

Journal of Visualized Experiments

Functionalization of silver/titanium dioxide composites in chitosan-based coatings and their egg preservation performances

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61850R1
Full Title:	Functionalization of silver/titanium dioxide composites in chitosan-based coatings and their egg preservation performances
Corresponding Author:	Yuanhao Wang Guilin University of Technology Guilin, Guangxi CHINA
Corresponding Author's Institution:	Guilin University of Technology
Corresponding Author E-Mail:	yuanhaowang@ppsuc-edu.cn
Order of Authors:	Xuandong Chen Winfred Zhong Wang Yuanhao Wang Shifeng Wang
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Shenzhen/Guang Dong province, China
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Chemistry
Please provide any comments to the journal here.	

TITLE:

Functionalization of silver/titanium dioxide composites in chitosan-based coatings and their egg preservation performances

AUTHORS AND AFFILIATIONS:

Xuandong Chen^{1,2}, Winfred Zhong Wang³, Yuanhao Wang^{4,5*}, Shifeng Wang^{6,7*}

¹ College of Civil and Architecture Engineering, Guilin University of Technology, Guilin, China.

² Guangxi Key Laboratory of New Energy and Building Energy Saving, Guilin, China.

³ Saint James School, 17641 College Road, Hagerstown, MD, USA

⁴ Guangxi Beibu Gulf Engineering Research Center for Green Marine Material, Guilin, China

⁵ Guangxi Engineering Research Center for Solid Waste Resources Development and Application, Guilin University of Technology, Guilin, China

⁶ Department of Physics, College of Science, Tibet University, Lhasa, China

⁷ Institute of Oxygen Supply, Tibet University, Lhasa, China

Correspondence to:

Yuanhao Wang at yuanhaowang@ppsuc.edu.cn

Shifeng Wang at wsf@utibet.edu.cn

KEYWORDS:

Ag/TiO₂ composite; Chitosan; Coating; Egg preservation; Synergistic effect; Shelf life

SUMMARY:

To enhance the stability and sterilizability of chitosan-based coatings and expand the application of specific nanoparticles into food preservation, Ag/TiO₂ composites are synthesized to functionalize chitosan coatings for egg preservation. The eggshell morphology, weight loss, Haugh unit and albumen pH are used to characterize the preservation performance of the coatings.

ABSTRACT:

Eggs are an excellent source of proteins, minerals, and vitamins, which have been popularly consumed in daily diet all over the world. The micro-pores and micro-cracks on the eggshells, however, lead to the loss of moisture and the escape of CO₂, resulting in the acceleration of egg deterioration and economic loss. To enhance the stability and sterilizability of the existing chitosan-based coating materials and to develop novel multifunctional nano-composites for anti-bacterial and egg preservation, silver/titanium dioxide (Ag/TiO₂) composites are synthesized and applied to modify chitosan for the extension of the egg shelf life. Electron microscope (SEM) images are used to analyze the structure and morphology of composite particles and the morphology of coated eggshells. The preservation performances of composite coatings are evaluated by various parameters: weight loss, Haugh unit, albumen pH and eggshell morphologies of the samples. Adoption of Ag/TiO₂ composites contributes to a synergistic effect to chitosan, which could prolong the preservation period further. The performances of chitosan coating, however, are presently limited by the existing particle species and concentration, which

requires optimization in future studies. Methods in this study examine novel coating materials, which could be created by adding specific nanoparticles into the coating precursor, to achieve the combinative effects of the nanoparticle and the precursor, as well as to prepare novel multifunctional coatings in the field of food preservation.

INTRODUCTION:

As excellent and popular sources of protein, inorganic salt and vitamins, eggs are popular suppliers for human nutrition, which are being produced and consumed worldwide at a large scale^{1,2}. Although eggshells are natural protective barriers, they are too fragile to retain their integrity during egg transportation and storage. The gas exchange and microbial penetration between the egg albumen and the environment, which can happen easily through tiny pores on the eggshells, would lead to CO₂ loss as well as the deterioration of egg quality^{3,4}. Furthermore, tiny cracks on the eggshells would increase the risk of microbial contamination. Therefore, effective egg preservation methods must be urgently developed for economic benefit and human health.

At present, there are two kinds of routes for egg preservation. The first way is to deactivate the microorganisms on the eggshells^{5,6,7,8}. The deactivation process extends the egg preservation period by clearing the eggshell surface away from the erosion of environmental microorganisms and moisture. On the other hand, coating the tiny pores and cracks on the eggshell with specific functional materials could also serve as an excellent method to prevent the loss of water vapor and CO₂ from the egg albumen, as well as to protect the eggshell from microorganism destruction. As they are simple, effective and energy-saving, coatings are attracting increasing attention for egg preservation. The primary principles that suitable coating materials should meet are chemical stability, effective permeability, wide availability and reliable safety. The most widely studied coating materials are oil^{9,10}, proteins¹¹, biopolymers³, and chitosan¹².

Chitosan has been regarded as a popular coating material because of its excellent properties of film formation, antibacterial activity, and safety¹³. The egg physicochemical changes and microbial contamination have been proven to be protected by a chitosan coating, which has served as an efficient way for egg preservation. However, as a hydrophilic polymer with poor water vapor barrier and moisture adsorption, chitosan is unstable in a high humidity environment, limiting the preservation effects and reducing the shelf life of eggs to a certain degree.

To overcome this problem and promote the chitosan preservation performance, specific nanoparticles have been used as an adulterant in chitosan-based coatings. Thereinto, as a nano filler with antibacterial character¹⁴, nano silver (Ag) has been doped to chitosan. The addition of Ag could not only enhance the barrier property of chitosan film, but also enhance its antibacterial effect, which has been proven to improve the preservation effect of the coating. The easy aggregation and simple structure of Ag particles, however, may decrease the stability and durability of the chitosan film, which have been verified to be improved by depositing specific nanoparticles. Titanium dioxide (TiO₂) is a typical metal oxide compound with excellent properties such as chemical stability, low toxicity as well as reasonable costs. These functional properties endow TiO₂ great potential in many research fields¹⁵. For example, TiO₂ particles could

serve as additives in medical devices and biomaterials due to their adhesiveness and bactericidal activities. The actual application of TiO_2 particles, however, is largely limited by their unstable thermodynamics and agglomerate trends. Therefore, doping specific functional materials into TiO_2 has been proposed to achieve the combinative effect of antibacterial activity, improved dispersibility and thermostability.

In this study, antibacterial Ag/TiO_2 composites are synthesized and applied into a chitosan coating for egg preservation. SEM images are used to analyze the structure and morphology of the Ag/TiO_2 particles and the eggshells. The preservation performance of the coating is evaluated and compared by weight loss, Haugh unit, albumen pH and eggshell morphologies. This study demonstrates the possibility and potential of nano composite blended chitosan coatings in food preservation.

PROTOCOL:

1. Synthesis of nano Ag/TiO_2 composites

1.1. To prepare the nano-silver sol, combine 100 mL of AgNO_3 solution, 100 mL of protective agent and 50 mL of NaBH_4 into a 500 mL breaker.

1.1.1. Mix acetic acid and methanoic acid (analytical grade) at the volume ratio of 1:1 to obtain 100 mL of complex acid solution as the precursor for the protective agent. Dilute the acid solution with deionized water ($18 \text{ M}\Omega\cdot\text{cm}$) to 500 mL as the protective agent.

1.1.2. Add AgNO_3 solution (0.3 mol/L) into the resultant protective agent under vigorous stirring, until AgNO_3 solutes are evenly dispersed in the protective solution. Add 0.4 g of NaBH_4 to obtain the well-dispersed Ag sol after reacting the mixture for 1 h at room temperature.

CAUTION: The particle size of nano-silver could be adjusted by the concentration of protective agent and the stirring rate in step 1.1.2.

1.2. Combine Ag into tetrabutyl titanate (TBOT)-ethanol solution under stirring and then add 80 mL of acid catalyst dropwise.

1.2.1. Combine 500 μL of benzenesulfonic acid (BA) and glacial acetic acid (AA) to obtain the mixed solution (BA and AA at the volume ratio of 1:2). Dilute the solution into 100 mL of deionized water ($18 \text{ M}\Omega\cdot\text{cm}$) to prepare the acid catalyst.

1.2.2. Add the resultant Ag sol into the pre-dispersed tetrabutyl titanate (TBOT)-ethanol solution (2.5 of TBOT in 100 mL ethanol solution) and stir for 1 h to obtain the mixed sol. After, add the sol dropwise into 80 mL of acid catalyst, and stir for 4 h at 70°C .

1.2.3. Continuously stir the mixture for 48 h at room temperature to produce the final Ag/TiO_2 composite.

CATION: Vigorous stirring may cause splashing of solution drops. Use protective devices to ensure safety, such as a protective oral-nasal mask, a lab gown, and gloves. There is no strict standard for the rotation speed in the abovementioned procedures.

2. Preparation of chitosan coating

2.1. Dissolve chitosan in 1% (vol) acetic acid and stir for 24 h at 25 °C to prepare the coating solution (make sure the chitosan concentration is 0.5% (wt) in the resultant solution).

2.2. Add Ag/TiO₂ particles into the suspension separately (0, 0.5, 1, and 1.5 g of Ag/TiO₂ into 50 g of chitosan solution, respectively), to obtain 0%, 1%, 2% and 3% (wt) Ag/TiO₂-chitosan solutions, denoted as Ag/TiO₂-CS0, Ag/TiO₂-CS1, Ag/TiO₂-CS2 and Ag/TiO₂-CS3, respectively.

CAUTION: There is no strict standard for the rotation speed in the abovementioned procedures.

3. Scanning electron microscopy (SEM) observation

3.1. Cut the experimental eggshell into pieces (square dimensions of about 2-3 mm).

3.2. Immobilize the eggshell pieces on a metal stub with a conductive adhesive (i.e., double-sided carbon conductive tape or other similar materials). Use gloves during the sample preparation to avoid any contamination of the sample from hands. Mark the sample (e.g., with an L-shaped scratch using a diamond pen cutter).

3.3. Alternatively, apply a sputtered coating with conductive material (~10 nm thick) to prevent charging effects.

3.4. Acquire at least three high-resolution SEM micrographs (ideally, a minimum of five) from a top view of the sample. Ensure that each image displays an area of at least 25 μm x 25 μm, with a resolution ratio of 20 μm. Avoid taking images from surface regions with macroscopic surface defects.

3.5. Use the following SEM parameters: operating voltage of 30 kV. The resolution of the second electron image can reach 2 nm by using a field emission electron gun in a high-grade scanning electron microscope (the ion beam current density is about 10⁵ A/cm²).

3.6. Note the exact position of each picture with a respect to the L-shaped marker.

4. Egg preservation experiments

NOTE: The freshly laid eggs are chicken eggs provided by a local farm in Shenzhen, China.

4.1. Screen experimental eggs by excluding eggs with cracks, macula or sands on their surfaces

to ensure a propitious egg preservation experimental process.

4.2. Divide the freshly laid eggs into five groups with 30 eggs in each group. Design the four coated groups, which are coated by the chitosan, Ag/TiO₂-chitosan doped with 0%, 1%, 2% and 3% (wt) as Ag/TiO₂-CS0, Ag/TiO₂-CS1, Ag/TiO₂-CS2 and Ag/TiO₂-CS3, respectively.

4.3. Carry out the coating process to immerse eggs in different coating solutions for 5 min and dry under ambient condition for 24 h. Set the water washed eggs (WE) as a control experiment. After the abovementioned treatments, store the treated eggs at 25 °C. Take the five marked eggs to measure the weight loss, Haugh unit, albumen pH and eggshell morphologies to evaluate and compare the preservation performance.

4.3.1. Obtain the weight loss (%) of the egg by calculating the weight difference in percentage of the egg compared to the first day. Measure the weight of eggs in each group every 5 days.

4.3.2. Calculate the Haugh unit to relate the egg weight with the thick of albumen (Equation 1)¹².

$$HU = 100 \log (H - 1.7W^{0.37} + 7.6) \quad (1)$$

where H represents the albumen height (mm) and W represents the egg weight (g).

4.3.2.1. According to the value of Haugh unit, classify the eggs to AA, A and B grade when the Haugh unit of an egg is above 72, between 71-60 and below 60, respectively (the United States Standards for Quality of Individual Shell Eggs).

4.3.3. Separate the albumen from the yolk and use a digital pH meter to measure the pH values of the albumen.

4.3.4. Observe the morphologies of the surfaces of eggshells using a scanning electron microscope after platinum sputtering of the samples.

CATION: The eggshells are brittle substances that cannot stand for violent impacts. Therefore, be careful to avoid any damage to the eggshells. Moreover, procedures in step 4.3.4 are the same as step 3.

REPRESENTATIVE RESULTS:

The particle size of the Ag/TiO₂ composites ranges from 100-300 nm, which is affected by the synthesis conditions (**Figure 1**).

[Place **Figure 1** here].

The weight losses of different egg samples during storage are shown in **Table 1**. Continuously increased weight loss is due to the escape of albumen CO₂ and water vapor through the pores on the eggshells, which leads to the deterioration of egg quality. The weight losses of WE eggs are much higher than for other groups, indicating the protective capacity of chitosan-based coatings for egg quality. After coating by chitosan, the cracks on the eggshell are visibly decreased, which

limits the loss of CO₂ and water vapor.

[Place **Table 1** here]

Moreover, chitosan coatings doped with Ag/TiO₂ particles are more effective at sealing the pores and forming dense layers, leading to considerably inhibited weight loss. The greater the dosage of the Ag/TiO₂ particles, the stronger the effect of the corresponding coating to reduce CO₂ and vapor loss (**Figure 2**).

[Place **Figure 2** here]

The Haugh unit is calculated by the age-related changes of the white proteins, reflecting the albumen thinning variation, which is closely related to the protein proteolysis and the albumin pH. The more rapid decrease and invariably lower values of the Haugh unit in the WE group than the chitosan coating groups indicate the effective protective capacity of chitosan. Eggs in chitosan treated groups maintain the superior grade A for 26 days, while the WE group degrades to grade B after day 6. The values of Haugh unit in Ag/TiO₂-CS1 are always the highest among all the treated groups, indicating that: (i) the addition of Ag/TiO₂ particles contributes to a synergistic effect with chitosan, which are more effective for the coating stabilization and bacterial control; while (ii) excess Ag/TiO₂ particles would destroy the layered structure of chitosan coating, leading to a poorer preservation capacity. According to the results in **Table 2**, chitosan doped with 1% (wt) Ag/TiO₂ particles exhibits the best performance to slow the deterioration of albumen proteins, thus extending the shelf life by up to 30 days.

[Place **Table 2** here]

The variation of albumen pH is caused by CO₂ evacuation, leading to a slow increase of pH values with storage time. The albumen pH of WE eggs increases sharply within 10 days, and reaches as high as 9.5 at day 30. The degradation of proteins into fat and peptone leads to a pH decrease. After being protected by a chitosan coating, the albumen pH present similar trends within 20 days, which are stabilized at around pH 8.0-8.2. After day 20, the pH values of Ag/TiO₂-CS0 and Ag/TiO₂-CS1 show slight fluctuation at around pH 8.2 and stabilize between pH 7.5-8.0 for Ag/TiO₂-CS2 and Ag/TiO₂-CS3. The relative stable albumen pH of treated groups compared with the WE group illustrates the effective reduction of CO₂ loss in albumen (**Figure 3**). The addition of Ag/TiO₂ particle promotes the stability of chitosan, which could maintain good stability until 31 days (**Figure 4**).

[Place **Figure 3** here].

[Place **Figure 4** here].

FIGURE AND TABLE LEGENDS:

Figure 1. SEM images of Ag/TiO₂ composite particles at different resolution ratios (500 nm).

Figure 2. SEM images of the raw eggshell surfaces and chitosan treated eggshell surfaces at

day 0, 11, 16 and 31. (A) the raw eggshell surfaces; (B) chitosan treated eggshell surfaces.

Figure 3. Changes in albumen pH of different eggs during storage time.

Figure 4. SEM images of Ag/TiO₂-CS coated eggshell surfaces at day 0, 11, 16 and 31. (A) Ag/TiO₂-CS1; (B) Ag/TiO₂-CS2; (C) Ag/TiO₂-CS3.

Table 1. The variation of weight loss of different eggs during storage time

Table 2. The variation of Haugh unit of different eggs during storage time

DISCUSSION:

Issues of egg protein quality preservation could be relieved by chitosan coating, which has been proven to be an effective way to extend the egg shelf life. The use of a single chitosan coating, however, creates several problems such as instability, limiting the preservation period and the actual application of chitosan-based coatings. Notably, doping specific antibacterial nanoparticles into chitosan has been proposed to extend the shelf life further. In this study, Ag/TiO₂ particles are successfully synthesized and doped into a chitosan coating, which could extend the preservation period to at least 30 days.

SEM images are used to analyze the structure and morphology of the Ag/TiO₂ particles, as well as the morphology of the coated eggshells. The preservation performances of composite coatings are evaluated by the weight loss, Haugh unit, albumen pH and eggshell morphologies of the samples. Adoption of Ag/TiO₂ composites contributes to a synergistic effect to chitosan, which could prolong the preservation period further.

The particle sizes of Ag/TiO₂ composites are in the range of 100-300 nm (controlled by the synthesis condition), which could block the pores on the top of eggshell and enhance the preservation performance. However, excess Ag/TiO₂ particles would destroy the layered structure of the chitosan coating, resulting in a lower preservation capacity.

At present, the performances of chitosan coating in this study, however, are limited by the existing particle species and concentrations, which requires optimization in future studies.

The methods in this study demonstrates novel coating materials, which could be mingled by specific nanoparticles into the coating precursor, to achieve combinative effects of nanoparticle and the precursor, as well as to prepare novel multifunctional coatings in the field of food preservation.

ACKNOWLEDGMENTS:

This work was supported by the Guangxi Key Laboratory of New Energy and Building Energy Saving Foundation (No. 19-J-21-17, 19-J-21-30), Guangxi Universities Scientific Research Project (2020KY06029), and Wuhan University of Technology-Tibet University Joint Innovation Fund (LZJ2020003).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Kuroli, S., Kanoo, T., Itoh, H., Ohkawa, Y. Nondestructive measurement of yolk viscosity in lightly heated chicken shelleggs. *Journal of Food Engineering*. **205**, 18-24 (2017).
2. Kostogrys, R.B. et al. Effect of dietary pomegranate seed oil on laying hen performance and physicochemical properties of eggs. *Food Chemistry*. **221**, 1096-1103 (2017).
3. Morsy, M.K., Sharoba, A.M., Khalaf, H.H., El-Tanahy, H.H., Cutter, C.N. Efficacy of antimicrobial pullulan-based coating to improve internal quality and shelf-life of chicken eggs during storage. *Journal of Food Science*. **80**, M1066-M1074 (2015).
4. Damaziak, K. et al. Effect of dietary canthaxanthin and iodine on the production performance and egg quality of laying hens. *Journal of Poultry Science* (2018).
5. Sert, D., Aygun, A., Demir, M.K. Effects of ultrasonic treatment and storage temperature on egg quality. *Journal of Poultry Science*. **90**, 869-875 (2011).
6. Yaceer, M., Aday, M.S., Caner, C. Ozone treatment of shell eggs to preserve functional quality and enhance shelf life during storage. *Journal of the Science of Food and Agriculture*. **96**, 2755-2763 (2016).
7. Viswanathan, K., Priyadharshini, M.L.M., Nirmala, K., Raman, M., Raj, G.D. Bactericidal paper trays doped with silver nanoparticles for egg storing applications. *Bulletin of Materials Science*. **39**, 819-826 (2016).
8. Aygun, A., Sert, D. Effects of vacuum packing on eggshell microbial activity and egg quality in table eggs under different storage temperatures. *Journal of the Science of Food and Agriculture*. **93**, 1626-1632 (2013).
9. Ryu, K.N., No, H.K., Prinyawiwatkul, W. Internal quality and shelf life of eggs coated with oils from different sources. *Journal of Food Science*. **76**, S325-S329 (2011).
10. Figueiredo, T.C. et al. Effects of packaging, mineral oil coating, and storage time on biogenic amine levels and internal quality of eggs. *Journal of Poultry Science*. **93**, 3171-3178 (2014).
11. Caner, C., Ydceer, M. Efficacy of various protein-based coating on enhancing the shelf life of fresh eggs during storage. *Journal of Poultry Science*. **94**, 1665-1677 (2015).
12. Xu, D., Wang, J., Ren, D., Wu, X.Y. Effects of chitosan coating structure and changes during storage on their egg preservation performance. *Coatings*. **8**, 317 (2018).
13. Kopacic, S., Bauer, W., Walzl, A., Leitner, E., Zankel, A. Alginate and chitosan as a functional barrier for paper-based packaging materials. *Coatings*. **8**, 235 (2018).
14. Sagnelli, D. et al. Cross-linked amylose bio-plastic: A transgenic-based compostable plastic alternative. *International Journal of Molecular Sciences*. **18**, 2075 (2017).
15. Megan, T., Robert, C.P., Ivan, P.P., Clara, P. Nanostructured titanium dioxide coatings prepared by aerosol assisted chemical vapour deposition (AACVD). *Journal of Photochemistry and Photobiology A-Chemistry*. **400**, 112727 (2020).

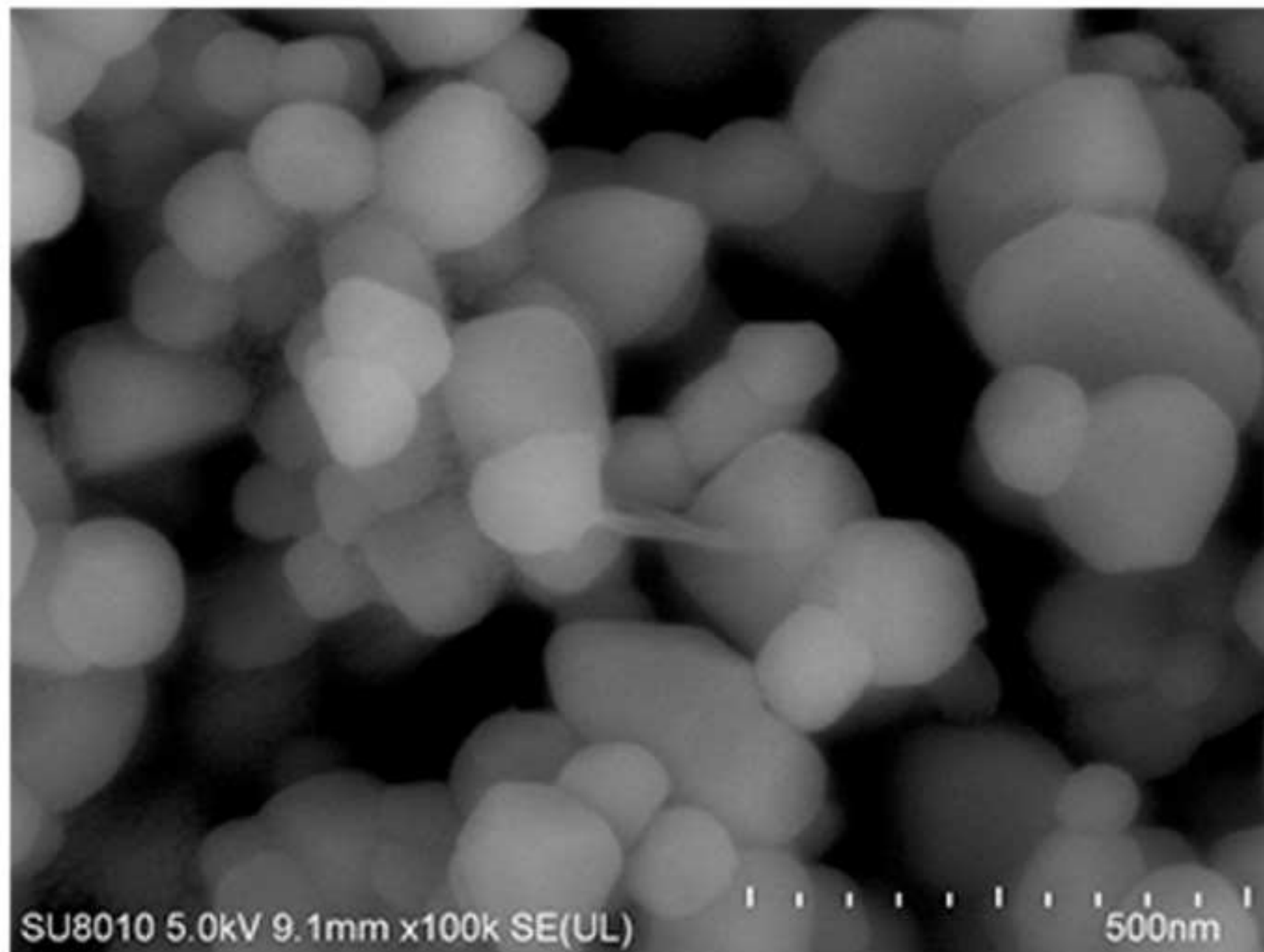
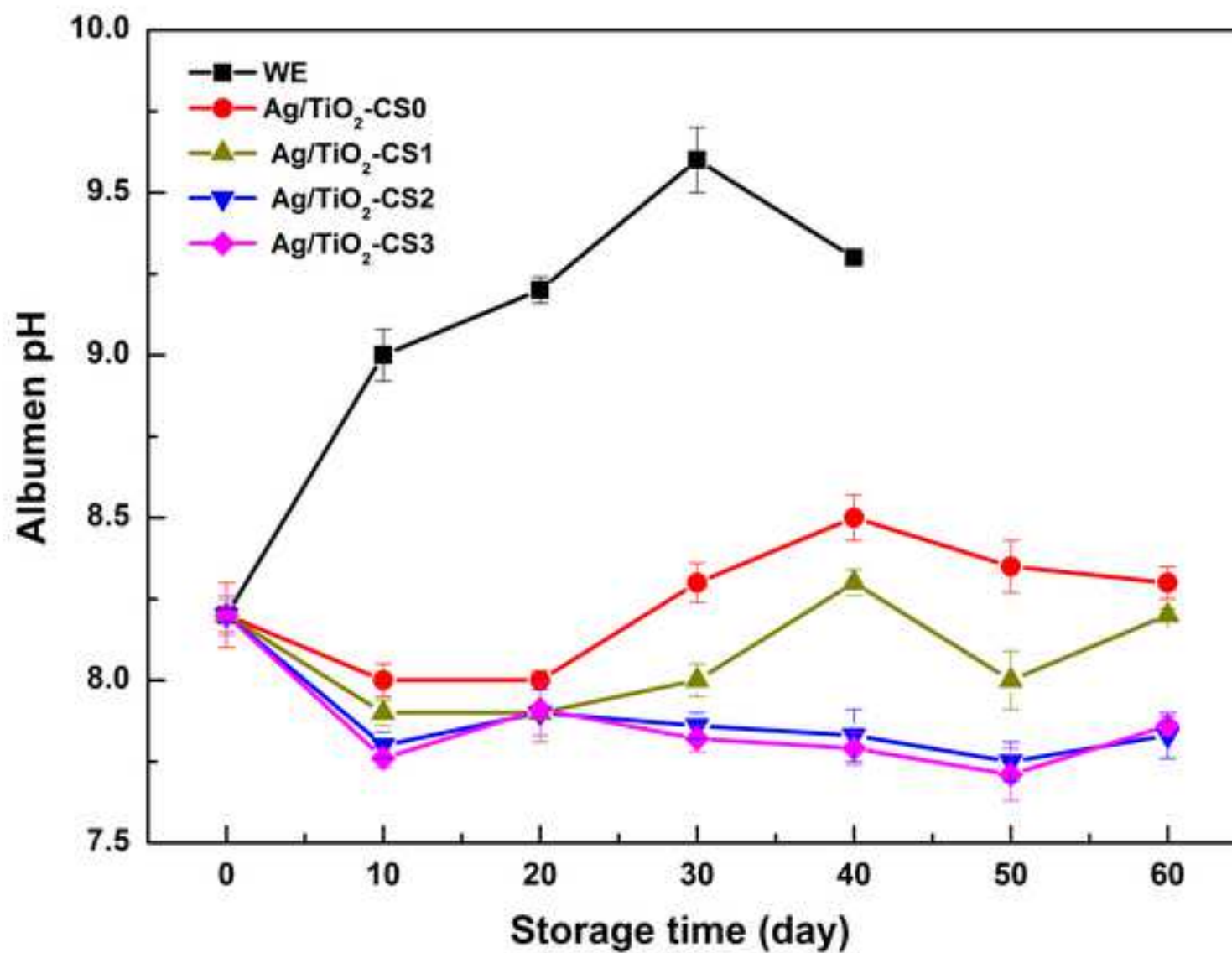
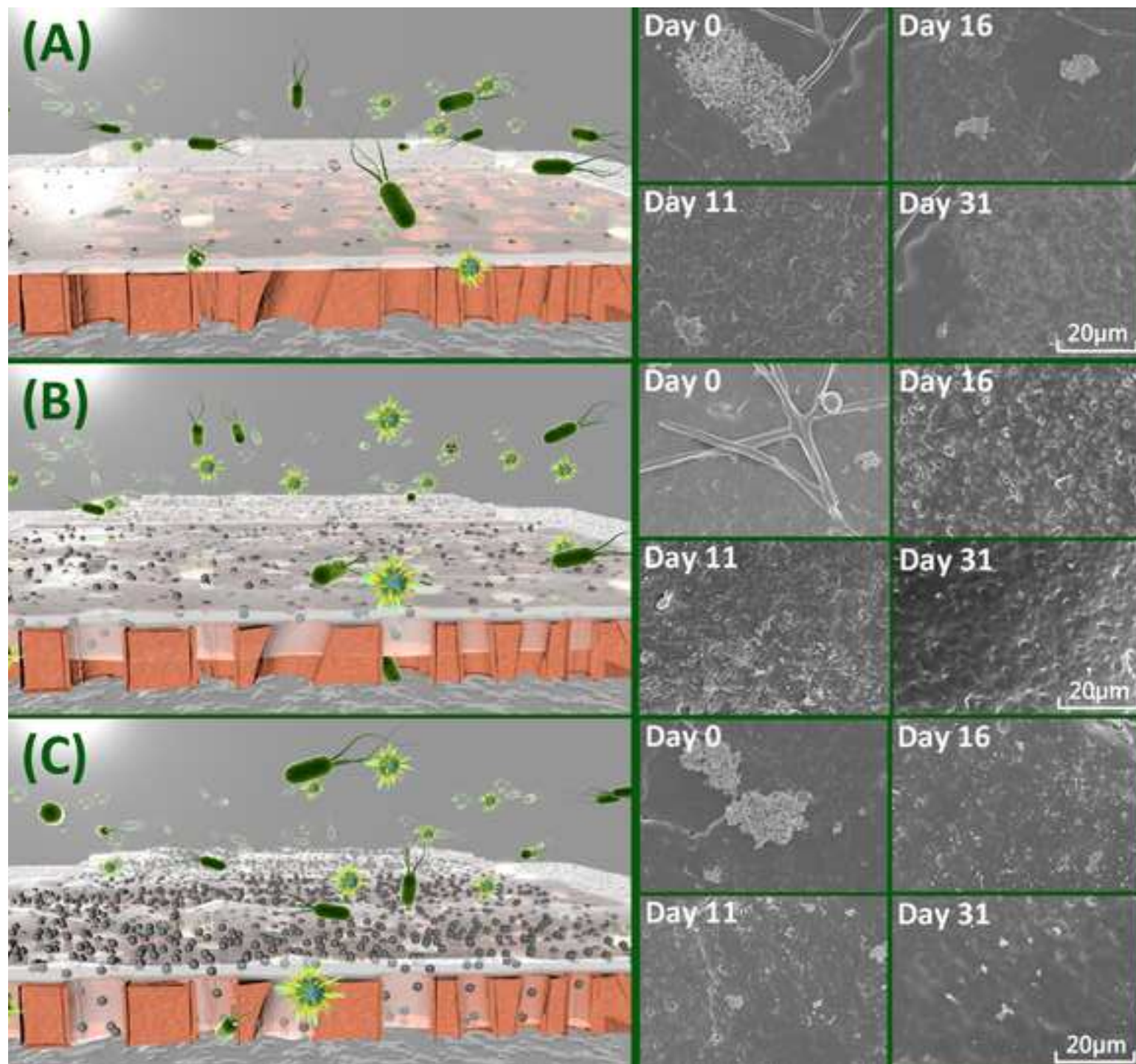




Figure 3





Storage time (day)	Weight loss (wt%)				
	WE	Ag/TiO ₂ -CS0	Ag/TiO ₂ -CS1	Ag/TiO ₂ -CS2	Ag/TiO ₂ -CS3
6	0.78±0.09c	0.69±0.09c	0.53±0.12a	0.49±0.21a,b	0.48±0.06a
11	1.85±0.13b	1.54±0.18c	1.34±0.15a	1.28±0.13a,b	1.26±0.21a
16	2.53±0.21b	2.34±0.27c	1.95±0.21b	1.93±0.35a	1.89±0.38a
21	4.01±0.25c	3.63±0.32b	3.21±0.09b	3.18±0.22a	3.09±0.16a
26	4.86±0.34b	4.18±0.25b	4.09±0.39b	4.05±0.29a	3.98±0.21a,b
31	5.62±0.41a	5.01±0.51b	4.76±0.48a	4.69±0.17a	4.58±0.35a

In the same row with different superscribed letters are significantly different.

Storage time (day)	Haugh Unit				
	WE	Ag/TiO ₂ -CS0	Ag/TiO ₂ -CS1	Ag/TiO ₂ -CS2	Ag/TiO ₂ -CS3
6	73.23 ± 0.68c	80.32 ± 0.59b	83.34 ± 0.12a, b	81.60 ± 1.41a	77.06 ± 0.35a
11	69.86 ± 3.25c	75.64 ± 1.27b	77.18 ± 2.45a, b	76.05 ± 3.13a, b	74.32 ± 1.41a
16	67.31 ± 2.43b	73.88 ± 2.06b	75.36 ± 1.34a	75.61 ± 2.15a	71.53 ± 2.18a
21	62.93 ± 5.32c	71.06 ± 3.88c	73.20 ± 3.09a	72.94 ± 3.52a	69.35 ± 1.34a,b
26	58.55 ± 2.89b	69.85 ± 1.53c	71.85 ± 2.39a	70.34 ± 4.19a, b	66.21 ± 2.10a
31	55.24 ± 3.04a	65.26 ± 0.51a	69.31 ± 3.18a	68.96 ± 1.17a	62.64 ± 4.03a

In the same row with different superscripted letters are significantly different

Name of Material/Equipment	Company	Catalog Number	Comments/Description
acetate	Aladdin	64-19-7	GR, 99.8%
Benzenesulfonic acid	Aladdin	11/3/1998	98%
Chitosan	Aladdin	9012-76-4	<200 mPa•s
Deionized water	prepared by ourselves	-	18 MΩ•cm
Electronic precision balance	Sartorius	BSA124S-CW	
Ethanol	Aladdin	64-17-5	≥99.8%
Formate	Aladdin	64-18-6	Standard for GC, >99%
pH meter	HeYi	PHS-25	
Scanning electron microscope	Hiatchi	SU8010	
Silver nitrate (AgNO3)	Aladdin	7761-88-8	≥99.9%
Sodium borohydride (NaBH4)	Aladdin	16940-66-2	98%
Temperature humidity chamber	YiHeng	LHS-50CH	
Titanium butoxide (TBOT)	Aladdin	5593-70-4	CP,98%

Dear Editor,

Thank you for your letter and for the reviewer's comments concerning our manuscript entitled "Functionalization of silver/titanium dioxide composites in chitosan-based coatings and their egg preservation performances" (ID: JoVE61850). The editor's and the reviewer's comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding to our researches. All of the criticisms have been taken into account in the revised manuscript. The related figures and references have been revised accordingly. Revised portions are marked red in the paper, and the "Respond to comments" is in next page. If you have any further questions, please don't hesitate to contact me.

Yours sincerely,

Y.H. Wang

Responds to the editor's and reviewer's comments

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thanks for the suggestion. We have revised the whole manuscript carefully and tried to avoid any grammar or syntax errors according to the editor's comments. Moreover, we have asked one who is skilled author of English language papers to check the English. The revised portions are marked in red in the revised manuscript. Please check it.

2. Figure 2/3: Please use the Greek symbol mu for the micron symbol instead of the letter U.

Response: Thanks for the comment. The symbols of “ μm ” have been corrected to the Greek form as Figure A and Figure B (corresponding to Figure 2 and Figure 3 in the revised manuscript, respectively). Please check it.



Figure A. SEM images of the raw eggshell surfaces and chitosan treated eggshell surfaces at day 0, 11, 16 and 31 ((a) the raw eggshell surfaces; (b) chitosan treated eggshell surfaces).

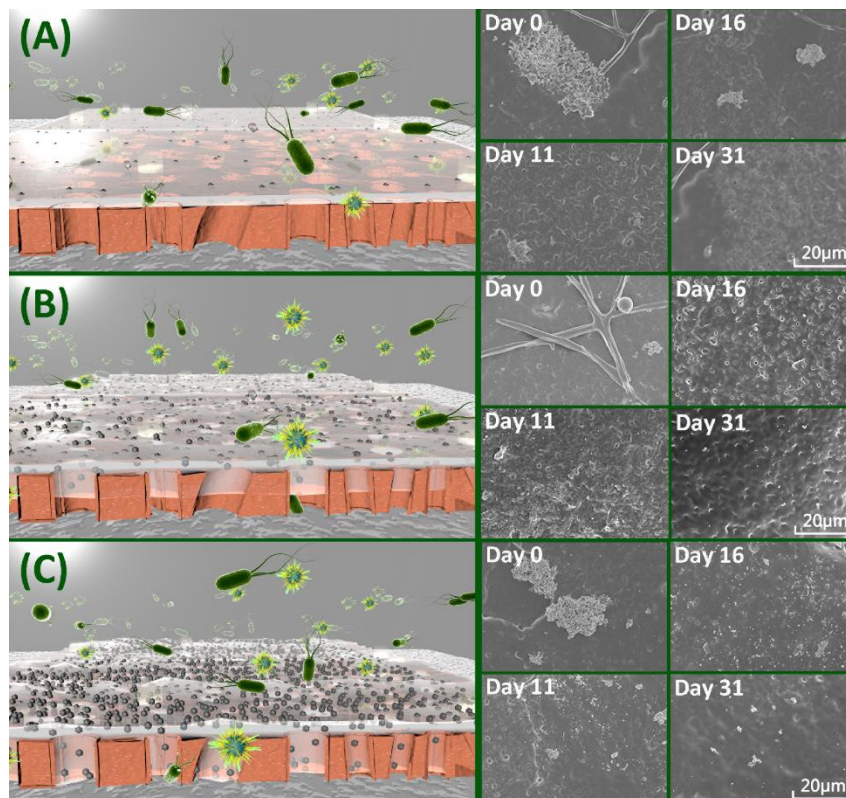


Figure B. Changes in albumen pH of different eggs during storage time.

3. Please specify any safety precautions if any.

Response: Thanks for the suggestion. In general, experiments in this manuscript are normal procedures without high temperature and/or high pressure conditions, or even strict safety requirements. In the protocol of Part 1, vigorously stirring may cause the splash of solution drops, which needs protective devices to ensure safety, such as protective oral-nasal mask, lab-gown and gloves. In Part 1.2.3, the long stir time needs constantly monitor to ensure safety. The procedures in Part 2 and Part 3 are normal procedures without any specific safety precautions. In Part 4, the eggshell are brittle substances that cannot stand for violent impacts. Therefore, procedures in Part 4 should be carefully conducted to avoid any damage of the eggshells.

Related descriptions have also been supplemented and added in the corresponding part of the revised manuscript (Page 2, lines 131-132; Page 4, lines 202-204). Please check it.

4. Please specify the SEM parameters.

Response: Thanks for the comment. The SEM procedures and parameters are specified into six points:

- 1) Cut the experimental eggshell into pieces with suitable size (squares with the side length of about 2-3 mm).
- 2) Immobilize the eggshell pieces on a metal stub with a conductive adhesive (i.e., double-sided carbon conductive tape or other similar materials). Use gloves during the sample preparation to avoid any contamination of the sample from hands. Mark the sample (e.g., with an L-shaped scratch using a diamond pen cutter).
- 3) Alternatively, a sputtered coating with conductive material (~10 nm thick) can be applied to prevent charging effects.
- 4) Acquire at least three high-resolution SEM micrographs (ideally, a minimum of five) of a top view of the sample. Each image display an area of at least $25 \times 25 \mu\text{m}$, with a resolution ratio of $20 \mu\text{m}$. Avoid taking images from the surface regions with macroscopic surface defects.
- 5) At present, the type of scanning electron microscope is SU8010, Japan. The resolution of the second electron image can reach 2 nm by using field emission electron gun in high grade scanning electron microscope (the ion beam current density is about 10^5 A/cm^2). The operating voltage is 30 kV.
- 6) Note the exact position of each picture with a respect to the L-shaped marker.

Related descriptions have also been added in the revised manuscript ([Page 3, lines 148-168](#)). Please check it.

5. 4.1: Chicken eggs?

Response: Thanks for the question. The freshly laid eggs are chicken eggs provided by a local farm of Shenzhen, China. Experimental eggs are screened by excluding the unqualified eggs with cracks, macula or sands on their surfaces to ensure a propitious preservation experimental process. Related descriptions about the egg sources and pretreatment methods have been added in the revised manuscript ([Page 3, lines 172-174](#)), please check them.

6. Please spell out the journal titles in the references.

Response: Thanks for the comment. The full names of journal titles has been supplemented in the [References](#) in the revised manuscript according to the editor's comment, please check them.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper is well documented and well written. Presented data are significant for the considered processes and materials. The prepared coatings are are very effective for egg protection

Major Concerns:

no major concerns

Minor Concerns:

no minor concerns

Response: Thanks for the comments.

Reviewer #2:

Minor Concerns:

The manuscript is clear and the protocol is helpful. Nevertheless, a style correction, mainly at the introduction, must to be done

Response: Thanks for the suggestion. The structure and style of the introduction have been adjusted according to the reviewer's suggestion. Moreover, the grammars and spells have also been checked to avoid any errors. Revised portions are marked red in the revised manuscript. Please check them.

Reviewer #3:

Manuscript Summary:

This research was well designed and conducted. The results are interesting.

Major Concerns:

The many grammar errors throughout the manuscript makes the paper very difficult to read and comprehend. Suggest the authors seek proof read by native English speakers and then resubmit the manuscript.

Response: Thanks for the suggestion. We have revised the whole manuscript carefully and tried to avoid any grammar or syntax errors according to the reviewer's comments. Moreover, we have asked one who is skilled author of English language papers to check the English. The revised portions are marked in red in the revised manuscript. Please check it.

Finally, thank you for your kind comments on our manuscript, and I hope this revised version could reach the criteria of *JoVE*.