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Mechanistic Study of Synergistic Antimicrobial Effects between Poly (3-hydroxybutyrate) Oligomer and Polyethylene Glycol

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Abstract:

We reported previously that poly (3-hydroxybutyrate) (PHB) oligomer is an effective antimicrobial agent against gram-positive bacteria, gram-negative bacteria, fungi and multi-drug resistant bacteria. In this work, it was further found that polyethylene glycol (PEG) can promote the antimicrobial effect of PHB oligomer synergistically. Three hypothetic mechanisms were proposed, that is, generation of new antimicrobial components, degradation of PHB macromolecules and dissolution/dispersion of PHB oligomer by PEG. With a series of systematic experiments and characterizations of HPLC-MS, it was deducted that dissolution/dispersion of PHB oligomer dominated the synergistic antimicrobial effect between PHB oligomer and PEG. This work demonstrates a way for promoting antimicrobial effect of PHB oligomer and other antimicrobial agents through improving hydrophilicity.

Keywords: Poly (3-hydroxybutyric acid); oligomer; polyethylene glycol; antimicrobial agent; synergistic antimicrobial effect.

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1. Introduction

Pathogens, e.g. bacteria, fungi and virus, can cause serious diseases due to their high reproductivity and adaptability[1]. Antibiotics have been commonly used to prevent the bacteria infection, whereas the overuse of them lead to antibiotic resistance, which has become a worldwide threat to human health. About 700,000 people die each year due to the infections of multi-drug resistant (MDR) bacteria[2, 3]. Inorganic nanoparticles, e.g. nano-silver particles[2], have been verified to be highly effective to MDR bacteria, nevertheless this type of antimicrobial agents have potential threats to human health and environment[4]. Many new antibiotics and antimicrobial peptides have been developed for dealing with continuously-emerging MDR bacteria, but the developing rate of novel antimicrobial agents cannot catch up with the enhancement of antibiotic resistance of MDR bacteria through bacterial surface modification, protease secretion and expression of efflux pumps[5, 6]. It has been found that many essential oils (Eos) were effective to kill MDR bacteria and can be applied with antibiotics for enhance the efficacy synergistically[7, 8]. However, the natural components of EOs cannot be controlled accurately, while the low durability and high volatility restrict their applications.

We have reported previously that the synthesized poly (3-hydroxybutyrate) (S-PHB) oligomer, made by open-ring polymerization of beta-butyrolactone, possessed excellent antimicrobial effect against gram-positive bacteria, gram-negative bacteria and fungi with a high reduction rate over 99.99%, as well as multi-drug resistant (MDR) bacteria (methicillin-resistant S. aureus, ATCC 43300) with reduction rate of 99.97% [9]. The PHB oligomer is degradable, durable, eco-friendly and safe, thus has significant advantages for the applications in healthcare field. Nevertheless, the material cost of synthesizing PHB (i.e. beta-butyrolactone) is relatively high compared with the fermented PHB powder from starch or sugar, in which the PHB oligomer can be extracted. The extracted PHB (E-PHB) oligomer exhibits a reduction rate of over 90% against gram-positive bacteria, gram-negative bacteria and fungi.

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In this work, we attempted to improve the antimicrobial property of E-PHB, and found surprisingly that purification reduced the antimicrobial effects of E-PHB, not increased it as expected. We noticed that the elimination of polyethylene glycol (PEG) from the PHB powder extract might be the main reason, a synergistic antimicrobial effect between the PHB oligomer and PEG might exist and should be revealed .

We then proposed three hypothetic mechanisms, including the generation of new antimicrobial components, degradation of PHB macromolecules and dissolution/dispersion of PHB oligomer by PEG during the process of purification. The final experimental results show that the hypothesis of dissolution/dispersion of PHB oligomer by PEG dominates the synergistic antimicrobial effect.

2. Materials and Methods

2.1 Materials

Poly (3-hydroxybutyrate) (PHB) powder was provided by TianAn Biologic Materials Co., Ltd. in Ningbo, Zhejiang, China. beta-butyrolactone (95.0%, TCI), aluminium isopropoxide (98.0%, TCI) and pyridine (99.0%, Acros) were used for chemically synthesizing PHB oligomer. PEG (M_w = 600, Acros Organics) is applied for synergistic antimicrobial effect with PHB oligomer. The solvents, including chloroform, ethanol, methanol, dichloromethane (DCM) and n-hexane, were supplied by Anaqua (Hong Kong).

2.2 Preparation of PHB oligomer by chemical synthesis

Beta-butyrolactone (0.86 g, 10 mmol) was added to a mixed solution of pyridine (2 mL) and aluminum isopropoxide (0.2 g, 1 mmol), then stirred at 65 °C under nitrogen atmosphere for 48 h[10]. HCl solution (2 m, 20 mL) was injected to quench the reaction,

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prior to an extraction with DCM. PHB oligomer was obtained after removal of the DCM by vacuum evaporation and separation by column chromatography (DCM/n-hexane = 5:1, v: v)[9].

2.3 Preparation of PHB oligomer by extraction (E-PHB) and compounding with PEG

Mixture of PHB powder (10 g) and chloroform (200 mL) was refluxed for 12 h, then 1 L of methanol was added into the viscous solution, followed by filtration for removing the PHB polymer with high molecular weight (collected as PHB bulk). Afterwards, the raw PHB oligomer was obtained after the removal of solvents through rotary evaporation and further purified by column chromatography (DCM/n-hexane of 1: 1 to methanol/DCM of 1:2). For compounding, the E-PHB was mixed with PEG (1: 1) and stirred under 150 °C for 4h.

2.4 Interaction of PHB powder and PEG

Bio-based PHB powder was mixed with PEG (1: 1) and stirred under 150 °C for 4h. Afterwards, ethanol was added to dissolve the mixture and residual PHB (solid) was removed by centrifugation. After removing the solvent, yellow oil was obtained as a mixture for synergistic antimicrobial test.

2.5 Notions of materials, products and experiments

The materials, products and experiments mentioned in this work are summarized in Table 1.

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Table 1 Notions of materials, products and experiments

PHB	Poly (3-hydroxybutyrate)
PHB powder	Raw material produced by fermentation
E-PHB	PHB oligomer extracted from PHB powder
EP-PHB	PHB oligomer extracted from PHB powder followed by further purification
S-PHB	PHB oligomer synthesized chemically by polymerization
PEG	Polyethylene glycol
EP-PHB & PEG	EP-PHB oligomer reacts with PEG (1:1, 150 °C, 4h)
PHB powder & PEG	PHB powder reacts with PEG (1:1, room temperature, 4h)
PEG heat treatment	Heat PEG at 150 °C for 4h
PHB bulk	PHB polymer extracted from PHB powder, mainly containing macromolecules.
PHB bulk & PEG	PHB bulk reacts with PEG at 150 °C for 4h
PHB powder heat treatment	Heat PHB powder at 150 °C for 4h
Extract of PHB powder (methanol)	Extract of PHB powder by methanol dissolution, filtration and evaporation.

2.6 Characterization of PHB and PEG

HPLC-MS tests were carried out by Thermo Fisher Orbitrap Fusion Lumos Mass Spectrometer. Solvent of methanol/dichloromethane (1: 1, v: v) was adopted as the eluent.

2.7 Antimicrobial Activity Tests

The antimicrobial property of the oligomer against Staphylococcus aureus (S. aureus) ATCC No. 6538, Klebsiella pneumoniae (K. pneumoniae) ATCC No. 4352, and

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Candida albicans (C. albicans) ATCC No. 10231 was tested according to the shake flask method[11] with concentration of 10 mg/mL[9].

3. Results and discussion

3.1 Discovery of synergy between PHB oligomer and PEG

For producing antimicrobial PHB oligomer with raw materials of lower cost, the extraction of PHB oligomer from PHB powder is preferable compared with chemical synthesis of PHB oligomer. However, the extracted PHB (E-PHB) oligomer possess relatively lower antimicrobial reduction against K. pneumoniae and C. albicans than the synthesized PHB (S-PHB) oligomer, i.e. 94.26% and 91.95%, respectively (Table 1). As the PHB oligomer is proved to be an effective antimicrobial agent previously, it is supposed that E-PHB oligomer with higher purity might achieve better antimicrobial effect, thus the E-PHB oligomer is further purified by column chromatography to obtain extracted and purified PHB (EP-PHB) oligomer. Nevertheless, the EP-PHB oligomer with higher purity doesn't possess better antimicrobial effect as expected. Contrarily, it achieves only antibacterial reduction of 49.6% against K. pneumoniae and has no effect against C. albicans.

For investigating the reason why higher purity leads to low antimicrobial effect, HPLC-MS tests are tested for both E-PHB oligomer and EP-PHB oligomer (Figure 3). It can be seen that a polymer component with repeating unit of 44 exists in E-PHB rather than EP-PHB, which is deduced as polyethylene glycol (PEG), as it has been found previously that there are only C, H and O elements exist in the sample[9]. Also, this component appears early (2 ~ 3.5 min) in the HPLC spectra, which means it has relatively high polarity and further confirm the deduction.

Therefore, it is hypothesized that the exist of PEG in PHB oligomer can promote the antimicrobial effect of PHB, whereas the elimination of PEG after purifying E-PHB decreases its antimicrobial performance. A verifying experiment was carried out to mix EP-PHB oligomer and PEG under 150 ^{o}C for 4 hours and test the antimicrobial effect

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of the prepared mixture, showing a large promoted antimicrobial reduction against K. pneumoniae and C. albicans, i.e. 82.60% and 85.60%, respectively. The antibacterial reduction of mixture of EP-PHB and PEG against K. pneumoniae (82.60%) is higher than that of individual EP-PHB (49.60%) and PEG (70.00%). Besides, the raw PHB powder has antibacterial reduction of only 60.28%, 0 and 82.40% against S. aureus, K. pneumoniae and C. albicans, whereas the mixture of PHB powder and PEG after stirring 4 h under 150 ºC achieve obviously promoted antibacterial effect, i.e. 92.22%, 99.44% and 87.70% for S. aureus, K. pneumoniae and C. albicans, respectively (Table 2 and Figure 2). We further test the antimicrobial effect of S-PHB (5 mg/mL) and S-PHB/PEG (10 mg/mL, 5 mg/mL for each). As seen in Table 2, compared with 10mg/mL S-PHB, the antimicrobial effect of the lower concentration of S-PHB (5mg/mL) was significantly reduced, especially *C. albicans*, whereas S-PHB (5mg/mL) achieve much better sterilization effect after mixed with PEG (5mg/ml). Therefore, these phenomena confirmed a synergistic antimicrobial effect between PHB oligomer and PEG.

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Table 2 Synergy between PHB oligomer and PEG

Sample	Concentration mg/ml	Degree of polymerization	Antimicrobial property		
			S. aureus	K. pneumoniae	C. albicans
S-PHB oligomer [9]	10	⩽6	>99.99%	>99.99%	>99.99%
S-PHB oligomer	5	⩽6	>99.99%	91.60%	61.50%
S-PHB oligomer and PEG	10 (5 for each)	N.A.	>99.99%	98.30%	96.15%
E-PHB oligomer [9]	10	8~15	>99.99%	94.26%	91.95%
EP-PHB oligomer	10	8~15	>99.99%	49.60%	0
EP-PHB oligomer and PEG (1:1,150 °C,4h)	10 (5 for each)	N.A.	>99.99%	82.60%	85.60%
PEG	10	≈14	0	70.00%	0
PHB powder	10	10000~20000	60.28%	0	82.40%
PHB powder and PEG (1:1, room temperature , 4h)	10 (5 for each)	N.A.	67.22%	79.02%	38.50%
PHB powder and PEG (1:1,150 °C,4h)	10 (5 for each)	N.A.	92.22%	99.44%	87.70%

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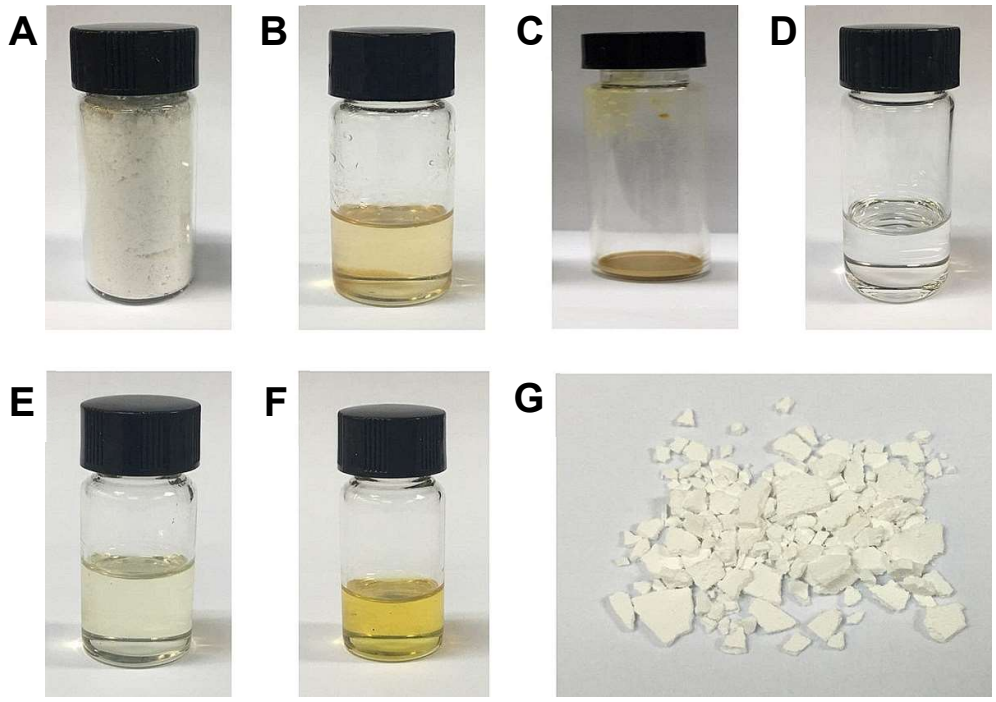

Figure 1 Images of PHB and PEG. (A) PHB powder. (B) S-PHB. (C) E-PHB or EP-PHB. (D) PEG. (E) Liquid product of reaction of PHB powder and PEG under room temperature. (F) Liquid product of reaction of PHB powder and PEG under 150 ºC. (G) PHB bulk

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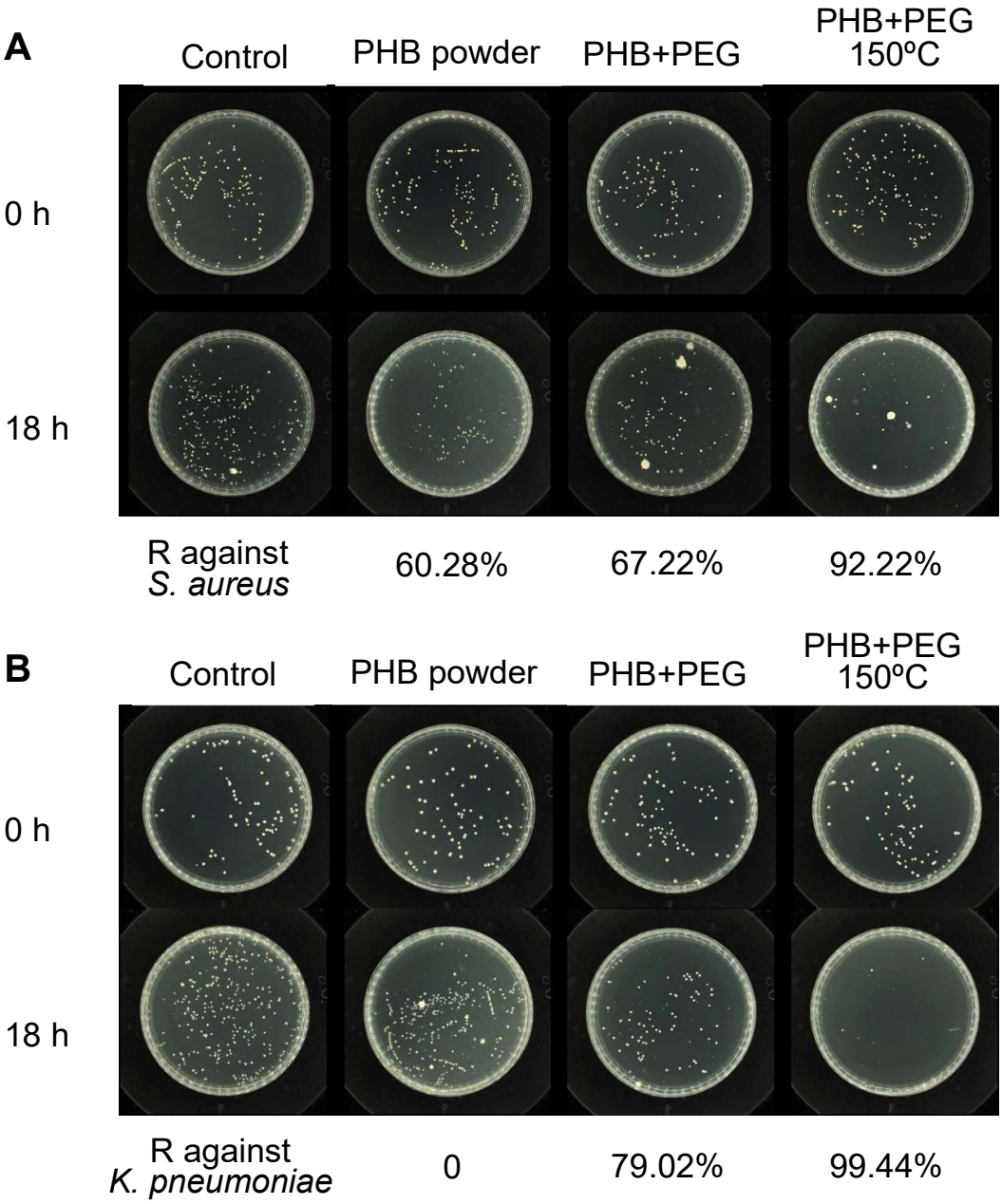

Figure 2 Antimicrobial test for PHB powder, mixture of PHB powder and PEG reacted under room temperature, and mixture of PHB powder and PEG reacted under 150 ºC. (A) Antimicrobial test against *S. aureus*. (B) Antimicrobial test against *K. pneumoniae*.

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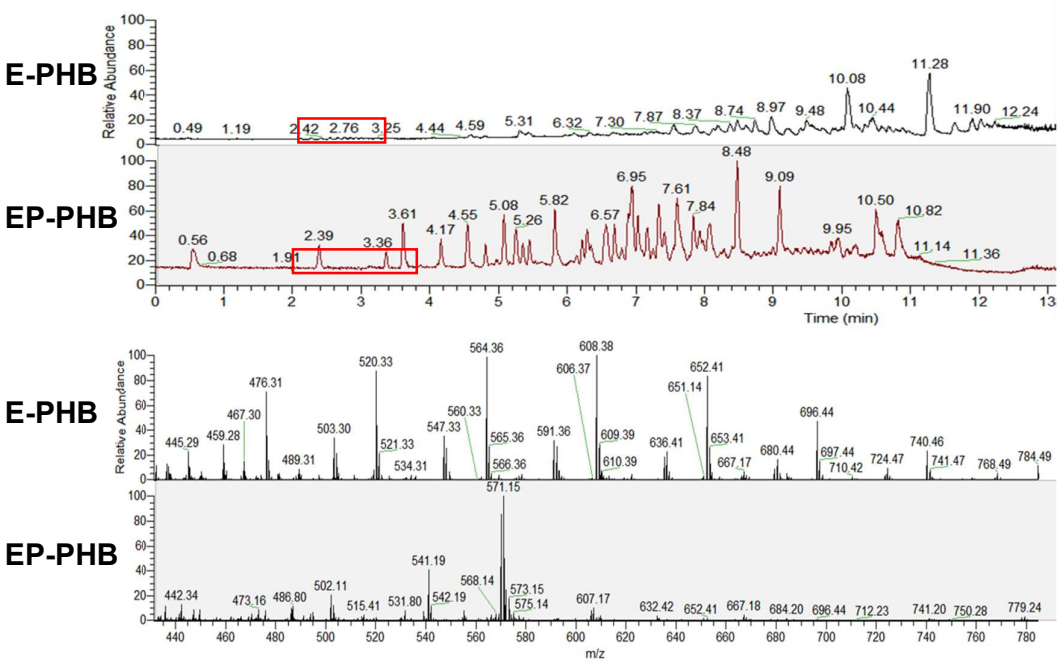

Figure 3 HPLC-MS spectrum of E-PHB and EP-PHB oligomer

3.2 Investigation on synergistic mechanism between PHB oligomer and PEG

After the discovery of synergy between PHB and PEG, three presumptions are raised as generation of new antimicrobial components, degradation of PHB macromolecules and dissolution/dispersion of PHB oligomer by PEG.

3.2.1 Hypothesis of generation of new antimicrobial components.

As the mixture of PHB powder and PEG after treatment under 150 ºC possess higher antibacterial reduction than each of the individual component, there might be new components are produced after the heat treatment, which possess better antimicrobial effect than both PHB oligomer and PEG. For verifying this hypothesis, the mass spectrum (MS) is carried out for the solid and liquid part of mixture of PHB powder and PEG after heat treatment, as well as the individual PHB powder and PEG after heat treatment under 150 ºC for 4 h. It can be seen from Figure 4A that there is no difference between PHB powder and the solid part of mixture of PHB powder and PEG after heat

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treatment, which means that no new solid antimicrobial component is produced after the reaction of PHB powder and PEG under high temperature. Similarly, the mass spectra of liquid part of mixture of PHB powder and PEG is compared with that of PEG in Figure 4B, showing no new chemical component is produced, whereas the average degree of polymerization of PEG increases approximately from 11 to 14 after heat treatment, illustrating the further polymerization of PEG rather than reaction with PHB power. Therefore, the hypothesis of generation of new antimicrobial components fails due to the constant chemical component observed by MS.

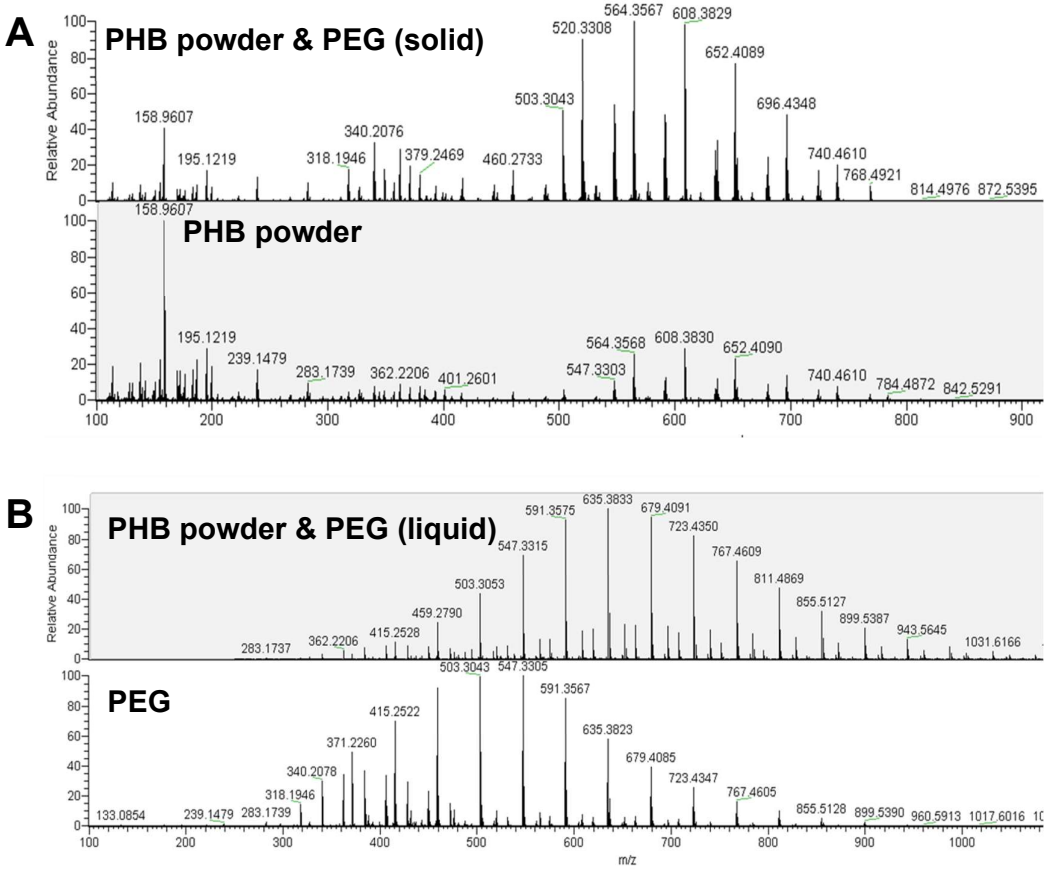

Figure 4 Mass spectrum of PHB powder and PEG after heat treatment. (A) Comparison between PHB power and the solid part of mixture of PHB powder and PEG after heat treatment. (B) Comparison between PEG and the liquid part of mixture of PHB powder and PEG after heat treatment.

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3.2.2 Hypothesis of degradation of PHB macromolecules

The second hypothesis is the degradation of PHB macromolecules, which can produce more PHB oligomer for better antimicrobial performance. Actually, it has been found previously that lower degree of polymerization facilitates better antimicrobial effect[9]. Therefore, PHB with higher degree of polymerization, i.e. PHB bulk, was extracted from the raw PHB powder, followed by reaction with PEG under 150 ºC for 4 h, to see if the PHB macromolecules can be degraded by PEG under high temperature. The results are present in the mass spectrum in Figure 5A. It is supposed that PHB oligomer with low degree of polymerization should exist in the liquid part of mixture of PHB bulk and PEG after treatment, whereas there is no peaks of degraded PHB in the spectra, showing almost the same peaks as the spectra of PEG, thus the hypothesis of degradation of PHB macromolecules is also not verified.

Actually, as the melting temperature of PHB is about 180 ºC[12] and the thermal degradation temperature is over 190 ºC[13], the heat treatment under 150 ºC is not sufficient for degrading the PHB macromolecules, even if in the powder state with high specific surficial area. Figure 5B exhibits the mass spectrum of liquid extract of raw PHB powder before and after heat treatment, showing that only PEG with low degree of polymerization exists, rather than the PHB oligomer through thermal degradation.

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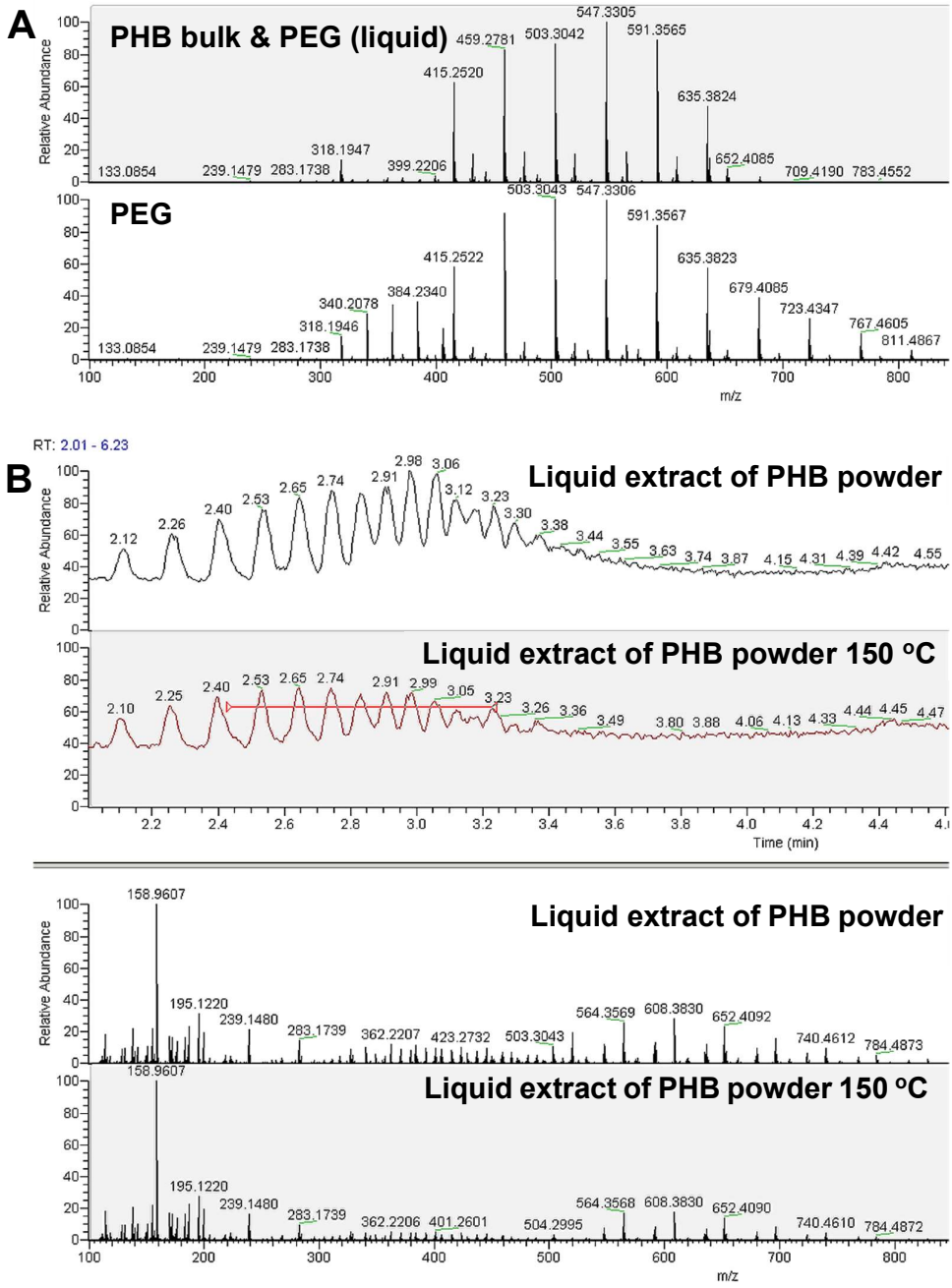

Figure 5 HPLC-MS analysis on hypothesis of degradation of PHB macromolecules. (A) Comparison between PEG and liquid part of mixture of PHB bulk and PEG after treatment under 150 °C for 4 h. (B) Extract of PHB powder before and after treatment under 150 °C for 4 h with solvent of methanol: dichloromethane = 1:1.

3.2.3 Hypothesis of dissolution/dispersion of PHB oligomer by PEG

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PEG has very good hydrophilicity and is commonly used for enhancing hydrophilicity of functional materials through compounding[14, 15] or grafting[16]. PHB oligomer is relatively hydrophobic material, especially for those with high degree of polymerization. The exist of PEG in raw PHB powder might facilitate the dissolution and dispersion of PHB oligomer therein into water, i.e. antimicrobial solution with bacteria.

For verifying this hypothesis, the PHB bulk (extracted from raw PHB powder with mainly macromolecules) was mixed with PEG (1: 1) prior to a heat treatment under 150 ^{o}C for 4 h. The solid part after centrifugation was tested by HPLC-MS with solvent of methanol: dichloromethane = 1: 1 (Figure 6A). Compared with the PHB bulk (same condition of treatment and test as above), new regular peaks appear during the eluting time between 6 min and 10 min, which are determined as PHB oligomer. This phenomenon illustrates that the PEG can promote the dissolution/dispersion of PHB oligomer in bulk into solvent/solution, thus confirm the hypothesis (Figure 7). Though the E-PHB oligomer can be dissolved in methanol, PHB oligomer cannot directly be extracted from raw PHB powder by methanol (only tiny PEG exists in the extract, Figure 6B). Therefore, it is concluded that larger dose of PEG facilitates better affinity of PHB oligomer to solvent by dissolution/dispersion.

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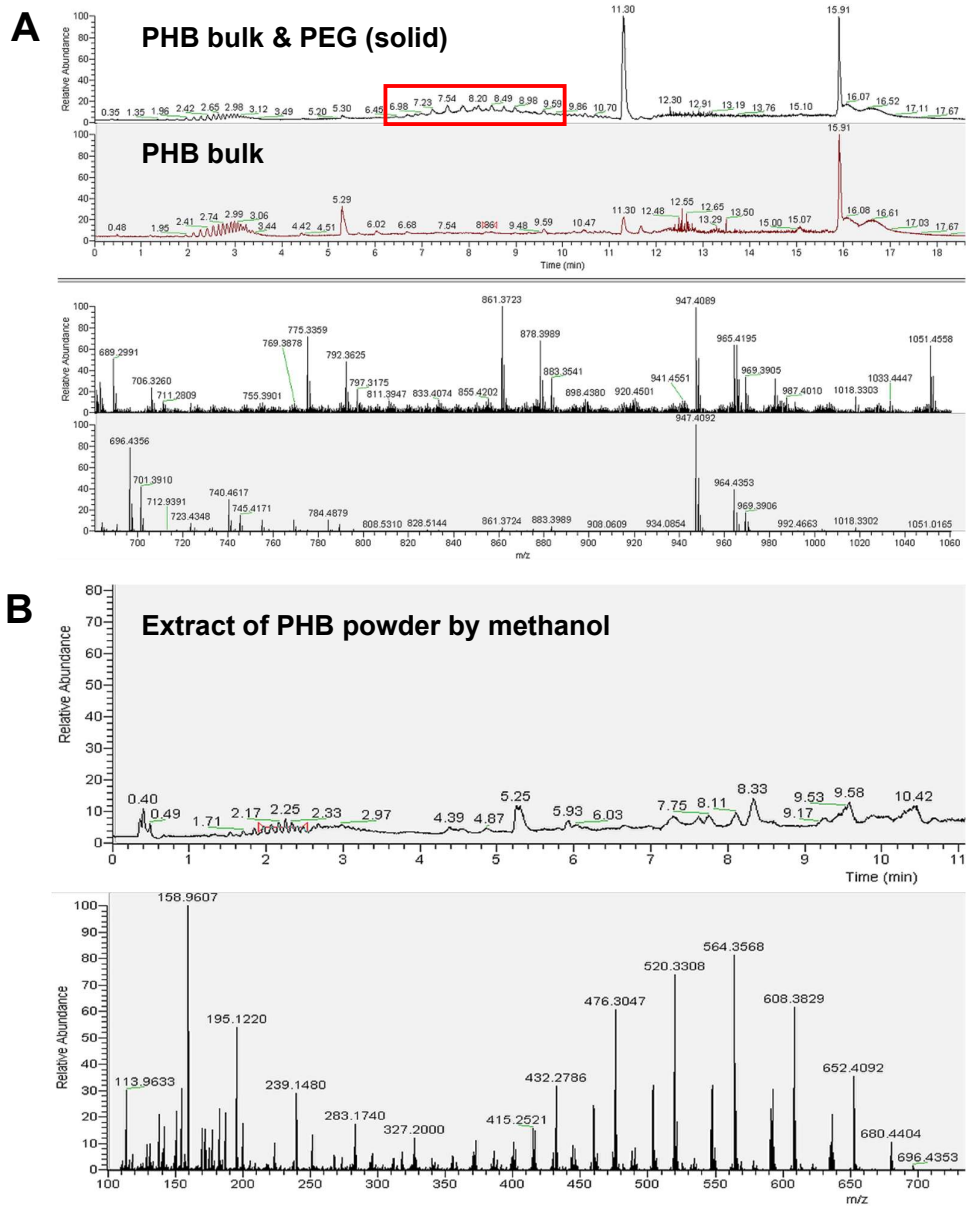

Figure 6 HPLC-MS analysis on hypothesis of dissolution/dispersion of PHB oligomer by PEG. (A) Comparison of PHB bulk and the solid part of mixture of PHB bulk and PEG after heat treatment under 150 ºC for 4 h. (B) Extract of PHB powder with methanol.

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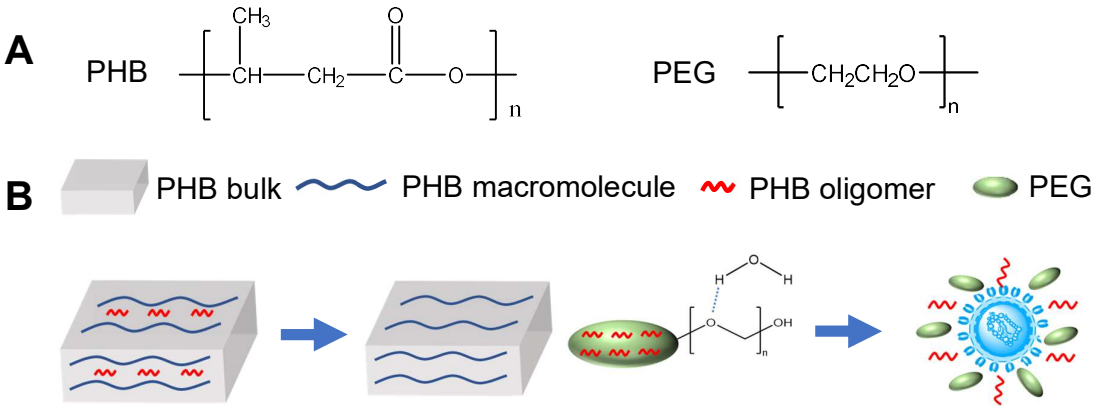

Figure 7 Schematic illustration of hypothesis of dissolution/dispersion of PHB oligomer by PEG. (A) Chemical structure of PHB and PEG. (B) Two steps are presented as dissolution/dispersion and synergistic antimicrobial effect.

4. Conclusions

A synergistic antimicrobial effect has been discovered between PHB oligomer and PEG after comparison of a series of antimicrobial tests against gram-positive bacteria, gram-negative bacteria and fungi, followed by HPLC-MS analysis. Three proposed hypothetic mechanisms have been tested by the experiments. The results show that there is neither new produced chemical component nor degradation/condensation of PHB after the compounding of PHB and PEG. The synergistic antimicrobial effect is due to the dissolution/dispersion of PHB oligomer by PEG, which has a good affinity to both the PHB and aqueous antimicrobial solution. This work reveals a new way to produce effective antimicrobial agent from bio-based PHB powder, reduce the minimal inhibitory concentration of pure PHB, and reduce the cost of production. It facilitates the cost-effective applications of bio-based PHB powder and synthesized PHB oligomer through improving the hydrophilicity.

Author Contributions: X.T. initiated, planned, and supervised the execution of the research. Z.Z. and J.L. implemented the extraction, chemical reaction and

characterization. L.M. and X.Y. conducted the antimicrobial tests. B.F. supervised the chemical analysis and synthesis work. P.H.M.L. supervised the microbiological study.

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Conflicts of Interest: The authors declare no conflict of interest.

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