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Preparation and Characterization of Electrospun Antimicrobial Fibrous Membranes Based on Polyhydroxybutyrate (PHB)

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Abstract: Poly [5,5-dimethyl-3-(3'-triethoxysilylpropyl)hydantoin] (PSPH), an *N*-halamine precursor was synthesized and employed to prepare antimicrobial biodegradable polyhydroxybutyrate (PHB) fibrous membranes by using an electrospinning technique. After exposure to chlorine bleach, the fibrous membranes could be rendered biocidal. The membranes were characterized by scanning electron microscopy (SEM), fourier transform infrared spectrometry (FT-IR), thermogravimetry (TG), and differential scanning calorimetry (DSC). The tensile strength, wetting property, UV light stability, and controlled release behavior were investigated. Biocidal efficacies of the chlorinated fibrous membranes against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* O157:H7 were evaluated by a modified AATCC Test Method 100-2004. The results showed that the prepared chlorinated membranes have excellent antimicrobial functions which could inactivate 92.10 % *S. aureus* and 85.04 % *E. coli* O157: H7 within 30 min of contact time, respectively. From this study, polyhydroxybutyrate (PHB)-based antimicrobial fibrous membranes may further expand the potential use as eco-friendly materials in a variety of applications such as food packaging and biomedical areas.

Keywords: Antimicrobial, Polyhydroxybutyrate, Biodegradable, *N*-halamine, Electrospinning

Introduction

Recently, biodegradable materials have attracted increasing attention with the improvement of environmental awareness. Aliphatic polyesters are known to degrade entirely in the natural environment without forming any toxic products due to the existence of ester bonds in its molecular structure. Generally, aliphatic polymers, which have been studied, can be classified into four groups: polylactides (PLAs), poly (hydroxyalkanote)s (PHAs), PLA/PHA blends, and other polyester blends. Polyhydroxybutyrate (PHB) is probably the most common type of PHA, which was discovered in 1925 from *Bacillus megaterium* by Lemoigne [1]. PHB has many excellent properties such as biodegradability, biocompatibility, optical activity, UV resistance, antithrombogenicity, piezoelectric property, and behaving similarly to polypropylene, which can be evaluated as a suitable material for a various possible applications including food packaging [2,3] and biomedical sectors [4-8] such as surgical sutures, wound dressings, controlled drug delivery, and tissue engineering scaffolds.

Microorganisms such as bacteria and fungi are everywhere in our daily life. Foods not only are of nutritional value to human but also are ideal media for microbial growth. The main function of food packaging is to maintain the quality and safety of food products, and to extend food shelf-life by preventing microbial contamination and other unfavorable conditions during storage and transportation [9]. The traditional food packaging materials such as polyethylene (PE), polypropylene (PP), polystyrene (PS), are difficult for decomposition and would cause serious environmental problems associated with their strong chemical stability. Besides, if medical products contain a certain amount of spoilage microorganisms, they may lead to infection for patients with the low defense function. Over the past years, researchers have mostly focused on the study of the improvement of physical and mechanical properties of PHB due to its high crystallinity and brittleness which limit its utilization [10,11], but have paid less attention to the antimicrobial modification. They have showed that blending PHB with poly(hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx) [12], poly(lactic acid) (PLA) [13], polycaprolactone (PCL) [14], and chitosan [15], etc. is an effective method to overcome its shortage. However, it is also important to increase its antimicrobial effectiveness in view of above mentioned values of PHB in food packaging and biomedical areas.

Generally, antimicrobial modification of materials can be obtained by incorporating antimicrobial agents by using techniques such as embedding, surface modification or coating [16]. For example, Narayanan has reported on antimicrobial food packaging by incorporating Eugenol into polyhydroxybutyrate (PHB) in conjunction with pediocin [17]. The antimicrobial activity was evaluated by challenging foodborne pathogens, spoilage bacteria, and fungi, and demonstrated a significant reduction within 24 h of contact time. However, its antimicrobial properties and efficacies are not durable, regenerable, and efficient. For the past two decades, *N*-halamines containing one or more N-Cl bonds

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have attracted growing attention as antimicrobial agents with their inherent advantages including long-term stabilities, regenerabilities and superior antibacterial functions. Particularly in these laboratories a series of *N*-halamines were synthesized and extensively applied onto cotton [18,19], polyacrylonitrile (PAN) [20], polyester (PET) [21], poly(lactic acid) [22], and other polymers. Among all of the *N*-halamines, poly [5,5-dimethyl-3-(3'-triethoxysilylpropyl)hydantoin] (PSPH) was demonstrated to have a wonderful antibacterial activity. Worley prepared this *N*-halamine siloxane monomers and polymers, and applied to functionalize the surfaces of paper, cotton and a polyurethane paint [23]. For the purpose of improving the antimicrobial efficacy, Liu attempted to add 3-(trimethoxysilylpropyl) octadecyl dimethyl ammonium chloride (SPODA), one of quaternary ammonium salts, into this *N*-halamine siloxane monomer- SPH coatings using two methods [24]. Li reported that coated PSPH onto cellulose together with titania nanoparticles showed excellent UV stability [25]. Nevertheless, there have been no reports of incorporation of PSPH in biodegradable PHB material.

Electrospinning is a simple, versatile, and effective fabrication technique, which can offer nano to microscale fibers with high porosity and large specific surface area to volume ratio [26]. Nano/microfibers have been widely employed in filtration [27], sensor [28], catalyst [29], molecular recognition [30], as well as biomedical science, such as tissue engineering scaffold [6,31] and drug delivery [32].

In this study, electrospinning was used to create the antibacterial fibrous membranes of polyhydroxybutyrate with the synthesized *N*-halamine compounds poly [5,5-dimethyl-3-(3'-triethoxysilylpropyl)hydantoin] (PSPH) for the first time. The membranes could be rendered biocidal by exposure to chlorine bleach. The prepared materials were characterized and analyzed by SEM, FT-IR, TG, and DSC. The antimicrobial performance against *Staphylococcus aureus* (ATCC 6538) as a typical Gram-positive bacterium and *Escherichia coli* O157:H7 (ATCC 43895) as a Gram-negative bacterium were evaluated. The tensile strength, wetting property, UV light stability and controlled release characteristic were also investigated in this study.

Experimental

Materials

Polyhydroxybutyrate with an average molecular weight (M_w) of 300,000 g/mol was purchased from Tianjin GreenBio Materials Co., Ltd. (GreenBio), China. 5,5-Dimenthylhydantoin and (3-chloropropyl)triethoxysilane were provided by Hebei Yaguang Fine Chemical Co., Ltd. and J&K Chemicals, Shanghai, respectively. Chloroform ($CHCl_3$), *N*,*N*-dimethylformamide (DMF), ethanol, acetic acid, household bleach (the active chlorine content was 5 %), and potassium iodide were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai. All reagents were used as received without further purification. The bacteria employed in this test were *S. aureus* ATCC 6538 and *E. coli* O157:H7 ATCC 43895 (American Type Culture Collection, Rockville, MD), and bacteria were grown in Trypticase soy agar (TSA) (Becton, Dickinson and Company, Detroit, MI).

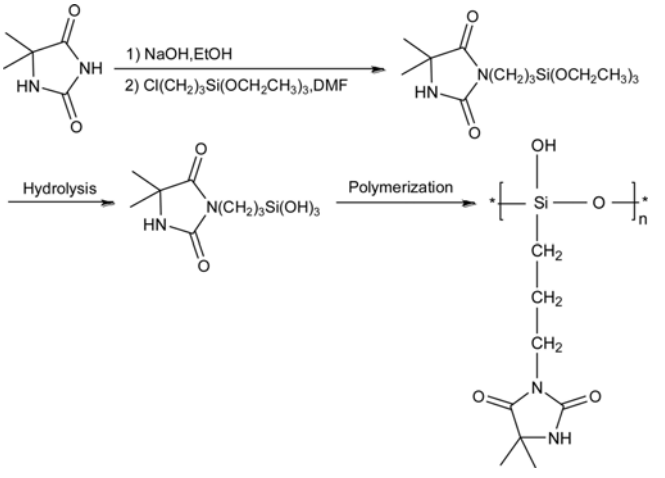
Instruments

The nanofibers morphology was studied using a SU-1510 scanning electron microscopy (SEM). The average fiber diameters and their size distributions were determined by measuring 200 fibers selected randomly from the SEM images using Nano Measurer software (Department of Chemistry, Fudan University). FT-IR spectra of PHB, PHB/PSPH, and PHB/PSPH-Cl were obtained by a NICOLET 10 FT-IR spectrometer. The thermal property was investigated by Differential Scanning Calorimetry (DSC) (TA-200) and Thermogravimetric analysis (TGA) (DTG-60 H).

Synthesis of Poly [5,5-dimethyl-3-(3'-triethoxysilylpropyl)hydantoin] (PSPH)

5,5-Dimethyl-3-(3'-triethoxysilylpropyl)hydantoin polymer was synthesized according to the methods reported earlier [23-25]. The starting material was prepared by mixing 5,5-dimethyhydantoin with an equimolar quantity of NaOH in ethanol and refluxing for 10 min at 90 °C. After drying for 2 days at 45 °C, the organic salt was dissolved in DMF, and followed by adding (3-chloropropyl)triethoxysilane with stirring at 95-100 °C for 8 h. The desired monomer 5,5-dimethyl-3-(3'-triethoxysilylpropyl)hydantoin was obtained through filtration and evaporation of the solvent DMF.

The precursor polymers were produced by the reaction of the above monomer, ethanol, and water in a mass ratio of 2:1:2, respectively. The reaction was continued at 85 °C for 5 h, and the pH was in the range of 3.4 to 5.5. The desired polymers were obtained by evaporation of the water and ethanol. The synthesis of PSPH is depicted in Scheme 1.



Scheme 1. Synthesis of PSPH.

Electrospinning

The electrospinning solution containing 10 wt% of PHB and 2 wt% of PSPH (based on weight of PHB) was prepared by dissolving appropriate amount of PHB in $CHCl_3$ and PSPH in DMF, respectively. The weight ratio of $CHCl_3$ and DMF was 11/1. The mixture was stirred until a homogeneous solution was formed. The prepared solution was filled in a 20 m*l* syringe equipped with a blunt steel needle of 0.7 mm inner diameter. Electrospinning was carried out at room temperature at 20 kV with a tip-to-collector distance of 17 cm and a flow rate of 0.3 m*l*/h. The obtained fiber mats were stored at room temperature in dark place.

Chlorination and Analytical Titration

The fibrous membranes were immersed in a 10 % aqueous solution of household bleach (0.5 % NaOCl) at pH 7 at room temperature for 1 h to produce the antimicrobial material. Then the chlorinated samples were washed thoroughly with deionized water and dried at 45 °C for 1 h to remove free chlorine. The loaded active chlorine on the membranes was determined by the modified iodometric/thiosulfate titration method. Briefly, about 0.1 g of chlorinated samples was suspended in ethanol/acetic acid (9/1, v/v) solution, and 0.5 g KI were added. The solution was titrated with 0.001 N sodium thiosulfate. The chlorine weight percent of the sample was calculated according to the equation below:

$$Cl^+ (\%) = \frac{N \times V \times 35.45}{W \times 2} \times 100$$

where Cl^+ (%) is the weight percent of oxidative chlorine on the sample, N and V are the normality (equiv./L) and volume (L) of the titrant sodium thiosulfate, respectively, while W is the weight (g) of chlorinated sample in test.

Tensile Properties Testing

Tensile tests of PHB, PHB/PSPH and chlorinated PHB/PSPH were carried out by using a universal testing machine (KD II-0.05) at a speed of 10 mm/min. Specimens dimensions were 100 mm×10 mm (length×width). The data presented are the average value of five measurements from each sample.

Contact Angle Measurements

The sessile drop method was used to determine the statistic contact angle of the fibrous membranes. Drops of distilled water were placed on the sample surfaces by a micrometer syringe, and the contact angle was measured for 5 times to calculate the average value for each mat.

UV Light Stability Testing

The stabilities of the chlorinated fibrous membranes were measured using UV-light produced by a model Accelerated Weathering Tester (The Q-LAB Company, USA). The modified samples were placed in the UV light (Type A, 315-400 nm) chamber for times in the range of 1-24 h. After a specific time of UV light irradiation, the membranes were removed from the UV chamber and titrated immediately, or rechlorinated and titrated.

Controlled Release Investigations

A quantitative evaluation of the controlled release of chlorine from the fibrous membranes was carried out according to the method reported previously [33]. In each test, a series of chlorinated PHB/PSPH films (1×1 cm^2) were immersed in 10 m*l* of distilled water in a closed container under constant shaking (50 rpm) at ambient temperature. After specific releasing time, 10 m*l* of the water were taken out and transferred into a 50 m*l* Erlenmeyer flask contained about suitable potassium iodide. After stirring under nitrogen gaseous atmosphere for 30 min, the solution was titrated with 0.0001 N $Na_2S_2O_3$ aqueous solution following the National Environmental Methods Index (NEMI) test method 4500-Cl B. The chlorine content of the distilled water was calculated with the following equation:

$$[C]_{Cl^+} = \frac{\Delta V \times 10^{-3} \times 0.0001 \times 35.45}{2 \times 10} \times 10^6$$

where $[C]_{Cl^+}$ was the active chlorine concentration in the solution (ppm), and ΔV was the volume of the titrant sodium thiosulfate (L).

Biocidal Efficacy Testing

Gram-positive *Staphylococcus aureus* (ATCC 6538) and Gram-negative *Escherichia coli* O157:H7 (ATCC 43895) were used to challenge the antibacterial functions for both of the chlorinated fibrous membranes and control samples according to a modified AATCC Test Method 100-2004. In this test, bacteria were suspended in pH 7, 100 mM phosphate buffer, and 25 μl of the bacterial suspensions were added to the middle of two pieces of one inch square films which were held in places by sterile weights. After exposure to the bacteria with contact times of 1, 5, 10 and 30 min, the samples were quenched with 5.0 m*l* of sterile 0.02 N sodium thiosufate solutions to remove all oxidative chlorine and vortexed. Serial dilutions of the quenched solutions were made with pH 7, 100 mM phosphate buffer, and plated on Tryticase soy agar plates. Then the plates were incubated at 37 °C for 24 h, and the bacterial colonies were recorded and enumerated for biocidal efficacy analysis.

Results and Discussion

Morphology of Electrospun Fibrous Membranes

Figure 1 shows the morphological characteristics of electrospun pure PHB, and electrospun PHB loaded with 2 wt% PSPH fibrous membranes. Obviously, PHB and PHB/PSPH fibers have a fairly uniform structure. In addition, the average diameters of PHB and PHB/PSPH are 676 nm± 83 nm and 689 nm±67 nm, respectively, according to the

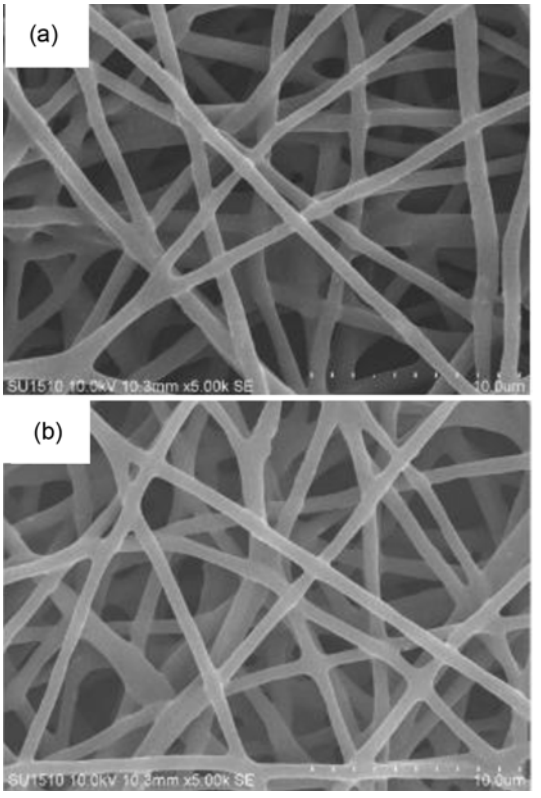


Figure 1. SEM images of electrospun (a) PHB and (b) PHB/PSPH fibrous membranes with magnification of 5,000×.

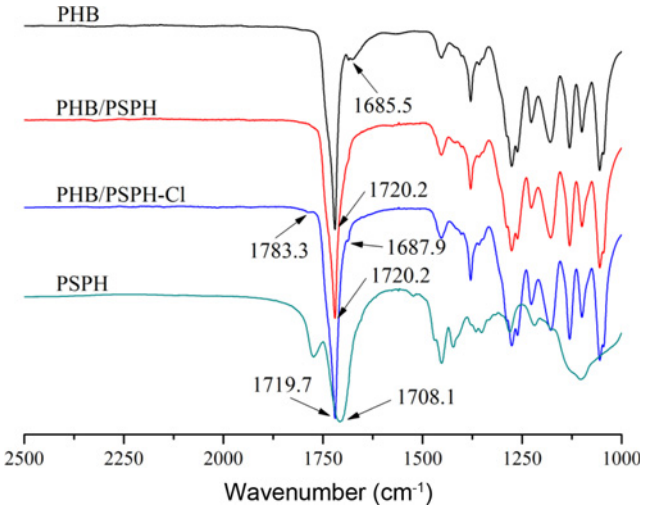


Figure 2. FT-IR spectra of PHB, PSPH, PHB/PSPH and chlorinated PHB/PSPH fibrous membranes.

Nano Measurer software.

Fourier Transform Infrared Spectroscopy

The FT-IR spectra of PHB, PSPH, PHB/PSPH, and chlorinated PHB/PSPH are shown in Figure 2. The spectrum of PSPH showed the characteristic absorption band at 1708.1 cm^{-1}, which corresponds to the carbonyl stretching vibration [34]. The characteristic bands of PHB appeared at 1720 cm^{-1} and 1685.5 cm^{-1}, which can be assigned to the C=O carbonyl group stretching vibration of the crystalline carbonyl group, and stretching of C=O (acetate group), respectively [6,35]. Compared with the FT-IR spectrum of PHB, the absorption peak at 1685.5 cm^{-1} in PHB/PSPH disappeared, which is attributed to the overlap of C=O carbonyl group stretching vibration (1720 cm^{-1}), stretching of C=O (1685.5 cm^{-1}) in PHB, and carbonyl stretching vibration (1708.1 cm^{-1}) in PSPH when loaded PSPH to PHB. After chlorination, the vibrational band of C=O shifts from 1708.1 cm^{-1} to 1783.3 cm^{-1}, which has been reported for other *N*-halamines [36,37], and the overlap disappeared.

Thermal Analysis

The thermal properties of the electrospun PHB, PHB/PSPH, and chlorinated PHB/PSPH fibrous membranes were examined by DSC and TG analyses. From the DSC curve of the chlorinated PHB/PSPH in Figure 3, a new exothermic peak at 180 °C could be clearly observed, which corresponds to the decomposition of N-Cl bonds. This is a characteristic peak of organic *N*-halamines, and has been reported previously by Chen *et al.* [33]. There is no significant difference observed in the DSC thermograph between PHB and PHB/PSPH films since the loading of PSPH was only 2 wt%. It can be predicted that the biodegradability of PHB should not be affected due to the small addition of PSPH since there is no covalent bond between them [22].

The thermograms (TGA) and the derivative mass loss (DTG) curves for PHB, PHB/PSPH fibrous membranes before and after chlorination were showed in Figure 4 and Figure 5, respectively. Only one sharp degradation step exhibited in the TGA curves of these three samples. The weight loss of these samples extended in the range of 200 to

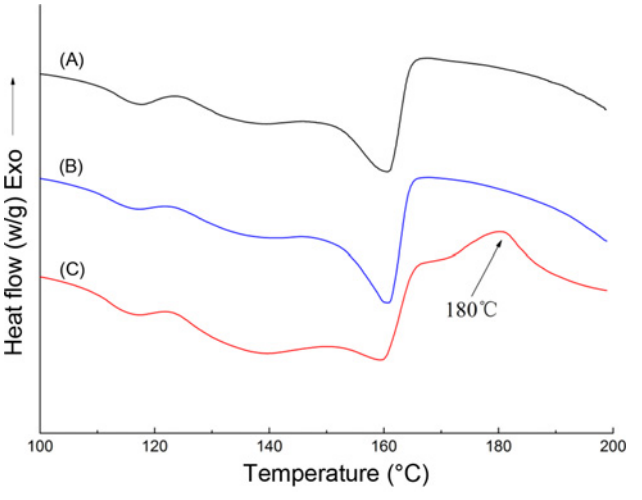


Figure 3. DSC curves of (A) PHB, (B) PHB/PSPH and (C) chlorinated PHB/PSPH fibrous membranes.

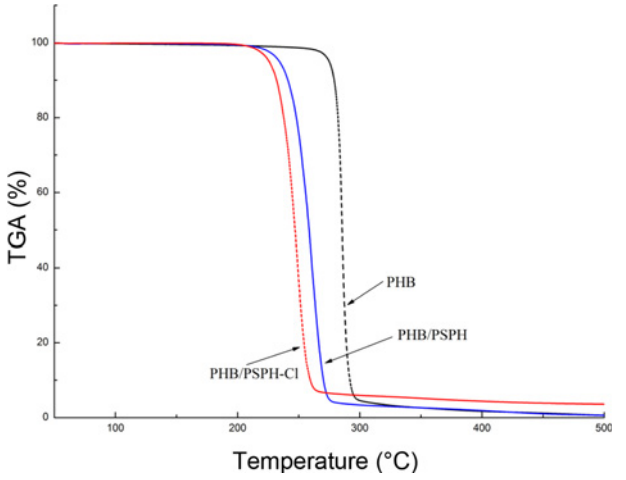


Figure 4. TGA curves of PHB, PHB/PSPH and chlorinated PHB/PSPH fibrous membranes.

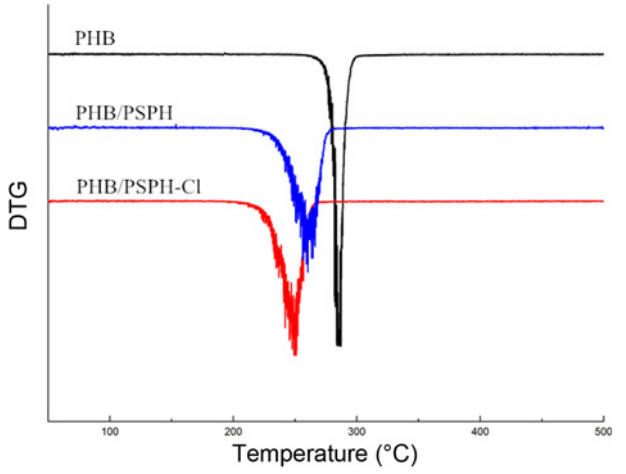


Figure 5. DTG curves of PHB, PHB/PSPH and chlorinated PHB/PSPH fibrous membranes.

330 °C, and the maximum decomposition temperature of PHB, PHB/PSPH, and chlorinated PHB/PSPH were located at 289 °C, 262 °C and 245 °C, respectively. After chlorination, the maximum decomposition temperature of PHB/PSPH fibrous membranes decreased from 262 °C to 245 °C, which was lower than those of the unchlorinated samples. The decomposition of the N-Cl bonds in the chlorinated PHB/PSPH samples may accelerate the thermal decomposition of films through a free radical process [33,36].

Tensile Testing

The results of tensile strength and elongation at break of the chlorinated and unchlorinated fibrous membranes loaded with PSPH are shown in Table 1. The incorporation PSPH into PHB will decrease the tensile strength from 48.46±1.18 MPa to 24.07±1.70 MPa, and the elongation at break from 94.15±1.28 % to 80.04±5.03 %. The PSPH in PHB fibers might cause poorer dispersion and crystal structure. Moreover, the addition of PSPH in PHB is believed to restrict the chain mobility, therefore, decreasing the ductile nature of PHB [15,38]. Upon chlorination, the tensile strength and elongation at break of samples decreased to 22.17±1.49 MPa and 78.04±3.4 5%, respectively. The chlorination made N-H bonds transform into N-Cl bonds so that intermolecular hydrogen bonds were broken.

Table 1. Results of mechanical properties of PHB, PHB/PSPH and chlorinated PHB/PSPH fibrous membranes

Sample	Tensile strength (MPa)	Elongation at break (%)
PHB	48.46±1.18	94.15±1.28
PHB/PSPH	24.07±1.70	80.04±5.03
PHB/PSPH-Cl	22.17±1.49	78.04±3.45

Figure 6. Statistic contact angle image of the chlorinated PHB/PSPH sample.

Contact Angle

The hydrophobicity of the samples was evaluated by measuring the water contact angles. The value of statistic contact angle is related to the surface hydrophilic/hydrophobic characters. During the experiment, we found that the PHB and unchlorinated PHB/PSPH fibrous membranes were wetted totally by distilled water, and no contact angle could be detected. But the water contact angle of the samples increased to 102.9 °±4.6 ° after chlorination (Figure 6). The improvement of hydrophobicity is due to the transformation of N-H to N-Cl and the breaking of the intermolecular hydrogen bonds [39].

UV Light Stability

Table 2 shows the UV light stability of PHB/PSPH fibrous membranes after chlorination. It can be seen that UV light irradiation induced a decrease in the chlorine loading over time. After 1 h irradiation, the active chlorine decreased from 0.31 % to 0.21 %. With the extension of irradiation time, the chlorine decreased slowly, and after 24 h exposure, only 0.06 % of the chlorine remained, which indicates that

Table 2. UV light stability of the chlorinated PHB/PSPH samples

Time (h)	Cl^+ (%)[a]
0	0.31
1	0.21
2	0.16
4	0.10
6	0.07
8	0.07
12	0.06
24	0.06
24 (rechlorination)	0.22

[a]Errors are estimated to be ±0.01.

most of the N-Cl bonds decomposed in the PSPH structure [19]. However, upon rechlorination of the samples following exposure to UV light for 24 h, 71 % of active chlorine could be regained, which exhibits good recoverability of antibacterial property.

Controlled Release Characteristics

According to the previous studies, we know that *N*-halamines could release active chorine into the environment from the dissociation of *N*-chloramine [33]. Generally speaking, the release behavior has two opposite effects. On the one hand, a fast release can lead to powerful and instant efficacy for biocidal action; on the other hand, the exhaustion of antimicrobial agents releasing to water in a short-term will cause environmental impacts. Therefore, it is very important to determine the amount of the active chlorine released from the antimicrobial materials in real applications [40].

Figure 7 presents the active chlorine levels released into deionized water from the chlorinated samples. It can be observed that the chlorine content released to solution increased dramatically in the initial 12 h, and then leveled off with the extension of releasing time. The positive chlorine released from the PHB/PSPH fibrous membranes was only 0.7 ppm after 120 h, which was lower than the current EPA maximum residual disinfectant level of 4 ppm in drinking water [41,42]. Besides, this result suggested that the chlorinated PHB/PSPH membranes are safe to the environment, since the N-Cl bonds in the *N*-halamine antimicrobial materials are quite stable [40].

Biocidal Test

The biocidal efficacy test results for the unchlorinated and chlorinated electrospun fibrous membranes against *Staphylococci aureus* and *Escherichia coli* O157:H7 at concentrations of about 10^6 cfu/sample are shown in Table 3. It is obvious that the unchlorinated PHB/PSPH films only caused a small degree reduction of *S. aureus* and *E. coli* within 30 min of contact time owing to the adhesion of the bacteria to the pores inside of the films rather than to inactivation [43-45]. Compared with the control samples, the chlorinated membranes with the chlorine loading of 0.31 % have significant improvement in antibacterial efficacy, and could inactivate 92.10 % *S. aureus* and 85.04 % *E. coli* O157: H7 within 30 min of contact time, respectively. We also observed that the chlorinated samples are more powerful in inactivation of *S. aureus* than *E. coli* O157: H7 within each contact time. The probable reason is the existence of an extra lipid layer on the outer cell membrane of the Gram-negative bacteria, which does not exist in Gram-positive bacteria. After chlorination, the hydrophobicity enhanced, which can be seen in the results of contact angle measurements in Figure 6, since the N-H bonds in *N*-halamines were converted into N-Cl bonds, which might affect the antimicrobial efficacies [39,46].

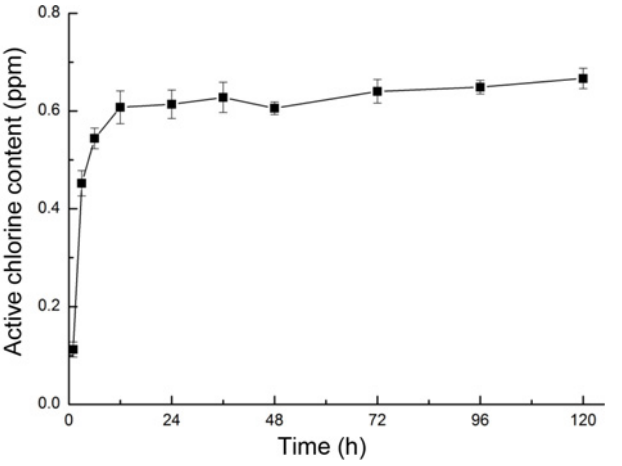


Figure 7. Active chlorine contents in the released solutions from the chlorinated PHB/PSPH membranes.

Table 3. Antibacterial property of the chlorinated PHB/PSPH fibrous membranes against *S. aureus* and *E. coli* O157:H7

Samples	Contact time (min)	Bacterial reduction (%)	
		S.aureus[a]	*E.coli* O157: H7[b]
PHB/PSPH	30	21.01	2.43
PHB/PSPH-Cl (0.31 % Cl^+)	1	13.83	2.43
	5	54.04	8.93
	10	77.74	21.94
	30	92.10	85.04

[a]The inoculum was 9.33×10^5 cfu/sample and [b]the inoculum was 1.03×10^6 cfu/sample.

Conclusion

Antimicrobial fibrous membranes were generated by electrospinning the mixed solution of Polyhydroxybutyrate (PHB) and 5,5-dimethyl-3-(3'-triethoxysilylpropyl)hydantoin

polymer (PSPH) and then chlorinating the as-prepared fibrous membranes. The surface morphology and structure were confirmed by SEM and FT-IR, respectively. According to TG and DSC data, the *N*-halamine additive has small effect on the thermal properties of electrospun PHB films. The chlorinated PHB/PSPH samples showed excellent antimicrobial efficacies which could inactivate 92.10 % *S. aureus* and 85.04 % *E. coli* O157: H7 within 30 min of contact time, respectively. Considering the powerful antimicrobial property of the as-prepared chlorinated fibrous membranes, they will have great potential for a number of applications including food packaging and biomedical materials.

Acknowledgements

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