# Structure-antimicrobial activity relationship and action mechanism of dimeric quaternary ammonium amphiphiles

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**Abstract.** The antibacterial activity of the five dimeric quaternary ammonium amphiphiles against *Escherichia coli* were evaluated by determining the minimum inhibitory concentration (MIC) and compared with one monomeric quaternary ammonium amphiphile. The antibacterial activity of dimeric amphiphiles is superior to the corresponding monomeric amphiphile. The action mechanism was investigated by measuring the activity of $\beta$-galactosidase from ***E. coli*** and scanning electron microscopy (SEM) imaging of ***E. coli*** cells. The result of $\beta$-galactosidase activity shows that 12-8-12 disrupts the membrane of ***E.coli*** leading to the releasing of intracellular contents and death of bacterial cells. This conclusion was supported by scanning electron microscopy, which imaged the holes on the cell membrane and irregular shape of cells caused by treatment of 12-8-12.

## Introduction

The appearance of bacterial strains with broad antibiotic resistance is becoming a global health concern, which provoked an urgent need to develop novel antimicrobial agents[1-2]. Among numerous antimicrobial agents, small-molecular-weight quaternary ammonium compounds (QACs) are extensively used as antimicrobial agents because of wide spectrum of antimicrobial activity against bacteria (both gram positive and gram negative), fungi, and certain viruses[3-5].

Dimeric amphiphiles, which are composed of two hydrophobic quaternary ammonium head groups and two hydrophilic chains, appear to be much better antibacterial activity than the corresponding conventional mono-QACs[6-7]. Among various types of dimeric amphiphiles, the set of dicationic quaternary ammonium compounds (often expressed as ***m-s-m***, where ***m*** and ***s*** denote the numbers of carbon atoms in the free alkyl chains and the spacer, respectively) have been probably the most widely studied [8-9], but a complete understanding of structure- antimicrobial activity relationship and the action mechanism for these dimeric amphiphiles are still lacking.

In the present study, five dimeric amphiphiles with different alkyl chain lengths and spacer lengths were chosen to explore the structure-antimicrobial activity relationship. The antibacterial activity of the amphiphiles against Escherichia coli were evaluated by determining the minimum inhibitory concentration (MIC). The disrupting of bacterial cell membrane by amphiphiles was investigated by measuring the activity of $\beta$-galactosidase from E. coli, and the direct visualization of damage to the bacterial membrane and changes to the bacterial morphology was found via scanning electron microscopy (SEM) imaging of E. coli cells.

## Experimental methods

Antibacterial activity was determined via slight modifications to literature procedures[10]. 20μL of the amphiphile solution with different concentration was added to a 96-well plate containing 180μL of ***E. coli*** cultures($10^6$ cfu/mL). The plate was then incubated at 37 °C for 24 h, and the ***MIC*** data was recorded by measuring the OD values at 600nm using a Thermo Electron Corporation multiskan spectrum.

 

Assay mixtures contained $5\times10^6$cfu/mL of *E. coli*, 2.5mmol/L ONPG and a series of concentrations of amphiphile in 2 mL PBS buffer, and the absorption intensity was measured at the wavelength of 420nm by a Thermo Electron Corporation multiskan spectrum in the kinetics mode.

Two *E. coli* suspensions were added different concentration of amphiphile solutions, and one suspension was left untreated as a control. The cells were fixed by glutaraldehyde and dehydrated sequentially with 20, 50, 80 and 100% ethanol. Then, 10μL of dehydrated cells was dropped on a small piece of aluminum foil and dried at room temperature. Images were recorded using FEI Nova NanoSEM 450 field-emission scanning electron microscopy.

## Result and discussion

### Structure-antimicrobial activity relationship

**Table 1 MIC and CMC values of cationic amphiphiles**

| | MIC(μM) | CMC(mM)* |
|---|---|---|
| DTAB | 35±1 | 14.3 |
| 12-4-12 | 1.2±0.1 | 1.01 |
| 12-6-12 | 1.1±0.1 | 0.97 |
| 12-8-12 | 0.9±0.1 | 0.79 |
| 14-6-14 | 1.0±0.1 | 0.15 |
| 16-6-16 | 6.4±0.3 | 0.042 |

* Values of CMC were determined by conductivity at 25°C.

Antibacterial activity of five dimeric amphiphiles against *E. coli* was determined and compared to one conventional monomeric amphiphile(DTAB). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent that inhibits bacterial growth, as recorded by measuring the OD600 after 24h of incubation at 37 °C. Table1 shows MIC and CMC values of the sis cationic amphiphiles. For the amphiphiles with same alkyl chain length ($C_{12}$), the MIC values of dimeric amphiphiles(12-s-12, s=4,6,8) are less than that of the monimeric amphiphile(DTAB), and the antibacterial activity of the dimeric amphiphiles followe a slight trend with the variation in the spacer chain length. It can be found that, the value of MIC decrease with the increase in the spacer length, which is similar with CMC. For the amphiphiles with same spacer, the value of MIC increases with the increase in the alkyl chain length, but the result of CMC is exactly opposite.

The generally accepted action mechanism of QACs is that the cationic head groups bind on the negatively charged bacterial cell membrane, thus disrupting the membrane with the aid of electrostatic and hydrophobic interactions leading to the release of cytoplasmic constituents and finally cell death [11-12]. So the antibacterial potency depends on the balance of hydrophilic and hydrophobic properties of the cationic amphiphiles. The higher antibacterial activity of dimeric amphiphiles compared to that of the corresponding monomeric amphiphile could be attributed to the greater number of positive charges as well as higher hydrophobicity of alkyl chains linked by spacer. To some extent, the interaction of cationic amphiphile with bacterial cell is similar to that negatively charged particle or polyelectrolyte. Owing to the electrostatic attraction between the oppositely charged amphiphile molecules and cell surface, the effective concentration of amphiphile around cells is higher than that in bulk phase, therefore much more amphiphile molecules will aggregate around the cell surface via hydrophobicity of alkyl chains. The value of CMC represents self-aggregation ability of amphiphile molecules, which means that the number of molecules binding on cell surface increase with the decrease of CMC value. As a result, as the number of charge head group and the alkyl chain length are the same (12-s-12, s=4, 6, 8), the trend of antibacterial activity depends on the value of CMC. The chain length of the alkyl chain of 12-6-12 and 14-6-14 did not play a remarkable role in the antibacterial activities against E. coli. The difference of MIC value is not significant. While, the much lower antibacterial activity of the dimeric amphiphile16-6-16 may be due to the poor solubility in water but greater tendency to form larger aggregates, thus leading to a greater binding affinity with a smaller number of cells.

## Activity of β-galactosidase

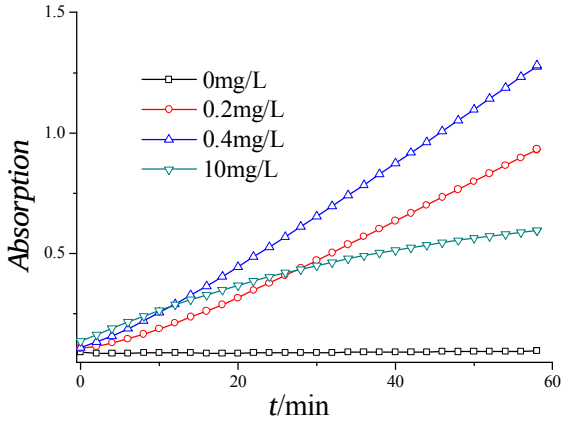


**Fig.1. Varying of absorption of ONP at 420nm with time under different amounts of *12-8-12***

Base on the above reuslt, we chose dimeric amphiphile 12-8-12 to explore the action mechanism.The substrate of ONPG can be hydrolyzed to form *O*-nitrophenol (ONP) under catalysis of *β*-galactosidase from *E.coli*. If the membranes of *E.coli* break, *β*-galactosidase will leak out of the cells, and further catalyzes the hydrolysis of ONPG in the solution. *O*-nitrophenol (ONP) has a characteristic absorption at 420 nm, so with spectroscopy method it can be determined whether the cells of *E. coli* are disrupted[13]. Three different amounts of 12-8-12 were added in to the *E.coli* cell suspensions containing ONPG respectively, and one control without 12-8-12 was used. The measuring results are shown in figure 1. It is seen that, the absorption of the control one has no change in 1h, indicating that ONPG was not hydrolyzed by *β*-galactosidase and the intact *E. coli* cells are permease-deficient. For the cell suspensions with 12-8-12, the absorption values at 420nm increase with time, which means clearly that the membranes of *E. coli* are disrupted by 12-8-12 and the intracellular contents including *β*-galactosidase are released. The breaking of cell membrane means the death of bacteria, so it can be concluded that the action of dimeric amphiphile 12-8-12 is on the cytoplasmic membrane, and its antibacterial effect is based on killing bacteria process.

Moreover, within the first 10min, the more the added amount of 12-8-12 is, the higher the destroying action to the cell membrane, and the stronger is the catalysis activity of *β*-galactosidase, that is, more cells are killed. While after 10min, for the one with higher concentration of 12-8-12(10mg/L), the absorption increase trend is slower, but the others keep the linear increase. It is known that *β*-galactosidase released from the broken cells keeps the activity to hydrolyze ONPG to ONP. When the concentration of 12-8-12 is much higher, the excessive amphiphile molecules will interact with *β*-galactosidase, resulting in the inactivation of enzyme.

## Scanning Electron Microscopy

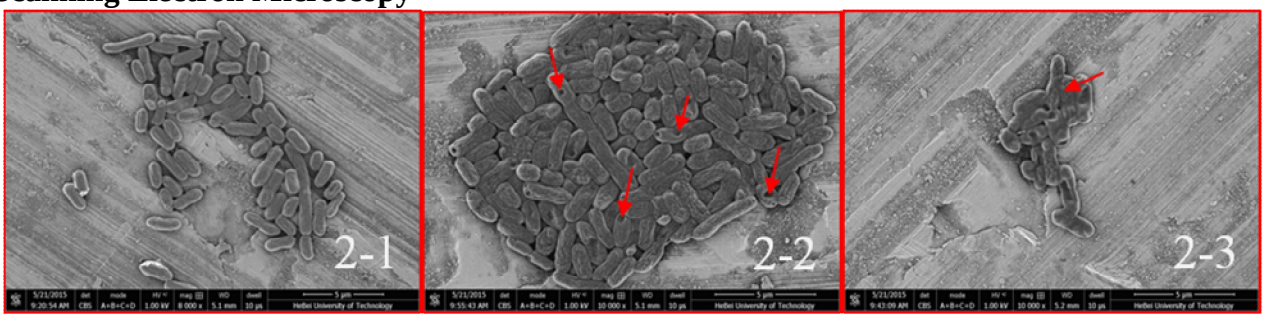


**Fig.2. Scanning electron microscopy (SEM) images of *E. coli*(2-1.untreated; 2-2. treated by 0.3mg/L 12-8-12; 2-3. treated by 5mg/L 12-8-12)**

To obtain visual insight into bacterial killing by dimeric amphiphile 12-8-12, a scanning electron microscopy (SEM) study was performed. The images of *E. coli* untreated (as a control) and treated by different concentration of 12-8-12 for 2h are shown in figure2. The untreated bacteria show the presence of normal cells with regular rod-like shape and the intact cell membranes with clear

boundaries (Fig.2-1). As the concentration of 12-8-12 is lower than the *MIC*, the shape of *E. coli* cells has no significant change, but some holes can be seen on the surface of cells, which indicate the destroying of cell membrane (Fig.2-2). Then the irregularly shaped and thus probably dead cells were observed upon treatment with a higher concentration of 12-8-12(Fig.2-3). With the results of the $\beta$-galactosidase activity and the images of *E. coli*, the antibacterial action of dimeric amphiphile 12-8-12 can be described as the process, that the amphiphile molecules interact with the *E. coli* cell membrane, make holes on the membrane, and then disrupt the cell membrane leading to the loss of cytoplasmic constituents and cell death.

## Conclusions

The antibcatrial activity of cationic dimeric amphiphiles was determined and compared to that of corresponding monomeric amphiphile. Due to the greater number of positive charges and higher hydrophobcity,the antibacterial activity of dimeric amphiphiles is superior to the corresponding monomeric amphiphile. The value of MIC decreases with the increase in the spacer length (12-s-12,s=4,6,8), and increases with the increase in the alkyl chain length (m-6-m,m=12,14,16). The result of $\beta$-galactosidase activity shows that 12-8-12 disrupts the membrane of *E.coli* leading to the releasing of intracellular contents and death of bacterial cells. This conclusion was supported by scanning electron microscopy, which imaged the holes on the cell membrane and irregular shape of cells caused by treatment of 12-8-12.

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