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The biocidal properties of anthraquininoid dyes

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ABSTRACT

A modified minimum inhibitory concentration test method was employed to determine the antimicrobial properties against both Gram-positive and Gram-negative bacteria of two series of cationic anthraquininoid dyes. The antimicrobial strength of the colorants increased with increasing carbon chain length up to C16; the activities of the colorants were improved by the use of elevated temperature as well as either high or low pH. However, the colorants were deactivated in the presence of both anionic surfactants and divalent cations.

1. Introduction

Quaternary ammonium salts (QAS) are surfactants containing a positively charged hydrophilic core and a long hydrophobic segment [1–6]. The positive head can interact with the surface of the microbes that normally carries negative charge. Therefore, the cationic centers on QAS can attack the outer cell wall and the hydrocarbon tail can further integrate with the lipid bilayer of cell membrane. This perturbation causes the leakage of the cell contents and then leads to the death of the microorganism [2]. Because of this antimicrobial mechanism, the biocidal efficacy of QAS can be affected by the electrostatic interaction between QAS and bacteria, the hydrophobicity of the tail, critical micelle concentration (CMC), solubility or any other factors affecting their diffusion behavior [2,7–9].

In previous studies, two series of antimicrobial colorants (mono-substituted, M series; di-substituted, Di series) with increasing aliphatic chain lengths and different numbers of QAS structures were synthesized [5]. The structures of the colorants are illustrated in Scheme 1. The colorants were named as M-4, M-8, M-12, M-16; Di-4, Di-8, Di-12 and Di-16, where the letter represents the series and the number stands for the hydrocarbon chain length. The colorants showed excellent biocidal efficacy in aqueous solution. Other research has shown that concentration, temperature, pH, anionic surfactants and divalent cations could affect the activities of QAS [10]. At the same time, different types of QAS could demonstrate diverse responses to a condition because of their unique structures. The aim of this research is to study the effect of the structure, time, concentration and the environmental factors on the antimicrobial activity of the colorants.

2. Materials and methods

2.1. Antimicrobial assessment

The antimicrobial activity of the colorants in aqueous solution was evaluated by a modified minimum inhibitory concentration (MIC) method [5,11] against both *Staphylococcus aureus* (*S. aureus*, Gram-positive, ATCC #12600) and *Escherichia coli* (*E. coli*, Gram-negative, K-12, UCD microbiology laboratory). Three replicas were made for each sample.

2.2. Effect of structure, concentration and time

The biocidal efficacy of the colorants was assessed at varied concentrations in 1 min. The colorants with octyl alkyl chain were tested from 1 ppm to 20 ppm; those with butyl, dodecyl and hexadecyl chains were examined from 1 ppm to 10 ppm. The biocidal dynamics of M-8 was conducted by varying the contract time from 15 min to 24 h against *E. coli* at 3–5 $\times$ 10^6 CFU/mL.

2.3. Effect of temperature, pH and alcohol

In this part, M-8 and Di-8 were chosen as representatives for these two series of colorants. The concentrations of M-8 and Di-8 were 15 ppm and 10 ppm, respectively. The antimicrobial activity of

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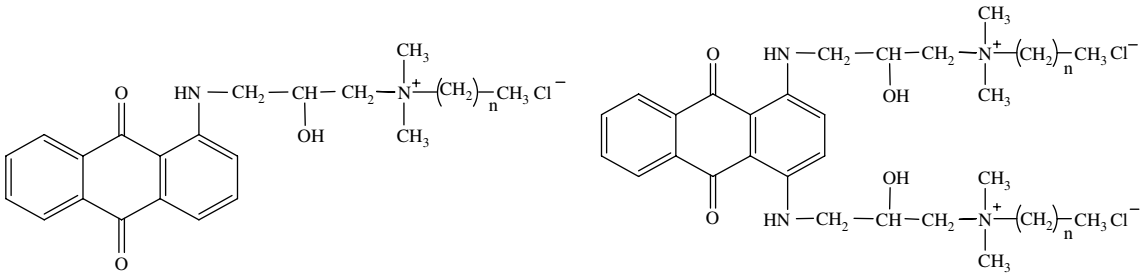

Scheme 1. Structures of the antimicrobial colorants ($n = 3$, 7, 11 or 15).

M-8 and Di-8 was tested from 25 °C to 45 °C. 5 M acetic acid and sodium carbonate were used to adjust the pH. Ethyl alcohol (10% by volume) was added to the colorant solutions to study the synergistic effect. Sterilized DI water under the same conditions was tested as a control.

2.4. Effect of anionic surfactant and divalent cations

M-4 and Di-4 were tested at 400 ppm and M-8 and Di-8 remained the same concentrations as in the experiments above. Sodium dodecyl sulfate (SDS) and sodium dodecyl benzene sulfonate (Dodecene-1 LAS, abbreviated as LAS) were added to the colorant solutions at 1:1 molar ratio to study the effect of anionic surfactants. The absorbances of the colorants at their maximum wavelengths were tested by using an Evolution 600 UV–vis spectrophotometer (Thermo Electronic). After the anionic surfactants were applied, the colorant solutions were centrifuged for 10 min, followed by standing for 24 h at room temperature till equilibrium. The absorbances of the supernatants were examined by the UV–vis spectrophotometer. The percentage of precipitation can be calculated by the following equation:

$$\text{Precipitation rate}(\%) = \frac{A_i - A_f}{A_i} \times 100\%$$

where A_i is the initial absorbance of the colorants; A_f is the absorbance of the supernatant after adding the surfactants.

The effect of divalent cations was assessed by adding divalent cations Mg^{2+} and Ca^{2+} in form of chloride salts to the colorant solutions. The concentrations of the cations in the solutions ranged from 1 to 5 mM.

3. Results and discussion

3.1. Structure–antimicrobial property relationship

The colorants at different concentrations were challenged with both *E. coli* and *S. aureus* and the results are listed in Table 1. As expected, M-4, Di-4, M-16 and Di-16 did not present noticeable elimination of the bacteria because the tested concentrations were lower than their MIC values (200 ppm for M-4 and Di-4; 60 ppm for M-16 and Di-16) [5]. Moreover, the antimicrobial efficacy of the each colorant increased as the concentration went up. At 1 ppm, no colorant demonstrated biocidal activity. At 5 ppm, the antimicrobial efficacy of the colorants was in the order of Di-12 > M-12 > Di-8 > M-8. At 20 ppm concentration, colorants with octyl and dodecyl chains could completely inactivate the bacteria. These results indicate that the activities of the colorants increase with their aliphatic chain lengths except for C16. The di-substituted colorants are stronger than the mono-substituted colorants because of the doubled QAS functional groups.

As seen above, the biocidal properties of the colorants increased to reach the maximum at C12. At C16, the antimicrobial activity dropped compared to C12. That is to say, the antimicrobial activity of the colorants is not linear with their hydrocarbon chain length. These results are consistent with the "cut-off effect" described in the literature [12]. Numerous theories have been postulated in regard to this effect. Devinsky et al. proposed that QAS with chain length higher than C16 have similar structure with the lipid bilayer of a cell, which causes less disruption to the cell membrane [12]. Janoff and Pringle attribute this effect to a lower solubility of QAS with longer hydrocarbon chain. They believed that the lipid tail with limited partition at the surface of microbes could not function efficiently to perturb the membrane [9]. A more plausible theory was based on the relationship between critical micelle concentration (CMC) and MIC of quaternary ammonium salts since they are surfactants [8,13]. At low chain length, the CMC of QAS is much higher than MIC. At this point, most QAS present in aqueous solution as monomers. As the chain length grows longer than 14 or 16, the CMC values become very low. In this case, most of QAS aggregate into micelles, vesicles or bilayers. As a result, the biocidal efficacy of the QAS is weakened and higher concentration needs to be produced in order to achieve the same effectiveness.

3.2. Biocidal dynamics of M-8

In practical applications of an antimicrobial agent, the bacterial elimination rate is a critical factor. The effect of contact time on the biocidal efficacy was evaluated on M-4, M-8, M-12, Di-4, Di-12 and Di-12 at 10 ppm against 10^6–10^7 CFU/mL of *E. coli*. It was found that M-4 and Di-4 showed no activity even after 24 h, whereas M-12, Di-12 and Di-8 destroyed all the bacteria in 15 min. However, M-8 with

Table 1
Biocidal efficacy of the colorants against *E. coli* and *S. aureus* at different concentrations.

Concentration (ppm)	Antimicrobial efficacy (log reduction)							
	M-4	M-8	M-12	M-16	Di-4	Di-8	Di-12	Di-16
1	0	0	0	0	0	0	0	0
5	0	0	4	0	0	1	6	0
10	0	0	6	0	0	2	6	0
15	–	3	–	–	–	6	–	–
20	–	6	–	–	–	6	–	–

Note: *E. coli* and *S. aureus* at 10^7–10^8 CFU/mL, contact time: 1 min.

mediocre biocidal properties demonstrated increasing log reduction throughout 24 h. As illustrated in Fig. 1, the log number of viable *E. coli* concentration decreased linearly during the tested period. This phenomenon is due to the nature of the growth or death of bacteria under this particular condition. According to bacteriology [14], microbial death is logarithmic or exponential, just like its growth. The bacteria population decreases by the same fraction at constant intervals, so a plot of log reduction over time is linear, which means a larger population of bacteria takes longer to sterilize. This phenomenon is of great importance in predicting the sterilization time at a given amount of bacteria. For example, in our case, to kill a one log (90%) of *E. coli*, it takes an average time of 3.8 h, and at least 23 h are needed to eliminate 10^6 CFU/mL bacteria.

3.3. Effect of temperature, pH and alcohol

Temperature is one of the critical environmental factors that will affect the growth of bacteria. All bacteria have a minimum temperature, an optimum temperature and a maximum temperature for growth. Bacteria cannot duplicate under the minimum temperature or above the maximum temperature. And they grow most rapidly under the optimum temperature. *E. coli* is mesophilic, with an optimum temperature between 42 and 44 °C [15]. Therefore, it is interesting to know whether temperature would affect their antimicrobial performance, particularly at the maximum growth temperatures of the bacteria. As listed in Table 2, the biocidal efficacy of M-8 and Di-8 increased significantly at 45 °C, but remained the same under 35 °C. It indicates higher temperature can facilitate the antimicrobial activity of the colorants.

E. coli is a neutrophile that can grow in a wide pH range of 5–9 [16], with optimal growing rates reached at pH 6 and 8. At too high or too low pH, the capability for growth is lost. The bacterium remains a constant growth within pH 7.5–7.9 and is intolerant to significant deviations from this value. As shown in Table 2, the alkaline or acidic solution without the colorants has no or little antimicrobial activity. However, after the base or acid was added to the colorants, the antimicrobial activities of the colorants were significantly improved. The log reduction of M-8 and Di-8 increased from 3 and 2 to 6 after the pH changed from 7 to 4 or 10, indicating that the bacteria are more susceptible to the colorants under either basic or acidic conditions. These results also implicate that antimicrobial effectiveness of the colorants could be enhanced by controlling the pH of the colorant solution.

Table 2 also shows the activities of M-8 and Di-8 after adding 10V% ethyl alcohol. It is well known that ethanol kills bacterial at a concentration higher than 60% by volume. Our results showed that 10V% ethanol itself exhibited no antimicrobial effect, but addition of the ethanol at the same concentration greatly improved the effectiveness of the colorants. This phenomenon is called the "synergistic effect". Normally, the bacteria cells swell in alcohol solution, so the surface area of the cell increases and cell wall becomes thinner [14]. According to the antimicrobial mechanism of QAS [20], this makes it easier for the colorants to break down the structure of the cells.

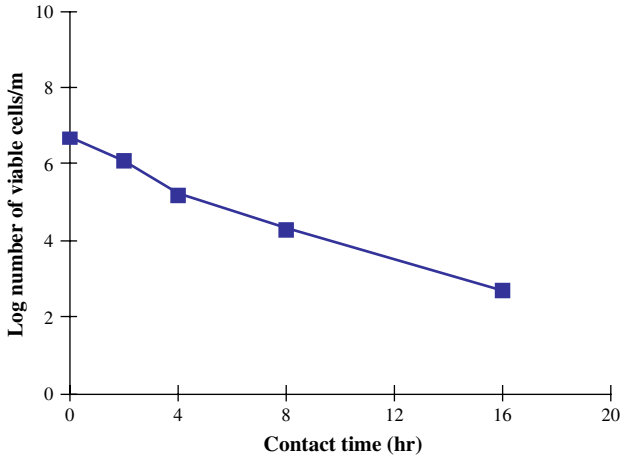

Fig. 1. Biocidal dynamics curve of M-8. (*E. coli*, same condition as Table 1).

Table 2
Biocidal efficacy of M-8 and Di-8 under different conditions.

		Log reduction		
		Control	M-8	Di-8
Temperature	25 °C	0	3	2
	35 °C	0	3	2
	45 °C	0	6	6
pH	4	1	6	6
	7	0	3	2
	10	0	6	6
Synergy	10% Alcohol	0	6	6

Note: *E. coli*, 10^7–10^8 CFU/mL, contact time: 1 min. Concentration: M-8, 15 ppm; Di-8, 10 ppm.

3.4. Effect of anionic surfactants

It has been reported that the antimicrobial action of QAS could be inactivated by anionic surfactants because of the ion-pairing of quaternary cations with the anions [17,18]. In our research, anionic surfactants sodium dodecyl sulfate (SDS) and sodium dodecyl benzene sulfonate (LAS) were added to the colorant solutions, and the log reductions are listed in Table 3. As shown, the antibacterial properties of the M series were completely impaired by both SDS and LAS at 1:1 molar ratio. The addition of SDS completed deactivated Di-4 and Di-8 and significantly decreased the activity of Di-12. Similarly, when LAS was added, the effectiveness of Di-4, Di-8 and Di-12 was weakened, though they still retained certain biocidal activity. These results indicate that anionic surfactants, especially SDS, can impair the antimicrobial activities of the colorants. This effect is caused by precipitations resulted from the electrostatic interaction between the cationic colorants and the anionic surfactants. After the colorants are dissolved in aqueous solutions, they completely dissociate into ions. The dissociated cationic species of the colorants in the solutions provide the biocidal properties. As the anionic surfactants are mixed with the colorant solutions, they will also dissociate into anionic and cationic species. The anionic ions can easily associate with the cationic part of the colorants to form ion pairs owe to the stronger ion interactions. The new ion pairs have strong interaction and thus low solubility in water, leading to the formation of precipitation. As listed in Table 3, all colorants formed precipitates with both surfactants and were removed from the solutions. SDS precipitated more colorants than LAS, thus

Table 3
Biocidal efficacy and precipitation rate of the colorants before and after addition of anionic surfactants.

	M-4	M-8	M-12	Di-4	Di-8	Di-12
Log reduction						
Blank	2	3	6	2	2	6
SDS	0	0	0	0	0	1
LAS	0	0	0	1	1	2
Precipitation rate (%)						
SDS	66.4	82.1	47.9	67.0	33.0	32.1
LAS	22.5	59.5	27.1	36.0	22.3	21.0

Note: *E. coli*, 10^7–10^8 CFU/mL, contact time: 1 min. Concentration: M-4 and Di-4, 400 ppm: M-8, 15 ppm; all others: 10 ppm.

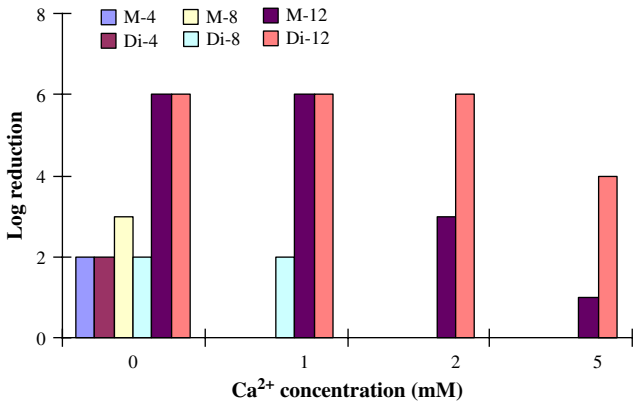

Fig. 2. Biocidal efficacy of the colorants before and after addition of Ca^{2+}.

deactivated the colorants more severely. This also implicate that SDS formed tighter ion-pairing with the colorants compared to LAS.

3.5. Effect of divalent cations

Several studies showed that the presence of divalent cations could affect the antimicrobial activity of QAS [10,19]. In this study, 1, 2 or 5 mM of Ca^{2+} and Mg^{2+} were added to the colorant solutions to study the effect of divalent cations. As shown in Fig. 2, M-4, Di-4 and M-8 were completely inactivated after adding 1 mM Ca^{2+}. The antimicrobial activities of Di-8, M-12 and Di-12 decreased rapidly as the concentration of Ca^{2+} became higher. The resistance of the colorants to Ca^{2+} was in the order of Di-12 > M-12 > Di-8 > M-8, the same with their antimicrobial effectiveness. This indicates that the colorants with stronger biocidal properties are less inactivated by the divalent cations. Fig. 3 reveals the same phenomenon. Comparing Fig. 2 to Fig. 3, we can see that Mg^{2+} was more poisoning to the colorants than Ca^{2+} at the same concentration. The reason for the deactivation could be explained by the antimicrobial mechanism of the colorants. As mentioned above, QAS destroy and eliminate bacteria by adsorption of the QAS onto the cell wall and then diffused through the physical barrier to disrupt the cell membrane [20]. Take *E. coli* as an example, the outer cell wall is composed of phospholipids, proteins, and lipopolysaccharides (LPS). The major role of the cell wall is to take a variety of environmental molecules away from the cell and to allow for selective uptake of other molecules. The divalent cations, i.e., Ca^{2+} and Mg^{2+}, are key players, which cross-bridge adjacent negatively charged LPS molecules in the membrane [21]. When the bacteria are exposed to the antimicrobial colorants, these cationic colorants will firstly adsorb onto the anionic sites on the membrane, then replace the divalent cations and eventually penetrate through the cell wall. The added Ca^{2+} and Mg^{2+} can compete with the QAS to the anionic sites on the cell wall, thus undermine the antimicrobial activity of the colorants.

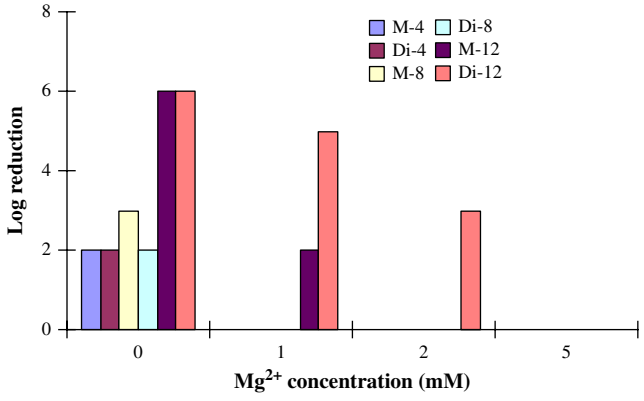

Fig. 3. Biocidal efficacy of the colorants before and after addition of Mg^{2+}.

4. Conclusions

The antimicrobial activity of the two synthesized colorants was assessed by a modified MIC procedure. The antimicrobial activity of both series increased with the hydrocarbon chain length and showed a "cut-off" effect when the chains were longer than 12 carbons. The log number of viable bacteria decreased linearly versus time after exposed to the colorants. This gives us a clue in predicting the elimination time for a given amount of microorganism. Higher concentration, elevated temperature and lower or higher pH facilitated the biocidal activity of the colorants. Addition of 10V% of ethyl alcohol improved the biocidal efficacies of the colorants. Both anionic surfactants and divalent cations could deactivate the colorants.

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