Antibacterial Effects of Chitosan and Its Water-Soluble Derivatives on *E. coli*, Plasmids DNA, and mRNA

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ABSTRACT: The antibacterial activities of chitosan and its water-soluble derivatives on *E. coli* were studied according to four influencing factors *in vitro*. The antibacterial study showed that chitosan, *O*-hydroxyethyl chitosan (O-HECS), and *O*-carboxymethyl chitosan (O-CMCS) could inhibit the growth of the microbial. To study the antibacterial mechanism, plasmid DNA pBR322 and pUC18 were selected to be the probes to find out the binding abilities of chitosans. Results showed that raw chitosan had a high binding ability with the plasmids and the influencing degrees were stable. The effects of chitosan derivatives on plasmids might be affected by space effect and static effect. With appropriate concentrations and molecular weights, the derivatives might have strong abilities to combine with DNA. The degree of influence of chitosan and its derivatives on plasmids had nothing to do with time. The experiment focusing on the relationship between chitosans and mRNA showed that O-CMCS would hinder the synthesis of mRNA, and this may give some proof to its antibacterial mechanism. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 3521–3528, 2007

Key words: chitosan; chitosan derivatives; plasmids DNA; binding ability; mRNA

INTRODUCTION

Chitosan is the deacylated product of the second natural polymer chitin and has been proved to be a good antibacterial material. Chitosan can inhibit the growth of a wide variety of bacteria and fungi, with several advantages, including higher antibacterial activity, broader spectra of activity, higher killing rate, and lower toxicity toward mammalian cells.[1–5] It has a variety of applications fields such as biomedical, food and chemical industries because of its good properties.[6–9] Though having the above advantages, the insolubility of chitosan in nonacidic solution limits its applications in many fields, while chitosan with a low molecular weight (M_w) is an exception.[6,10,11] Interestingly, antifungal activity has been found for modified chitosan derivatives,[10,12–14] which can broaden the applications. Interestingly, the antifungal activity of modified chitosan derivatives further broadens their applications. There can be a lot of derivatives of Chitosan, which has hydroxyl and amino groups on their backbones. *O*-carboxymethyl chitosan (O-CMCS) and *O*-hydroxyethyl chitosan (O-HECS), which are being discussed in this paper, are water-soluble in a wide pH range. The substitutions of chitosan take place mainly on hydroxyl group, so its amino groups, which are crucial for its especial property, are a little affected.

There have been a large number of reports discussing chitosan's antimicrobial mechanism. The theories in those papers propose two different mechanisms by the different targets on the cells. Some researchers thought that the polycations on chitosan could interfere with negatively charged residues of macromolecules at cell surface. Chitosan may interact with the cell membranes and change the cell permeability. UV-absorption studies indicated that chitosan could weaken the membranes of bacteria and cause considerable leakage of amino acid and protein.[15,16] Another study demonstrated that chitosan, which bind with bacteria could form a polymer membrane, inhibits its respiration and keep nutrients out. This behavior rendered the bacteria impaired.[17,18] The other proposed mechanism involves the binding of chitosan with DNA to inhibit the synthesis of mRNA. When chitosan was released from the cell wall of fungal pathogens by plant host hydrolytic enzymes, it penetrates the nuclei of fungus and interferes the synthesis of RNA and protein.[19] It has been proved that a relatively small quantity of chitosan could remove major proportions of nucleic acid of the bacterial suspensions.[20–24]

Until recently, the mechanism of how chitosan and its derivatives acted upon bacteria has not been elucidated clearly. In this paper, the antibacterial activities of chitosan and its water-soluble derivatives with low M_w on *E. coli* are investigated. Other methods such as binding abilities of chitosans on plasmids pBR322 and pUC18 and the influences of chitosans on the

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synthesis of mRNA are studied to give some proof to its antibacterial mechanism.

METHODS

Materials

Chitosan was provided by Jiangnan University (Jiangsu Province, China; weight average M_w 3–5, 5, 8, 10, and 20 kDa; degree of deacetylation > 85%) and Zhejiang University (Hangzhou, Zhejiang Province, China; M_w = 2 kDa; degree of deacetylation > 85%). Bacteria *E. coli* cloned with gene *NahG2* was provided by the School of Life Science, Nankai University (Tianjin, China), and was stored at 4°C. Biochemical reagents such of tryptone, yeast extract, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). Plasmid reagent boxes of pBR322 and pUC18 were purchased from Huamei Biomaterial Co. (Henan Province, China). Agar power was from Bethesda Research Laboratories Technologies Ltd. (Bethesda, MD). The Tris saturated hydroxybenzene was obtained from TBD Biomaterial Co. (Beijing, China). Trizol reagent was provided by No.1 Central Hospital of Tianjin (Tianjin, China).

Methods

Preparation of O-CMCS

5 g raw chitosan was placed in a 250-mL flask equipped with a magnetic stirring bar and 50 mL NaOH (42%) was added. The mixture was then stirred in ice bath for 2 h to let chitosan swell sufficiently. A total amount of chloroacetic acid [chitosan:chloroacetic acid = 3:2.3 (w : w)] was added bit by bit in about 1 h. After 48 h, adjust to pH = 7 with HCl. The pure offspring was obtained by dialysis for three days and lyophilized.

Preparation of HECS

Five grams of raw chitosan was mixed with 50 mL (42%) NaOH, and the solution was frozen overnight. 20 mL isopropanol was added after unfreezing. The solution was then stirred and 50 mL 2-chloroethanol was placed into the solution dropwise. The mixture needed to react for 24 h under 75°C. The reactant was dialyzed and lyophilized.

Characterization of O-CMCS and HECS

Infrared (IR) spectrum was measured to study the structure of the derivative by Bio-Rad FTS 135. The degree of substitution was determined by traditional potentiometric titration. Advancing contact angles were measured in films to study its water solubility.

Antibacterial activities of chitosan and O-CMCS

E. coli was used as the test organism. After two successive transfers of the test organism into nutrient broth (LB) at 37°C for 24 h, the activated culture was inoculated again into 50 mL LB at 37°C for 16 h. For the experiment of antibacterial activities of chitosans, solutions of chitosan and its derivatives with LB nutrient were first prepared. 75 μL inoculum of *E. coli* was added into 5 mL nutrient broth containing chitosans and incubated at 37°C with shaking at 110 rpm for a period of 16 h. The viable population of the test organisms was determined by turbidity every 2 h using UV-absorber (unico and UV-2000 UV-spectrophotometer) at 610 nm.

Abilities of chitosan and its derivatives to binding with plasmid DNA

Solutions of chitosan and its derivatives were prepared with bidistilled water. 13.5 μL of each solution and 1.5 μL of plasmids DNA pBR322 or pUC18 were blended and then the mixture was preserved at 4°C. Samples of the above solutions were withdrawn in predecided intervals. Effects were confirmed by electrophoresis on a 0.7% agarose-gel with Tris-acetate (TAE) running buffer at 80 V for 40 min. DNA was visualized with ethidium bromide (EtBr) (0.5 mg/mL). The results were analyzed by UV Transilluminator. All the experiments were carried out in triplicate to ascertain the reproducibility.

The effects on mRNA by chitosan and O-CMCS

Bacteria *E. coli* with gene *NahG2* was inoculated into LB liquid added with chitosan or O-CMCS (5000 ppm) and incubated at 37°C for 24 h. RNA plot-hybridization was carried out after mRNA being extracted by Trizol reagent, the photo of which was exposed to view the results.

RESULTS AND DISCUSSION

Preparation of O-CMCS and HECS

Figure 1(a–c) are IR spectra of chitosan, O-CMCS, and HECS, respectively. There are two characteristic peaks of chitosan, 3380 and 1070 cm^{-1}, representing primary hydroxyl groups. In the spectrum of O-CMCS [Fig. 1(b)], the peak around 3300 cm^{-1} became broader, which indicated wild vibrations of O—H of carboxyl acid. The sharp peak at 1045 cm^{-1} in Figure 1 (c) represents vibrations of primary hydroxyl C—O(H). Peaks of 1300–1500 cm^{-1} represent methylene of hydroxylethyl groups. The peaks at 1597 and 883 cm^{-1} represent primary amido groups, indicating that synthesis took place on hydroxyl groups and most of the amide groups were reserved.

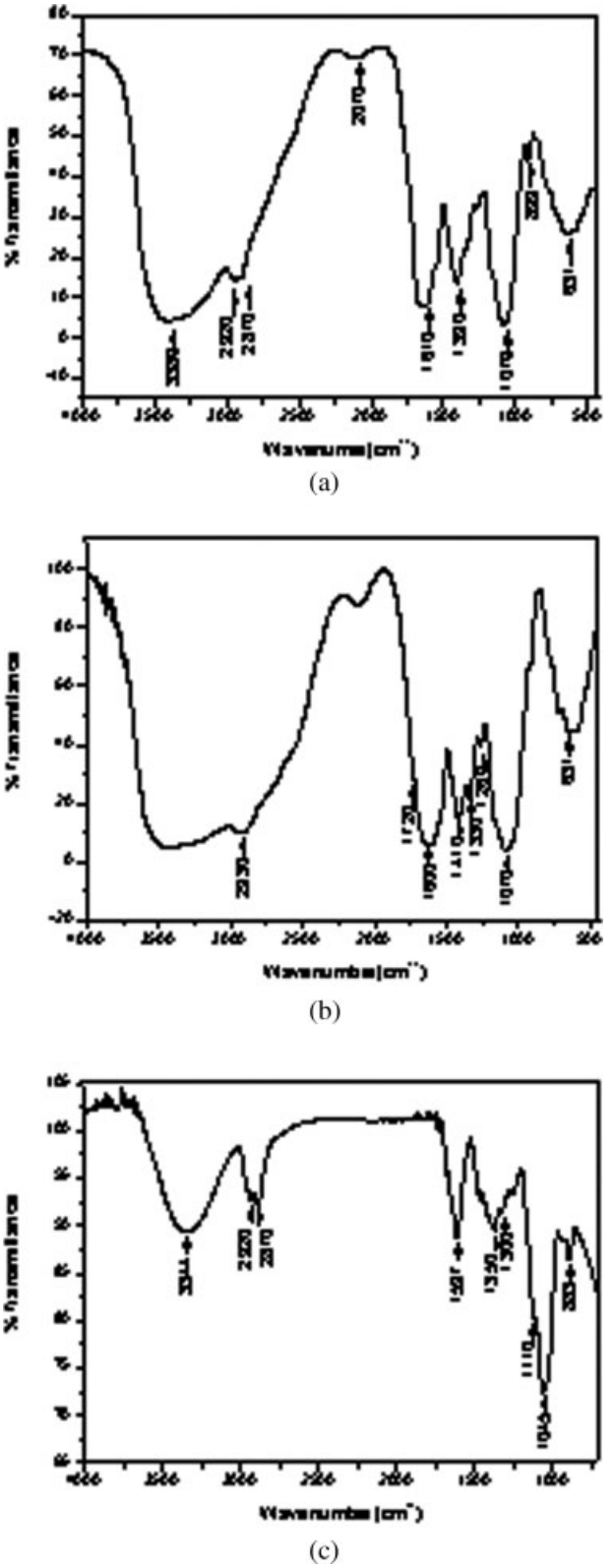


Figure 1 IR spectrum of chitosans. (a) chitosan; (b) O-CMCS; (c) HECS.

Table I gives out the advancing contact angles of chitosan and its derivatives. The smaller the dynamic contact angle, the higher the hydrophilicity of the chemical. So it is shown from the data that the hydrophilicity of O-CMCS and O-HECS is better than that of the raw chitosan. In addition, the hydrophilicity of O-CMCS is lower than that of O-HECS mainly because —COOH can enhance the hydrogen bonds that enhance the interactions between the molecules.

TABLE I
Advancing Contact Angle (°) of Chitosan and its Derivatives

Sample	Advancing contact angle (°)
Chitosan	87.51
O-CMCS	61.73
HECS	37.97

Through traditional potentiometric titration method, the degree of substitution (DS) of the O-CMCS is determined as 90%.

Antibacterial effects on *E. coli* and plasmids of chitosan and its derivatives

Figure 2 shows the optical density (OD) versus culture time for chitosan (M_w = 3–5 kDa), O-CMCS (original chitosan M_w = 3–5 kDa, %DS = 90%), and O-HECS (original chitosan M_w = 3–5 kDa) against *E. coli*. Results indicated that the change of OD influenced by O-HECS was close to that of chitosan. Near antibacterial activity of O-HECS was observed compared with that of raw original chitosan. However, the OD of bacteria which was added with O-CMCS was lower than that of chitosan, and the antibacterial activity was slightly enhanced.

O-HECS is a product where —OH groups on chitosan are substituted by $—CH_2CH_2OH$ groups. So, compared with raw chitosan, its $—NH_2$ content is almost identical, and its antibacterial activity is kept. O-CMCS is the substitution of chitosan with $—CH_2COOH$ mostly to —OH, its amount of $—NH_2$ is merely changed. Moreover, its —COOH groups may have reacted with the $—NH_2$ groups intra- or intermolecular, and charged these $—NH_2$ groups. So, in the same condition, the number of $—NH_3^+$ groups of O-CMCS

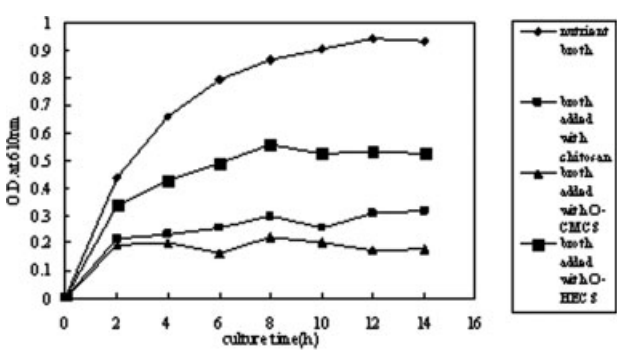
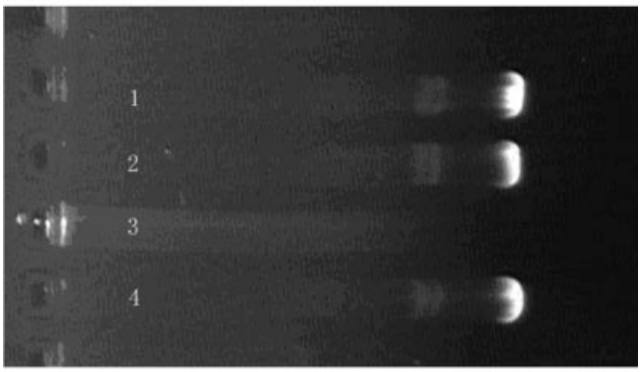


Figure 2 OD versus culture time for chitosan and its derivatives (M_w = 3–5 kDa, 5000 ppm) against *E. coli.*

(a)



(b)

Figure 3 The binding abilities of chitosan and its derivatives of different kinds (M_w = 3000–5000) with plasmid DNA. (a) agarose-gel assay of pBR322; (b) agarose-gel of pUC18; lanes 1–3 were plasmids bind with O-CMCS, HECS, chitosan with M_w of 3000–5000 and lane 4 represented control experiment, the concentration of chitosans in each experiment was 5000 ppm, reactive time was 4.5 h.

is larger than that of raw chitosan. Therefore, the antibacterial activity of O-CMCS increases.

The different DNA-binding abilities of chitosan and its derivatives with plasmids are investigated by agarose-gel assay and the results are shown in Figure 3. The luminance of the plasmids bound by chitosan could not be observed in both figures. It can be explained that all negative charges of DNA had been counteracted by chitosan and they will not able to move in electric field accordingly. The amino groups of the chitosans that possess positive charges would attract the negative phosphate groups of DNA. And we consider the quantity of amino groups is the dominant factor.

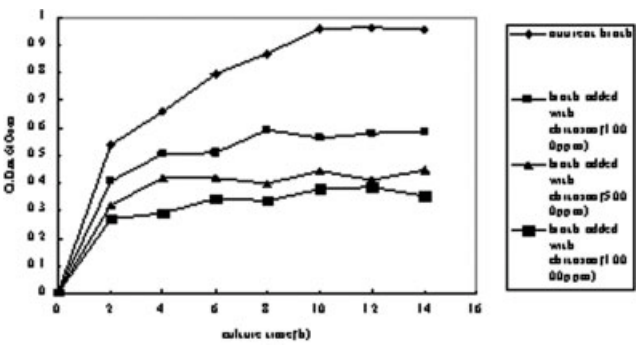
Figure 3(a) and Table II display the photo and data of chitosans' influence on plasmids pBR322, where the degree of influence indicated the abilities of chitosan, O-HECS, and O-CMCS to bind with pBR322. Compared with chitosan, the abilities of O-HECS and O-CMCS to bind with pBR322 decreased.

But the binding abilities on plasmids were not all the same when the probe was pUC18. It is obvious that chitosan had the same effect that the luminance of the plasmids disappeared, but the results of both derivatives did not have many differences from that of the control experiment [Fig. 3(b)]. There revealed no change appeared to be electrostatically neutral paired with the original plasmid. It seemed that neither derivatives had bound with the probe (Table II).

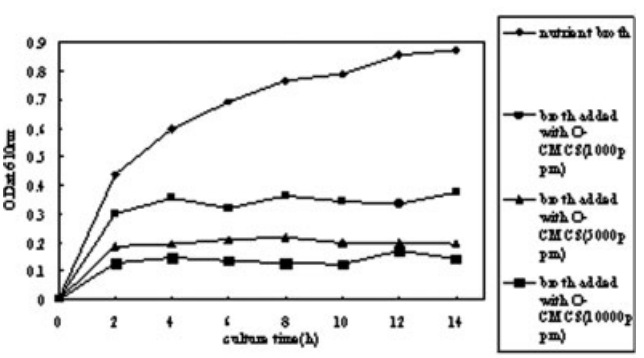
Plasmids pBR322 and pUC18 are about 4363 bp and 2686 bp in length (about 10–100 nm), respectively. Raw chitosan with low M_w (3–5 kDa in our experiments) has a tiny volume (less than 10 nm) compared to the plasmids, so the space restriction effects can be neglected. The negative charges on the plasmids are counteracted easily by chitosans results that the brightness of the corresponding bands weakened, even disappeared. But for the derivatives, because of the introduction of functional groups, volumes of chitosan become larger and the space conformation of the chain segments become more complicated, resulting in huge space effect. But with such a low molecular weight, the positive charges on the derivatives lack enough electrostatic force to overcome the space effect. And the amino groups on

TABLE II
The Degree of Influence on Plasmids

	pBR322		pUC18	
Sample	Degree of grey	Degree of influence (%)	Degree of grey	Degree of influence (%)
Control	39387.9	–	65855.2	–
Chitosan	0	100	0	100
HECS	23507.7	40.32	71543.5	–
O-CMCS	27533.7	30.1	73813.5	–



(a)



(b)

Figure 4 OD versus culture time for chitosan and O-CMCS of concentration ranging from 1000 ppm to 10,000 ppm against *E. coli.* (a) chitosan; (b) O-CMCS.

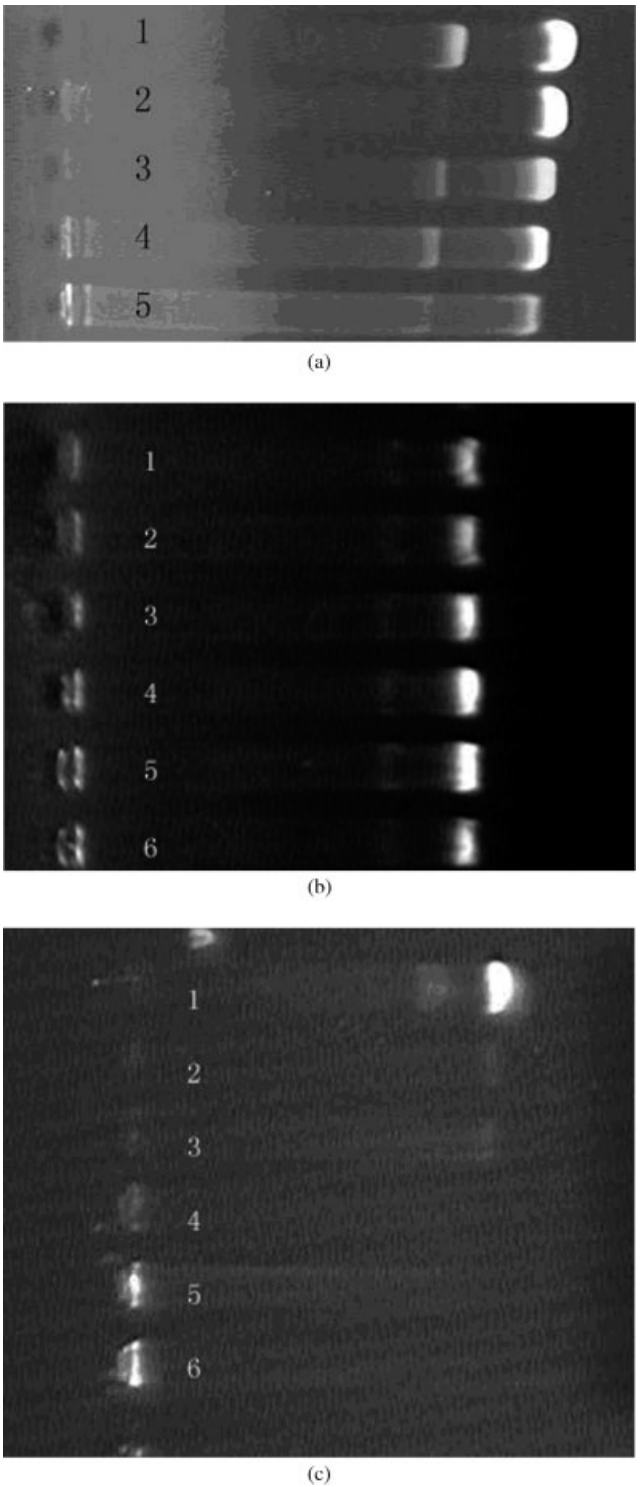


Figure 5 The binding abilities of chitosan and its derivatives of different concentrations with plasmid DNA ($M_w = 3$–5 kDa). (a) agarose-gel assay of pBR322 bound by O-CMCS; (b) agarose-gel assay of pUC18 bound by O-CMCS; (c) agarose-gel assay of pUC18 bound by raw chitosan; lane 1 on each picture was control and from top to bottom the concentration was 500 ppm, 1000 ppm, 5000 ppm, 10,000 ppm, and 20,000 ppm, reactive time was 4.5 h.

the derivatives may be partly replaced by functional groups. The reduction of the quantity of amino groups is probably another important reason contributed to the decrease in binding with DNA.

Effects of chitosan and O-CMCS with different concentrations on *E. coli* and plasmids

Figure 4 shows O.D. versus culture time for chitosan and O-CMCS with different concentrations against *E. coli*. The figures give out the influence of concentration on antimicrobial activity of chitosan and O-CMCS. As it can be seen, the activities of both chitosan and O-CMCS would increase if the concentration increased. As the concentration of chitosans in the medium indicates the concentration of $—NH_2$ groups, the above results evidenced that the inhibitory effects on bacteria depended on the amount of $—NH_2$ and was strengthened with the $—NH_2$ concentration in the experimental range.

The results of electrophoresis of plasmids also pointed out that concentration was an important influencing factor (Fig. 5). The brightness of the plasmid bands weakened gradually as concentration increased, showing the aggravation of the interactions.

Figure 5(a,b), and Table III were results of O-CMCS functioned on both two plasmids. To pBR322, as the concentration increased, the binding abilities of O-CMCS with it increased gradually. This increasing may have something to do with the amount of $—NH_2$ on O-CMCS to the whole. The quantity of amino groups is augmented, and the electrostatic effect of O-CMCS with pBR322 is remarkably larger when concentration is high enough (concentration larger than 5000 ppm in our experiment). The same regular phenomena was observed in the behavior of chitosan acted on pUC18, the difference was that the remarkable concentration only needed to be 500 ppm [Fig. 5(c) and Table IV].

To pUC18, the influence of O-CMCS having on it was not obvious when concentration was lower than 10,000 ppm. It can be concluded as the binding ability of O-CMCS with pUC18 was lower. This is mainly because of the space restriction effect. As mentioned

TABLE III
The Degree of Influence of O-CMCS on Plasmids

	pBR322		pUC18	
Concentration (ppm)	Degree of grey	Degree of influence (%)	Degree of grey	Degree of influence (%)
Control	104716	–	34503.4	–
500	85770.3	18.09	45673.6	–
1000	62138.5	40.66	38860.9	–
2000	50147.2	52.11	37753.8	–
5000	16092.2	82.63	25110.3	27.22
10,000	–	–	24248.8	29.72

TABLE IV
The Degree of Influence of Chitosan on pUC18

Concentration (ppm)	Degree of grey	Degree of influence (%)
Control	52,492.8	–
500	1520.24	97.1
1000	2051.92	96.09
2000	0	100
5000	0	100
10,000	0	100

above, O-CMCS with M_w of 3–5 kDa does not have enough positive charges to conquer the space effect.

Effects of O-CMCS with different M_ws on *E. coli* and plasmids

In our previous study of effects on *E. coli* and plasmids acted by chitosan with different M_ws (>10 kDa), the antibacterial activity and binding ability with DNA were not varied with M_w. But in the same study on O-CMCS, some interesting results were obtained.

Figure 6 shows the results of study on antibacterial activity of O-CMCS with different M_ws. As M_w increased gradually from 2 kDa to 8 kDa, the antibacterial activity of O-CMCS slightly enhanced, but the change was not much obvious.

However, the study of binding abilities of plasmids with O-CMCS was so interesting When the probe was pBR322 [Fig. 7(a)], its binding ability decreased while M_w increased (when $M_w < 8$ kDa). The bands of plasmids could not be observed when M_w was larger than 10 kDa (lanes 6–9). But to pUC18, visible effects could be seen when M_w was 2, 5, 8, and 10 kDa. But bands had a little change when M_w was 3–5 kDa.

The combinations of high M_w O-CMCS ($M_w > 20$ kDa) and plasmid pBR322 were so firm that no bands could be observed. Of O-CMCS with lower M_w ($M_w < 10$ kDa), the ability decreases as M_w increases. As M_w increases from 2 kDa to 8 kDa, volume of O-CMCS becomes larger and larger so that the combinations get to be more and more difficult because of the increasing space effect caused by both of plasmid and derivative. But with such low M_ws, the few amino groups cannot give enough electrostatic force to overcome the space restriction and therefore binding with DNA is difficult. When M_w is as high as 10 kDa, the total amount of positive charge is adequate and the space restriction effect is minor.

However, this phenomenon could be observed in the study of O-CMCS to pUC18 when M_w was only 5 kDa. As can be seen from Figure 7(b) and Table V, when M_w was 2 kDa, the binding ability of O-CMCS with the plasmid could be observed. And when M_w

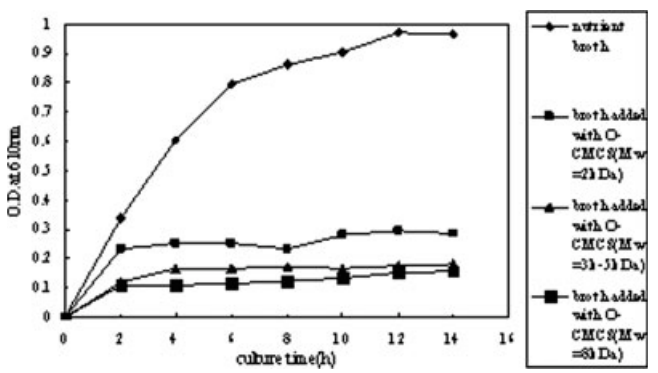
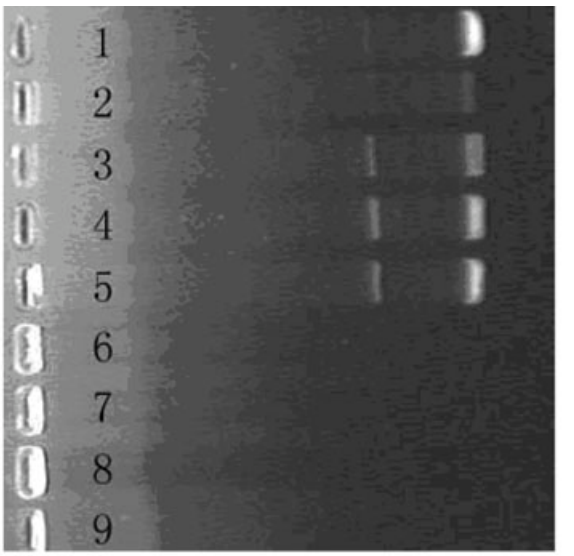
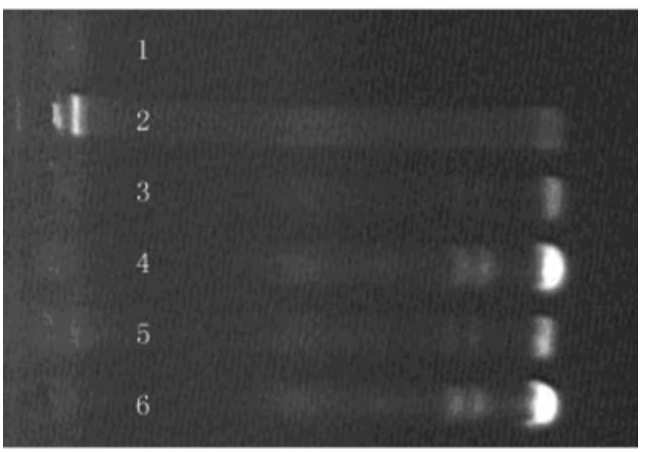


Figure 6 OD versus culture time for O-CMCS with M_w ranging from 2 kDa to 8 kDa against *E. coli.*



(a)



(b)

Figure 7 The binding abilities of O-CMCS of different M_ws with plasmid DNA. (a) agarose-gel assay of pBR322 bound by O-CMCS, lane 1 is control and lanes 2–9 M_ws were 2, 3–5, 5, 8, 10, 15, 18, and 20 kDa, respectively; (b) agarose-gel assay of pUC18 bound by O-CMCS, the concentration was 5000 ppm; lane 6 is control and lanes 5–1 M_ws were 2, 3–5, 5, 8, 10 kDa, respectively; reactive time was 4.5 h.

TABLE V
The Degree of Influence of O-CMCS on Plasmids

	pBR322		pUC18	
M_w (kDa)	Degree of grey	Degree of influence (%)	Degree of grey	Degree of influence (%)
Control	37,609.9	–	45,070.2	–
2	3172.02	91.57	16,689.6	62.97
3–5	14,105.9	62.49	45,915	–
5	21,691.7	42.32	8988.25	80.06
8	24,202.8	35.65	3579.42	92.06

was above 5 kDa, the degree of influence of O-CMCS on pUC18 became larger along with increasing M_w. That might be due to that the increasing amount of amino groups was big enough to conquer and counteract the space effect. Under this condition, the space effect was minor. But when M_w was 3–5 kDa, the influence was not obvious.

Effects of chitosan and O-CMCS on plasmids for different reacting times

Though the antibacterial activity was affected by time, the binding between chitosan and its derivative, as we know, begin with the counteraction of charges, which should take a short time and not be affected by time. The results of different reaction times (Fig. 8 and Table VI) gave out the proof.

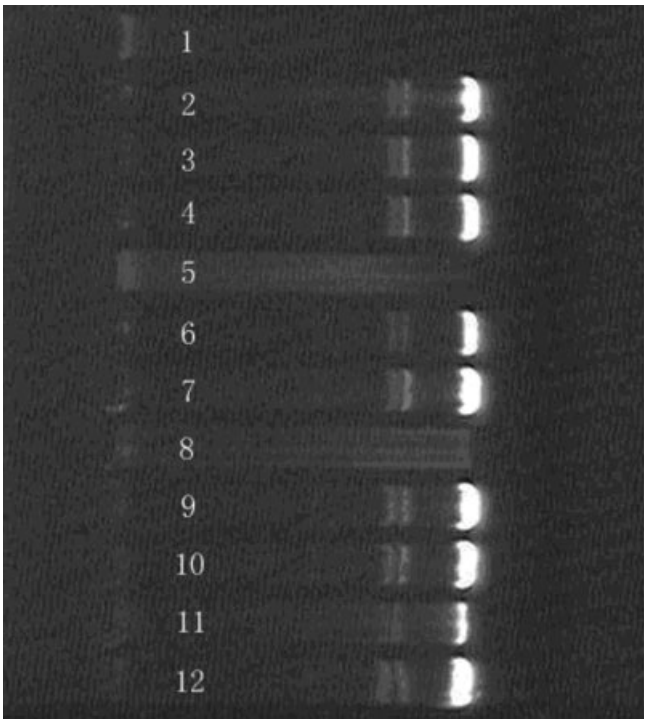


Figure 8 The effects of chitosan and its derivatives on plasmid DNA for different reactive times (lanes 1, 2–4 were control, agarose-gel assay of pUC18 bind with chitosan and O-CMCS for 3, 6, 9 h, respectively; lanes 3–1 were control, agarose-gel assay of pUC18 bind with O-CMCS and chitosans for 12 h).

TABLE VI
The Degree of Influence of O-CMCS on pUC18

Reactive time (h)	Degree of grey		Degree of influence (%)
	Control	O-CMCS	
4	46,371.9	41,014.5	8.9
8	32,363	35,516.2	11.55
10	28,937.4	35,213.8	–
12	51,278	46,663.4	–

Effects of chitosan and O-CMCS on mRNA

As above, O-CMCS has a better antibacterial activity as raw chitosan has. But the binding ability with DNA of O-CMCS was not consistent with its antibacterial activity. So the antibacterial mechanism of O-CMCS should not be the same as that of raw chitosan. The effects of chitosans acted on mRNA would give out some other proof (Table VII). The principle of hybridization is based on the complementary base pairing theory. The microbial and probe DNA used in the experiment were *E. coli* cloned with gene *NahG2* and PCR products of gene *NahG2*, respectively. The probes were marked by isotope ^{32}P; it would combine with mRNA if there were mRNA that had the same genes. The result then appeared to be a black dot on the film, whose gray degree represented the concentration of mRNA. Figure 9 shows the results of RNA Northern plot hybridization.

The combination of chitosan or O-CMCS with the single-chain structure of mRNA begins with charges attracting to each other. The single chain of mRNA pairs with one chain of DNA according to base pairing theory and is the template of protein duplication.

There may be two reasons why the degrees of influences were different. One, the pH of LB broth that incubated *E. coli* is 7.2, a neutral condition. In such circumstance, the protonation of $—NH_3^+$ is slow and somehow incomplete, causing low attachment between chitosan and mRNA. With a lower acidity than that of phosphate, the carboxyl of O-CMCS could protonate amino groups. The protonated ones then could attract mRNA. Also it could be explained in terms of the space restriction effect. Chitosan and its derivative

TABLE VII
The Effect of Chitosan and O-CMCS on mRNA

Sample	Degree of grey	Degree of influence (%)	Statistical result
Control	227.717	–	–
Chitosan 1	186.134	18.26	31.96
Chitosan 2	123.741	45.66	
O-CMCS 1	71.758	68.49	84.24
O-CMCS 2	0	100	

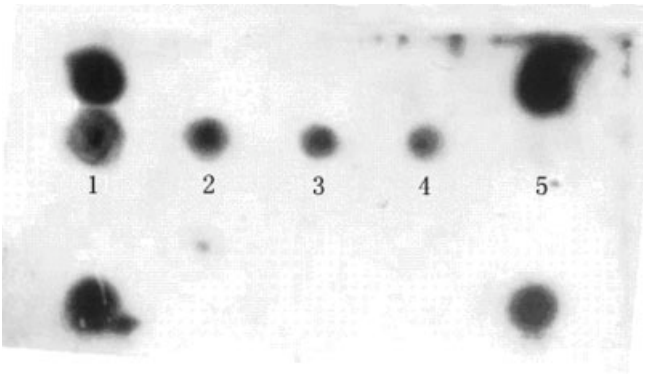


Figure 9 The result of northern plot (plot 1: control; Plot2: chitosan with $M_w = 3$–5 kDa; Plot 3: chitosan repeated; Plot 4: O-CMCS with $M_w = 3$–5 kDa; Plot 5: O-CMCS repeated).

can form outer membrane to cover the RNA the moment they contact it. These membranes inhibit the contact of mRNA and outside DNA so that the bases cannot pair with each other. The duplication is hindered. As a derivative, O-CMCS has a larger volume than chitosan has, which markedly decreases the contact.

CONCLUSIONS

The antibacterial activities on *E. coli* and binding abilities with plasmid DNA of chitosan and its water-soluble derivatives were studied in detail. Experimental figures and data presented in this paper provided several strong evidences that both chitosan and its derivatives had antibacterial activity and O-CMCS had a higher one than raw chitosan had.

The influences study of chitosan on plasmids showed that raw chitosan could bind with DNA intensively; indicating the amino groups on chitosan would attract the negative phosphate groups on DNA. And the ability was not affected by concentration, M_w and reactive time in our research range. The influences O-CMCS and HECS had on plasmids were apparently lower than that of raw chitosan. Especially when probe was pUC18, there seemed that both derivatives had not bound with it. This might be due to the substitution on amino groups and space restricting effect. Such influences could be better explained in the study of O-CMCS with different M_w on plasmids. For pBR322, as the space effect was larger than the static effect, the binding ability got smaller while M_w increased from 2 kDa to 10 kDa; while for pUC18, the degree of influence of O-CMCS on pUC18 became larger with increasing M_w (beyond 5 kDa). That might be due to that the increasing amount of amino groups was big enough to conquer the space effect. Compared with chitosan, O-CMCS could hinder synthesis of mRNA and *E. coli* transformation heavier, both of which would contribute to its antibacterial ability.

O-CMCS and other water-soluble derivatives of chitosan may have good potential as antibacterial agents. However, this paper is only a preliminary study; other parameters such as the N/P ratio may affect the binding and should be investigated in future.

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