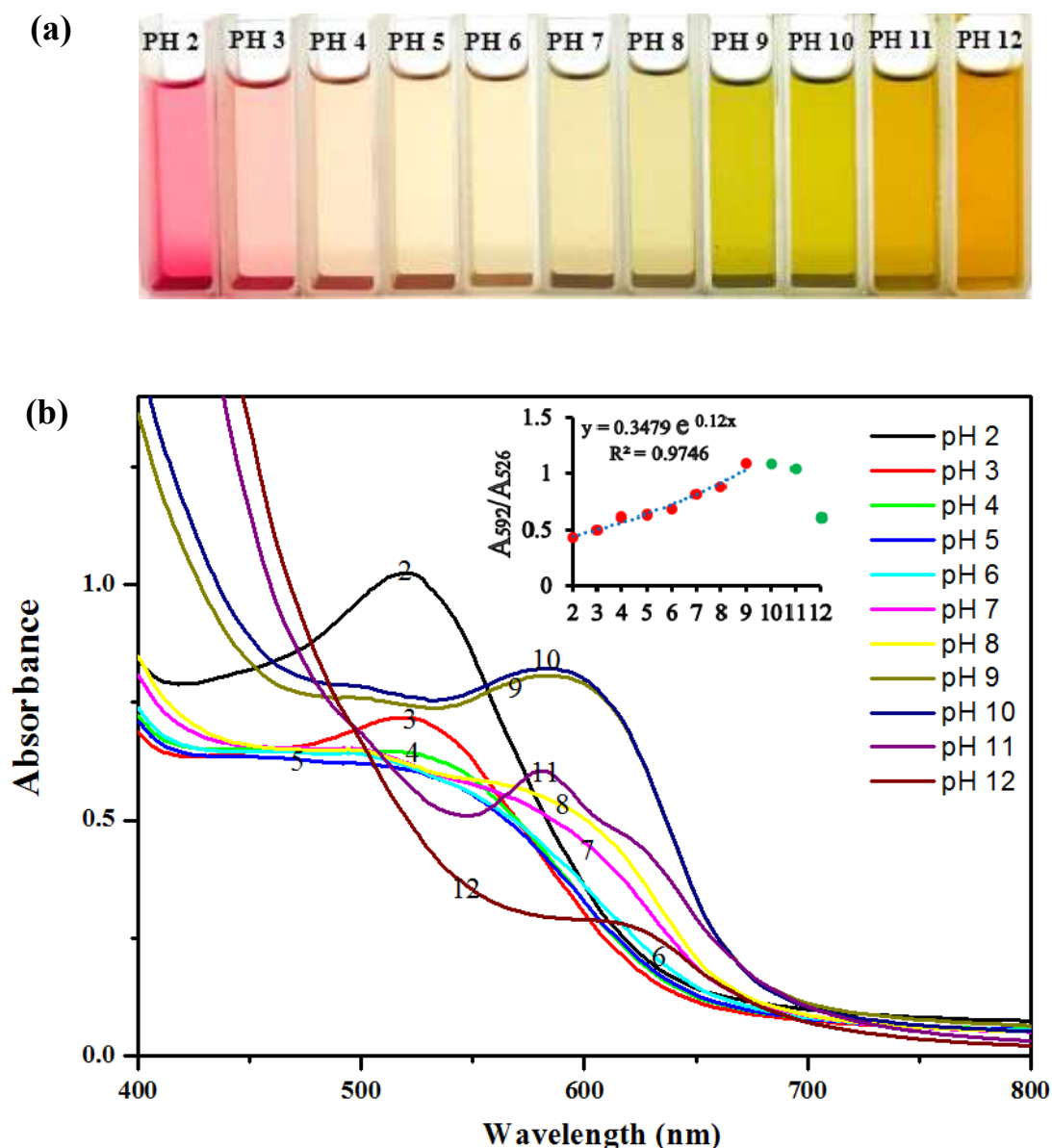
167 Fig. 1a shows the color changes of MBA solution under different pH conditions. The MBA solution

168 presented a remarkable color change from light pink to colorless at pH 2-6. When the pH increased from
169 7 to 10, the color of the MBA solution changed gradually from light green to yellow-green and presented
170 an orange color at pH 11-12. The corresponding UV-Vis spectra of the MBA solution in different pH
171 buffer solutions are shown in Fig. 1b. When the pH increased from 2 to 6, the maximum absorption peak
172 (MAP) of the MBA solution at 526 nm gradually decreased, and then underwent a shift from 526 nm to
173 592 nm when the pH increased from 6 to 7. The MAP at 592 nm gradually increased when the pH
174 increased from 7 to 10, and then decreased sharply at pH 10-12. The intensity of green compared to red
175 color could be expressed by the absorbance ratio at 592 nm versus 526 nm (A_{592}/A_{526}). The inset in Fig. 1b
176 shows an exponential relationship between A_{592}/A_{526} and pH 2-9. Expressing the following formula:

$$177 \quad y=0.3479e^{0.12x} \quad (5)$$

178 where x is the pH and y is the absorbance ratio at 592 nm versus 526 nm:

179 The value of A_{592}/A_{526} increased when the pH increased from 2 to 9, indicating a deeper green color. The
180 values decreased with the pH increased from 10 to 12. Similarly, Zhai, et al. (2018) also reported that the
181 absorbance ratio of red radish anthocyanins had good exponential relationships with the pH values among
182 2 to 9, and then the values decreased with the pH increase from 10 to 12. These results indicated that
183 MBA could be used as the natural pH-sensitive pigments for colorimetric films.

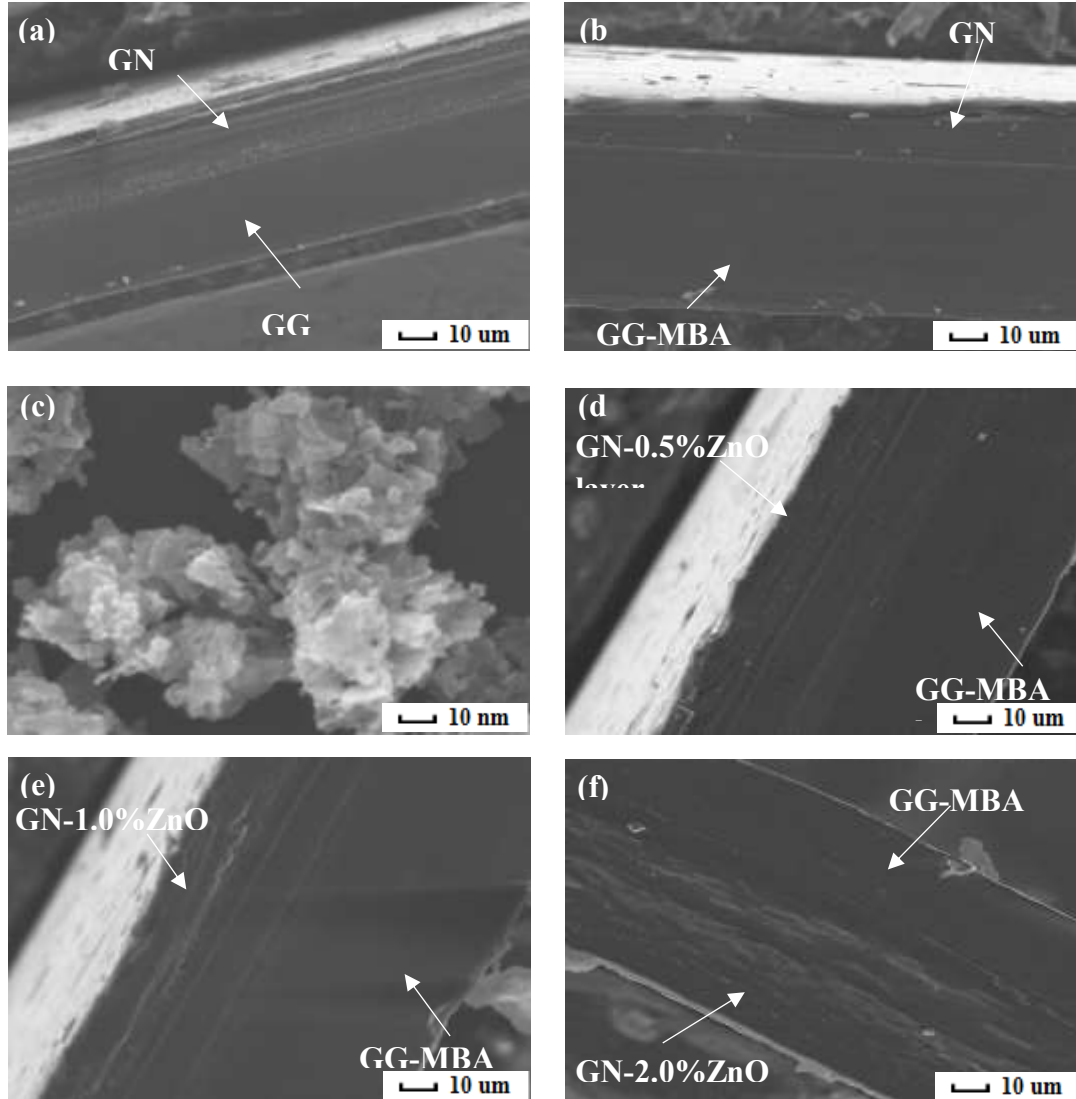


184 Figure. 1. Color changes (a) and UV-Vis spectra of MBA solutions at different pH (b).

185 **3.2. Morphological and EDX analysis**

186 The cross-section of the GG layer was comparatively smooth and homogeneous, while the GN layer was
 187 less homogeneous with some cracks (Fig. 2a). A bilayer structure could be clearly seen from the GG/GN
 188 film. This could be due to GG molecules and calcium ions form a stable gel structure, making GG
 189 immiscible with GN during the thermal drying process. The GG and GN layer represented a continuous
 190 matrix with good structural integrity, and there was no obvious gap between the two layers. This could
 191 be due to that GN and GG can be partially cross-linked through hydrogen bonding force. Fig. 2b shows
 192 that the cross-section of the GG layer altered little with the addition of the MBA. The sheet polyhedral
 193 shapes ZnO nanoparticles were agglomerated to clusters as observed in the FE-SEM (Fig. 2c). Fig. 2d
 194 shows that little granular protrusion was observed in the cross-section of GG-MBA/GN-0.5% ZnO,
 195 indicating that the ZnO nanoparticles were well distributed and have good miscibility with the GN. With
 196 the increase of ZnO content, the GN-ZnO layer indicated some irregularities representative of semi-
 197 crystalline structures (Fig. 2e and 2f), which are related to the presence of the degree of crystallinity of
 198 ZnO nanoparticles. All the GG-MBA/GN-ZnO films showed that the interface of the GG-MBA layer

199 and GN-ZnO layer maintained well-crosslinked, and the ZnO nanoparticles in GN-ZnO layer did not
200 move to the GG-MBA layer. The EDX analysis was also performed to identify the element composition
201 of the bilayer film (Fig. S1). The signal related to Zn and O was found in the spectrum, which confirmed
202 the presence of ZnO in the bilayer film. These results revealed that we developed the GG-MBA/GN-ZnO
203 bilayer films with satisfactory distribution and integrity.



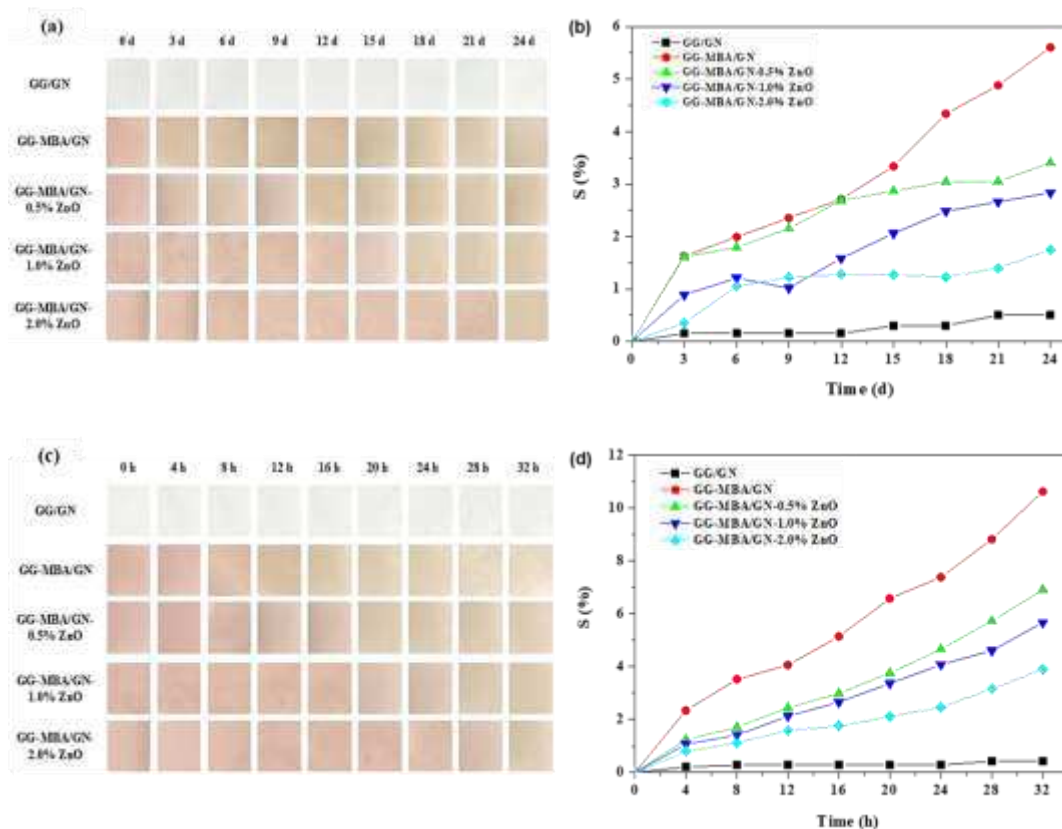
204
205 Figure. 2. SEM images of cross section of GG/GN (a), GG-MBA/GN (b), ZnO nanoparticles (c), GG-
206 MBA/GN-0.5% ZnO (d), GG-MBA/GN-1.0% ZnO (e) and GG-MBA/GN-2.0% ZnO film (f).

207 3.3. Optical property and color stability of the bilayer films

208 The UV/vis transmission spectra of the bilayer films are shown in Fig. S2. The transmission of the
209 GG/GN film was > 61.6% at 400-800 nm, and decreased slightly with the incorporation of the MBA.
210 The visible light transmittance (400-650 nm) of colorimetric film furtherly decreased with the
211 incorporation of ZnO. The decrease in the visible light transmission of the ZnO-incorporated colorimetric
212 film was mainly attributed to the ZnO nanoparticles in the bilayer film that could hinder the passage of
213 light (Kanmani & Rhim, 2014; X. Liu, Chen, Ren, Chang, He, & Zhang, 2019; Wang, Gong, Miao, Guo,
214 Liu, Fan, et al., 2019). It is interesting to note that the ZnO-incorporated colorimetric film presented
215 excellent UV-blocking property, and this property of the film further improved with the increasing
216 concentration of ZnO. The blocking of the UV spectrum was due to the ZnO in the upper layer film with

217 band-gap energy of 3.2 eV, that can absorb a larger fraction of UV light (Lupan, Postica, Pauporté, Hoppe,
218 & Adelung, 2019; Shankar, Teng, Li, & Rhim, 2015; Wang, et al., 2019). Such UV-blocking properties
219 have also been observed with other biopolymer films (Lizundia, Ruiz - Rubio, Vilas, & León, 2016; Oun
220 & Rhim, 2017; Shankar & Rhim, 2017).

221 The color stability of the anthocyanins embed in the colorimetric film is essential for practical
222 application. Fig. 3a shows the color change of the films exposed to visible light at 25 °C for 24 d. During
223 the storage, the color of the GG/GN film was well-preserved. All colorimetric films were faded to yellow
224 after exposure to visible light for 24 days. The S value of the film was determined as the measure of the
225 degree of discoloration. As can be seen in Fig. 3b, the S value of the GG/GN film exposed to visible light
226 remained almost constant (< 1%). This indicated that GG/GN as a composite material was light-stable.
227 As for bilayer colorimetric films, the S value increased slightly during the storage period. At the end of
228 the storage, the S value of GG-MBA/GN film was increased to 5.61%. However, the S value of the GG-
229 MBA/GN-0.5% ZnO film was only 3.41%, and when the content of ZnO increased to 2.0%, the S value
230 of the bilayer films further decreased to 1.75%. This was in agreement with the fact of the light-blocking
231 property (Fig. S2) and oxygen barrier property (Table S1) of ZnO that protected the anthocyanins from
232 degradation. Fig. 3c shows the color change of the films exposed to UV light at 25 °C for 32 h. The
233 GG/GN film exhibited a stable gray color during the storage period. After 32 h of storage, the color of
234 bilayer colorimetric films faded to yellow. Meanwhile, all colorimetric films presented high S values
235 under the UV light (Fig. 3d), indicating that the stability of mulberry fruit's anthocyanins was
236 significantly affected by UV light (Aramwit, Bang, & Srichana, 2010). During the storage period, the S
237 value of the GG-MBA/GN-ZnO films was lower than the GG-MBA/GN film, and decreased with the
238 increasing concentration of ZnO. This was due to the UV-light absorption function of the ZnO
239 nanoparticles (Fig. S2) (Kubacka, Fernandez-Garcia, & Colon, 2011). The UV light absorbing ability
240 could decrease the degradation of MBA in the lower layer film. The above-mentioned results indicated
241 that ZnO nanoparticles could be used as a UV blocking agent to enhance the color stability of the film.



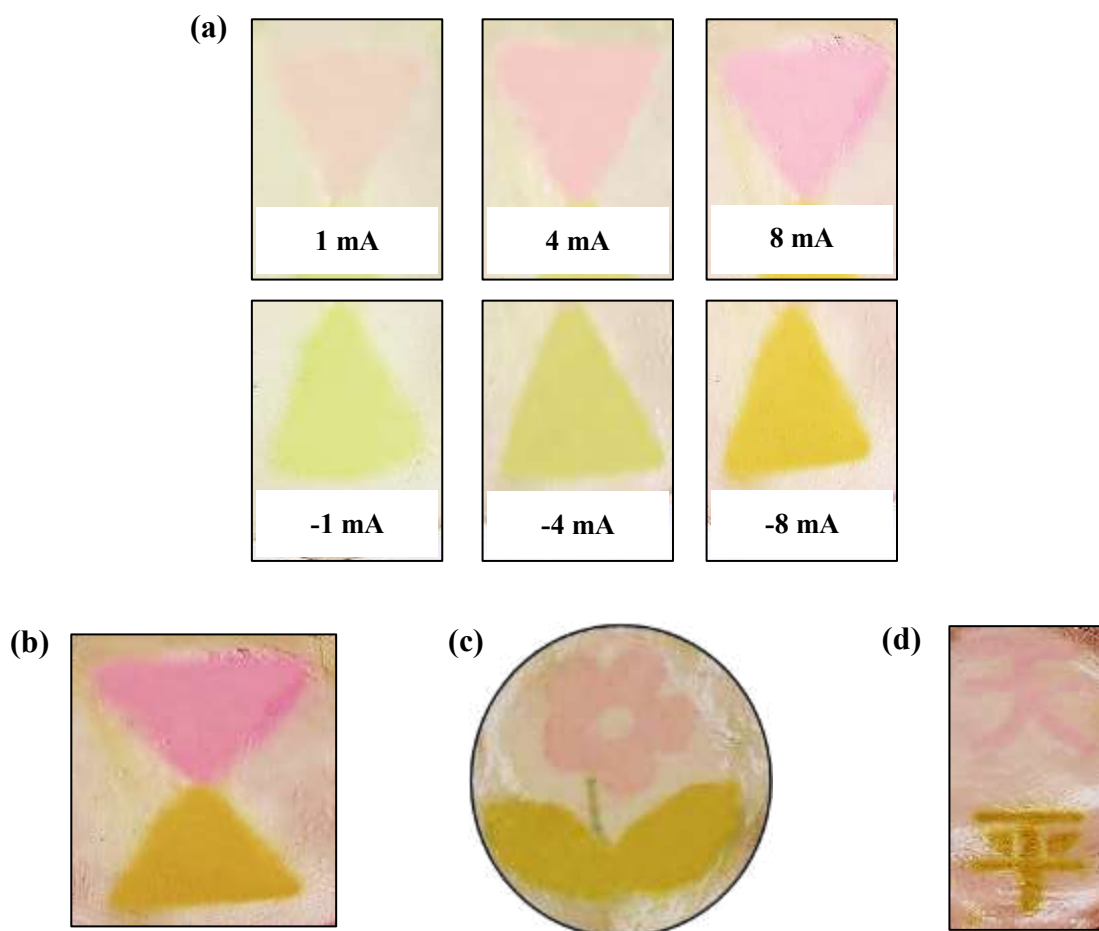
242

243 Figure 3. Transparency spectrum of the bilayer films (a); the color changes (b) and S value (c) of the
 244 bilayer films under visible light, and the color changes (d) and S value (e) of the bilayer films under
 245 UV light.

246 3.4. Electrochemical writing on bilayer film

247 A bilayer hydrogel with excellent structural stability was firstly produced in this work. The picture of the
 248 triangle pattern written on the GG-MBA/GN-2.0% ZnO film under different positive currents (the
 249 positive current direction is from Pt needle to Pt plate) and negative current (the negative current direction
 250 is from Pt plate to Pt needle) are shown in the Fig. 4a. The triangle pattern was light pink when the current
 251 was 1 mA, and turned to pink with the current increase to 8 mA. This accord with the fact that the surface
 252 of the GG-MBA hydrogel will generate hydrogen ions due to the anodic water electrolysis reaction while
 253 it connected with the Pt needle. Thus, the structure of MBA in the GG hydrogel transformed from
 254 anhydrobase to flavylum ion, and the MBA changed to light pink. With the increase of the current, the
 255 water electrolysis reaction on the surface of the hydrogel became more intense; thus, generating more
 256 hydrogen ions so that more anhydrobase transformed to flavylum ion. Finally, the color of MBA embeds
 257 in the film changed to pink. With the current increased from -1 mA to -8 mA, the triangle pattern
 258 gradually changed from light green to yellow-green and finally a yellow color. This accord with the fact
 259 that the surface of the GG-MBA hydrogel will generate hydroxide ions due to the cathodic water
 260 electrolysis reaction while it connected with the Pt needle. Hence, the structure of MBA in the GG
 261 hydrogel transformed from anhydrobase to anhydrobase anion, and the MBA changed to light green.
 262 With the increase of the current, the cathodic water electrolysis reaction on the surface of the hydrogel
 263 became more intense; thus, generating more hydroxide ions so that more anhydrobase transformed to
 264 anhydrobase anion. Finally, the color of MBA embeds in the film changed to yellow. As shown in Fig.
 265 4b, 4c, and 4d, the two-color hourglass, multicolor flower, and the Chinese characters were successfully

266 written on the bilayer colorimetric films. This electrochemical writing process is reliable and
267 programmable. Meanwhile, the electrochemical writing on the film will also broaden the application of
268 the film.



269
270 Figure 4. The images of triangle pattern written on the GG-MBA/GN-2.0% ZnO film under different
271 currents (a); the images of hourglass (b), flower (c), and the Chinese characters (d) written on the GG-
272 MBA/GN-2.0% ZnO film.

273 3.5. NH₃ sensing analysis

274 The gas-sensing ability of the colorimetric film is essential for practical application in monitoring the
275 freshness (J. Zhang, Zou, Zhai, Huang, Jiang, & Holmes, 2019). Hence, the response of the bilayer
276 colorimetric film to NH₃ was tested in this work. The color changes (inset) and the corresponding S value
277 of the bilayer colorimetric films toward different concentrations of NH₃ are shown in Fig 5a. With the
278 increasing concentration of NH₃ (0-0.10 mM), all the films gradually turned from pink to green and
279 finally a yellow color. This was due to the fact that volatile NH₃ could combine with H₂O embedded in
280 the colorimetric film and hydrolyze into OH⁻ ions and produced an alkaline environment. Finally, the
281 color of the film was changed due to the structure of anthocyanins transformed from flavylum ion to
282 anhydrobase anion in the presence of hydroxyl ions (J. Liu, Wang, Guo, Li, Chen, Jiang, et al., 2019).
283 When the bilayer colorimetric films reacted with different concentrations of NH₃, no obvious difference
284 was observed in terms of their color change, corresponding S value, and corresponding UV-Vis spectra
285 (Fig. 5b, S3, S4 and S5). The aforementioned results indicated that the incorporation of ZnO
286 nanoparticles had no negative effect on the NH₃-sensing ability of colorimetric film. Moreover, the UV-
287 Vis spectra of the GG-MBA/GN-2.0% ZnO film when reacted with different concentrations of NH₃ is

288 shown in Fig. 5b. With the increasing concentration of NH₃, the absorption peak at 497 nm decreased
289 from 0.44 to 0.37, and the absorption peak at 614 nm gradually increased from 0.29 to 0.32. This
290 indicated that the GG-MBA/GN-2.0% ZnO film gradually transferred to be more basic. The intensity of
291 green color compared to red color was represented by the absorbance ratio at 614 nm versus 497 nm
292 (A_{614}/A_{497}). As can be seen in the inset of Fig. 5b, the A_{614}/A_{497} shows a linear relationship with NH₃
293 concentration (0-0.08 mM). Expressed the following formula:

$$294 \quad y=3.0x+66, R^2=0.99 \quad (6)$$

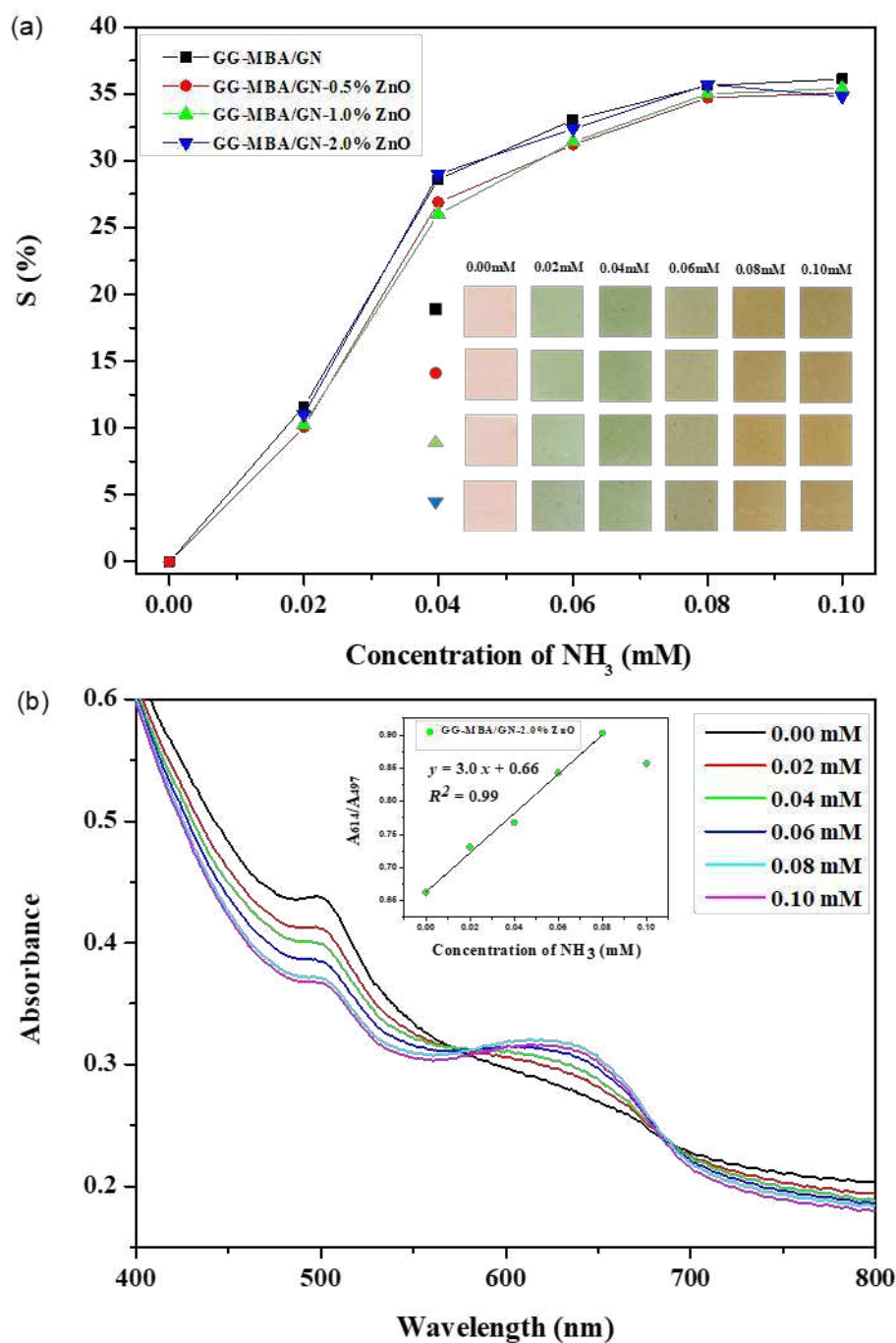
295 where x is the NH₃ concentration and y is the absorbance ratio at 614nm versus 497 nm:

296 The absorbance ratio at 614nm versus 497 nm of GG-MBA/GN-2.0% ZnO film gradually increased with
297 the increase of NH₃ concentration (0-0.08 mM), while it decreased when NH₃ concentration was up to
298 0.10 mM. Meanwhile, the limit of detection (LOD) of GG-MBA/GN-2.0% film toward NH₃ was also
299 determined by the following formula:

$$300 \quad LOD=\frac{3K}{N} \quad (7)$$

301 Where K is the standard deviation of blank measurements ($n = 15$) and N is the slope of the calibration
302 curve.

303 The LOD of GG-MBA/GN-2.0% ZnO film to NH₃ was 0.01 mM, indicating the GG-MBA/GN-2.0%
304 ZnO film could be used as a gas sensor to monitor the NH₃.



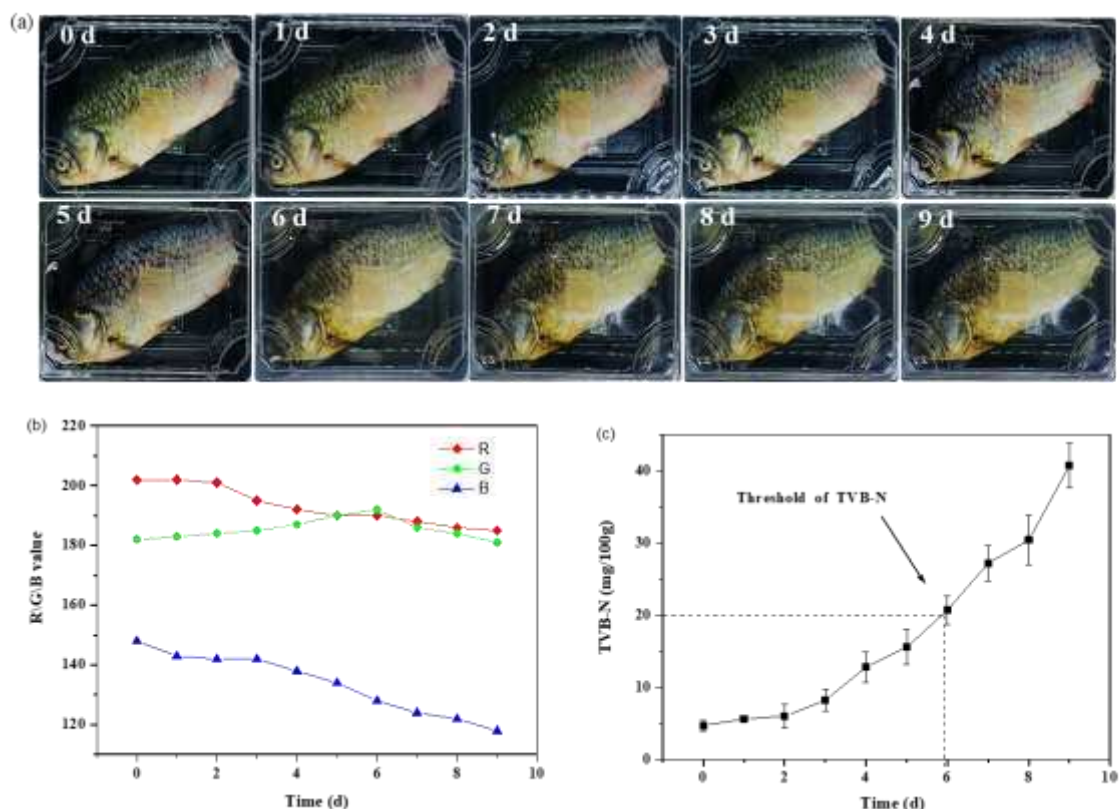
305

306 Figure 5. The S value (a) and color changes (inset) of the bilayer colorimetric films after exposure to
 307 different concentrations of NH₃, and the UV-Vis spectra of GG-MBA/GN-2.0% ZnO film after
 308 exposure to different concentrations of NH₃ (b).

309 3.6. Application of film for indicating fish spoilage

310 In our initial trial, the tensile strength (TS), elongation at break (EB), water vapor permeability (WVP)
 311 and oxygen permeability (OP) of the bilayer films were measured (Table S1). Although the GG-
 312 MBA/GN-1.0% ZnO presented a quite higher TS, the GG-MBA/GN-2.0% ZnO film presented the
 313 optimal performance on EB, UV-blocking property, color stability, and NH₃-sensing ability. Hence, the

314 GG-MBA/GN-2.0% ZnO film was selected to monitor the crucian spoilage. The rectangular pattern was
 315 printed on GG-MBA/GN-2.0% ZnO film by means of electrochemical writing. As shown in Fig. 6a, 6b,
 316 and 6c, the color change of the film, corresponding color parameters (R, G, and B) and corresponding
 317 TVB-N content were changed with the change of the crucian freshness at 4 °C. The initial TVB-N value
 318 of the fresh crucian was 4.7 mg/100g and the color of the film was pink. After ~6 days of storage, the
 319 TVB-N value was increased to 20.7 mg/100g. Meanwhile, the color of the film was changed to light
 320 green, and the corresponding R, G and B values were recorded as ~190, ~192 and ~128, respectively.
 321 The limit of TVB-N content of fish in Chinese standard specification GB/T 5009.228 (2016) is
 322 20 mg/100 g, indicated that the crucian was inedible at this stage. The TVB-N value reached 40.7
 323 mg/100g at 9 d and the film reached a final yellow-green color, and the corresponding R and B value of
 324 the GG-MBA/GN-2.0% ZnO film decreased to 185 and 118, which was consistent with the color change
 325 of the film. It is interesting to note that the rectangular pattern formed by electrochemical writing was
 326 maintained clearly during the storage period, indicating the stability of electrochemical writing
 327 information. These results showed that the GG-MBA/GN-2.0% ZnO film with written patterns was
 328 expected to be a biological sensor for detecting the spoilage of crucian.



329

330 Figure 6. Images of GG-MBA/GN-2.0% ZnO film with a written pattern for monitoring the spoilage
 331 of crucian (a), corresponding R, G, and B value changes of the film (b), and the TVB-N level of the
 332 crucian (c).

333 3.7. Safety and cost of the film

334 The bilayer colorimetric film is cost-effective and presented low toxicity. GG is a natural extracellular
 335 polysaccharide, which has currently listed as an edible additive in many food products by both U.S. FDA
 336 and the EU (E418). GN as a biopolymer derived from collagen has been widely used in the food industry
 337 (Farshchi, Pirsá, Roufegarinejad, Alizadeh, & Rezazad, 2019). In addition, ZnO nanoparticle is an

338 inorganic compound, which has received U.S. FDA approval for application as a generally safe (GRAS)
339 material and has previously used as an antimicrobial additive (Espitia, Soares, Teófilo, Coimbra, Vitor,
340 Batista, et al., 2013). The cost of the chemicals involved for one thousand bilayer hydrogels is merely
341 \$19.21 (Table S2). Hence, the bilayer colorimetric film is a safe and economical sensor for smart food
342 packaging.

343 **4. Conclusions**

344 Bilayer colorimetric films with electrochemical writing ability were developed for monitoring the fish
345 freshness. SEM images indicated that the bilayer film was fabricated successfully. The addition of ZnO
346 nanoparticles significantly improved the illumination stability of the bilayer film, while did not produce
347 interference on the NH₃-sensing ability of the films. The LOD of GG-MBA/GN-2.0% ZnO film to NH₃
348 was 0.01 mM. The bilayer colorimetric films performed visible color changes with the crucian spoilage,
349 and the patterns written on the films were well-maintained. Thus, the developed colorimetric film with
350 written patterns was expected to be a safe, low-cost and non-destructive indicator for monitoring the fish
351 spoilage during storage.

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358 **Supplementary data**

359 Some essential tables and figures were added.

360

361 **Reference**

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