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► **To cite this version:**

Zhen Yang, Jean-Regis Degorce-Dumas, Hu Yang, Eric Guibal, Aimin Li, et al.. Flocculation of *Escherichia coli* Using a Quaternary Ammonium Salt Grafted Carboxymethyl Chitosan Flocculant. *Environmental Science and Technology*, 2014, 48 (12), pp.6867-6873. 10.1021/es500415v. hal-02914228

**HAL Id: hal-02914228**

**<https://hal.science/hal-02914228v1>**

Submitted on 30 Sep 2024

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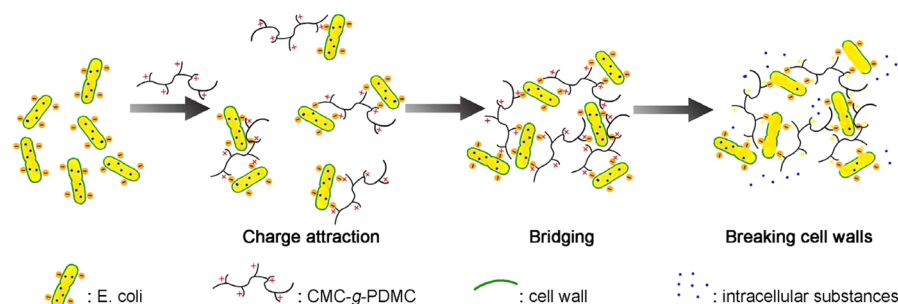
# Flocculation of *Escherichia coli* Using a Quaternary Ammonium Salt Grafted Carboxymethyl Chitosan Flocculant

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**ABSTRACT:** Only few studies are available on bacteria removal efficiencies and antibacterial properties of flocculants, which is one of the important requirements in water treatment work. *Escherichia coli* (*E. coli*) was selected as an example of a Gram-negative bacteria for testing the flocculating properties of a quaternary ammonium salt grafted chitosan (carboxymethyl chitosan-graft-poly[(2-methacryloyloxyethyl) trimethylammonium chloride] copolymer; i.e., CMC-g-PDMC). The effect of various flocculation parameters, including flocculant dosage, initial bacterial density, nutrient medium content, and pH were successively investigated. The experimental results indicated that, besides flocculation effects, CMC-g-PDMC also exhibited a bactericidal effect (not requiring additional treatment facilities). Moreover, the flocculation mechanisms were investigated via zeta potential measurements, floc observation, and three-dimensional excitation–emission matrix spectra analysis. Apart from its flocculating and settling effect, this chitosan-based material has bactericidal action through the breaking of bacterial cell walls by grafted quaternary ammonium salt.

## INTRODUCTION

Many water treatment plants in China currently use a conventional treatment process with combined technologies of flocculation, sedimentation, filtration, and disinfection.<sup>1</sup> Such a “combined” process is applicable for most raw water (not subject to overgrowth of bacteria and other biomass), but when facing raw water contaminated by excessive eutrophic substances and large amounts of bacteria, the efficiency of the aforementioned treatment might be significantly reduced for three main reasons. First, the macromolecular eutrophic substances and bacteria’s metabolites may consume large amounts of flocculants and induce some flocculation difficulties.<sup>2</sup> The decanted sludge may be maintained in sedimentation tanks for several hours before being discharged. During this period, depending on conditions, the bacteria in the sludge can continue growing. As a consequence, the microbial contamination of the water would be enhanced. In addition, high bacterial density in the water requires large dosages of disinfectants, engendering risk of high levels of disinfection byproducts. It may be helpful to combine the coagulation–flocculation operation with disinfection properties to simplify

the process and avoid adding supplementary treatment modules. However, investigations on this topic are sparse.

Traditional flocculants, such as inorganic salts and synthetic polymeric flocculants, have been proven to yield high flocculation efficiencies for raw water without enormous amounts of bacteria. However, development of environmentally friendly, biodegradable, and wide-sourced new flocculants are still very necessary.<sup>3</sup> Thus, as an alternative, much attention has been given to natural-materials-based flocculants in recent years,<sup>4–6</sup> such as chitosan, starch, and so on. Among them, chitosan, the deacetylation product of chitin, appears to be one of the most promising candidates.<sup>7</sup>

The unique properties of chitosan mainly come from the primary amine groups present on the macromolecular backbone.<sup>8,9</sup> Under acidic conditions, the molecular chains are positively charged: this makes the biopolymer quite efficient for

flocculating common contaminants that bear negative surface charges. This fundamental property would clearly provide a benefit to bacterial removal because most bacteria normally carry negative charges on the outside of their cell wall. In addition, previous researchers have demonstrated that chitosan shows antibacterial ability to some degree.<sup>10–14</sup> However, the actual antibacterial ability of chitosan is not easily predictable. Indeed, it depends to a certain extent not only on the type of bacteria but also on the properties and characteristics of chitosan and its dosage.<sup>13</sup> Nevertheless, there are still mainly two shortcomings of chitosan as a flocculant to overcome: The first one deals with its poor solubility.<sup>15</sup> Most of chitosan can be dissolved only in acidic solutions, leading to partial degradation and a potential decrease of flocculation efficiency.<sup>16</sup> Carboxymethylation is an easy modification method for improving the solubility of polysaccharides.<sup>17</sup> The other challenge to overcome is that the cationic property of primary amino groups is not strong enough and might be largely depressed in the presence of excessive eutrophic substances and salts.<sup>8,18</sup> Taking into account this weak point, grafting of strongly cationic quaternary ammonium groups onto a chitosan backbone might provide a solution for improvement of both the positive charge and the molecular weight of the modified biopolymer.

On the basis of the aforementioned considerations, a novel chitosan-based flocculant, carboxymethyl chitosan-graft-poly-[(2-methacryloyloxyethyl) trimethylammonium chloride] copolymer (i.e., CMC-g-PDMC) was synthesized. In our previous work,<sup>19</sup> CMC-g-PDMC has shown superior flocculation performance in treating high turbid raw water containing high content of humic substances in comparison to conventional flocculants. However, little is known about the bacterial removal efficiency of this novel chitosan-based flocculant. Actually, the flocculation mechanism of its precursor, chitosan, remains debatable: several mechanisms can be involved such as electrostatic patch and/or bridging effect.<sup>23</sup> Strand et al.<sup>13</sup> found that purely electrostatic interactions were not playing a dominant role in the flocculation of Gram-negative bacteria. The flocculation performance of CMC-g-PDMC for removal of bacteria is expected to be enhanced after grafting quaternary ammonium salts on the biopolymer.

The present study investigated the flocculation properties of the chitosan-based flocculants for bacterial decontamination of water. *Escherichia coli* (*E. coli*) was used here as a model bacteria,<sup>8,14</sup> which has the advantages of clear genetic background, simple operation, and facile culture condition. The flocculation mechanism was studied in detail through zeta potential (ZP) and three-dimensional excitation–emission matrix (3DEEMs) spectra analysis. Besides, the effects of the flocculant dosage, the initial bacterial density, the nutrient medium content, and the pH on both flocculation performance and viability of bacteria were systematically assessed.

## ■ MATERIALS AND METHODS

**Materials.** The chitosan-based flocculant, CMC-g-PDMC, was synthesized in the authors' lab. The synthesis route is described in detail in Supporting Information Figure S1 and Text S1. The methodologies for the characterization of CMC-g-PDMC, including solubility, Fourier transform infrared (FTIR), <sup>1</sup>H nuclear magnetic resonance (<sup>1</sup>H NMR), X-ray diffraction (XRD), and ZP measurements, are presented in Supporting Information Text S2.

Luria Broth (LB) powder from Becton, Dickinson, and Company was composed of 20 wt % of yeast extract, 40 wt % of

tryptone, and 40 wt % of sodium chloride. Agar powder was purchased from Sigma-Aldrich. Ultrapure water was used in all experiments.

**Bacterial Culture.** *E. coli* (pUC18 (ATCC 37253)) was cultured in liquid LB medium (LLB, Difco; LB Broth, Miller) at 37 °C in 500 mL presterilized glass flasks for 24 h before tests. LLB was prepared by dissolving 25 g of LB powder into 1 L of water, adjusting the pH to pH 7 using dilute sodium hydroxide solution, and then sterilizing in an autoclave at 121 °C for 15 min beforehand.

**Bacterial Flocculation Experiments.** Standard jar tests were performed in 1 L jars and a six-place paddle flocculator (Jar Test, model no. 10409, Fisher Bioblock Scientific) at 20 ± 1 °C. Stock solution of CMC-g-PDMC was always freshly prepared by dissolving 0.1 g of the flocculant into 100 mL of water before each test. Synthetic wastewater containing a given amount of LLB was also freshly prepared by diluting *E. coli* culture solution using 0.9 wt % NaCl solution or LLB, whereas that without LLB was prepared by harvesting *E. coli* using centrifugation (6000 rpm, 5 min), twice washing, and resuspension in 0.9% NaCl solution (under stirring). After the pH of synthetic wastewater was adjusted to the designed value with 0.1 mol/L HCl or NaOH, a known amount of flocculant stock solution was added into each jar under rapid mixing at 200 rpm for 5 min, followed by a slow mixing step at 50 rpm for 15 min. At last, the flocs were kept still to settle down without stirring for 90 min. After that, samples were collected at a depth of 2 cm in the supernatant for further analysis. Each measurement was triplicated, and relative errors of results in this work were less than 6.0%.

**Supernatant Analysis.** Turbidity was measured by a HACH DR/2000 Direct Reading Spectrophotometer. Optical density at a wavelength of 600 nm (OD<sub>600</sub>) was measured by a SHIMADZU UV-160A UV–vis Recording Spectrophotometer. ZP was measured by a MALVERN Nano-Z Zetasizer.

Relative turbidity and relative OD<sub>600</sub> were calculated through subtraction of the corresponding blank sample value from the absolute value of supernatant. The blank sample was prepared by removing *E. coli* using centrifugation (6000 rpm, 5 min) from the corresponding raw wastewater. Hence, the relative turbidity and relative OD<sub>600</sub> can be considered as the representative values of the specific contribution of bacteria.

Bacteria density was determined using the plate counting method. After diluting the supernatant in 0.9 wt % NaCl aqueous solution to a suitable degree, 0.1 mL of the diluent was spread and inoculated onto solid LB medium (SLB) in disposable Petri dishes. Generally, to ensure accuracy of colony counting, dilutions were determined to make the order of magnitude of the colony number in Petri dishes between 10<sup>2</sup> and 10<sup>3</sup>. Then, the dishes were incubated at 37 °C for 24 h, and the colony number was counted by an INTERSCIENCE Scan-1200 Automatic Colony Counter. The bacteria density was expressed as colony-forming units per milliliter (CFU/mL). The SLB used here was prepared by suspending 25 g of LB (Difco) powder and 15 g of agar (Difco) powder into 1 L of water, adjusting the medium to pH 7, and then sterilizing in an autoclave at 121 °C for 15 min beforehand.

The analysis of 3DEEMs spectra was carried out using a HITACHI F-7000 fluorescence spectrophotometer. The emission spectra were recorded from 280 to 550 at 1 nm increments by varying the excitation wavelength from 200 to 500 at 5 nm sampling intervals. The width of both excitation slit and emission slit was maintained at 10 nm, and the scanning

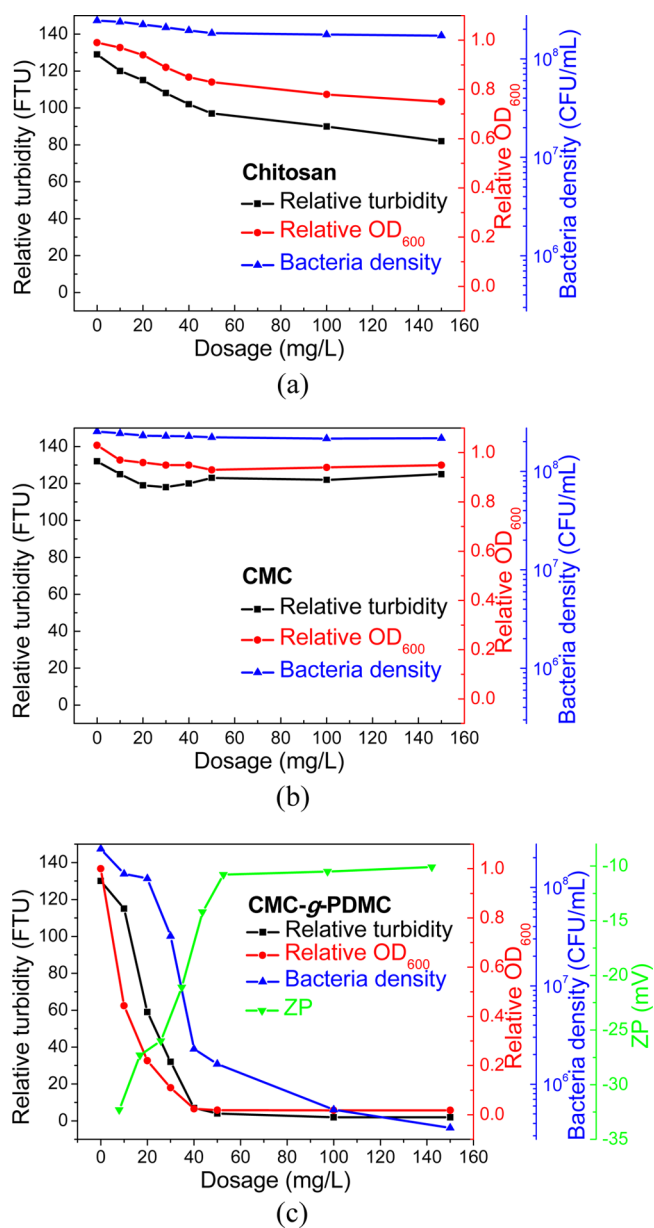
speed was set at 2400 nm/min. By dividing the integrated intensity area of the Raman water curve at excitation wavelength of 350 nm in emission wavelength range from 370 to 450 nm, the intensity of EEM spectra was normalized.<sup>20</sup> The 3DEEMs data was processed using MatLab 8.2 (MathWorks, Inc.).

## RESULTS AND DISCUSSION

**Characterization of CMC-g-PDMC.** Solubility tests of the novel flocculant were first carried out using the Chen and Park's method.<sup>21</sup> In comparison with the chitosan used in the current study, which can be only dissolved at pH lower than 3, CMC and CMC-g-PDMC were soluble in the tested pH range from 1 to 13, due to the introduction of carboxymethyl groups. The enhanced solubility was expected to induce positive effects on removing bacteria, as more effective flocculation sites were available when flocculants were under their soluble form. In addition, characterization results of FTIR, <sup>1</sup>H NMR, XRD, and ZP measurements were illustrated and extensively discussed in Supporting Information (Figure S2 and Text S3, respectively). The NMR spectroscopy confirmed the molecular structure of CMC-g-PDMC: the substitution degree of carboxymethyl groups was evaluated to 48.3% and the grafting ratio of PDMC to 251% (calculated from <sup>1</sup>H NMR spectra). As depicted in Figure S2d (see Supporting Information), CMC-g-PDMC with PDMC branches behaves like a strong-cationic polyelectrolyte: this contributes to improve charge attraction properties of the biopolymer for *E. coli* (which bears negative surface charges).

**Effect of Flocculant Dosage.** Flocculant dosage plays a vital role in the flocculation efficiency.<sup>22</sup> Hence, flocculation performance of CMC-g-PDMC and its precursors was investigated as a function of flocculant dosage at the beginning. As plotted in Figure 1a, the ability of chitosan for bacterial removal came from its primary amino groups. But the ability was relatively weak in the tested dosage range due to its extremely high molecular weight (the average molecular weight of chitosan is  $8.34 \times 10^5$  g/mol); this is in agreement with other published work.<sup>8,11,14</sup> However, the bactericidal efficiency became worse after carboxymethylation as shown in Figure 1b, because the negatively charged carboxymethyl groups on CMC backbone reduced charge attraction between flocculant and bacteria. After PDMC branches were grafted onto the chitosan backbone, CMC-g-PDMC exhibited notably high removal efficiencies in the aspects of relative turbidity, relative OD<sub>600</sub>, and bacteria density, as shown in Figure 1c. This improvement was attributed to both the increase of effective flocculation sites and the strong-cationic characteristics of CMC-g-PDMC, as mentioned above. Therefore, charge attraction might be, at least, one of the main flocculation mechanisms, and a discussion of the ZP–dosage profile is found in Supporting Information (see below). However, other mechanisms were probably involved in the process. Indeed, the removal efficiency–dosage curves in Figure 1c reached a plateau when the flocculant was overdosed (in the measured dosage range); this is contradictory with the conventional restabilization (occurring with the reversal of surface charges: attraction/repulsion).<sup>17,23</sup>

**Flocculation Mechanism.** To give an in-depth study of the flocculation mechanism, ZP measurements, floc observations, and 3DEEMs spectra were performed. ZP of the supernatant as a function of CMC-g-PDMC's dosage was illustrated in Figure 1c also. When the dosage was lower than 50 mg/L, ZP rose



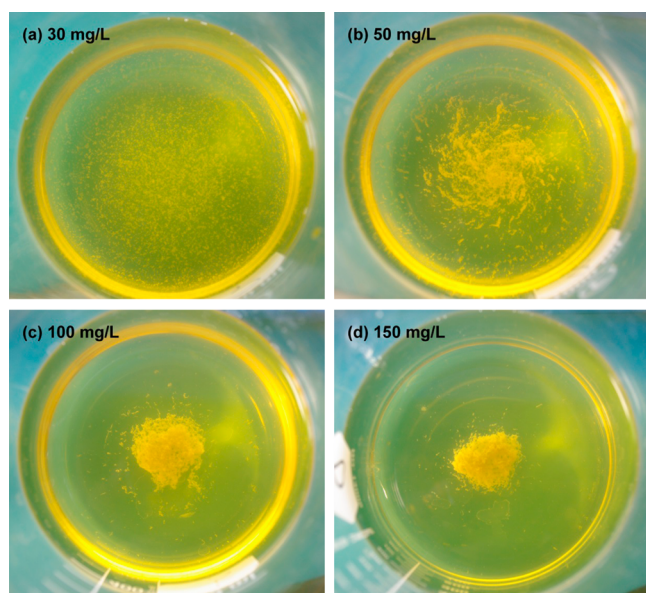
**Figure 1.** Effect of dosage on the removal of *E. coli* by chitosan (a), CMC (b), and CMC-g-PDMC (c).

rapidly with the increase of flocculant dosage: this confirmed the significant effect of charge attraction in flocculation. Surprisingly, above 50 mg/L, ZP remained stable even with further increase of flocculant dosage. For wastewater with simple composition, for example kaolin suspension,<sup>17</sup> when a strong cationic flocculants was excessively dosed, ZP of the supernatant changed to positive value due to adsorption of excessive flocculant onto the surface of flocs: the positive charges at the surface of the flocs leads to their restabilization (repulsion of positively charges agglomerates). However, the present results showed quite different trends, meaning that other effects, such as the bactericidal actions of quaternary ammonium salts, may contribute to consumption of the positively charged flocculant. This effect will be discussed in detail in the following section.

Photographs of flocs formed with various flocculant dosages were taken after the sedimentation period during jar tests. An interesting phenomenon was observed from Figure 2:



increasing the flocculant dosage led to larger and more compacted flocs.



**Figure 2.** Images of flocs formed by various dosages of CMC-g-PDMC.

At the dosage of 150 mg/L, the flocs came to a single aggregate. Such phenomenon showed a bridging effect of the polymeric flocculant contributing to the flocculation performance. The grafted PDMC branches largely enhanced the approachability of flocculant chains to *E. coli* cells. In addition, this observation also brought useful information for practical application. The cost of chemicals increases with the amount of flocculant used, but larger and denser flocs are quite appreciable for subsequent disposal in water treatment units.<sup>24</sup> Consequently, evaluation of flocculation performance of some flocculants should comprehensively consider a balance between removal efficiency and floc properties.

The 3DEEMs spectra of the supernatant were recorded in Figure 3. According to the location of 3DEEMs peaks and literature reports,<sup>25</sup> the characteristic peaks corresponding to LLB in Figure 3b were ascribed to Region I (aromatic proteins I; excitation wavelength: 220–250 nm; emission wavelength: 280–330 nm). The main substances in Figure 3c appeared in Region II (aromatic protein II; excitation wavelength: 220–250 nm; emission wavelength: 330–380 nm): they can be associated with metabolites released from the bacterial cells after *E. coli* was cultured in the medium. After adding 50 mg/L of CMC-g-PDMC (corresponding to the beginning of the plateau in removal efficiency) the spectra of supernatant (Figure 3d) hardly changed. However, when excessive flocculant (100 mg/L in Figure 3e and 150 mg/L in Figure 3f) was added, the 3DEEMs spectra became obviously different: (i) Strong fluorescence signals appeared in Region I and II (excitation wavelength: 220–250 nm; emission wavelength: 280–380 nm), indicating a large amount of aromatic proteins were released into water from the inside of the *E. coli* cell; (ii) a new peak in Region IV (excitation wavelength: 250–300 nm; emission wavelength: 280–380 nm) was assigned to soluble microbial byproduct-like substances, which also confirmed the release of intracellular substances. On the basis of 3DEEMs spectra, it can be concluded that overdosed CMC-

g-PDMC was partially consumed in the breaking of the cell wall of *E. coli*, due to the bactericidal properties of quaternary ammonium groups<sup>1</sup>, in addition to the antimicrobial effects of chitosan itself. Therefore, unlike common flocculation of inorganic suspended particles<sup>15,17,19</sup> or dye solution,<sup>22</sup> a “breaking-cell-wall” effect was observed in the flocculation mechanism in addition to charge attraction and bridging effects.

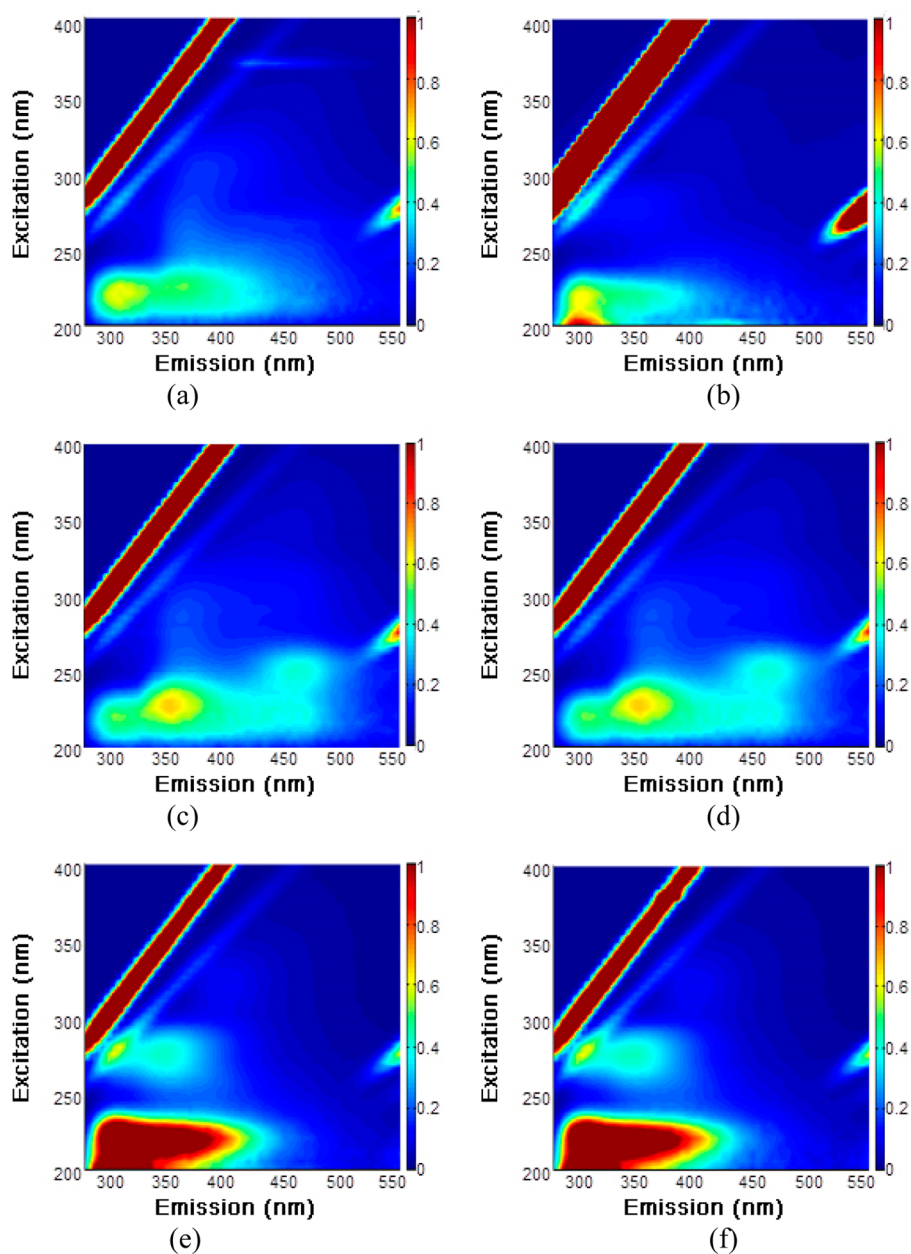
The suggested flocculation mechanism was described in Figure 4. Charge attraction predominated at the very beginning, causing a sharp increase of ZP. Then, flocs tend to aggregate together to form larger and denser ones through bridging effect of the flexible PDMC branches of overdosed CMC-g-PDMC. Lastly, the *E. coli* cell walls may be (at least partially) disrupted or damaged, leading to the decrease of the cell population through the “breaking-cell-wall” effect associated with quaternary ammonium groups on PDMC branches.

Hence, based on this discussion, the two problems aforementioned at the beginning of the Introduction section have been solved. The development of CMC-g-PDMC may be potentially significant as a promising alternative flocculant, which (a) had an additional bacterial removal effect (or bactericidal action) and (b) may reduce the supplementary costs resulting from the installation of additional treatment facilities (for further sterilization).

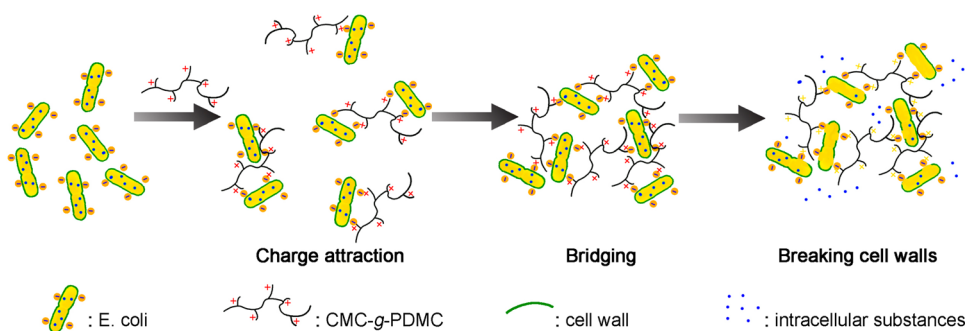
**Effect of Initial Cell Density.** In addition to the wastewater with the initial cell density of approximately  $10^8$  CFU/mL in Figure 1c, two other kinds of wastewater with different initial cell density of approximately  $10^7$  and  $10^6$  CFU/mL, respectively, were also carried out to determine the effect of *E. coli* cell density on the flocculation efficiency of CMC-g-PDMC (see Supporting Information Figure S3). CMC-g-PDMC exhibited high *E. coli* removal efficiency over the wide two-log-unit range of cell density (from  $10^6$  to  $10^8$  CFU/mL). Differing from classic stoichiometric relationship between initial contaminants concentration and optimal flocculant dosage occurring when the process adhered to the charge neutralization mechanism,<sup>26</sup> the initial *E. coli* density had no obvious effect on the flocculation efficiency. This finding agreed well with the flocculation mechanism concluded in this work (i.e., besides charge attraction, bridging and “breaking-cell-wall” effects may have significant contribution to flocculation).

An abnormal increase in both relative turbidity and relative  $OD_{600}$  at low flocculant dosage was observed in Figure S3b (see Supporting Information). An alternative explanation may be proposed for this phenomenon: when insufficient dosage was added, primary flocs were formed but too loose to settle down owing to low cell density. Thus, the suspended primary flocs gave rise to the abnormal turbidity.

**Effect of Nutrient Medium Content.** To investigate the effect of nutrient medium content on the flocculation process, several kinds of synthetic wastewater with various contents of LLB but the same initial cell density ( $2.5 \times 10^7$  CFU/mL) were used (Figure S4, see Supporting Information). Based on Figure S4, the effect of LLB volume percentage on the required flocculant dosage was illustrated in Figure 5a: two regions were found. When the LLB volume percentage was lower than 5%, nutrient medium had almost no effect on flocculation. On the other hand, when the LLB volume percentage was higher than 5%, the increase of LLB content in water triggered off a significant upturn of required flocculant dosage. This indicated that large content of LLB might also consume parts of the flocculant through mutual adsorption.



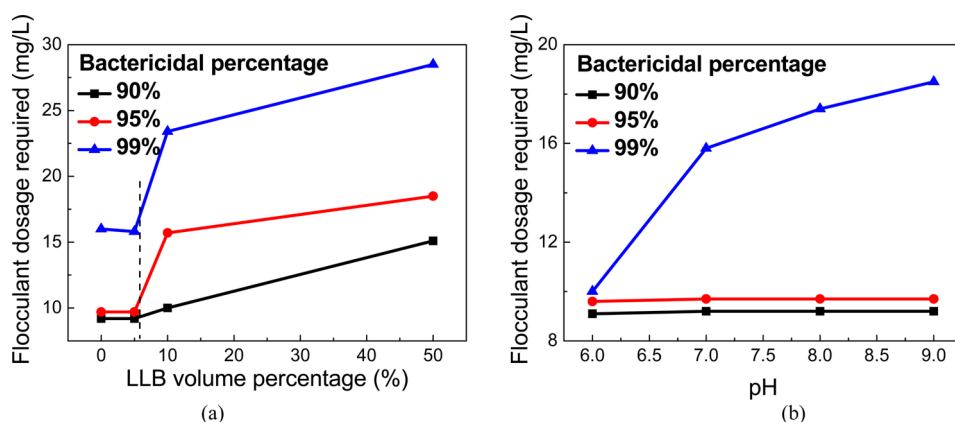
**Figure 3.** Three-dimensional excitation–emission matrix spectra of blank NaCl solutions (a), LLB (b), supernatants after flocculation with flocculant dosages of 0 (c), 50 (d), 100 (e), and 150 (f) mg/L, respectively.



**Figure 4.** Schematic diagram of flocculation mechanisms.

**Effect of pH.** Because pH is another important factor affecting flocculation performance,<sup>27</sup> various kinds of synthetic wastewater with different initial values of pH but the same

initial cell density ( $2.5 \times 10^7$  CFU/mL) were tested (see Supporting Information, Figure S5). The ionic strength was kept constant. The pH range was set between 6 and 9 to



**Figure 5.** Effects of nutrient medium content (a) and solution pH (b) on the required flocculant dosage.

maintain viable conditions for *E. coli*. After each test, the pH of the supernatant was also systematically measured. The pH value after flocculation always remained stable compared to the initial one. The results in Supporting Information (Figure S5) were then summarized in Figure 5b. Interestingly, for 90% and 95% bactericidal percentage, no pH effect was observed, whereas for 99% bactericidal percentage, the required flocculant dosage increased as pH rose. As shown in Supporting Information (Figure S2d), ZP of CMC-g-PDMC declined from pH 6.0 to 9.0. Hence, in the tested pH range, charge attraction between the flocculant and *E. coli* would reduce with the increase of pH. Such reduction was not so effective for relatively low bactericidal requirement, because the flocculants can easily collide with *E. coli* cells (for high populations of bacteria) and then form flocs to settle down. But when very high bactericidal efficiency was required, the situation was significantly different. After most of the *E. coli* cells had aggregated and settled down (at the beginning, as mentioned above), further collision between the flocculant and the small number of residual *E. coli* did not easily take place. In this situation, the strength of charge attraction strongly affected collision frequency and efficiency of treatment. Hence, charge attraction at the lower pH was stronger, resulting in a lower flocculant dosage requirement.

**Significance of the Current Work and Environmental Implication.** The present results demonstrated that the novel chitosan-based flocculant had highly additional bacterial removal efficiency in water treatment plants without requiring complex and expensive equipment facilities after grafting abundant quaternary ammonium salt onto chitosan. This work also showed that the target performance of the flocculants could be achieved by suitable chemical modifications based on the structure–activity relationship. CMC-g-PDMC displayed significant flocculation performance in a wide range of initial bacterial density. *E. coli* could be efficiently flocculated, prompted to settle down mainly via charge attraction and bridging mechanisms, and then killed through the “breaking-cell-wall” effect. A high concentration of nutrient medium required relatively high flocculant dosage to reach the desired bactericidal efficiency. The solution pH did not affect the required flocculant dosage at bactericidal percentage lower than 95%. However, for much higher levels of bactericidal requirement, the optimal flocculant dosage would increase with the rise of pH.

Moreover, it is well-known that disinfection byproducts in the disinfection process in water treatment plants may be very

harmful for human health.<sup>28</sup> In this work, the chitosan backbone is biodegradable. After the chitosan backbone has degraded, the short graft chains of PDMC fell into pieces of quite low molecular weight with quite low toxicity. It was expected that the novel chitosan-based flocculant could reduce secondary-pollution risk of the disinfection byproducts also when CMC-g-PDMC is employed as a substitute to commercial flocculants. It was due not only to its own biodegradable properties and environmentally friendly characteristics<sup>16</sup> but also to the reduction of the required disinfectant dosage in the disinfection process (for high bacteria removal efficiency). Although the current cost for preparation of CMC-g-PDMC is still high, the natural polymer-based flocculants would be applicable in the long-term and large scale for their significant advantages when low-cost preparation technologies have been fully developed.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Synthesis route of the chitosan-based flocculant (i.e., CMC-g-PDMC, Figure S1) and its detailed preparation description (Text S1). Structural characterization methods (Text S2) and results (Text S3 and Figure S2) of CMC-g-PDMC. Flocculation performance of CMC-g-PDMC for the treatment of synthetic wastewater with various influencing factors such as initial cell density, LLB volume percentage, and solution pH (Figure S3–S5). This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (grant no. 51378250 and 51073077), the Scientific Research Foundation of Graduate School of Nanjing University (grant no. 2012CL06), and the Research and Innovation Project for Graduates of Jiangsu Province (no. CXZZ12\_0034). The research of Z.Y. at Ecole des mines d’Alès was made



possible by the Doctoral Students' Visiting Scholar Program of Nanjing University. We are grateful for the financial support.

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