CENTER for PRODUCE SAFETY

CPS 2017 RFP
FINAL PROJECT REPORT

Project Title
Rechargeable antimicrobial and antifouling plastics for improved cleaning and sanitation of plastic bins and totes

Project Period
January 1, 2018 – December 31, 2019

Principal Investigator
Nitin Nitin
University of California, Davis
Department of Food Science and Technology
One Shields Avenue
Davis, CA 95616
T: 530-752-6208
E: nnitin@ucdavis.edu

Co-Principal Investigators
Gang Sun
University of California, Davis
Materials Science and Engineering
T: 530-752-0840
E: gysun@ucdavis.edu

Glenn M. Young
University of California, Davis
Department of Food Science and Technology
T: 530-752-5292
E: gmyoung@ucdavis.edu

Objectives

1. *Demonstrate continuous sanitation of reusable plastic container (RPC) food contact surfaces using a novel rechargeable antimicrobial plastic surface.*
2. *Develop non-fouling functionality in polymer films and evaluate the influence of this modified composition on: (a) attachment of Listeria to the plastic surface; and (b) formation of biofilms on the RPC surfaces under simulated conditions.*
3. *Combine non-fouling functionality with rechargeable antimicrobial functional material to improve sanitation of RPCs and conduct pilot-scale testing in collaboration with industry groups.*

Funding for this project provided by the Center for Produce Safety through:
WSDA SCBGP grant# K2307 & CPS Campaign for Research

FINAL REPORT

Abstract

Sanitation of reusable plastic containers (RPCs) is a significant challenge and inadequate sanitation can lead to cross-contamination of fresh produce. These cross-contamination events can result in a foodborne disease outbreak as well as reduce the shelf life or quality of the product. To address this challenge, this project was aimed at developing rechargeable antimicrobial and antifouling plastic material(s) and then evaluating the materials for eliminating bacterial contamination from various sources and reducing biofilm formation. The antimicrobial properties of the developed material can be recharged by simply using a diluted chlorine (bleach) solution. This novel material can be used as a rechargeable liner attached to existing RPCs and/or development of new RPCs with this novel plastic material. This project also briefly evaluated the impact of the antimicrobial materials on produce quality after extended contact. In summary, this project addresses a significant unmet need in the industry to improve sanitation of RPCs.

Background

Contamination of fresh produce during post-harvest handling when using reusable plastic containers (RPCs) can lead to the spread of pathogens from an initial contaminated batch (including fresh produce, soil and organic plant debris, and/or residual water) to a larger number of uncontaminated fresh produce batches. Spreading of pathogens increases the risk for cross-contamination, which can result in major outbreaks of foodborne illnesses as well as nationwide recalls that can have significant impact on the fresh produce industry. The risk of cross-contamination is further exacerbated when sanitation conditions during product handling are sub-optimal. Although there is significant scientific evidence corroborating the increased risk of post-harvest cross-contamination of pathogens during handling and packaging of fresh produce, the current approaches to control this cross-contamination are limited. This challenge is well illustrated in multiple reports by other researchers (Warriner, University of Guelph; Suslow, University of California, Davis) who showed that significant numbers of viable bacteria can be detected on the interior surface of RPCs and over 30% of the RPCs examined were found to have bacterial loads of greater than 5 log CFU on the interior surface [1–3]. Furthermore, the results revealed a small fraction of containers having bacterial loads greater than 6 log CFU based on a swab test. These studies clearly indicated the limitations of current sanitation practices and the necessity to develop novel materials for self-decontaminating containers.

Research Methods and Results

1. Prevention of cross-contamination of fresh produce by halamine films and evaluation of the effect of RPC films on the quality attributes of leafy greens

a) *Demonstrated self-cleaning property of antimicrobial N-halamine films:* The self-cleaning activity of chlorine-charged antimicrobial N-halamine films was demonstrated by extended incubation of antimicrobial N-halamine films after the contact with a spinach leaf inoculated with 10^5 log CFU/leaf of *Listeria innocua*. After the contact of N-halamine films with contaminated leaves, the contaminated films were incubated at room temperature for 5–20 min. Figure 1 shows that the bacterial population on the charged films decreased as a

function of time, and bacteria were not recovered from the charged N-halamine films after 20-min incubation. The concentration of bacteria on the uncharged films did not decrease significantly ($p > 0.05$).

b) *Enhanced prevention of cross-contamination of fresh produce:* In this study, a texture analyzer was used to precisely control the contact force and contact time. To simulate the cross-contamination between leaf and liner materials, lower force (1 N) and high force (9.8 N) were applied in this assay, respectively. Figures 2 and 3 demonstrate that the chlorine-charged N-halamine film can significantly reduce the bacterial transfer from contaminated leaf to plastic surface. When the exposure time was more than 20 min, the charged N-halamine films can eliminate the bacterial transfer to uncontaminated leaves. In addition, the consumption of chlorine on the antimicrobial N-halamine films was quantified using X-ray photoelectron spectroscopy (XPS). The results in Figure 4 and Table 1 suggest that although a spinach leaf itself consumes chlorine charged on a N-halamine film surface upon contact, there was still a significant amount of chlorine content on the surface of the N-halamine film after contact with leaves.

c) *Demonstrated insignificant effect on the produce quality:* The color and texture analyses of fresh spinach leaves after the contact are presented in Figure 5. The contact was done for 20 min at an applied force of 9.8 N, and the results after the contact with uncharged and charged films were compared. Overall, the results show that there is no significant influence on spinach leaves by contact with a chlorine-charged halamine film.

2. Development of rechargeable antibacterial N-halamine plastic films with antifouling function

a) *Demonstrated high concentration of active chlorine and rechargeability:* In this study, we developed a novel rechargeable antibacterial halamine film with antifouling function by chemically incorporating zwitterionic moieties (SBMA) onto surfaces of the existing halamine films (HAF). The new bi-functional films (SBMA@HAF) exhibit integrated properties of high transparency, robust mechanical property, great hydrophilicity, ease of chlorine recharging (>250 ppm), and long-term stability (Figure 6).

b) *Improved antimicrobial properties:* The antimicrobial tests demonstrated improved biocidal properties of the new film (SBMA@HAF) against both gram-positive and gram-negative bacteria as compared to the original N-halamine film (HAF) without antifouling function (Figure 7).

c) *Incorporated antifouling functions:* This surface modification approach was effective in reducing bacterial adhesion to the plastic surface and preventing the formation of biofilm. Field-emission–scanning electron microscopy (FE-SEM) results demonstrate that the new bi-functional film (SBMA@HAF) can effectively reduce bacterial adhesion in contaminated water (Figure 8). When more bacterial cells were loaded onto the film surface, the new film exhibited better bacteria-releasing behavior compared with the chlorine-charged halamine film (Figure 9). The significant differences between the HAF film and the SBMA@HAF film proved the success in development of multifunctional biocidal and antifouling films as a liner material for fresh produce.

3. Development of rechargeable antimicrobial plastic films by immobilization of N-halamine functionalized bio-based carriers on the surface

a) *Enhanced chlorine content on PVA-co-PE films:* In this study, yeast cell wall particles (YCWPs) were used as a bio-based carrier to encapsulate halamine-binding food-grade polymer, ε-polysine (EPL). The YCWPs with and without encapsulated EPL were then immobilized onto PVA-co-PE film by a chemical conjugation approach. The modified PVA-

co-PE films can be charged with 1% chlorine. The results in Figure 10 show that the chlorine content of the modified PVA-co-PE films was greater than 700 nmol/cm^2 (Figure 10a). The stability and rechargeability were also demonstrated in Figure 10b and 10c, respectively.

b) *Enhanced antimicrobial activity:* The antimicrobial activity of native and modified PVA-co-PE films was tested in this assay (Figure 11). Both YCWPs-modified and YCWPs@EPL-modified films can inactivate 5 log of bacteria cells in water without organic content (COD = 0 mg/L) after 2-min exposure. In the presence of organic load (COD = 500 mg/L), YCWPs@EPL-modified films can achieve 5-log reductions of *E. coli* and *L. innocua* by exposure for 20 min and 10 min, respectively. The results demonstrated a significant enhancement of antimicrobial activity as compared to native PVA-co-PE films.

c) *Demonstrated prevention of cross-contamination of fresh produce:* Prevention of cross-contamination by modified PVA-co-PE films was demonstrated by performing the assay as described in section 1(b). The results in Figure 12 show that both YCWPs-modified and YCWPs@EPL-modified films significantly reduced bacterial transfer from the plastic films to spinach leaves. No bacteria were detected on modified films after the second 10-min contact with leaves.

4. Development of bio-based antimicrobial coatings for preventing RPC contamination

a) *Inactivation of L. innocua on RPC surface:* A bio-based antimicrobial coating was developed for preventing cross-contamination on RPCs. We developed a simple formulation by combining a food-grade compound with a chitosan solution. The antimicrobial solution (particles + chitosan) was applied on the RPC surface using an airbrushing system with air compressor. The RPC was sprayed with the antimicrobial solution (at ambient temperature, 22–25°C) at ~5 cm from the sprayer to form a uniform coating over the RPC surface. After the coating treatment, the RPC was immersed in chlorine solution (1% active chlorine), then drained for 1 min, and then washed three times with sterile Milli-Q water to remove any free chlorine in solution. The sprayed RPC was allowed to dry under a biosafety hood for 2 h.

   A bacterial suspension of gram-positive strain *L. innocua* (ATCC 33090, Manassas, VA, USA) was selected as a surrogate for gram-positive human pathogenic *L. monocytogenes.* For inoculation, 10 spots of 10 µL (total of 100 µL) of bacterial suspension (10^9 CFU/mL) were placed on the RPC surface (area of 4 cm^2). After contact (5–30 min), the presence of the inoculated bacterial strain was detected by swabbing the control surface (not modified) and treated surface (formulation + chlorine solution) to detect cross-contamination of the surface contaminated with organic matter (COD = 1000 or 2000 mg/L). Cells on the surface of the RPC were swabbed with a 3M™ Sponge-Stick (hydrated sponge with 10 mL of neutralizing buffer) to estimate bacterial populations on the RPC surface. Each swab was transferred to a bag containing 40 mL of maximum recovery diluent (MRD). A 1:50 dilution of the cells present on the RPC surface was made by adding 40 mL of MRD, and then the sample was pummeled for 120 s in a stomacher and allowed to stand for 2 min. The sample liquid was serially diluted and triplicate spread plated on agar. Bacterial counts were determined after overnight incubation at 37°C. The results in Figure 13 and Figure 14 show that this bio-based sanitizer delivery system can inactivate 6 log of pathogenic bacteria in different organic loads: in 10 minutes (for 1000 COD) or 20 minutes (for 2000 COD).

b) *Cross contamination from contaminated fresh produce to RPC – Simulated leaf-to-RPC cross contamination: L. innocua* was selected as a surrogate for the gram-positive human pathogen *L. monocytogenes*. Baby spinach leaves were purchased from a local market and stored at 4°C until further use, up to 4 days. Outer leaves were removed, and the leaves without any visual tissue damage were selected. The selected leaves were washed with sterile deionized water prior to the experiments. Bacterial inoculation was performed with a modified protocol for the leaf surface compared to the RPC surface. Briefly, each spinach

leaf was placed in a Petri dish (100-mm diameter), and then 10 spots of 10 µL (total of 100 µL) of bacterial suspension (10^9 CFU/mL) were dropped by pipetting and spread with a sterile cell spreader over the adaxial (upper) leaf surface; the Petri dish was sealed with parafilm (Fisher Scientific) and stored at 4°C for 24 h to allow bacterial adhesion to the leaf surface. After 24-h storage, 3-cm diameter disks were cut from each leaf with a sterile cork borer. Leaf disks were incubated at room temperature on the coated and chlorine-charged RPC (treated) and control RPC (coated but not charged) for 5–15 min. After each incubation time, the level of bacterial transfer to the RPC surface from the contaminated leaf was quantified by using the swabbing method outlined in section 4a. The results in Figure 15 show that within 5 min of incubation the contaminated leaves transferred more than 5 log of bacteria to the control RPC surface while the treated RPC surface reduced the transfer to ~3 log of bacteria. After 15 min of incubation no bacteria from the contaminated leaves were detectable on the treated surface, while the control surface had ~5 log of bacteria. Overall, these results illustrate the potential of bio-based coatings on RPCs to significantly reduce cross-contamination risks from contaminated fresh produce.

5. Evaluation of resisting effect of zwitterion (SBMA)

a) *Improved surface area to amplify adsorption capacity:* In earlier experiments, we successfully developed a bi-functional SBMA@HAF plastic film and proved antifouling functions, including both bacterial resisting and releasing. We further investigated the antifouling process to understand the antifouling mechanism of the zwitterionic structures on surfaces of halamine polymers. Due to the limited surface area on solid plastic films and to demonstrate the resisting process on the surfaces of the polymer, an electrospun SBMA@HAF nanofibrous membrane (SBMA@HAF NFM) was employed that exhibits a much greater surface area and larger adsorption capacity than the plastic film (Figure 16).

b) *Inhibited non-specific adsorption of protein (resisting function):* With the presence of zwitterionic moieties (SBMA), the membrane was effective in reducing non-specific protein adsorption to the surface and in further preventing the formation of biofilm. Bovine serum albumin (BSA) protein was employed to provide the non-specific adsorption interaction. The adsorption kinetic behavior was explained by the pseudo second order, with $R^2 = 0.99$:

$$\frac{1}{q} = \frac{1}{q_e} + \frac{1}{k_2 q_e^2} * \frac{1}{t}$$

where q = amount of adsorbed BSA protein at time t (mass/mass of film)
q_e = amount of adsorbed BSA protein at equilibrium state (q_e = 0.08 (mg/50mg))
k_2 = adsorption rate constant of pseudo second-order model ($k2$ = 0.61 (50 mg/mg/min))

The adsorption mechanism fellows the Langmuir isotherm model within the low BSA concentration range (0.5–2 mg/mL), with $R^2 = 0.97$ and $R^2 = 0.98$ for the SBMA@HAF NFM and HAF NFM, respectively:

$$\frac{1}{Cas} = \frac{1}{Ka\ Cs} * \frac{1}{Ca} + \frac{1}{Cs}$$

where *Ka* = Langmuir isotherm constant (vol/mass)
Cs = maximum adsorption capacity for forming single layer (mass/vol)

Both *Ka* and *Cs* values of SBMA@HAF NFM (*Ka* = 0.47 mL/mg and *Cs* = 0.35 mg/50 mg) are smaller than those of HAF NFM (*Ka* = 0.56 mL/mg and *Cs* = 2.99 mg/50 mg), indicating that SBMA could decrease the affinity between membrane and protein and also limit the protein adsorption capacity (Figure 17).

Outcomes and Accomplishments

1. Rechargeable antimicrobial halamine films

- Demonstrated self-cleaning property of antimicrobial halamine films (inactivates more than 3 log of inoculated bacteria on surface in 20 min)
- Enhanced prevention of cross-contamination of fresh produce (more than 2 log inactivation compared to controls)
- Demonstrated insignificant effects on the produce quality (color and texture)

2. Rechargeable antibacterial halamine plastic films with antifouling function

- Demonstrated high concentration of active chlorine and rechargeability
- Reduced attachment of bacteria to the halamine plastic films
- Improved efficiency of bacterial removal from the halamine plastic films

3. Coating approaches to deliver bio-based sanitizers on food contact surface

- Demonstrated prevention of cross-contamination of food contact surfaces such as RPCs
- Improved efficiency of bacterial inactivation, especially in high organic loads (more than 5 log inactivation)

Summary of Findings and Recommendations

- Developed and demonstrated efficacy of antimicrobial and antifouling plastic surfaces for improving the sanitation of food contact surfaces as well as reducing cross-contamination of fresh produce.
- The antifouling materials significantly (>1 log) reduce attachment of bacteria to plastic surfaces.
- To translate these materials to reusable-plastic-container (RPC) applications, partnership with tote/bin manufacturers is needed.

APPENDICES

Publications

Ma, Y, J Li, Y Si, K Huang, N Nitin, and G Sun. 2019. Rechargeable antibacterial *N*-halamine films with antifouling function for food packaging applications. ACS Applied Materials & Interfaces 11(19):17814–17822.

Presentations

Yi, J., Huang, K., Young, G. M., Ma Y., Sun, G., & Nitin, N. A novel antimicrobial film for preventing cross-contamination of fresh produce. Oral presentation: Developing Scientist Award Competition Finalist. IAFP 2019 Annual Meeting, Louisville, KY, July 2019.

Budget Summary

The total funds awarded to this project were $281,967, through the WSDA Specialty Crop Block Grant Program ($250,000) and CPS Campaign for Research funds. The team anticipates that all funds will be expended by the end of the grant period.

References Cited

1. Warriner, K. 2013. Microbiological Standards for Reusable Plastic Containers within Produce Grower Facilities. Available at: https://cccabox.org/in-the-news/wp-content/uploads/2015/10/RPC_Report_August_2013.pdf.

2. Warriner, K. 2014. Containers Used to Ship Produce Fall Short of Safety Standards for Two Consecutive Years. Available at: https://cccabox.org/in-the-news/wp-content/uploads/2015/10/Press-Release_CCCA-Oct27.pdf.

3. Suslow, T. 2015. Minimizing Microbiological Risks in Multiple-Use Containers. Food Quality & Safety, April 13. Available at: http://www.foodqualityandsafety.com/article/minimizing-microbiological-risks-in-multiple-use-containers.

Figures and Table (see below)

Figures 1–17 and Table 1

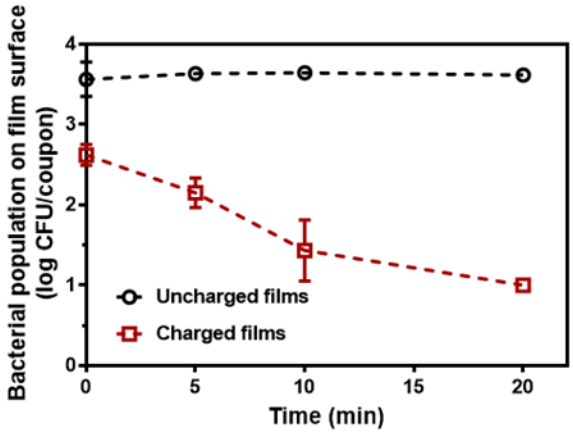

Figure 1 Self-cleaning activity of antimicrobial halamine films.

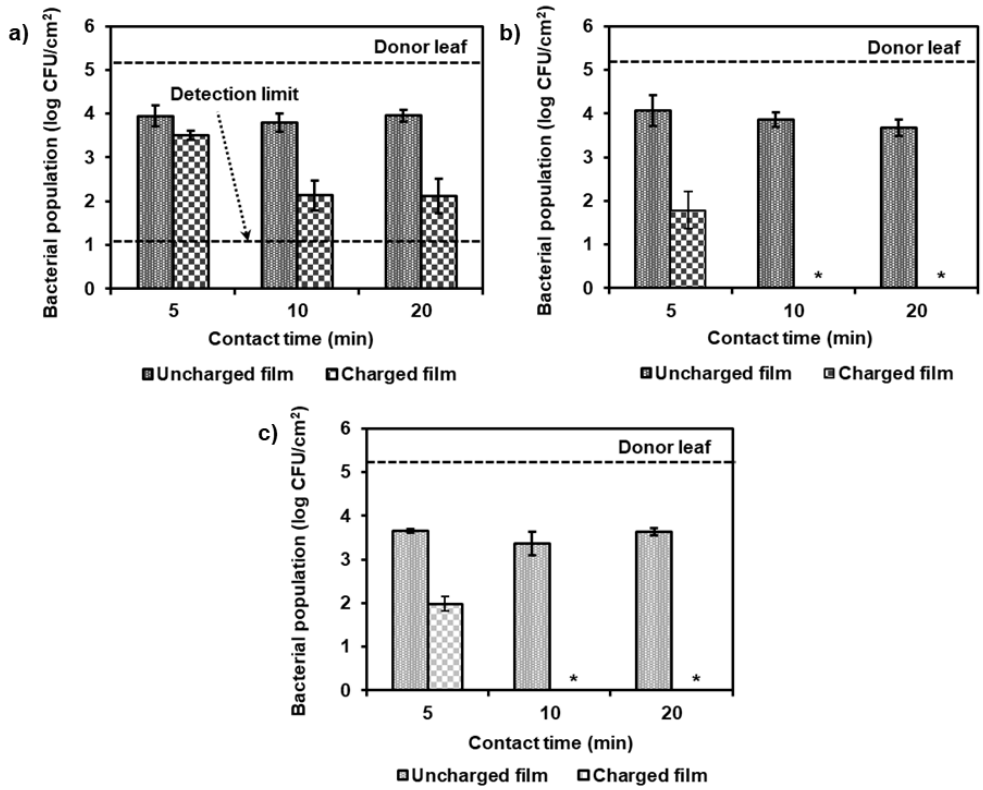

Figure 2 Prevention effect of modified halamine films against cross-contamination of *L. innocua* on spinach leaves for different contact times at an applied force of 1 N: Bacterial population on a) film after 1st contact, b) film after 2nd contact, and c) leaf after 2nd contact. The initial load on a donor leaf was 5.19–5.30 log CFU/cm^2 and the detection limit was 1.10 log CFU/cm^2.

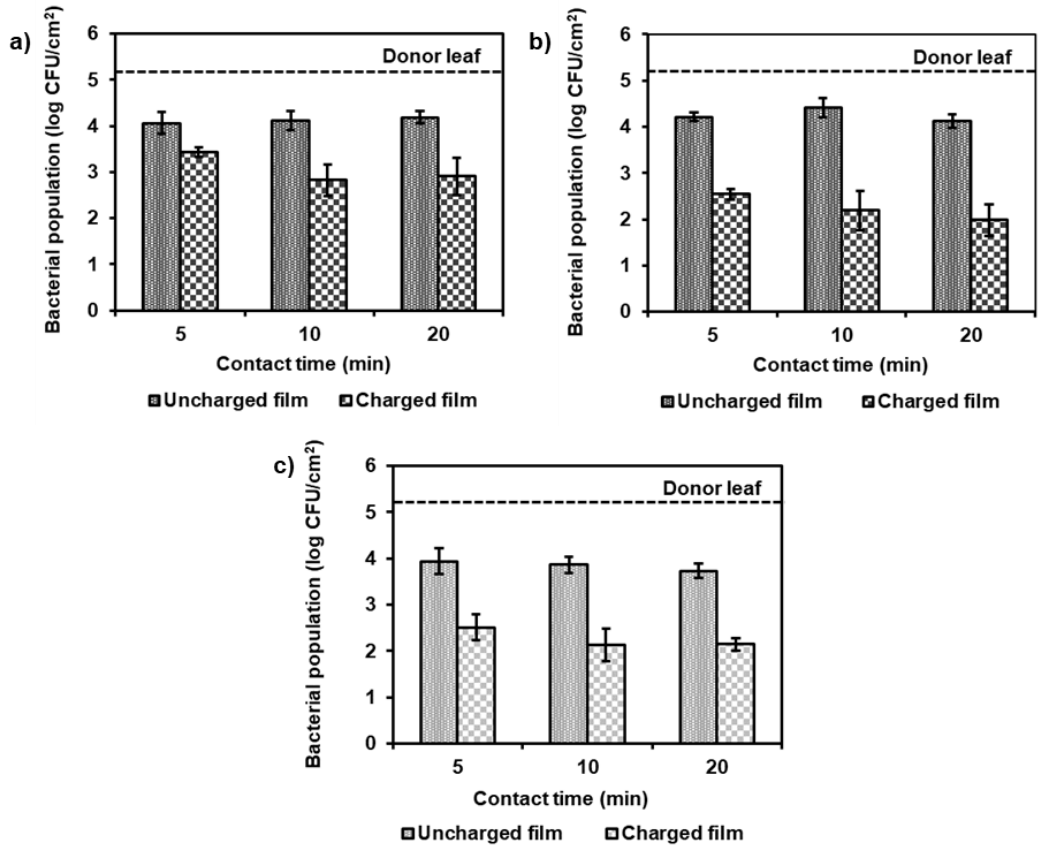

Figure 3 Prevention efficacy of modified halamine films against cross-contamination of *L. innocua* on spinach leaves for different contact times at an applied force of 9.8 N: Bacterial population on a) film after 1st contact, b) film after 2nd contact, and c) leaf after 2nd contact. The initial load on a donor leaf was 5.19–5.30 log CFU/cm^2 and the detection limit was 1.10 log CFU/cm^2.

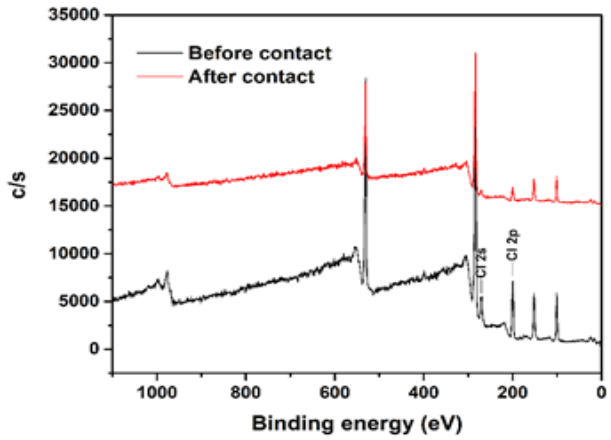

Figure 4 X-ray photoelectron spectroscopy (XPS) survey scan of antimicrobial PVA-co-PE films before and after contact with spinach leaves.

Table 1 Atomic composition of top ~5 nm of antimicrobial halamine films as determined by XPS*

	% atomic composition				
Sample	O	C	Si	Cl	N
Before contact	44.90±6.07a	30.40±5.28a	13.16±5.91a	9.53±1.06a	2.00±0.66a
After contact	50.67±4.80a	29.17±3.67a	14.97±1.86a	3.87±0.71b	1.33±0.47a

*Different letters within the same column indicate statistical significance at ($P < 0.05$) representing SD of n = 3 values.

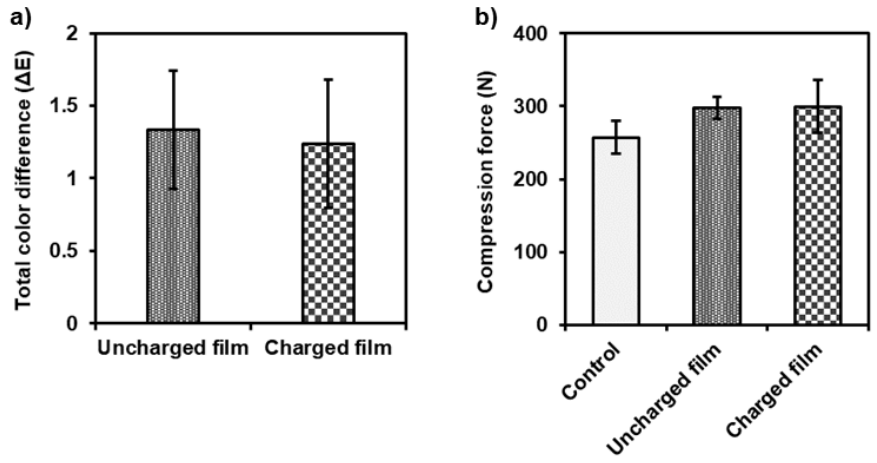

Figure 5 Quality tests of a fresh spinach leaf after a 20-min contact with a modified N-halamine film at an applied force of 9.8 N: a) color, b) texture.

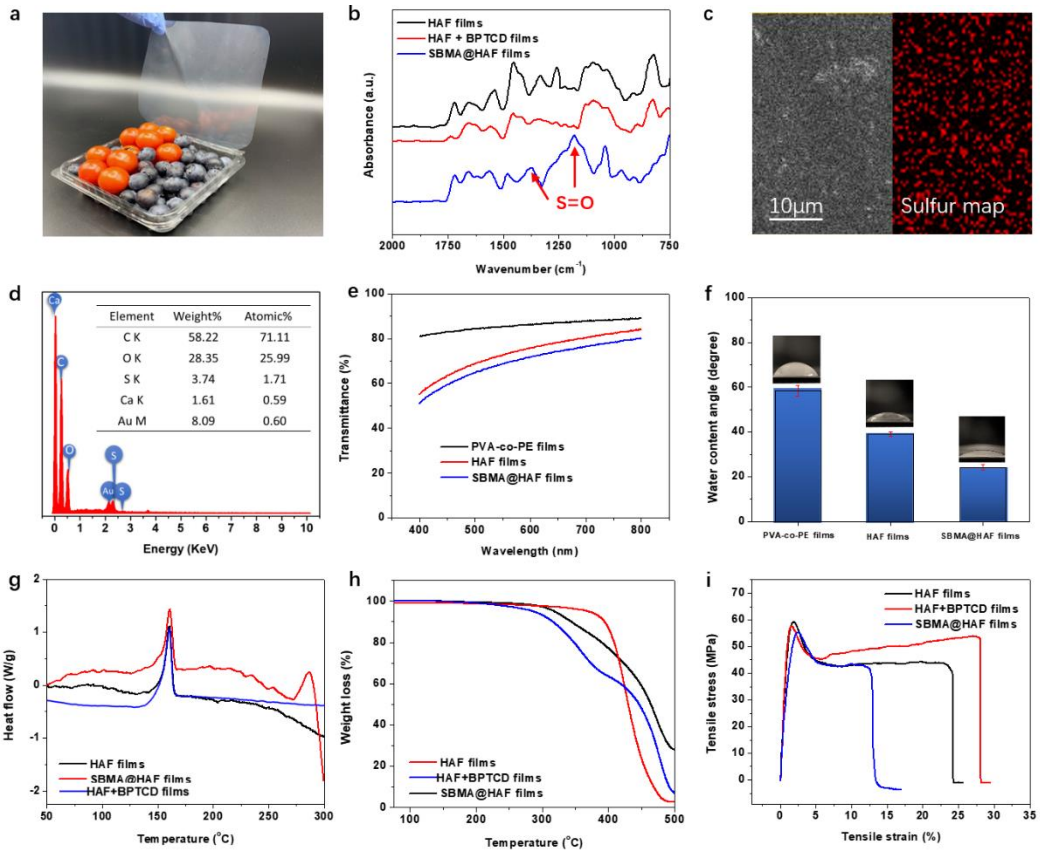

Figure 6 a) Optical image of a bi-functional halamine (SBMA@HAF) film with the thickness of 0.05 mm. b) FT-IR spectra of HAF, intermediate (HAF+BPTCD) and SBMA@HAF films, c) and d) EDX results of SBMA@HAF films, e) UV-vis transmittance spectra and f) water contact angles of PVA-co-PE film, HAF films, and SBMA@HAF films, g) DSC curves and h) TGA curves of HAF, HAF+BPTCD, and SBMA@HAF films, and i) Tensile stress-strain curves of HAF, HAF+BPTCD, and SBMA@HAF films.

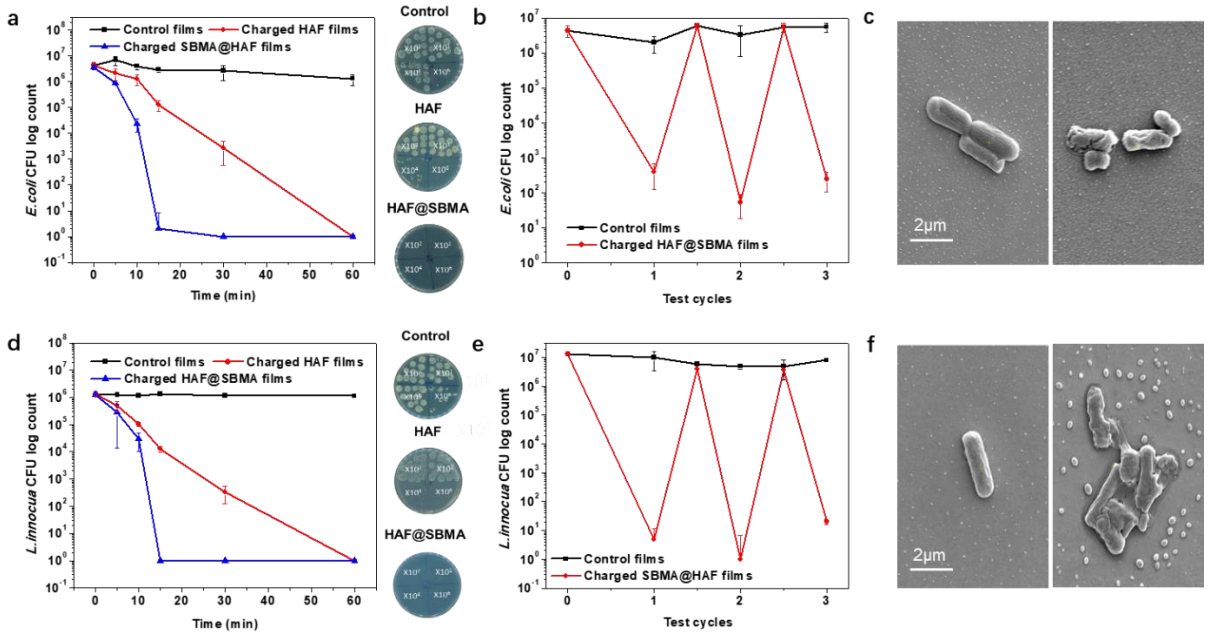

Figure 7 Antimicrobial activities of control films (unchlorinated SBMA@HAF films), chlorinated HAF and SBMA@HAF films against: a) *E. coli* and d) *L. innocua*, respectively. The right images exhibit viable bacteria on agar plates after 15-min exposure. Three repeated chlorination-decontamination tests of chlorinated SBMA@HAF films against: b) *E. coli* and e) *L. innocua*, FE-SEM images of c) *E. coli* and f) *L. innocua* before and after exposing to control films and chlorinated SBMA@HAF films for15 mins.

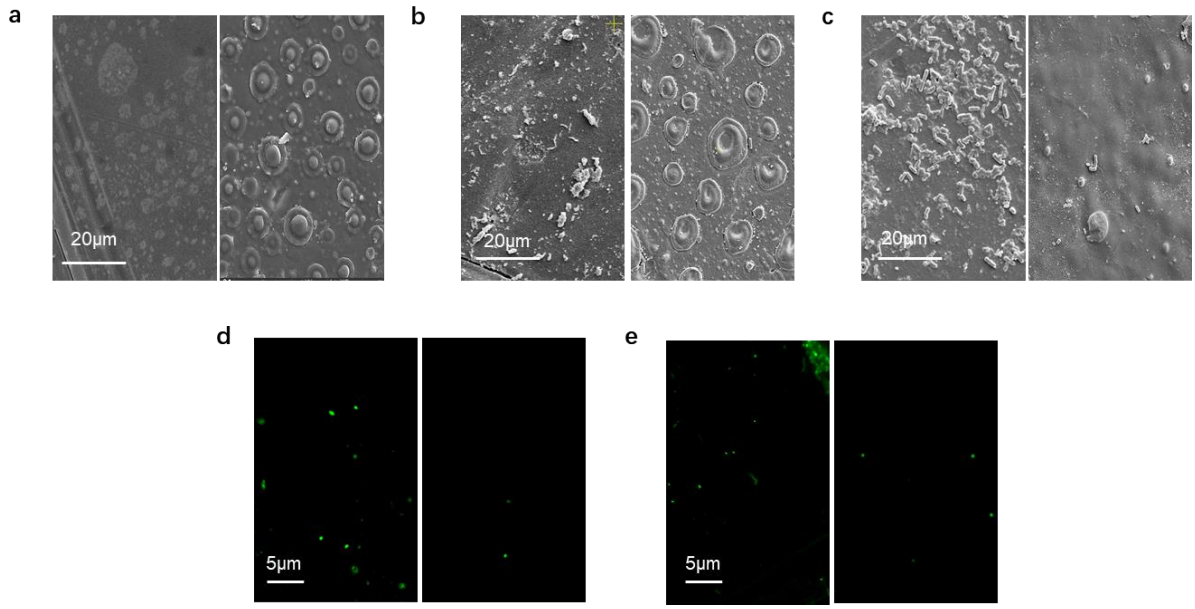

Figure 8 a) FE-SEM image of HAF and SBMA@HAF films. FE-SEM images of unchlorinated HAF and SBMA@HAF films after incubating with b) E. coli and c) L. innocua suspensions, respectively. SG stained fluorescence microscopy images of unchlorinated HAF and SBMA@HAF films surfaces after incubating with c) E. coli and d) L. innocua suspensions, respectively.

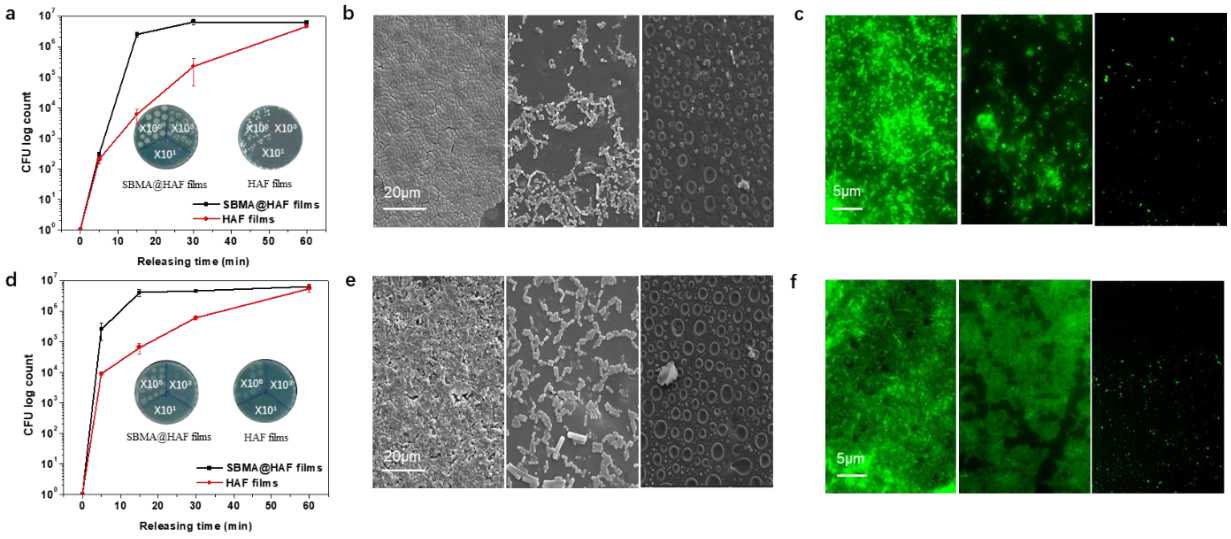

Figure 9. Releasing behavior of unchlorinated HAF and SBMA@HAF films against a) E. coli and d) L. innocua via plate counting method. The insert images exhibit the plate counting results after 15-min releasing. b) FE-SEM images and c) SG stained fluorescence microscopy images of E. coli before and after releasing on unchlorinated HAF and SBMA@HAF films. e) FE-SEM images and f) SG stained fluorescence microscopy images of L. innocua before and after releasing on unchlorinated HAF and SBMA@HAF films.

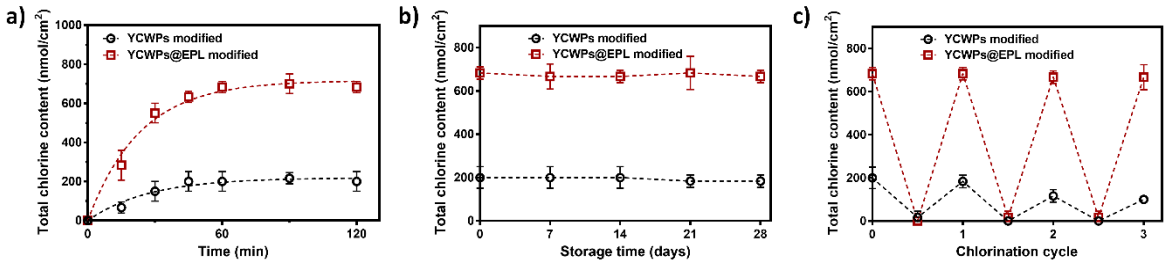

Figure 10 Characterization of chlorine content of modified PVA-co-PE films. (a) Chlorination of YCWPs modified and YCWPs@EPL modified films; (b) Storage stability of YCWPs modified and YCWPs@EPL modified films; (c) Rechargeability of YCWPs modified and YCWPs@EPL modified films.

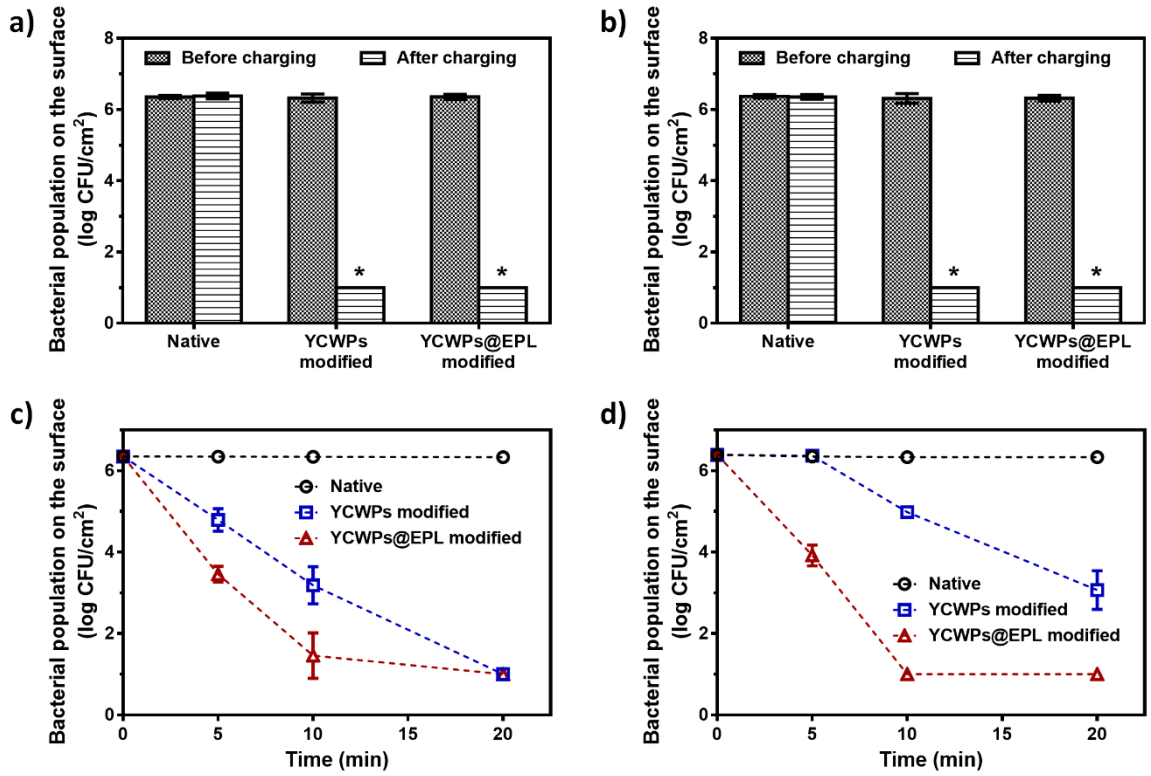

Figure 11 Inactivation of bacteria in clean water (COD = 0 mg/L) after 2-min treatment: (a) *E. coli* O157:H7; (b) *L. innocua*. Inactivation kinetics of planktonic cells on charged films with organic load (COD = 500 mg/L): (c) *E. coli* O157:H7; (d) *L. innocua*.

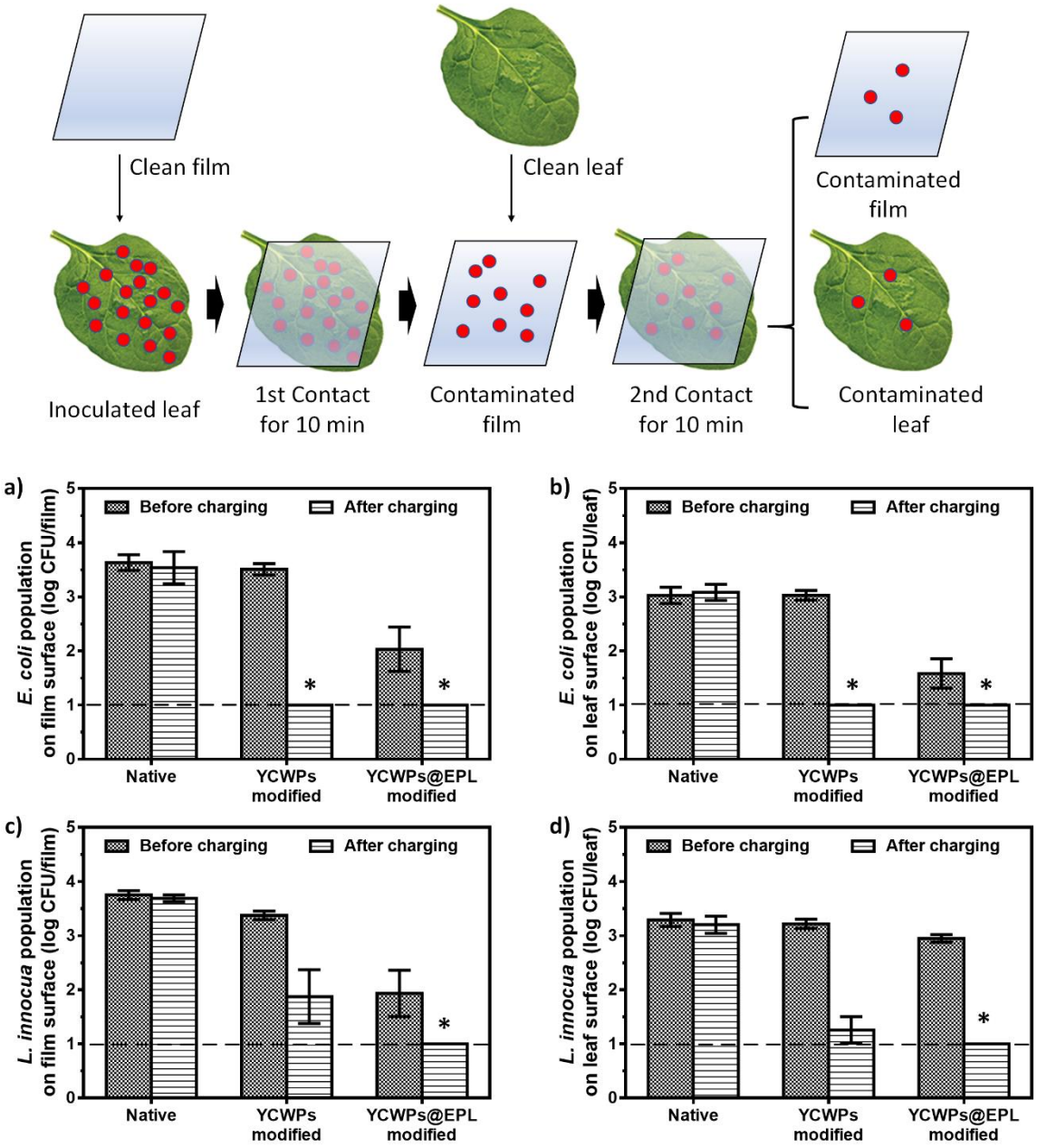

Figure 12 Prevention effect of native and modified films against cross-contamination of spinach leaves: a) *E. coli* population on the film surface after 2nd contact; (b) *E. coli* population on the leaf surface after 2nd contact; (c) *L. innocua* population on the film surface after 2nd contact; (d) *L. innocua* population on the leaf surface after 2nd contact.

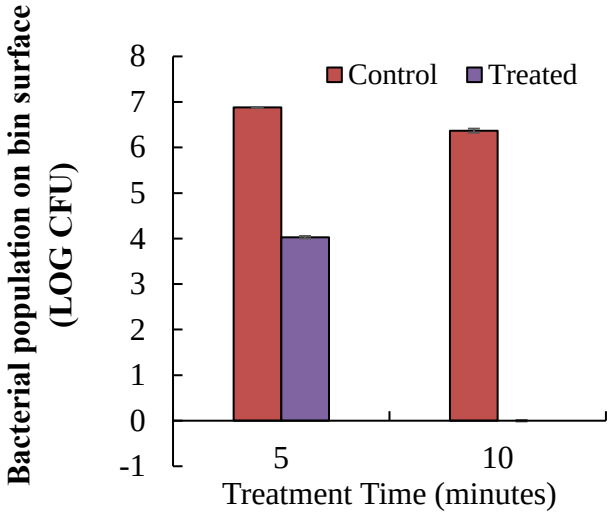

Figure 13: Inactivation of *L. innocua* on reusable plastic container (RPC) (COD = 1000 mg/L).

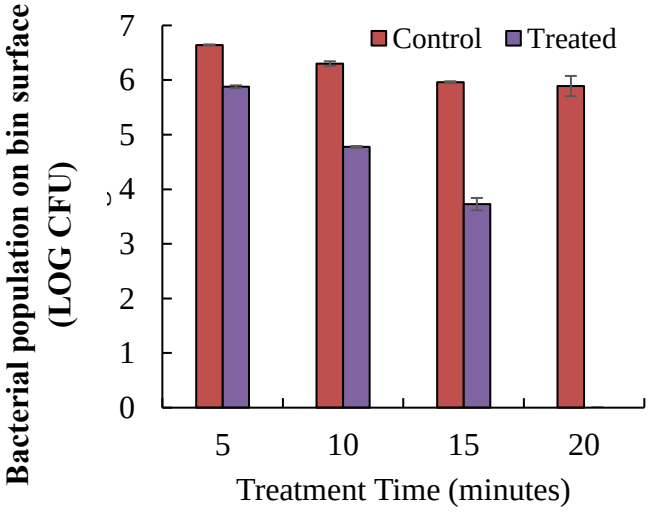

Figure 14: Inactivation of *L. innocua* on reusable plastic container (RPC) (COD = 2000 mg/L).

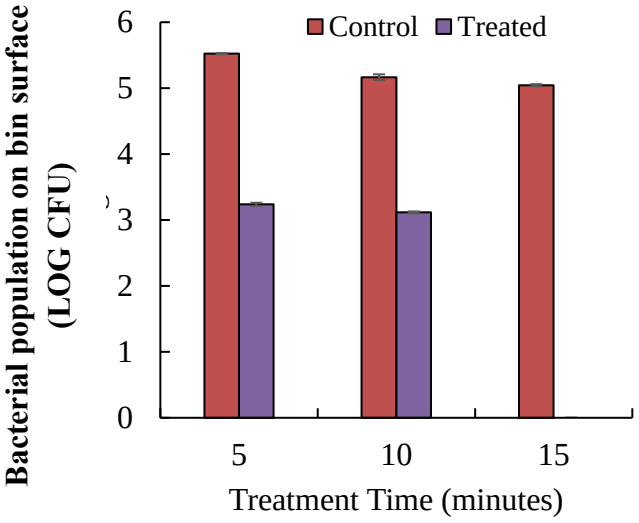

Figure 15: Leaf-to-RPC cross contamination. Bacterial transfer from a contaminated leaf to a non-contaminated RPC coated with bio-based sanitizer. A fresh spinach leaf was contaminated with 8-log CFU/ml suspension of *Listeria innocua.*

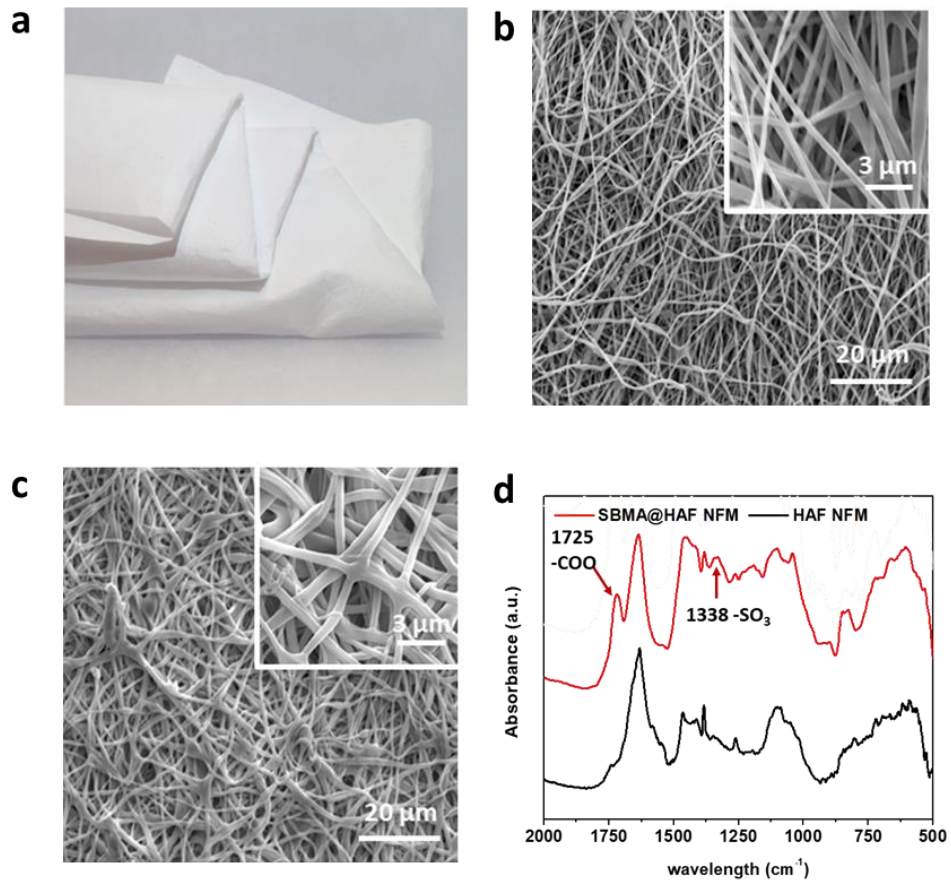

Figure 16 (a) Optical image of HAF nanofibrous membrane. SEM images of HAF NFM (b) and SBMA@HAF NFM (c). (d) FT-IR spectrum of SBMA@HAF NFM and HAF NFM.

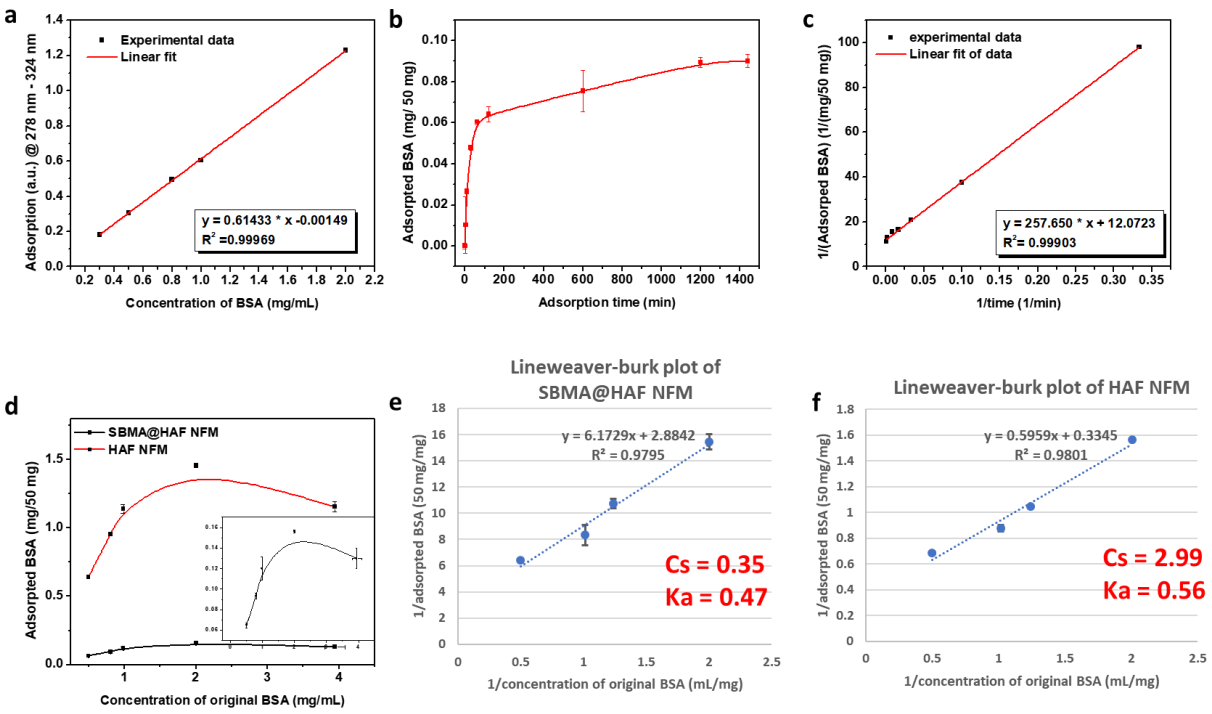

Figure 17 (a) The standard curve of BSA concentration respecting to the UV-vis adsorption at wavenumber of 278 nm. (b) The BSA adsorption kinetic curve of SBMA@HAF nanofibrous membrane at natural pH condition with initial BSA concentration of 1 mg/ml. (c) The BSA adsorption kinetic linear-fitting curve of SBMA@HAF NFM based on the pseudo second order model. (d) The change of BSA adsorption capacity as a function of various initial BSA concentrations, the insert figure refers to the amplification of SBMA@HAF NFM. (e) and (f) show the Lineweaver-Burk plots of SBMA@HAF NFM and HAF NFM respectively.