Preparation of Amphiphilic Chitosan and Their Antimicrobial Activities

BYUNG-OK JUNG,[1] CHUN-HO KIM,[2] KYU-SUK CHOI,[1] YOUNG MOO LEE,[1] JAE-JIN KIM[2]

[1] Department of Industrial Chemistry, Hanyang University, Seoul, 133-791, Korea

[2] Biomaterial Research Center, Division of Polymer Science and Engineering, Korea Institute of Science and Technology, Seoul, 130-650, Korea

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ABSTRACT: Two anionic soluble monomers, mono(2-methacryloyl oxyethyl)acid phosphate and vinylsulfonic acid sodium salt, were grafted onto chitosan to obtain copolymers with zwitterionic property. Graft reaction improved the antimicrobial activities of chitosan. Antimicrobial activities of chitosan and graft copolymers depended largely on the amount and type of grafted chains as well as changes of pH, against *Candida albicans, Trichophyton rubrum,* and *Trichophyton violaceum.* The most excellent antimicrobial activity among tested samples was shown at pH 5.75 with demonstrating strain selectivity against *Candida albicans* and *Trichophyton violaceum* due to the difference in affinity between cell wall of fungi and chitosan or its derivatives. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 72: 1713–1719, 1999

INTRODUCTION

Chitin is derived from the shell of crab or shrimp, and has a similar structure to cellulose. It is also known to be biocompatible, biodegradable, and nontoxic, and therefore, has been used in drug delivery systems and also in biomedical applications. However, because of its crystalline nature, chitin is difficult to be dissolved in organic solvents. Since the discovery of dilute organic solvents to dissolve chitin, it has been utilized as films, membranes, fibers etc., because of its many advantageous features. Chitosan, a deacetylated product of chitin, is easy to be dissolved, even in dilute acetic acid, and thus, has been more widely used in comparison with chitin.[1–6]

After the discovery of antimicrobial activities of chitosan and its salts by Allan,[7] Kendra,[8] and Uchida,[9] many researchers have continued studies in this field.[10] Growth inhibition of chitosan against bacteria and fungi depended on the molecular weight of chitosan.[11] More recently, antibacterial activities of α-methyl styrene-based polymeric salts had been evaluated with various molecular weights and type of counter ions.[12–14] Antifungal or antimicrobial activities of chitosan are believed to originate from the polycationic nature of chitosan that can bind with anionic sites in proteins, thus resulting in selective antimicrobial activities toward fungi or bacteria. Researchers showed that the antimicrobial activities, which were not discovered in chitin, were mainly depended on the type of functional groups in chitosan and the molecular weight of the base chitosan. We reported that the chain length of alkyl group in *N*-alkylated chitosan linearly affected the antimicrobial activities.[15,16]

The mechanism behind the antimicrobial activities of chitosan can be summarized as follows. (1) The cationic nature of chitosan binds with sialic acid in phospholipids, consequently restraining the movement of microbiological substances.[17] (2) Oligomeric chitosan penetrates into the cells of micro-organism and prevents the growth of cells

Correspondence to: Y. M. Lee.

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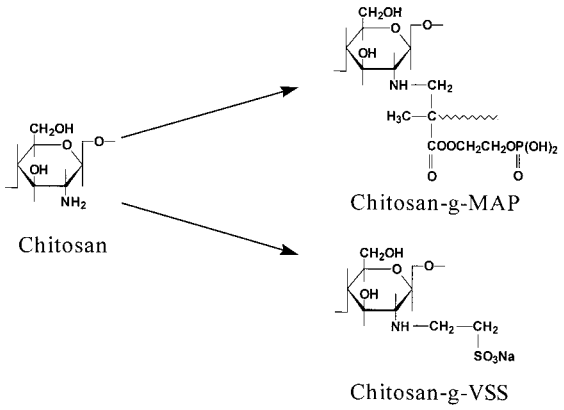

Scheme 1 Preparation of grafted chitosan copolymers.

by prohibiting the transforming DNA into RNA.[18] As described above, the antimicrobial activities are generated from the free amino groups in chitosan in an aqueous acidic environment.

In this study, we would like to prepare water-soluble anionic chitosan moieties and to investigate their antimicrobial activities for possible application in posttreatment of fibers. There have been no reports on the antimicrobial activities of high molecular weight water-soluble chitosan derivatives. To prepare water-soluble chitosan derivatives, mono(2-methacryloyl oxyethyl) acid phosphate(MAP) and vinylsulfonic acid sodium salt (VSS) were grafted onto chitosan (Scheme 1).

EXPERIMENTAL

Reagents

Mono(2-methacryloyl oxyethyl)acid phosphate (MAP) and vinylsulfonic acid sodium salt (VSS) were obtained from Tokyo Kasei Co. Ceric ammonium nitrate (CAN) was obtained from GFS Chemicals.

Chitosan from the shell of *Chionoecetes opilio* was provided from Shin Young Chitosan Co. in Seoul, Korea. It has a deacetylation degree of 70%. It was repeatedly treated three times with acids and bases according to the method of Mima et al.[19] Chitosan was dissolved in acetic acid and filtered with a G4 glass filter to remove any insoluble residues. Then, it was precipitated by adding sodium hydroxide solution. The precipitates were filtered and washed with water to reach neutral pH, with ethanol and ether, and dried at 60°C under vacuum. Final products were found to have a deacetylation degree of 90%, according to potassium poly(vinyl sulfate) (PVSK) method.[19]

Graft Reaction of Chitosan

Chitosan (2 g) and a predetermined amount of monomer (see Table I) were added into 500-mL reactor with 200 mL of 0.5 wt % acetic acid, and stirred for 1 h under nitrogen atmosphere with heating to 40°C. CAN (5.48 mg) dissolved in 100 mL of 1 *N* HNO_3 was slowly added into the reactor to initiate the graft polymerization. Reaction products were precipitated in 300 mL of acetone, filtered, and dried at 50°C for 6 h. Homopolymers were extracted in a Soxhlet apparatus by influxing in methanol for 24 h, and dried under vacuum at 50°C for 6 h. Graft percentage, efficiency of grafting and total conversion (%) were calculated using the following equations,[20]

$$\text{Percentage of grafting (\%)} = \frac{W_{gm}}{W_c} \times 100 \quad (1)$$

Table I Graft Polymerization of MAP and VSS onto Chitosan at 40°C

Monomer	Conc. of Monomer $\times 10^{-2}$	Percentage of Grafting (%)	Efficiency of Grafting (%)	Total Conversion (%)
MAP	0.2	42.0	79.1	99.1
MAP	1.2	78.2	84.1	36.9
MAP	2.0	113.9	85.2	28.2
MAP	3.2	99.1	82.5	18.0
VSS	0.3	45.5	91.5	123.1
VSS	1.0	61.2	92.1	68.5
VSS	1.5	93.6	95.7	38.2
VSS	2.5	81.2	85.8	18.8

Reaction time; 4 h, concentration of CAN: 3.5×10^{-3} *M*.

$$\text{Efficiency of grafting (\%)} = \frac{W_{gm}}{W_{gm} + W_{hm}} \times 100 \quad (2)$$

$$\text{Total conversion (\%)} = \frac{W_{gm} + W_{hm}}{W_m} \times 100 \quad (3)$$

where W_{gm}, W_{hm}, W_c, and W_m represent weight of grafted monomer, weight of the homopolymer, the weight of the charged chitosan, and the weight of the charged monomer, respectively.

Characterization

Structural analysis of chitosan and its derivatives were conducted using FTIR (Nicolet Model 5DX), a solid-state CP/MAS ^{13}C-NMR spectrometer (Bruker ARX-300) with a mass spin rate of 4 kHz and 512 number of scanning, and elemental analysis (Foss Heraeus Analysentechnik Gmblt).

Antimicrobial Test

Test cells were *Candida albicans* (ATCC 10231), *Trichophyton rubrum* (ATCC 44766), and *Trichophyton violaceum* (ATCC 8376) from the Korea National Institute of Health. These cells were selected because they were the representative cells causing tinea or tinea pedis in the human feet. *Candida albicans* was cultured in potato dextrose agar (Difco Co.) at 25°C for 48 h, while *Trichophyton rubrum* and *Trichophyton violaceum* were cultured at 28°C for 72 h.

Antimicrobial tests were conducted using chitosan and its derivatives in 0.5 wt % acetate buffer solution at various pH values. Each dilute solution (0.5 mL) was incubated in potato dextrose agar. Then, viable cells were counted after 48–72 h. Blank test was done without a cell.[21]

$$\text{Antimicrobial activity (\%)} = \frac{\text{CFU}_b - \text{CFU}_s}{\text{CFU}_b} \times 100$$

where CFU_b and CFU_s represent a colony-forming unit (CFU) of the blank and sample, respectively.

RESULTS AND DISCUSSION

Identification of Chitosan-*g*-MAP and Chitosan-*g*-VSS Polymer

The graft copolymerization of chitosan with MAP and VSS was identified by FTIR spectroscopy.

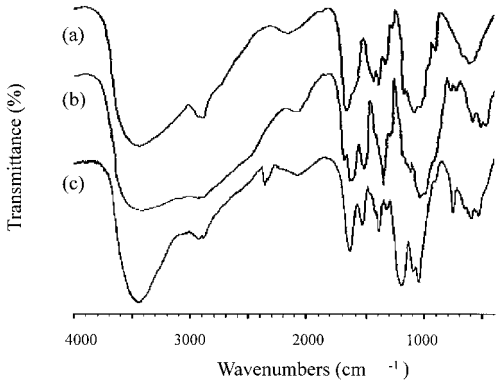

Figure 1 FTIR spectra of (a) chitosan, (b) chitosan-*g*-MAP, and (c) chitosan-*g*-VSS.

There were three characteristic spectral peaks of chitosan at 3455, 1093, and 661 cm^{-1} [Fig. 1(a)]. Characteristic FTIR spectrum of chitosan-*g*-MAP was observed at 1100 cm^{-1} due to the presence of phosphate group [Fig. 1(b)]. There were characteristic absorption peaks of chitosan-*g*-VSS [Fig. 1(c)] at 770, 1047, and 1197 cm^{-1} due to symmetric and asymmetric stretching S═O in sulfonate anion. As the grafting yield of chitosan derivatives increased, the peak intensity also increased (not shown here).

The grafting yield of chitosan derivatives, determined by changes in weight before and after the graft reaction, was summarized in Table I. The maximum grafting yields of MAP and VSS were obtained at the monomer concentration of 2.0×10^{-2} *M* and 1.5×10^{-2} *M*, respectively. The reason for the presence of optimum monomer concentration for maximum grafting yield might be that the rate of homopolymerization of monomer itself was faster than that of graft copolymerization with chitosan when monomer concentration exceeded 2.0×10^{-2} *M*.

^{13}C-NMR Analysis of Chitosan Graft Copolymer

Figure 2 illustrates the solid-state CP/MAS ^{13}C-NMR spectra for chitosan, chitosan-*g*-MAP, and chitosan-*g*-VSS. The carbon peaks due to C═O and CH_3 were found at 167.1 and 22.8 ppm in chitosan, respectively. For chitosan sample with approximately 70% deacetylation degree, about 30% of acetamino group still remain in C2 of the glucose unit in chitosan. When we grafted MAP in the amino group of chitosan, chemical shifts at 167 and 173 ppm appeared due to the carbonyl group in chitosan and the new ester carbonyl in

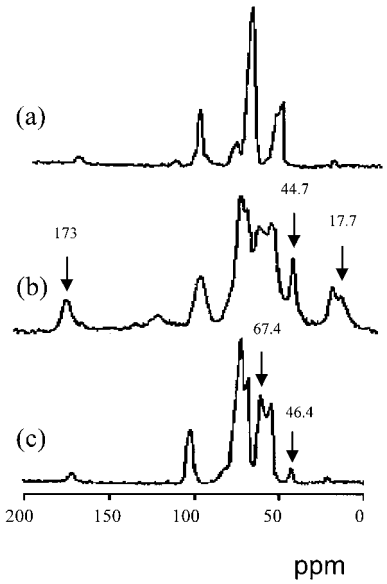

Figure 2 Solid-state CP/MAS ^{13}C-NMR spectra of (a) chitosan, (b) chitosan-*g*-MAP, and (c) chitosan-*g*-VSS.

the MAP, respectively. Strong and intensive peaks at 17.7 and 44.7 ppm were obtained due to the presence of methyl carbon and CH_2 units in the MAP. In the case of the chitosan-*g*-VSS copolymer, new chemical shifts at 46.4 and 67.4 ppm were noticed, resulting from the presence of CH_3 carbon and CH carbon in the VSS.

Antimicrobial Activities

Effect of pH

Figures 3–5 represent the effect of pH on the antimicrobial activities against *Candida albicans, Trichophyton rubrum,* and *Trichophyton violaceum,* respectively, with the dosage of 6.25×10^{-4} g. In Figure 3, for chitosan samples having a deacetylation degree of 70% (DA-70) and 90% (DA-90), antimicrobial activities of highly deacetylated chitosan dropped further as pH increased. This illustrates that free amino groups in chitosan binds with fungi, and thus, influences the binding ability between chitosan and the surface of the wall in a cell.

For graft chitosan derivatives, pH dependence of antimicrobial activity is rather drastic at a pH between 5.00 and 5.75. Chitosan-*g*-MAP showed 95% antimicrobial activity, while chitosan-*g*-VSS had 73% antimicrobial activity. If the pH changed to 6.20, antimicrobial activities of both copolymers dropped to 10–15%, which was much less than the antimicrobial activity of chitosan. In this experiment, we noticed that the growth of fungi such as *Candida albicans* increased as the pH of the medium became alkaline. However, the antimicrobial activity of chitosan-*g*-VSS had a similar pH dependence with that of chitosan-*g*-MAP.

In Figure 4, the antimicrobial activities of DA-70 and DA-90 at 6.25×10^{-4} g against *Trichophyton rubrum* were greater than that of chitosan-*g*-MAP. The trends of antimicrobial activities of three samples were similar. However, antimicrobial activity of *Trichophyton violaceum* in Figure 5 and that of *Trichophyton rubrum* in Figure 4 for chitosan and its copolymers were different. Anionic chitosan-*g*-MAP showed much enhanced antimicrobial activities against *Trichophyton violaceum,* compared with those of DA-90 and DA-70. At pH 5.75, antimicrobial activities against all microbials are better than those at the rest of the pH regions tested. Antimicrobial activities of materials we tested were selective toward a certain type of microbials, obviously resulting from the structural affinity between the wall of microbial and the chitosan or its derivatives. A possible reason might be that the wall of microbial was consisted of chitin, chitosan, or β-glycan.[22]

Effect of Concentration of Microbials

In Figures 6–8, effects of concentration of chitosan or its copolymers on the antimicrobial ac-

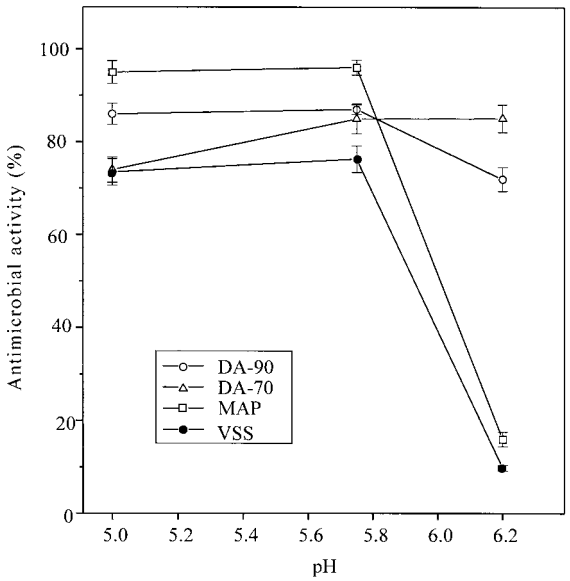

Figure 3 Effect of pH on the antimicrobial activity against *Candida albicans* at the dosage of 6.25×10^{-4} g.

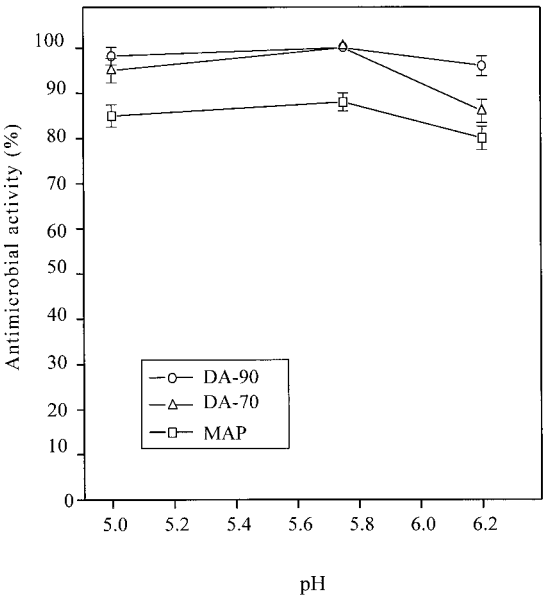

Figure 4 Effect of pH on the antimicrobial activity against *Trichophyton rubrum* at the dosage of 6.25 $\times 10^{-4}$ g.

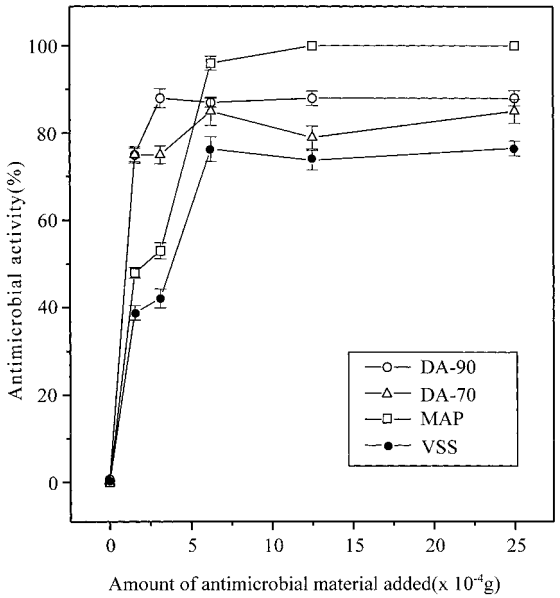

Figure 6 Effect of the dosage of antimicrobial material on the antimicrobial activity against *Candida albicans* at pH 5.75.

tivities for three microbials are summarized. At pH 5.75, an optimum pH as seen above, chitosan with a high deacetylation degree showed high antimicrobial activities toward *Candida albicans,* which was similar to the trends shown in Figure 3. Clearly, this must be due to the specific interactions between the fungi and graft copolymers. Antimicrobial activities of DA-70 and DA-90 against *Trichophyton rubrum* were above 92% at the sample concentration of 1.56×10^{-4} g (Fig. 7). If the sample concentration increased to 6.25

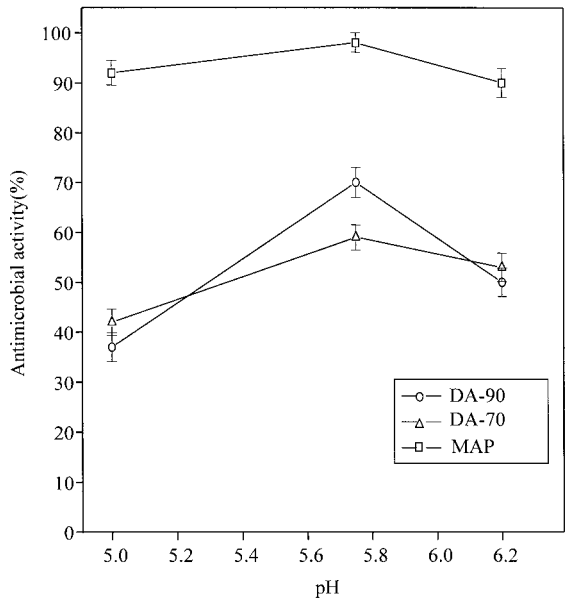

Figure 5 Effect of pH on the antimicrobial activity against *Trichophyton violaceum* at the dosage of 6.25 $\times 10^{-4}$ g.

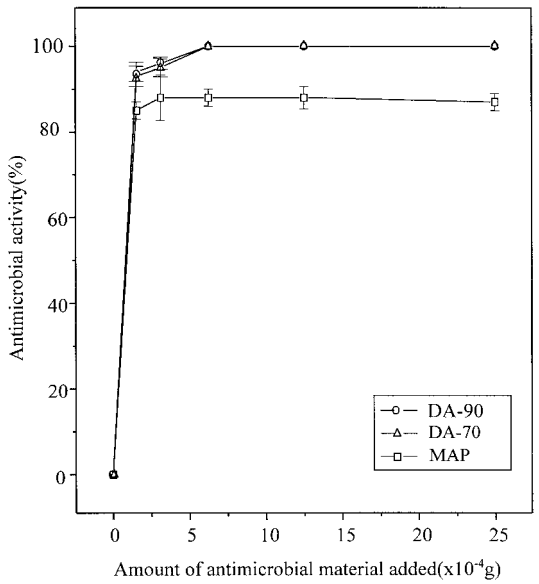

Figure 7 Effect of the antimicrobial material on the antimicrobial activity against *Trichophyton rubrum* at pH 5.75.

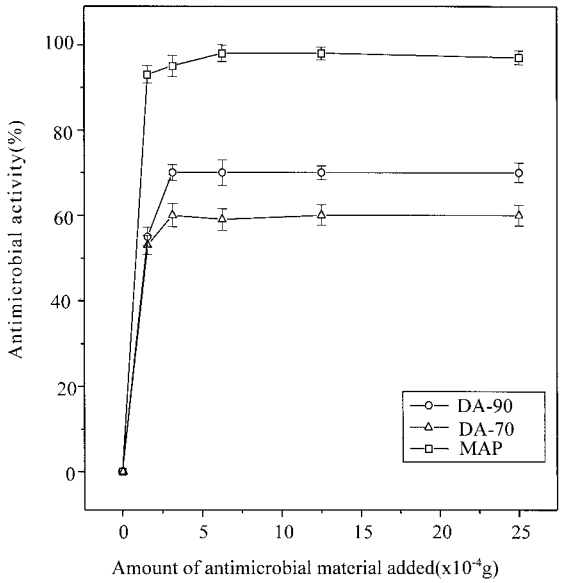

Figure 8 Effect of the dosage of antimicrobial material on the antimicrobial activity against *Trichophyton violaceum* at pH 5.75.

$\times 10^{-4}$ g, DA-70 and DA-90 samples exhibited almost 100% antimicrobial activities. Anionic chitosan-*g*-MAP samples showed less antimicrobial activities (85%) at the sample concentration of 3.12×10^{-4} g, and did not decrease at all at higher concentrations.

Against *Trichophyton violaceum,* chitosan-*g*-MAP showed much more enhanced antimicrobial activities compared with nongraft chitosan samples (Fig. 8). That is, DA-70 and DA-90 samples exhibited 60–70% antimicrobial activities at the above 3.12×10^{-4} g concentration. Antimicrobial activities did not change much even at higher concentrations. In this case, we can say that the chitosan with a high free amine content exhibits an enhanced antimicrobial activity. In the case of chitosan-*g*-MAP, only 1.56×10^{-4} g of the sample concentration exhibited more than 90% antimicrobial activities. As the graft reaction further proceeded, the pyranose ring was broken and the free amino group content thus decreased.[23] However, although the free amino group content was reduced as the graft reaction was progressed, the antimicrobial activities increased. This could be explained by the fact that there might be structural interactions between the phosphorous group containing chitosan-*g*-MAP and phospholipid in the cell wall of the *Trichophyton violaceum.*[24]

CONCLUSIONS

From this study we can conclude that we confirm the incorporation of MAP and VSS onto chitosan through FTIR and ^{13}C-NMR spectra. In general, the graft reaction into chitosan improved the antimicrobial activities. Results on antimicrobial activities of chitosan samples against three microbials showed that an optimum pH for the best antimicrobial activities, i.e., pH = 5.75, existed against *Candida albicans* and at pH 5.75, the antimicrobial activities of chitosan-*g*-MAP, DA-90, DA-70, and chitosan-*g*-VSS were 95, 85, 82, and 75%, respectively. Against *Trichophyton rubrum,* the antimicrobial activities of all the samples except chitosan-*g*-MAP were above 95%. However, chitosan-*g*-MAP showed selective antimicrobial activities (95%) against *Trichophyton violaceum,* while DA-70 and DA-90 had only 55 and 70%, respectively. The antimicrobial activities of all the samples against *Candida albicans* and *Trichophyton rubrum* at above 6.25×10^{-4} g exhibited above 80%. For *Trichophyton violaceum,* DA-70 and DA-90 showed 60–70% antimicrobial activities, while chitosan-*g*-MAP had remarkable 95% or above antimicrobial activities.

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