Preparation and Antimicrobial Activity of Konjac Glucomannan Modified with Quaternary Ammonium Compound

Wanxue Lei,[1,2] Qinhuan Yang,[1] Xingang Jia,[1] Tingyou Zhang[1]

[1]*National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, China*
[2]*Department of Chemistry, Henan Institute of Education, Zhengzhou 450001, China*

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ABSTRACT: Seven kind of graft copolymerization Konjac Glucomannan with quaternary ammonium group have been prepared, using Konjac Glucomannan (KGM) and methacryloxylethyl alkyl dimethyl ammonium bromide with c_8–c_{18} alkyl and benzyl in water, ceric ammonium nitrate as initiator, the reaction temperature of 348 K, and the reaction period of 3 h. The structures were confirmed by FTIR. The 15 min inhibitory rates of all the graft copolymerization KGM against *Escherichia coli* and *Staphylococcus aureus* reached 99.99%, against *Candida albicans* somewhat lower, but 30 min inhibitory rate still reached 99.02% for graft copolymerization KGM with quaternary ammonium group having 14 alkyl. The antibacterial mechanism of the graft copolymerization KGM has been investigated by adsorption ability to *E. coli*, measure of 260 nm absorbing materials and SEM micrographs. Firstly, the bacteria were fastly adsorbed by graft copolymerization KGM. Interactions between bacterial membranes and antibacterial product cause fundamental changes in both membrane structure and function, induced leakage of cytoplasmic contents is a classic indication of damage to the bacterial cytoplasmic membrane. The loss of the connection between the outer membrane and the underlying peptidoglycan induces the abnormality of nodular structures and bleb formation of the cell envelope of *E. coli*. The antibacterial mechanism is in accordance with microbiologic findings identifying surface blebbing as the first morphologic change occurring in the permeability barrier of gram-negative bacteria under mild heat stress and laser irradiation, etc. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 3453–3459, 2010

Key words: Konjac Glucomannan; graft copolymerization; quaternary ammonium compound; antibacterial

INTRODUCTION

Konjac glucomannan (KGM), which is a polysaccharide derived from the fruit of amorphophallus konjac, has been widely applied to the biomedicine, pharmacology, and agriculture as a kind of reactive and functional polymer.[1] KGM has excellent biocompatibility, biodegradability, and hydrophilicity, also possesses good film-forming ability, etc., but has the common flaw of natural polymers, low mechanical properties, poor antimicrobial activity and water solution rheology, poor film surface quality, and water-resistance, etc., so its modifying is necessary. The different modified product has different performance and purpose. The graft copolymerization of KGM by acrylonitrile, acrylic acid, acrylamide, vinyl acetate, methyl acrylate, butyle acrylate, etc. have been reported[2–8]

The graft copolymerization onto KGM by methacryloxylethyl benzyl dimethyl ammonium chloride[9] and methacryloxylethyl tetradecyl dimethyl ammonium bromide[10] have been reported by us.

But there are few published articles on graft copolymerization onto KGM by quaternary ammonium bromide with different alkyl chain length in the literature. It is clear that bromide is more environmentally friendly than chloride, and the modified products with different long alkyl have considerable property difference each other, such as antimicrobial activity, film-forming ability, film quality, water-resistance, blend compatibility , and so on, which are very important for application, specially for making blend films. Making blend films with modified KGM and other macromolecule materials for packaging and biomedical materials is the main application trend of KGM.

In this manuscript, we report the primal work, preparation, antimicrobial activity, and mechanisms of graft copolymerization onto KGM with c_8–c_{18} alkyl and benzyl quaternary ammonium bromide.

Correspondence to: T. Zhang (suli-zhangtymail@163.com).
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The antimicrobial activity is one of the important application properties of modified KGM.

The bactericidal activity of the insoluble bactericides depends on kind and concentration of the antibacterial groups, accessibility of active centers, nature of the spacer and nature of the carrier.[11]The carrier structure influences clearly the activity of antibacterial groups bound on the carrier. The situation of graft copolymerization nature polysaccharide is similar. Although KGM, chitosan, starch, and cellulose, all of them belong to polysaccharide, their structures are also different, even if using the same graft copolymerization monomer, the properties of their modified products are not identical.

EXPERIMENTAL

Materials

KGM (food grade, molecular weight 220KD) was purchased from Xieli Co. (Chengdu, China). *N*,*N*-dimethylaminoethyl methacrylate was kindly donated by Yili New Technique Development Company of the research institute of Qilu petrochemical Co. (Shandong, China). Lecithin was purchased from Sigma-Aldrich Co. (USA). Tween-80, CAN and other chemicals were purchased from Chengdu Kelong Chemical Reagent Factory (China), which were of analytical grade.

Tested microorganisms

Tested microorganisms included the gram-negative bacteria *Escherichia coli* (*E. coli*, 8099), Gram positive bacteria *Staphylococcus aureus* (*S. aureus*, ATCC6538), and fungus *Candida albicans* (*C. albicans*, ATCC10231).

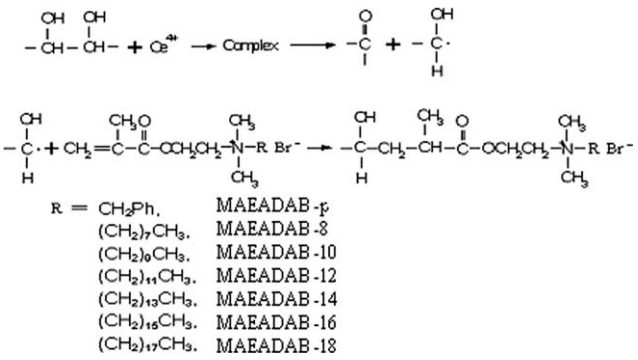
Synthesis of methacryloxylethyl alkyl dimethyl ammonium bromide (MAEADAB)

The quarternary salts of dimethylaminoethyl methacrylate with a higher alkyl were prepared referring to the reference.[12] *N*,*N*-dimeythylaminoethyl methacrylate and alkyl bromide (molar ratio of 1 to 1) were added into in a flask with acetone, thereafter the mixture was at 313 K refluxed for 20 h. After removal of acetone, anhydrous ether was added, and the precipitated solid was obtained by filtration.

Synthesis of KGM-MAEADAB

KGM-MAEADAB stands for the graft copolymerization KGM by MAEADAB.

KGM (1 g) was dissolved with 100 mL distilled water in a three-necked flask fitted with a stirrer, which was heated in a thermostat water bath. At 348 K and in nitrogen atmosphere, added 0.5 g of MAEADAB and 0.7 mmol L^{-1} of CAN, 3 h later, added ethanol and water (1 to 1 $v.v^{-1}$), precipitate was filtered and washed with distilled water. This reaction proceeds according to the scheme 1.



Scheme 1 Reaction scheme of Synthesis of KGM-MAEADAB.

The content of nitrogen element of KGM-MAEADAB was measured by BÜCHI k-437 and BÜCHI 339, on the basis of which the weight of quaternary ammonium of KGM-MAEADAB (W_g) was obtained by calculation, the percent grafting (PG) was calculated as follows:

$$PG\% = W_g \times 100/(W - W_g)$$

Here W is the weight of KGM-MAEADAB.

FTIR analysis

FTIR spectra were recorded on a FTIR spectrometer PerkinElmer, USA.

Evaluation of antimicrobial activity

MIC of MAEADAB

The minimum inhibitory concentration (MIC) of MAEADAB was evaluated by the solution dilute method. MAEADAB solution (100 g/L) was autoclaved for 25 min at 394 K and a serial concentration of MAEADAB were prepared by duplicate serial dilution with nutrient broth. The bacteria suspension was diluted with physiological saline solution to approximate 10^8 cfu·mL^{-1}. 1 ml of 10^8 cfu·mL^{-1} of *E. coli* were fed into tubes, respectively, then the same volume of MAEADAB of different concentrations were added, respectively, hereafter, were incubated at 310 K for 24 h. The MAEADAB concentration in which no bacterium is found is MIC value of MAEADAB.

Inhibitory bacteria rates of KGM- MAEADAB

To assess the inhibitory bacteria rate of KGM-MAEADAB, gram-negative bacteria *E. coli* (8099),

Gram positive bacteria *S. aureus* (ATCC6538) and the fungus *C. albicans* (ATCC 10,231) were investigated by the quantitative suspension method.[11,13]

KGM-MAEADAB (0.5 g) was added into a 250 mL Erlenmeyer flask containing 1 mL of $\approx 10^8$ cfu·mL^{-1} of bacteria and 50 mL of physiological saline solution. The flask was shaken at 310 K in a Burrell wrist action shaker for 5, 15, and 30 min, respectively. The mixture solution was serially diluted, and 1 mL of the diluted solution was dropped on a piece of agar plate, was incubated at 310 K for 24 h, the colonies were counted, and the inhibitory bacteria rate of KGM-MAEADAB was caculated.

Antibacterial mechanisms of KGM-MAEADAB

The target site of free cationic disinfectants is a cytoplasmic membrane of bacteria, they must pass through a cell wall to react their target site. At the present time, however, it is unclear whether the immobilized form of the biocides reaches their target sites, disorganizes the membrane structure and causes the death of the cells.

When the cationic biocides are covalently attached to the surfaces of the immobilized form of the biocide with short spacers, it is unlikely that they penetrate through the cell walls of bacterial cells, but the immobilized cationic biocides may reach their target sites through long, flexible spacers. Depression of biological activities of bacteria (*E. coli* and *Azotobacter agile*) and enzymes was observed when the cells were adsorbed on the materials. The phenomena were interpreted in terms of depression of specific surface area of the cells because of adsorption and subsequent interference with substrate transport. Furthermore, in the case of *E. coli* adsorbed on a resin, morphological changes of the cells were observed. The bacteria cells are generally insensitive to external stimulation because of the presence of the rigid layer (cell walls). If bacterial cells suffer from the partial breakdown of the cell walls resulting from the adsorption, it may be lethal. Therefore, the fate of the bacteria adsorbed on the immobilized surface of biocides may originate from strong interaction between the cell surface and the biocide and, consequently, lysis.

KGM-MAEADAB is a new kind of immobilized cationic macromolecule antibacterial product, graft copolymerization of MAEADAB onto KGM. The primary antibacterial experiment against to *E. coli* and *S. aureus* revealed its antibacterial ability is excellent, but we don't know how the depression of biological activities of bacteria was inflicted by KGM-MAEADAB, so that to choose KGM-DMAEMAB-14, which has excellent antimicrobial activity, against *E. coli* as a sample, the adsorption ability of KGM-MAEADAB to bacteria and the cell membrane integrality of bacteria were tested, the morphologies of bacteria were observed by SEM.

Adsorption ability of KGM- MAEADAB to bacteria

A certain quantity of KGM-DMAEMAB-14 was fed into a 250 mL flask, then 90 mL of physiological saline solution and 10 mL of approximate 10^8 cfu·mL^{-1} of *E. coli* were added, shaken at 310 K, the samples (fluid) were taken, serially diluted, the colonies N were counted. The adsorption ability was described in Log N_t/N_0.

Measure of 260 nm absorbing materials

The bacterial suspension was separated into several flasks. A certain quantity of KGM-DMAEMAB-14 was added to each flask except the control. 1.5 mL of sample was removed from flasks for definite interval. The specimens were immediately filtered with 0.2 μm syringe filters to remove the bacteria. The supernatan was then diluted appropriately and optical density at 260 nm was recorded with a UV-Vis spectroscope lambda 25, PerkinElmer.

SEM micrographs of bacteria after quantitative suspension test

The piece of crust leather disinfected by ultraviolet was coated with a suspension of antibacterial agent KGM-DMAEMAB-14 (10P.of 0.5 g KGM-MAEMAB-14/100 mL and 90P. of water) and dried at room temperature.

The piece of coated leather was placed into a 250 mL Erlenmeyer flask containing 10 mL of $\sim 10^6$ cfu/mL *E. coli* suspension liquid and 90 mL of sterilized water, the flask was vibrated for definite time period at 298 K, drain, the piece of leather was washed with distilled water, and dried at room temperature.

The morphologies of bacteria on the dried piece of antibacterial crust leather were observed as follows: The specimens were fixed with glutaraldehyde and dried by supercriticalcarbondioxidefluid, thereafter, the specimens were sputter coated and examined in a JSM-5900LV Field Emission Scanning Electron Microscopy (FE–SEM).

RESULTS AND DISCUSSIONS

Spectra of FTIR

Figure 1 shows FTIR spectra of KGM and graft copolymerization KGM.

Comparing FTIR of KGM with that of the graft copolymerization KGM, it can be seen that a band at 1384 cm^{-1} appears, which is attributed to the methyl group. The absorption band at 1729 cm^{-1} in graft copolymerization KGM is referenced as ester group, and the absorptions band at 809, 766, and 715 cm^{-1}

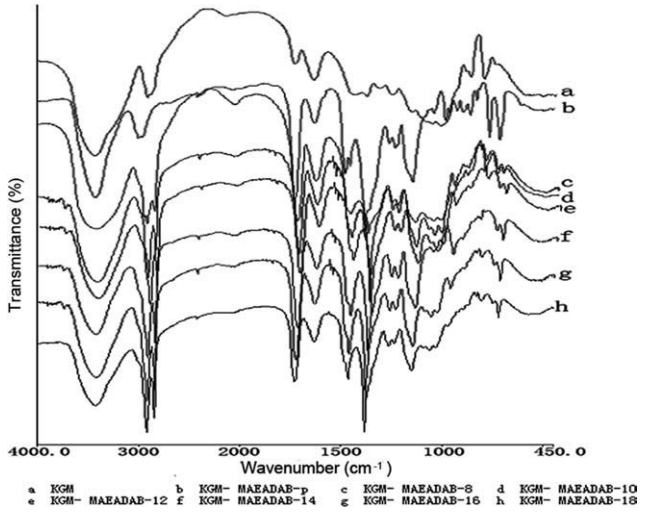


Figure 1 FTIR spectrum of graft copolymerization KGM.

are ascribed to the benzene ring in which one of the hydrogen atoms has been replaced. The new absorption of 1236 cm^{-1} (C—N) can be detected from the KGM-DMAEMAB FTIR spectrum. These are the characteristic absorptions of the graft copolymerization KGM, which confirmed the graft copolymerization of DMAEMAB onto KGM.

Antimicrobial activity

MIC of MAEADAB

As given in Table I, these quaternary ammonium shalts with higher alkyl show antimicrobial activity, MIC was only 6.25 g/L or below it by the qualitative suspension method, most of them below 0.2 g/L.

Inhibitory rate of KGM-MAEADAB

The inhibitory rates of KGM-MAEADAB for 5, 15, and 30 min against *E. coli*, *S. aureus* and *C. albicans* are shown in Table II.

TABLE I
The MIC of the Quaternary Ammonium Salts[a]

Quaternary ammonium salts[b]	MIC (g/L)
MAEADAB-p	6.2500
MAEADAB-8	3.1250
MAEADAB-10	0.1953
MAEADAB-12	0.0488
MAEADAB-14	0.0122
MAEADAB-16	0.0977
MAEADAB-18	0.3906

[a] Experiment temperature 293 K, approximate 10^8 cfu/mL of *E. coli.*
[b] All the PG of KGM-MAEADAB were 35.2–38.5%

The inhibitory rate of graft copolymerization KGM against *E. coli* and *S. aureus* is high, all of 15 min inhibitory rates already reached 99.99%, despite MIC values of MAEADAB, graft copolymerization monomer, are different. That is to say the excellent antimicrobial activity of MAEADAB is not reduced after graft copolymerization onto KGM. The 15 min inhibitory rates of graft copolymerization KGM against *C. albicans* were 91.26–98.12%, 30 min inhibitory rates 93.1–99.02%, somewhat lower, but the inhibitory rate of graft copolymerization KGM with fourteen alkyl still reached 99.02%.

Antibacterial mechanism of KGM-MAEADAB

Adsorption behavior

Adsorption behavior of KGM-MAEADAB-14 to *E. coli* is shown in Figure 2.

Figure 2 stands for adsorption kinetic curve of graft copolymerization KGM to *E. coli*. It shows that when there were 0.5 g of KGM-MAEADAB-14 and 10 mL of approximate 108 cfu·mL^{-1} of *E. coli*, the number of active colonies was decreased by about 104 for only 5 min, the adsorption was increased

TABLE II
Inhibitory Rate of KGM-MAEADAB[a]

Microorganism	Time (min)	Inhibitory rate against bacteria (%)						
		A	B	C	D	E	F	G
E. coli	5	99.10	98.62	98.56	99.48	99.72	99.34	99.28
	15	99.99	99.99	99.99	99.99	99.99	99.99	99.99
	30	99.99	99.99	99.99	99.99	99.99	99.99	99.99
S. aureus	5	98.25	98.60	99.11	99.61	99.87	99.42	99.51
	15	99.99	99.99	99.99	99.99	99.99	99.99	99.99
	30	99.99	99.99	99.99	99.99	99.99	99.99	99.99
C. albicans	5	88.06	86.37	91.66	92.86	93.64	93.31	92.18
	15	91.26	92.44	94.51	95.82	98.12	97.66	96.23
	30	93.10	93.89	95.18	97.45	99.02	98.72	98.02

[a] Temperature 293 K. A, B, C, D, E, F, and G stand for KGM-MAEADAB-p, KGM-MAEADAB-8, KGM-MAEADAB-10, KGM-MAEADAB-12, KGM-MAEADAB-14, KGM-MAEADAB-16, and KGM-MAEADAB-18, respectively. All the data of inhibitory rate were the means of three parallel experiments, of which the Standard deviation were less than 3%. All the blank tests cfu/mL were 1.6–4.5 $\times$ 10^7, all the PG of KGM-MAEADAB were 35.2–38.5%.

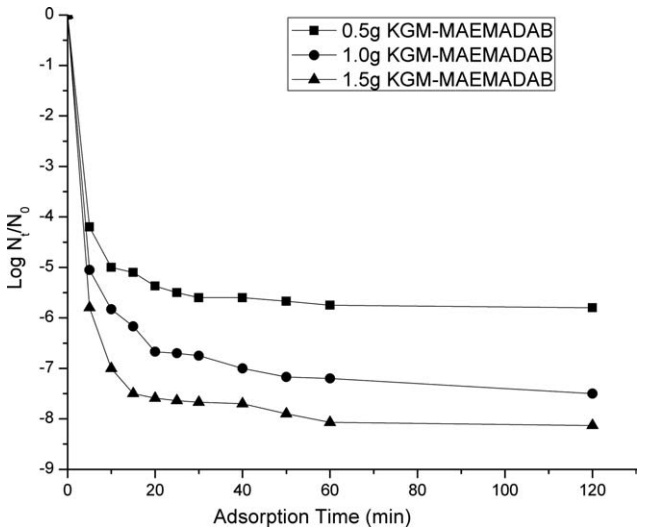


Figure 2 Adsorption behavior of KGM-MAEADAB-14 to bacteria.

from 0.5 to 1.5 g KGM-MAEADAB-14. The adsorption ability of graft copolymerization KGM to the bacteria is very strong. The antibacterial procedure of graft copolymerization KGM is that the bacteria are adsorbed by it, firstly.

260 nm absorbing materials

The bacterial membrane serves as a structure component,[14,15] which may become compromised during a biocidal challenge such as exposure to a cationic biocide. Therefore, release of intracellular components is a good indicator of membrane integrity. Small ions such as potassium and phosphate tend to leach out first, followed by large molecules, such as DNA, RNA, and other materials. As these nucleotides have strong UV absorption at 260 nm, they are described as "260 nm absorbing materials". It is very easy to detect whether there are any 260 nm absorbing materials using a UV-vis spectrophotometer and this method is widely used in determining membrane integrity.[9,10] The leakage of potassium ions correlates well with growth inhibition and a bacteriostatic effect, the inorganic phosphate and the release of 260 nm absorbing material corresponds to biocidal activity.[16]

Figure 3 shows release of 260 nm absorbing materials from *E. coli*.

There was significant 260 nm absorbing for only 5 min, and the absorbing value was increased with the quantity of applied antibacterial material KGM-MAEADAB-14 from 0.5 to 1.5 g, and the optical density of *E. coli* suspension at 260 nm reached a plateau quickly, after 20 min the value was little changed. This quick release of 260 nm absorbing materials was in good agreement with fast antibacterial kinetics of KGM- MAEADAB-14 showed in Table II.

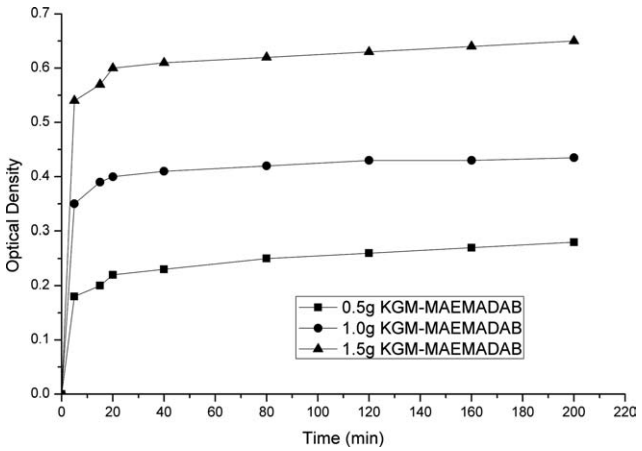


Figure 3 Release of 260 nm absorbing material from *E. coli*.

The cytoplasmic cell membrane undoubtedly is the target for many antimicrobial agents. Interactions between bacterial membranes and biocides frequently cause fundamental changes in both membrane structure and function.[17] These changes indicative of damage to cytoplasmic membrane have been observed in bacterial populations trite with bacteriostatic and bactericidal levels of cations or polycations. Leakage of cytoplasmic contents is a classic indication of damage to the bacterial cytoplasmic membrane.

SEM micrographs

Figure 4 shows visible injury patterns inflicted on *E. coli* when *E. coli* exposed to KGM-MAEADAB-14 for only 5 min. Figure 5 is SEM micrographs for 15 min, indicates the injury increased dramatically with the contact time of *E. coli* with KGM-MAEADAB-14.

The cells showed surface alterations in terms of protuberances like blebbing and formation of irregular nodular structures of variable size covering the cell bodies. Furthermore, coagulated phenomenon

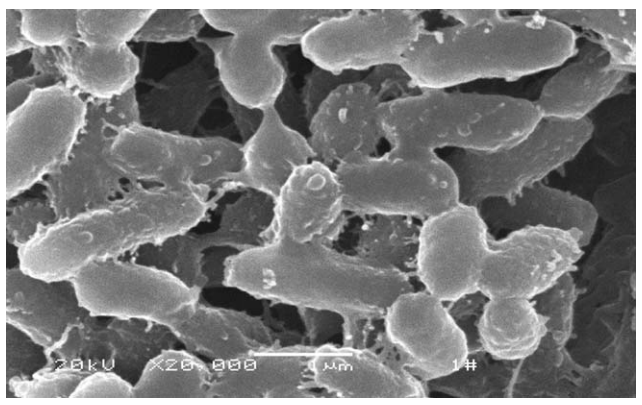


Figure 4 SEM micrographs of *E. coli* treated 5 min with KGM- MAEADAB-14, ×20k.

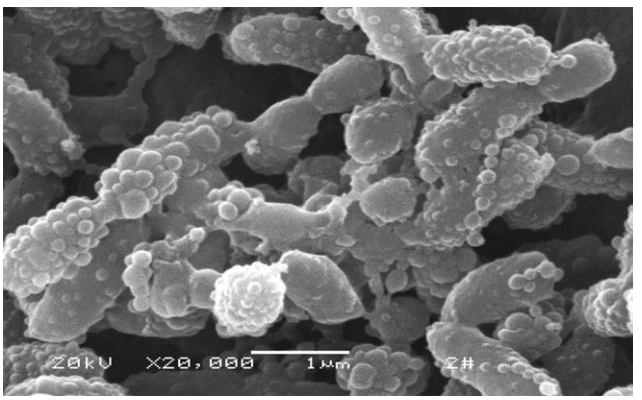


Figure 5 SEM micrographs of *E. coli* treated 15 min with KGM- MAEADAB-14, ×20k.

was seen, in which the shape of the bacteria merged with one another. The cell bodies seemed to be partly fused together. No morphologically intact bacteria were discernible

E. coli is a gram-negative rod possessing the typical cell envelope, its peptide glycan or murein layer has a diameter of only about 2 nm, with only 15–20% of the cell wall being made up of it. The murein is intermittently crosslinked and surrounded by a complex outer membrane structure.[18,19] Lipoproteins, which constitute about 58% of the cell envelope, stabilize the outer membrane and anchor it to the murein. The lipopolysaccharides of the gram-negative cell envelope form part of the outer leaflet of the outer membrane.[20]

A. Moritz reported[21] *E. coli* showed striking perturbations of the cell envelope by laser irradiation, even at "subtherapeutic" settings, the cell envelope of *E. coli* showed nodular structures and bleb formation and this structural alteration is in accord with microbiologic findings identifying surface blebbing as the first morphologic change occurring in the permeability barrier of gram-negative bacteria under mild heat stress.[22,23] Katsuij[24] suggested that blebbing results from the heat-induced release of membrane lipids and that the blebs consist of outer membrane materials. He deducted that the loss of the connection between the outer membrane and the underlying peptidoglycan induces this abnormality. Studies of mutant strains with a genetic defect of these very lipoproteins revealed that the consequences of this structural abnormality resulted in the formation of outer membrane blebs and physiological deficiencies such as decreased growth rate and hypersensitivity to detergents.[25,26]

In evidence, there is no producing of heat stress when *E. coli* contacts with the antibacterial agent KGM- MAEADAB, so that the breaking of the cell is also not produced by heat stress here, but surface blebbing of the cell as the first morphologic change occurring in the permeability barrier of gram-negative bacteria is the same as under heat stress and laser irradiation, etc.

CONCLUSION

Seven kind of graft copolymerization Konjac Glucomannan with quaternary ammonium group have been prepared, using Konjac Glucomannan (KGM) as carrier and methacryloxylethyl alkyl dimethyl ammonium bromide with c_8–c_{18} alkyl and benzyl (MAEADAB) as graft copolymerization monomer in water, CAN as initiator, the reaction temperature of 348 K, the reaction period of 3 h. The graft copolymerization KGM exhibited excellent antibacterial activity, all of the inhibitory rates for 15 min against *E. coli* and *S. aureus* reached 99.99%, the excellent antimicrobial activity of MAEADAB is not reduced after graft copolymerization onto KGM. The 15 min inhibitory rates of graft copolymerization KGM against *C. albicans* were 91.26–98.12%, 30 min inhibitory rates 93.1–99.02%, somewhat lower, but the inhibitory rate of the graft copolymerization KGM with 14 alkyl still reached 99.02%.

The antibacterial mechanism is that the bacteria are adsorbed speedily by graft copolymerization KGM firstly. Interactions between bacterial membranes and antibacterial product cause fundamental changes in both membrane structure and function, induced leakage of cytoplasmic contents is a classic indication of damage to the bacterial cytoplasmic membrane. The loss of the connection between the outer membrane and the underlying peptidoglycan induces the abnormality of nodular structures and bleb formation of the cell envelope of *E. coli* It is the same as under heat stress and laser irradiation, etc.

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