A new antimicrobial agent: poly (3-hydroxybutyric acid) oligomer

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Abstract

In this work, it is first reported that the poly (3-hydroxybutyric acid) (PHB) oligomer with a few degrees of polymerization possesses effective antibacterial and antifungal properties. Two preparation methods for the PHB oligomer are described, namely, one-step ring-opening polymerization of -butyrolactone and extraction from the fermented PHB polymer. An appropriate amount of the synthesized PHB oligomer shows no physiological toxicity to the skin and major organs of mice. Topological application of the synthesized PHB oligomer imparts antimicrobial ability to non-antibacterial fabrics with washing resistance. The synthesized PHB oligomer offers effective sterilization and promotes wound healing in infected nude mice. Most importantly, the PHB oligomer is also reactive to drug-resistant bacteria. These results suggest that the PHB oligomer is not only a great candidate for antimicrobial modification but also a promising one for biomedical applications. Finally, the antimicrobial mechanisms of the PHB oligomer are revealed, and these include disruption of biofilm and the bacterial wall/membrane, leakage of the intracellular content, inhibition of protein activity, and change in the trans-membrane potential.

Key words: Poly (3-hydroxybutyric acid), oligomer, antimicrobial agent, extraction, synthesis, antibacterial mechanism

Introduction

The prevention of microbial transmission and infection hasbeen a major public health and safety concern worldwide [1-3]. Microbial infections not only cause diseases and wound pain [4-5], but also lead to increased morbidity and mortality in severe cases [6-8]. Antimicrobial agents

have been widely applied in agriculture, the environment, medicine, textiles, food, food packaging and other industries to protect people individuals against the microorganisms. Among the three types of commonly used antimicrobial agents, the inorganic ones exhibit high antimicrobial efficiency and good heat stability [9-12]. However, there is an increasing concern on their potential perniciousness to the environment and human health from the leaching of heavy metals [12]. Organic antimicrobial materials are commonly used and include quaternary ammonium [13-14], polybiguanides [15], and N-halamine materials [16-17]. However, their functional modification on the surface of materials shows poor durability [18]. The natural antimicrobial agents are safe, bio-active, nontoxic and environment-friendly [19], hence attract increasing attentions.

Antibiotics are the first natural antimicrobial agents to be discovered and are mainly produced by microorganisms; traditional antibiotics are found to be effective to plank-tonic bacteria in the free-floating state but are less effect for most bacteria in biofilm state [21-23]. Biofilm is formed by bacteria self-produced extracellular polymeric substances (EPS), when bacteria adheres to solid surfaces and aggregate [23]. It act as a diffusion barrier to limit antibiotics access to bacteria, results in antibiotic resistance [24-25]. Additionally, the non-judicious use of antibiotics has caused the emergence of multi-drug resistance (MDR) bacteria. Subsequently, varieties of new natural antibiotics have been developed to deal with MDR. Antimicrobial peptides (AMPs) are a common kind of nature antibiotics that produced small molecules containing 10~50 amino acid residues. AMPs has excellent antimicrobial, anti-parasitic and antiviral activities [26-33]. Most AMPs can cause bacterial cell membrane damage by electrostatic interactions between the amino acid residues and cell surfaces. Despite the rapid development of various new types of AMPs, the resistance of the bacterial species has been enhanced by means of bacterial surface modification, expression of efflux pumps and protease secretion [34].

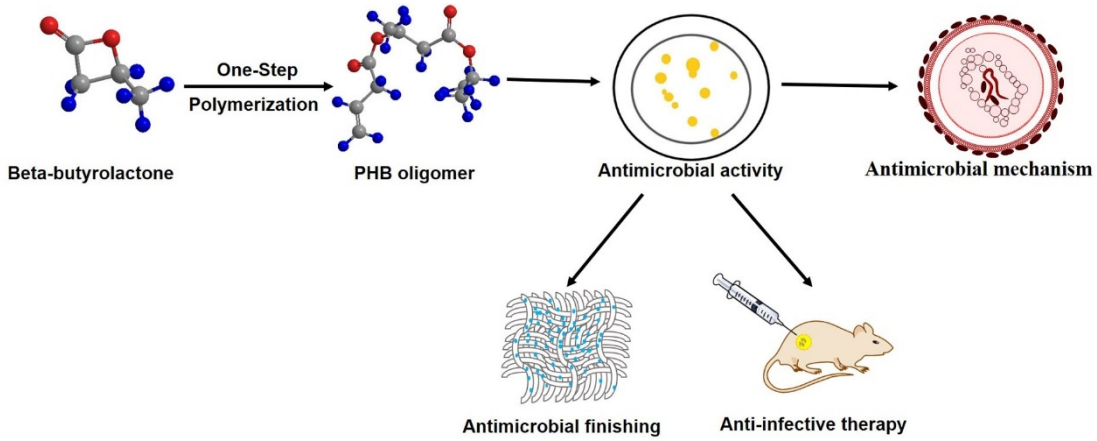
Natural antimicrobial extracted from plants are also effectively inhibit microbial growth. Essential oils (EOs) typically represent plant antimicrobial contents, and the components include phenolics, terpenoids, flavones, and esters [35-39]. Recently, studies have reported that EOs can destroy the biofilm, control MDR and are effective against bacterial resistance mechanisms [40], which may be associated with hydrophobic antibacterial mechanism. The hydrophobic action of EOs can non-specifically lead to effects such as the destruction of membrane fluidity and permeability, disturbance of membrane proteins, and suppression of respiration[41-45]. However, the plant origin is limited by region. Plant growth can greatly affect the extract of EOs, and is also influenced by many physical factors, such as sunlight,

temperature, and humidity. The maximum application limit of EOs is high volatility and poor durability [46-47].

With the emergence of drug-resistant bacteria, developing novel and natural antimicrobial agents is urgently needed. A new medical antimicrobial agent not only has excellent antimicrobial ability but also need to consider other conditions such as non-toxicity to individuals, bio-degradation, environmental-friendliness, renewable energy potential, and high yield. Poly (3-hydroxybutyric acid) (PHB) is a bio-synthesized and biodegradable linear aliphatic polyester from renewable sources. It has been used in industries based on its excellent mechanical property, optical activity, nontoxicity, biocompatibility and ultraviolet (UV) resistance [48-49]. Because of its bio-degradability, PHB long-chain polymer also has been a focus of research interest in biomedical discipline, including controlled drug delivery, surgical structure, wound dressing, vascular graft, blood vessel, orthopedic devices, tissue engineering and skin substitute[50-52]. To date, studies on antibacterial properties of PHB have involved the PHB with addition of other antibacterial agents or with chemically grafted antimicrobial moieties [53-58].

In 2016, we reported that fabrics made from polylactide acid/poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV/PLA)-blend filament fibers exhibited broad-spectrum bactericidal effects [59]. In this work, we separated ingredients in the PHBV/PLA fabrics by extraction and analyzed their compositions by infrared spectroscope (IR), nuclear magnetic resonance spectroscopy (NMR) and mass spectrometer (MS). We then identified Poly (3-hydroxybutyric acid) PHB oligomer with a low degree of polymerization (DP) as the antimicrobial agent (scheme 1). A hydrophobic and non-polar uncharged molecule that is surprisingly different from the common poly-cation type of antimicrobial with multiple positive charges to endow water solubility as well as electrostatic attraction to the anionic bacterial cell membrane[59]. Inspired by this finding, we obtained the PHB oligomer through extraction from biologically fermented PHB powder and verified its antimicrobial ability. Subsequently, the PHB oligomer was synthesized by one-step open-ring polymerization of -butyrolactone, which is a simple and rapid organic synthesis method. To verify the antimicrobial modification applicability of S-PHB (i.e., synthesized PHB), one example of durable antimicrobial modification of PHB oligomer is demonstrated by repeated washing of non-antimicrobial staple fiber fabrics. Furthermore, to test its potential in medical applications, skin sensitization and infected wound cure in mice treated with PHB oligomer were observed.

Finally, the antimi- crobial mechanism of the synthesized PHB was revealed.



Scheme 1. Schematic illustration of the investigation: preparation of the PHB oligomer, antimicrobial activity in the in vitro and in vivo applications, and the antimicrobial mechanism.

Results

PHB oligomer produced by extraction and chemical synthesis

PHB oligomers (E-PHB) were obtained by extraction from bio-based PHB powder and were characterized by MS (**Figure 1**). Number of repeating units was observed as 86 by MS of E-PHB, indicating that the major component of E-PHB was a mixture of a series of PHB oligomers with DP ranging from 8 to 15. Antimicrobial tests were performed against *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Candida albicans* for E-PHB and the PHB powder with high DP (**Table 1**). The PHB powder with high DP displayed no bactericidal effect, but E-PHB oligomers with a DP from 8 to 15 showed an excellent antimicrobial rate greater than 90% against the three microorganisms. These results confirmed that the extracted PHB oli- gomers with low degrees of polymerization could kill bacteria and fungi effectively at the test concentration.

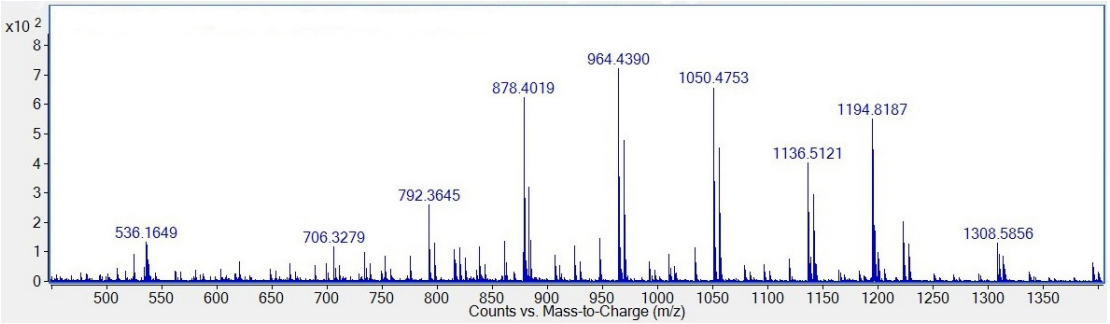


Fig. 1 Mass spectrum of the E-PHB oligomer

Table 1 Antimicrobial rates of PHB with different DP

Sample	Polymization degree	*S. aureus*	*K. pneumoniae*	*C. albicans*
PHB powder	10000~20000	0	0	0
E-PHB	8~15	>99.99%	94.26%+1.15%	91.95%+1.53%

The PHB oligomer was prepared using the one-step open- ring polymerization synthesis method (S-PHB) (**Figure 2**a). The obtained E-PHB and S-PHB were characterized by IR and NMR (Figure 2b and Figure S2, Supporting Information). In the IR spectra of E-PHB, typical stretching vibration peaks of C-H were evident at 2930 and 2853 cm^{-1}. The stretching vibrations of C=O were observed at 1725 cm^{-1}. Typical C=O and C-C stretching at 1710 and 1660 cm^{-1} were also found in the IR spectrum of S-PHB. Characteristic chemical shifts of C=O in E-PHB were evident between 168 and 178 ppm (Figure S2, Supporting

Information). In the NMR spectra of S-PHB, chemical shifts of C=O were found at 165 to 175 ppm that were similar to E-PHB. The characteristic chemical shifts of C=C were also observed at the range of 120–150 ppm, indicating the existence of carbon-carbon double bond. Additionally, the MS result of S-PHB, which contains repeating units of 86, matches very well with the theoretical $[M+1]^{+}$ values (Figure S3, Supporting Information). These data further confirmed the chemical structure of S-PHB, and the terminal groups of the oligomer should be a carbon-carbon double bond and isobutyl ester group, indicating that S-PHB with a DP from 1 to 6 was successfully prepared and S-PHB is a chiral oligomer and belongs in the racemate (Figure S4, Supporting Information).

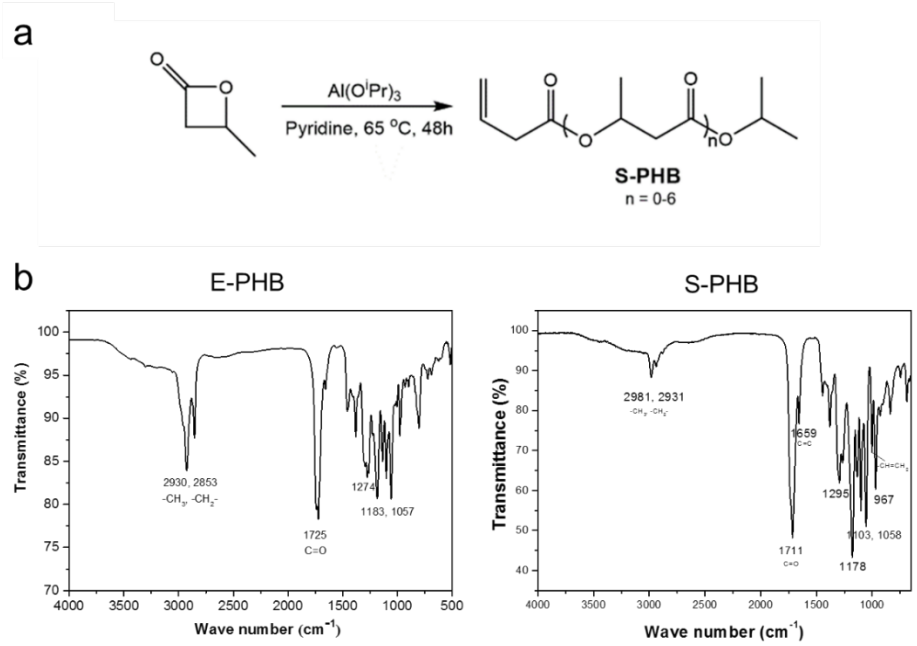


Fig. 2 a) Synthetic method of S-PHB. b) IR spectrum of E-PHB and S-PHB.

Antimicrobial properties of S-PHB

The as-synthesized S-PHB was tested for its antimicrobial activity using quantitative and qualitative analyses. After incubating for 18 h, the number of bacterial colonies treated with S-PHB decreased to 0 (Figure S5, Supporting Information). Ourresults showed that the antimicrobial effects of S-PHB against S. aureus, K. pneumoniae and C. albicans were up to 99.99%, indicating that S-PHB had broad-spectrum antimicrobial properties. This result was consistent with our previous work where the PHB oligomers extracted from bio-synthesized PHB polymers possessed antimicrobial ability (Table 1). Meanwhile, compared with E-PHB (DP from 8 to 15), S-PHB (DP from 1 to 6)

at the same concentration showed better antibacterial activity against K. pneumoniae and C. albicans. This indicates that the degree of polymerization is an important factor affecting the antibacterial properties of PHB, and the reduction of the degree of polymerization enhances the antibacterial proper- ties of PHB. To further examine the best antimicrobial effect of S-PHB, growth kinetics analysis of S. aureus and K. pneumoniae treated with different concentrations of S-PHB was performed. The results showed that S-PHB could completely inhibit the growth of S. aureus at a concentration of 6.25 mg mL^{-1}. By con- trast, at the same concentration, S-PHB could only inhibit the growth of K. pneumoniae within 12 h. Hence, the minimum inhibitory concentrations (MICs) of S-PHB against S. aureus and K. pneumoniae were 6.25 mg mL^{-1} and 12.5 mg mL^{-1}, respectively, indicating that S-PHB exhibited a stronger anti- bacterial effect against Gram-positive bacteria (Figure 3). On the other hand, a higher S-PHB concentration was needed to completely inhibit the growth of C. albicans. The MIC of S-PHB against C. albicans was 25 mg mL^{-1}, which was four times that of S. aureus.

For qualitative analyses, acridine orange (AO) and ethidium bromide (EB) were used as the fluorescent dyes to stain the bacterial cells, and corresponding micro-graphs were acquired using con-focal laser scanning microscopy (LSM). AO stained both viable and dead cells and produced green fluorescence, while EB stained dead cells and produced red fluorescence. Most of the untreated S. aureus and K. pneumoniae bacterial cells showed green fluorescence and little red fluorescence, indicating that most cells were viable in the control groups (Figure S6, Supporting Information). Bacterial cells treated with S-PHB showed more red fluorescence than the control group, indicating that more bacteria were killed by S-PHB. The inhibition effect increased with increasing concentration of S-PHB, resulting in no bacterial survival after treatment with 4 MIC of S-PHB. These results further confirmed the ability of PHB to kill bacteria. Additionally, because EB dyes only penetrated cells with damaged cell membranes to produce red fluorescence, these results suggest that the antimicrobial mechanism of S-PHB is related to the destruction of cell membranes.

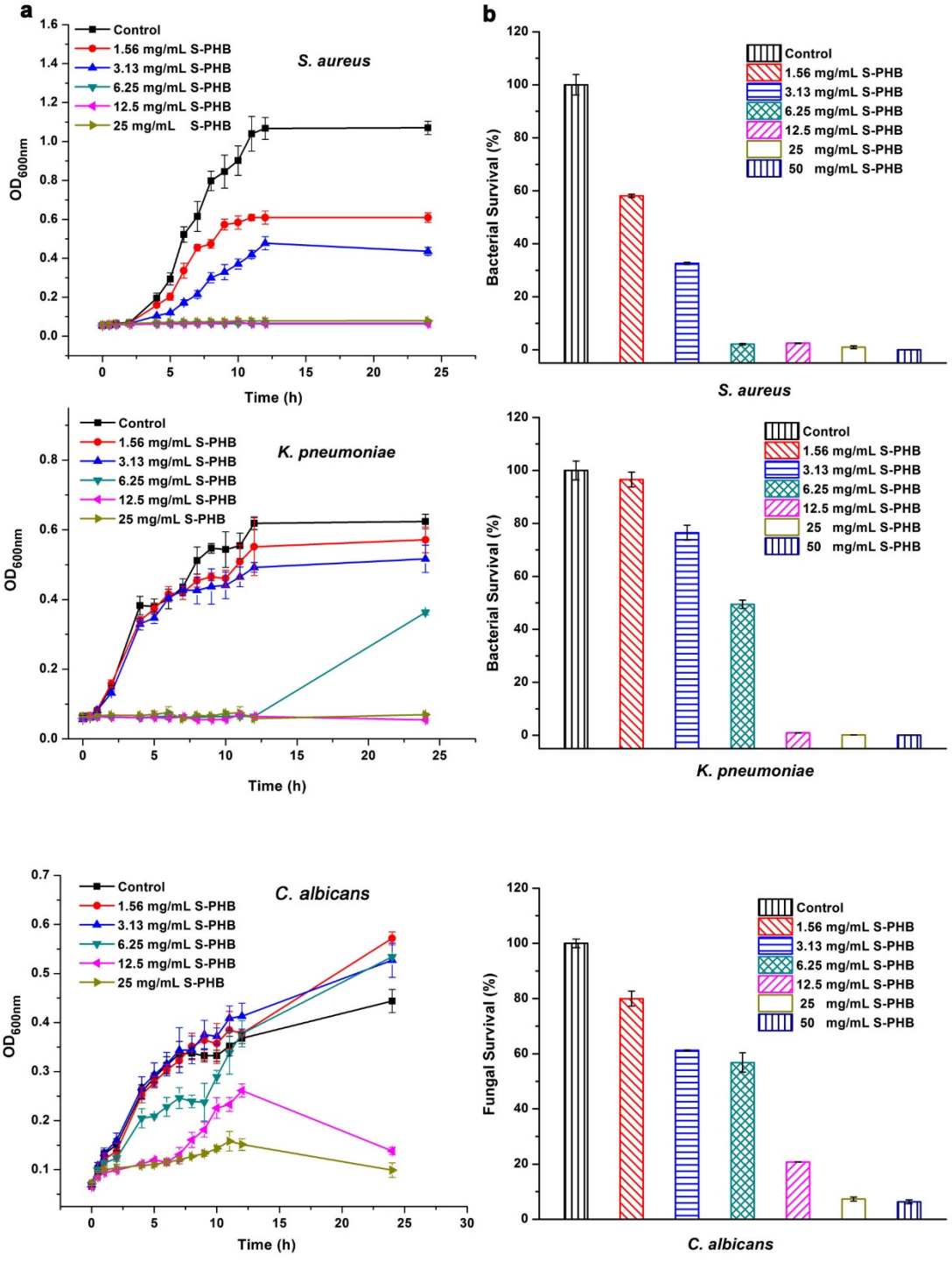


Fig. 3 (a)The growth curve of microorganisms treated with different concentrations of S-PHB. (b) Survival rates of microorganisms treated with the different concentration of S-PHB after 18 h, PBS solution is as control group.

Antimicrobial ability of S-PHB in medicated textiles

As an antibacterial medicated agent, the material should not cause skin sensitivity. First, histological evaluations were performed to detect any irritation effect of S-PHB on BALB/c mouse skin using H&E staining. From H&E staining images (Figure S7, Supporting Information), using various treatment concentrations of S-PHB, no significant difference was detected on skin between the S-PHB groups and wild-type (WT) mouse groups. This result indicates that S-PHB is up to the physiological standard of an antimicrobial modification agent. SEM images showed that the addition of S-PHB has no effect on the surface structures of the fabric and fibers (Figure 4a). The onset decomposition temperatures of the untreated and S-PHB treated fabrics were 272 and 247 ℃, respectively. For untreated PLA/PHBV fabric, the weight loss (30%) between 257 and 290 ℃ results from the thermal decomposition of PHB, and the decomposition of PLA occurs at the range of 290–373 ℃. The TGA curve of the treated fabric was similar to that of the untreated fabric, but it started to decrease at 167 ℃, mainly due to the decomposition of S-PHB (Figure 4b). Additionally, PLA/PHBV fabric treated with S-PHB exhibited an antimicrobial effect (Table 2). After washing five times, this fabric can kill bacteria effectively, and the antimicrobial rates against S. aureus, K. pneumoniae and C. albicans were 99.99%, 90%, and 87.5%, respectively.

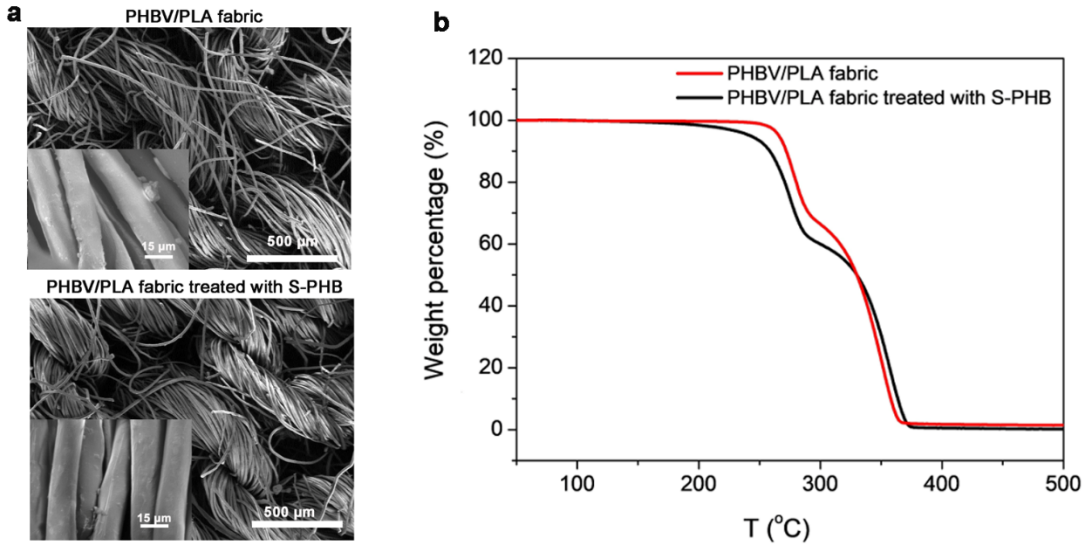


Fig.4 a, SEM micrographs of PHBV/PLA fabric treated with S-PHB, b, TGA of PHBV/PLA fabric treated with S-PHB.

Table 2. Antimicrobial rates of fabrics

Materials	*S. aureus*	*K. pneumoniae*	*C. albicans*
PHBV/PLA fabric	0	0	0
PHBV/PLA fabric treated with S-PHB (no washing)	>99.99%	>99.99%	>99.99%
PHBV/PLA fabric treated with S-PHB (washed 1 time)	>99.99%	97.46%+1.90%	90%+8%
PHBV/PLA fabric treated with S-PHB (washed 5 times)	>99.99%	93.75%±5%	87.5%+3.75%

The potential application in biomedicine

SD rats were subcutaneously injected with S-PHB at various concentrations from 6.25 to 100 mg mL^{-1} to evaluate skin irritation. No roseola or edema was observed when the concentration of S-PHB was lower than 50 mg mL^{-1} after inoculating for 72 h, as shown in Figure S8, Supporting Information. However, the skin treated with 100 mg mL^{-1} of S-PHB became obviously red, swollen, and edematous. Furthermore, toxicity experiments of S-PHB in nude mice in vivo were performed to further evaluate the skin irritation and histologic sections of haematoxylin- eosin (H & E)-stained major organs, including the heart, lung, liver, kidney, and spleen (Figure S9, Supporting Information,and Figure 5). Compared with the wild-type (WT, no treat-ment) mouse group, the skins of the treated groups showedno change. The organ tissues incubated with 6.25 and 12.5 mgmL^{-1} of S-PHB maintained their normal structures, demonstrating that S-PHB has no noticeable toxicity to the majororgans of mice within the experimental dosage range. However,the PHB oligomers may be slightly toxic considering the relatively low LC50/MIC value. In other words, an appropriate doseof S-PHB will not cause skin allergy and toxicity; thus, it can beapplied as a suitable antimicrobial agent for in vivo applications

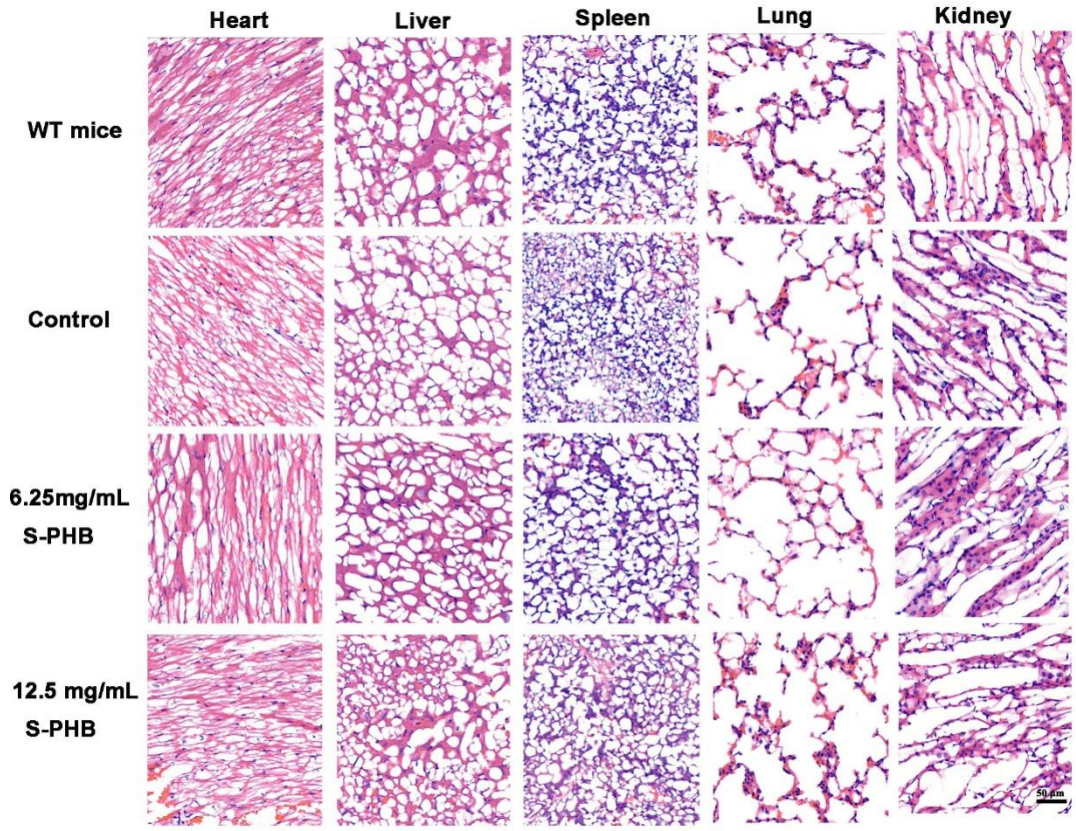


Fig.5 Histological evaluation of the main organs after nude mice were treated with S-PHB for 72 h. Wild type (WT, no treatment) mice are used and Nude mice treated with PBS solution are as control group.

To evaluate the effectiveness of S-PHB in vivo, the antibacterial activity in mice was further tested. Nude mice were inoculated and infected with *S. aureus* under the skin for 4 days. Thewound caused by the injection showed signs of redness, edemaand ulceration due to infection with the bacteria. The mice weredivided into three groups, and each was treated with PBS buffersolution (control), 6.25 mg mL^{-1} S-PHB (1 MIC) and 12.5 mgmL^{-1} of S-PHB (2 MIC), respectively. **Figure 6**a illustrates thatthe wound size increased during the first 4 days for the controland S-PHB treated groups, suggesting effective wound infection. After 11 days, no significant difference was observed inwound healing between the control and 1 MIC of S-PHB group.In contrast to the above two groups, 2 MIC of S-PHB injection promoted wound healing to a remarkable extent and reducedthe amount of *S. aureus* distinctly, thereby revealing a significant therapeutic effect (Figure 6a). Additionally, the expression levels of white blood cells (WBCs) and neutrophil granu-locyte cells (NEUTs) were monitored at day 11 (Figure 6b,c).The normal counts of WBCs and NEUTs in wild-type mice were measured as 1.89 $\pm$ 0.27 10^9 per L and 0.375 $\pm$ 0.055 10^9 per L, respectively. However, the concentrations of these two cells remained at a high level in both the control and 1 MICof S-PHB group because there was no effect against bacterial infection. In comparison, the counts of WBCs and NEUTs weresignificantly decreased and recovered to their normal levels in the 2 MIC of S-PHB group, implying the sufficiently high concentration of 2 MIC of S-PHB can kill *S. aureus* and promote wound healing ability in the skin infection model. Moreover, histological evaluations were subsequently performed to further compare the effect of S-PHB dosage on skin repair performance using H&E and Masson's trichrome staining. From the H&E-stained images (Figure 6d), the

surfaces of the bare woundin all the treatment groups were covered with an enclosed scab marked with red arrows. Large areas of subcutaneous tissue were infected with *S. aureus* (black arrows) in the control group. After treating with 1 MIC of S-PHB, the number of bacteria was obviously reduced. Nevertheless, no significant difference was found in the wound sizes between the 1 MIC and control group, mainly due to the insufficient dosage of S-PHB. When the concentration of S-PHB was increased to 2 MIC, most of the bacteria under the skin were killed, and accelerated healing was also observed in the epidermal tissue. Collagen remodelingand maturation were estimated by Masson's trichrome staining (Figure S10, Supporting Information). The images revealedthat subcutaneous tissue was damaged by most bacteria in the control group. After treating with S-PHB, collagen fibers were present, in varying degrees, in the wound sites (yellow arrows). Additionally, more evenly distributed and mature collagen was regenerated in the wound site after treatment with 2 MIC of S-PHB compared with 1 MIC of S-PHB. These results demonstrate the strong antibacterial ability and excellent wound healing effects of S-PHB.

Finally, S-PHB showed outstanding performance against drug-resistant bacteria (methicillin-resistant S. aureus, ATCC 43300) as illustrated in Table S1, Supporting Information. The PHB oligomer is also active against drug-resistant bacteria with an antibacterial rate surpassing 99.97% with a concentration of 6.25 mg mL^{-1}.

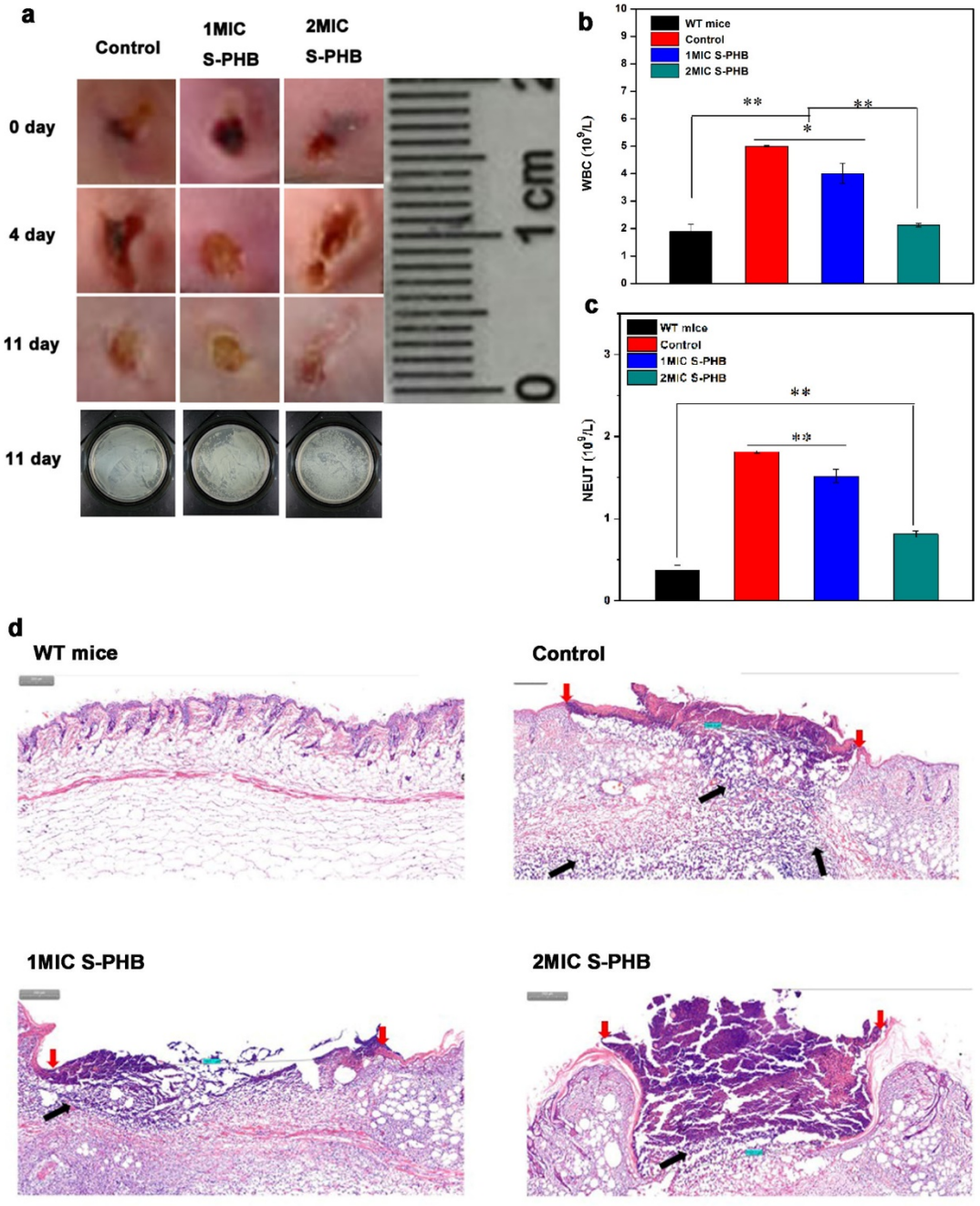


Fig. 6 a) Photos of bacterial infected wound treated with control (PBS solution) group, 1MIC of S-PHB and 2 MIC of S-PHB; b) WBC determination at day 11th after S-PHB treatment; c) NEUT determination at day 11th after S-PHB treatment; d) Histological evaluation of infected

wound using H&E stains, including subcutaneous tissue (black arrows), wound boundary (red arrows)

Antimicrobial mechanism

Biofilm formation under S-PHB was directly observed by SEM. The results indicated that the untreated S. aureus, K. pneumoniae and C. albicans were covered with bright and intact biofilms (Figure 7). The addition of different concentrations of S-PHB destroyed the biofilms of the three microbes as shown in the SEM images. Untreated S. aureus, K. pneumoniae, and C. albicans showed globular or rod-shaped morphology with smooth and complete cell walls. C. albicans formed mycelium that may cause infection and pathogenicity. After treatment with S-PHB for 1 h, the cell walls were wrinkled and dam- aged, while the formed mycelium of C. albicans also faded away. S-PHB caused more destruction and shrinkage of the cell membrane of S. aureus and K. pneumoniae with increased concentrations, indicating the serious leakage of the intracellular contents. Overall, the results indicated that S. aureus, K. pneumoniae, and C. albicans treated with S-PHB show the disrupted integrity of biofilms and damaged cell walls/membranes, implying that S-PHB may destroy the permeability of the membranes and induce the death of the bacteria or fungus.

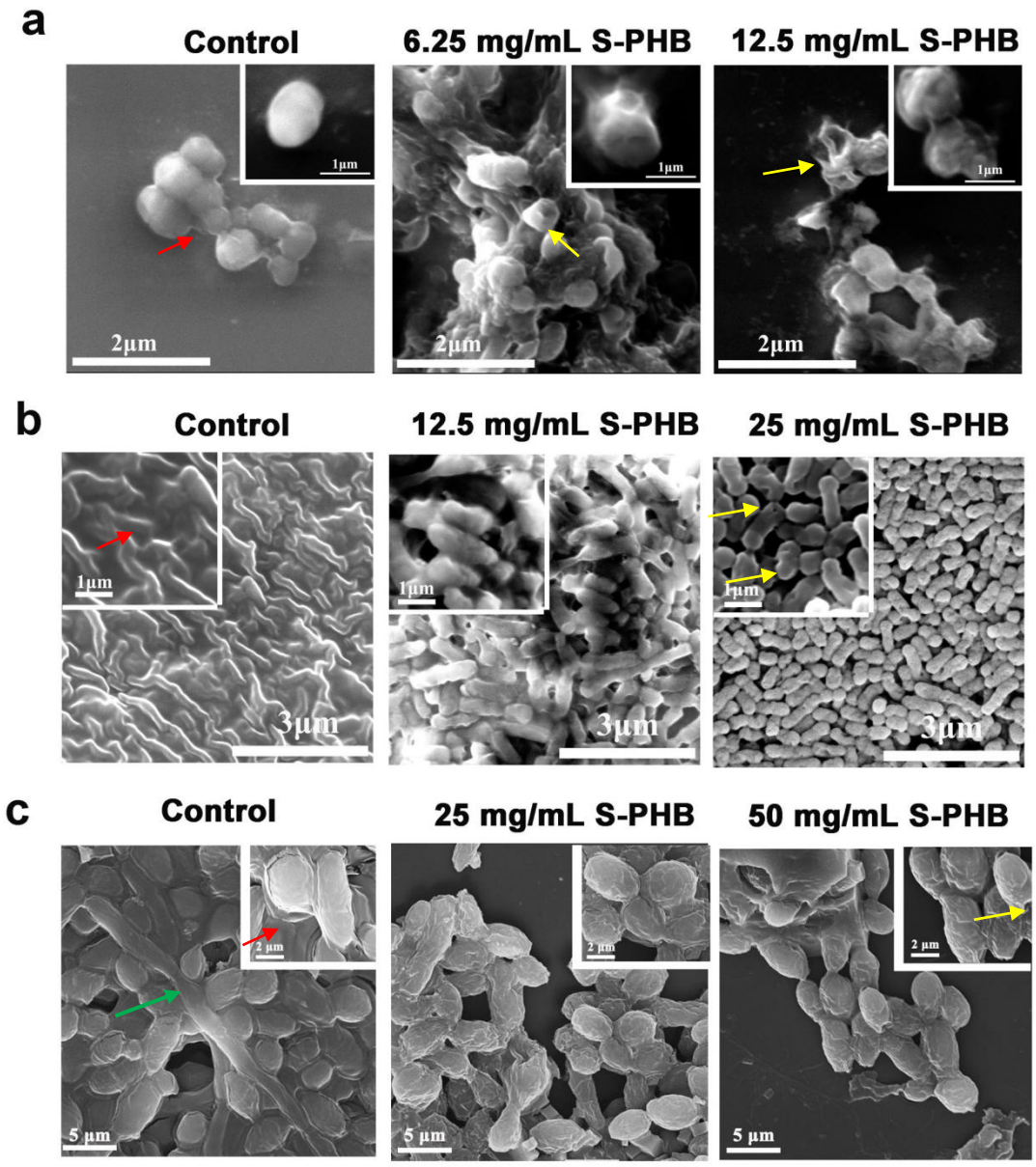


Fig.7 SEM micrographs of S. aureus, K. pneumoniae and C. albicans treated with different concentrations of S-PHB for 1h: (a) S. aureus: control, 6.25 mg/ml S-PHB, 12.5 mg/ml S-PHB; (b) K. pneumoniae: control, 12.5 mg/ml S-PHB, 25 mg/ml S-PHB; (c) C. albicans: control, 25 mg/ml S-PHB, 50 mg/ml S-PHB. The control groups were done without S-PHB. There are biofilm (red arrows), the hole in the cell membrane (yellow arrows) and mycelium (green arrows).

Furthermore, the disruption of cell walls and cell membranes was investigated via the leakage of intracellular contents. The proteins released in the microbial suspension were determined using the BCA protein assay kit. The increase in the endogenous proteins in suspension were found when the microbe was treated with S-PHB (Figure 8a). These results further suggested that S-PHB may damage the integrity of the cell walls and membranes, resulting from the hydropho- bicity

of S-PHB. The effect of S-PHB on the cytoplasmic membrane potential was inves- tigated using the 3,3-dipropylthiadicarbo-cyanine iodide DiSC3(5) dye, a lipophilic fluo- rescent probe that changes its fluorescence intensity when a membrane-permeabilizing agent is added. Compared with the untreated control groups, the fluorescence intensity of the microbe with S-PHB was dramatically increased. This result proved that the addition of S-PHB induced a transmembrane potential change (Figure 8b). The ROS concentrations of S. aureus, K. pneumoniae, and C. albicans treated with S-PHB were also measured to determine the ROS accumulation in the microbes, possibly leading to oxidative damage and death (Figure S11, Supporting Information). The results showed that S-PHB did not induce a distinct change in cellular ROS, indicating that the antimicrobial mechanism of S-PHB may not involve an oxidative damage-related pathway. To further investigate the antimicrobial mechanism of S-PHB, the total proteins of S. aureus, K. pneumoniae, and C. albicans were extracted and migrated to form separate bands at different positions by polyacrylamide gel electrophoresis (PAGE) according to the corresponding molecular weight of proteins (Figure 8c). Some bands corresponding to macromolecular protein showed faint brightness and even disappeared (black arrows) after incubating with S-PHB compared with the protein band of normal bacteria or the fungus. Additionally, the clear bands, which represent new protein with low molecular weight, were visible in the S-PHB group (red arrows). These results demonstrated that S-PHB could induce the disassembly of macromolecular proteins. The effect of S-PHB on DNA plasmid (Figure S11, Supporting Information) was monitored as well, and no change was observed in the band brightness and position on PAGE between the pure DNA plasmid and S-PHB groups, indicating that S-PHB did not noticeably affect the DNA plasmid.

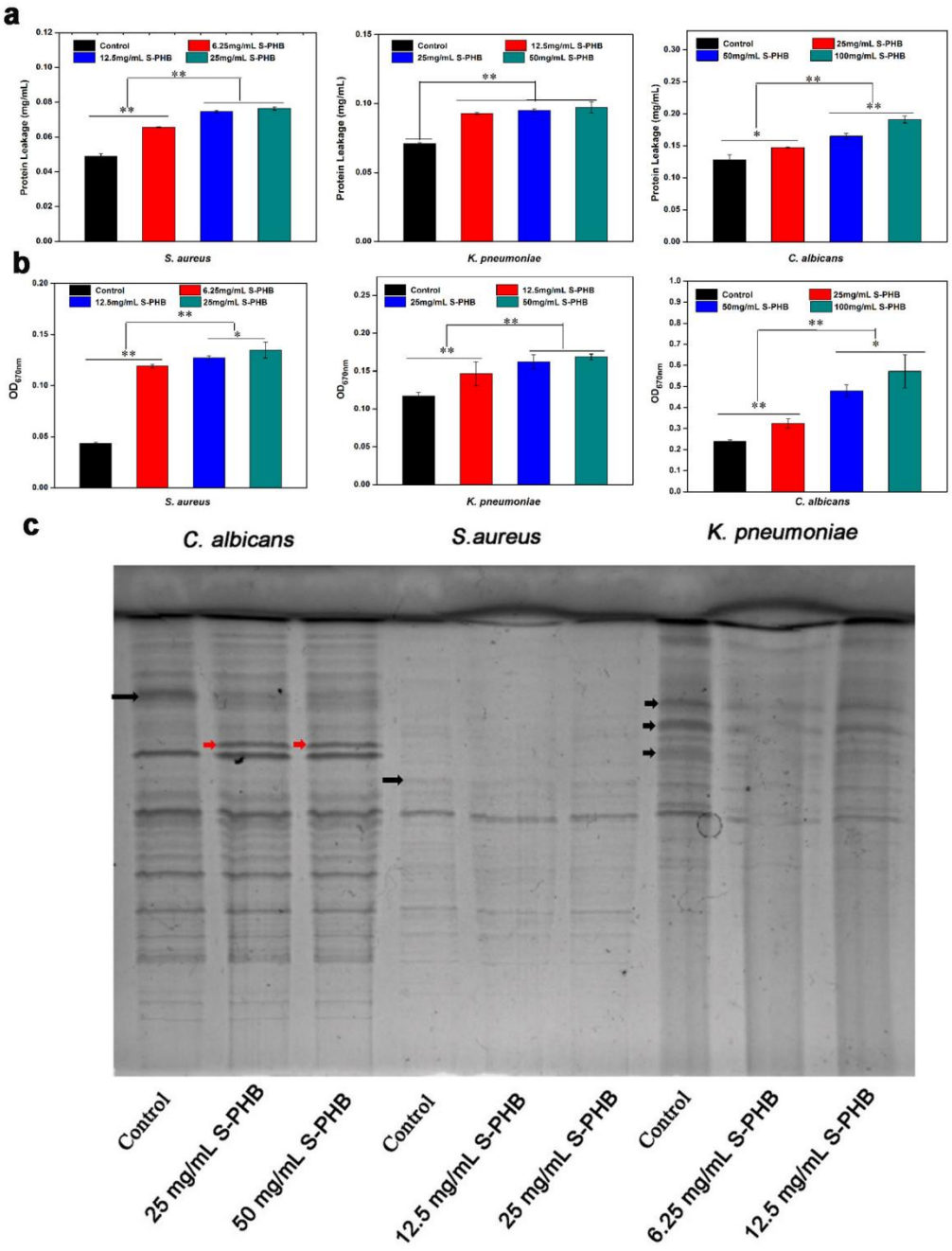


Fig.8 a) Protein leakage related to the membrane integrity; b) Transmembrane potential change was determinate by $DiSC_3(5)$ staining; c) Gel electrophoresis of protein (*S. aureus*, *K. pneumoniae* and *C. albicans*) treated with S-PHB. Macromolecular protein shows faint brightness and even disappeared (black arrows), and new protein with low-molecular weight are visible (red arrows).

Discussion

Natural antimicrobial agents are safe, bioactive, and non- toxic; hence, they have attracted increasing attention. Essential oils extracted from plants are common natural antimicrobial agents. However, based on the awareness of environmental protection and energy conservation, recent studies are more inclined to develop new antimicrobial agents produced by the fermentation of microorganisms. In this work, we extracted the PHB oligomer (E-PHB) from fermented PHB polymer, and the chemical structure of this PHB oligomer was identified as an ester. Subsequently, the E-PHB with a few DPs exhibited anti- microbial properties against a wide spectrum of bacteria and possibly leading to the increased antibacterial concentration. The high concentration hampers the applications of S-PHB in antibacterial agents or drugs, but they could find promising applications in antibacterial textiles and healthcare. Compared with S. aureus, a higher concentration of S-PHB was needed to completely inhibit the growth of K. pneumoniae. It is generally recognized that Gram-negative bacteria are less susceptible to antimicrobial agents than Gram-positive bacteria due to their more complex cell wall and membrane[60-61].The narrow porin channels in the cell membrane of Gram-negative bacteria limitsthe inward fluidity of hydrophobic products.[62]. Additionally, a higher concentration of S-PHB is needed to completely inhibit the growth of *C. albicans*; the MIC of S-PHB against *C. albicans* is 25 mg mL^{-1}, four times that of *S. aureus*. The MIC valuesof S-PHB against microbes are increased several orders ofmagnitude compared with those of the antimicrobial esters in essential oils.[63]. However, in the skin stimulation experiment, these drug concentrations caused no allergies in mouse skin. The staining of the bacterial cells further confirms the excellentantibacterial ability of S-PHB and suggests that the antimicrobial mechanism of S-PHB is related to the destruction of cell membranes.

The strong physiological adaptation properties induce the intrinsic resistance of microorganisms against antimicrobial products[64]. Biofilms are the important products of microbesin adaptation microenvironments and one of primary causes of microbial resistance[40]. In the SEM images, S-PHB was demonstrated to destroy the biofilms of bacteria and the fungus within a short time (Figure 7). Additionally, the destruction andshrinkage of cell membrane were clearly observed, a finding that is consistent with the phenomenon in the Live-Dead cell staining test. The increase in microbial endogenous proteins with the addition of S-PHB further proves that S-PHB candamage the integrity of the cell walls and membranes, likely resulting from the hydrophobicity of S-PHB. This mechanism should be similar to that of essential oils, which have been confirmed to break the permeability of the cell membranes throughhydrophobic interaction [47, 65], This result was also consistent with the degree of polymerization being an important factor affecting the antibacterial properties of PHB. More hydrophobic PHB oligomers increase with the decrease in the DPof the PHB polymer, thus enhancing the

antimicrobial ability. MDR is also a physiological adaptation behavior of microorganisms against antimicrobial products. It can be acquired by de novo mutation or from other resistance gene-carrying microorganisms.[66]. Resistance genes may express enzymes against antimicrobial agents, produce a metabolic pathway to avoid theeffects of antibiotics, or modify the drug target site[67]. However, the PHB oligomer seems to offer hope to control MDR becauseof its nonspecific antibacterial mechanism based on hydrophobicity. Moreover, other antibacterial mechanisms of the PHB oligomer are well studied, as well as the possible mechanisms for the antibacterial leakage of the intracellular content, inhibition of protein activity, and change in the transmembrane potential

Finally, topological coating of non-antimicrobial fabrics with the synthesized PHB oligomer has been demonstratedwith an outstanding bactericidal effect even after washing five times. Moreover, in vivo testing of nude mice showedno physiological toxicity with subcutaneous injection of an appropriate amount of the PHB oligomer, indicating that the PHB oligomer can provide effective sterilization and promote the wound healing of infected nude mice. Most importantly,we have discovered that the PHB oligomer is also active against drug-resistant bacteria. These results suggested that the PHB oligomer not only can be a great candidate in topological antimicrobial finishing but also has promising prospects in the biomedical field. Future studies could investigate the chemical modification of PHB oligomers to improve the water solubility and antibacterial activity. For example, the carbon–carbon double bond could be oxidized to carboxyl or other hydrophilic groups to increase the water solubilityor the PHB oligomer could be grafted on other polymers through click-reactions. Additionally, new synthesized agents could be tested for the selectively antibacterial property of certain bacteria or cells.

4. Methods

Materials

Poly (3-hydroxybutyrate) (PHB) powder was obtained from TianAn Biologic Materials Co., Ltd. in China. Starting materials for chemical synthesis, such as *beta*-Butyrolactone butyrolactone (purity 95.0%, TCI), aluminum isopropoxide (98.0%, TCI) and pyridine (99.0%, Acros), were used as received without further purification. The solvents including chloroform, methanol, ethanol, dichloromethane (DCM) and *n*-hexane were purchased from Anaqua (Hong Kong).

Extraction of PHB oligomer (E-PHB) from fermented PHB powder

10g PHB powder was added in 200 ml chloroform and the mixture was refluxed overnight. Then the viscous solution was poured into 1 L ethanol and filtered to remove the high molecular weight PHB. Subsequently, raw PHB oligomer was collected after the solvents were removed by rotary evaporation and further purified by column chromatograph using eluents from DCM/n-hexane (1:1) to methanol/DCM (1:2).

Synthesis of PHB oligomer (S-PHB)

To a solution of aluminum isopropoxide (0.2 g, 1 mmol) and pyridine (2 ml), *beta*-butyrolactone (0.86g, 10 mmol) was added under an inert N_2 atmosphere. The solution was stirred at 65 °C under N_2 for 48h [68]. After cooling down, diluted HCl solution (2M, 20 ml) was added to quench the reaction followed by extraction with DCM (20 ml x 3). The DCM layers were collected and rotary evaporated to remove the solvents after which the residue was eluted with DCM/n-hexane (5:1, *v:v*) in silica gel column to afford light-yellow oil.

Characterization method

NMR spectra were obtained on a Bruker Ultrashield 400 MHz FT-NMR spectrometer in $CDCl_3$ with tetramethylsilane (TMS) as the internal standard. HPLC-MS spectra were measured on Thermo Fisher Orbitrap Fusion Lumos Mass Spectrometer. Infrared spectra were collected on Perkin Elmer Spectrum 100 FTIR spectrometer.

Antimicrobial activity tests

Staphylococcus aureus (S. aureus) ATCC No. 6538, *Klebsiella pneumoniae (K. pneumoniae) ATCC No. 4352* and *Candida albicans (C. albicans) ATCC No. 10231* were used as the gram-positive, gram-negative and general fungus respectively to investigate the antimicrobial property of the oligomer according to the shake flask method[69]. Drug-resistant bacteria

(methicillin-resistant *S. aureus*, ATCC 43300) was used for the additional test.

The microorganisms were cultured overnight in Luriae Bertani (LB) solution at 37 ºC℃ and then diluted to about 10^5 CFU/ml with PBS and incubated with 0.01 g/ml PHB on a shaking bed for 18 h at 24 ºC. The bacteria incubated without the addition of PHB served as the negative control group. Then the bacteria were diluted to 10^2 CFU/ml, spread on a solid agar plate and cultivated for 18 or 48h at 37 ºC. The antibacterial rate was calculated by the following equation:

$$Antibacterial_{rate} = (1 - \frac{CFU_{PHB}}{CFU_{control}}) \times 100\%$$

The growth kinetics of bacterial treated with S-PHB was detected by a broth microdilution method [30]. 100 μl microorganisms cultivated overnight (10^6 CFU/ml) and 100 μl PHB solution with different concentrations were transferred to a 96-well polypropylene (PP) plate and further incubated for 1, 2, 4, 6, 8, 10, 12 and 24 hours at 37 ºC on a shaking bed. Antimicrobial kinetics was determined by OD_{600nm} using a microplate reader (Multiskan GO, Thermo, USA).

Minimum inhibitory concentration (MIC) is the minimum material concentration at which the growth of the bacterial is inhibited. In our work, MIC was determined by the CCK-8 assay (CCK-8, Beyotime, China). After 10^6 CFU/ml of the bacteria or fungus suspensions were treated with different concentrations of S-PHB for 18 hours, the viability of the bacterial or fungal cells were measured by CCK-8 assay.

Live/dead cell staining

1.2 ml of logarithmic-phase bacteria (OD_{600nm}0.5) was collected by centrifugation at 5000 rpm for 5min and rinsed three times with PBS solution. 4 ml PBS was added into the remaining bacteria and divided into 4 groups. After that, 100 μl bacteria solution was treated with 100 μl of PHB with various concentrations (1MIC, 2MIC and 4 MIC) on a shaking bed for 2 hours. The fluorescent dyes were prepared according to the literature[70]. After staining in the dark for 15 mins, the bacteria were centrifuged for recollection, washed three times with PBS and resuspended in PBS solution. The morphology of bacteria was imaged by laser scanning confocal microscope (LSM-700, Carl Zeiss, Germany).

***In vivo* safety assay**

In vivo safety evaluations of S-PHB were implemented by skin stimulation experiment[71]. Initially, BALA/c mice were shaved back hear, disinfected and coated with S-PHB with the different concentrations from 6.25 mg/ml to 100mg/ml MIC for evaluation of skin irritation. After 72 hours, the mice were euthanized, and the skin were removed, fixed and stained with hematoxylin & eosin (H&E) stain.

Mice model of wound infection

In this study, the animal experiments were carried out according to the Institutional Animal Care and Use Committees (IACUC) guidelines. BALB/c nude mice (male, weighting 18-20g) were narcotized and disinfected. The mice were sorted randomly into four groups and three of which were injected10 μl of suspension containing 10^7 CFU of *S. aureus* in the right hind leg. After four days, different treatments were conducted to the mice: Group I (No infection, non-treatment), Group II (Infection, non-treatment), Group III (1MIC of S-PHB) and Group IV (2MIC of S-PHB), respectively. S-PHB was injected on the wound site every three days. To determine *S. aureus* burden at the site of infection wound, the images of mice wound were recorded and the bacteria in the skin tissue/PBS solution (6.75 mg/ ml) were collected and diluted to an appropriate concentration, spread on an agar plated and cultured for 18 h. The data of white blood cells (WBC) and neutrophil granulocyte cells (NEUT) were obtained from the blood near the wound site. At last, the mouse of each group was euthanized, then the abscessed skin lesions were removed, fixed and stained with hematoxylin & eosin (H&E) and Masson's trichrome staining.

Antimicrobial mechanism

50 μl of microorganisms were cultivated overnight (10^6 CFU/ml) and 50 μl PBS solution with different concentrations of PHB were transferred to a 96-well PP plate and continually incubated with shaking at 37 ºC for 2 hours. The bacteria were fixed with 4% paraformaldehyde in the dark at 4 ºC for 4 hours and washed by sterile water. Subsequently, the bacteria were dehydrated with gradient ethanol. The morphology of bacterial cells was observed using SEM (TM-1000, Hitachi, Japan). In addition, the proteins released in bacterial or fungal suspension were studied with the BCA protein assay kit (sigma, US).

The extracted proteins and plasmids were incubated with S-PHB at various concentrations for 30 minutes. The untreated proteins and plasmids were used as control groups. Gel

electrophoresis was applied to investigate the influence of S-PHB on the proteins and plasmids and the results were analyzed by gel imaging and analysis system (Gel Doc 2000, Bio-Rad, US).

After 10^6 CFU/ml of bacteria or fungal were treated with S-PHB for 3 h, the reactive oxygen species (ROS) was detected by ROS assay kit (sigma, US). The incubated bacterial or fungal cells were stained with 20, 70-dichlordihydrofluorescein diacetate (DCFH-DA) for 30 min in the dark. After rinsing with PBS, the number of cells were determined by measuring the absorbance in $OD_{495\ nm}$ using a microplate reader. Moreover, the bacterial or fungal cells were incubated with 4 mM Disc3(5) for 1 hour. The excess Disc3(5) was removed and KCl was added to balance the ions. After that, 10^6 CFU/ml of bacteria or fungal were treated with S-PHB for 3 hours and the fluorescence absorbance were measured at 622 nm and 670 nm.

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Declarations

Tao XM initiated, planned and supervised the execution of research. Li J synthesized S-PHB oligomer and characterized it. Zhang ZH carried out extraction and determination of the E-PHB as well as the topological application. Ma LL conducted antimicrobial tests, mechanistic study and medical applications. Fei B supervised the chemical analysis and synthesis work. Leung P supervised the microbiological study.

Ma LL, Li J and Zhang ZH are the equal first authors.

The prior ethical approval had been obtained for the biological tests by University Ethics Committee of Hong Kong Polytechnic University and for the animal tests by Donghua University, China.

There is no financial interest or any other potential conflict of interests by the team members.

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