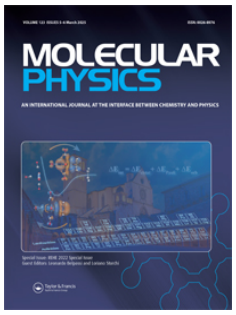

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Oligomerization can enhance synergistic activity of antimicrobial peptides

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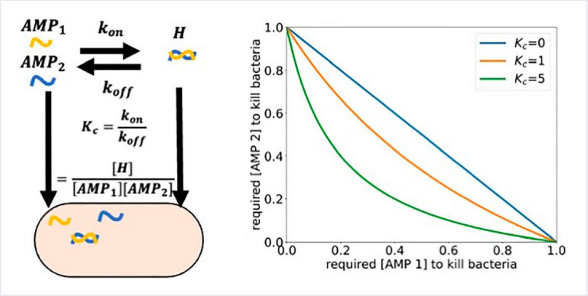
ABSTRACT

Antimicrobial peptides (AMPs) have emerged as promising therapeutic agents against antibiotic-resistant bacteria. It has been observed that different types of AMPs might reversibly associate in solutions before binding to bacteria and form larger molecules known as AMP hetero-oligomers. Still, it remains unclear how these chemical reactions are influencing antimicrobial activity. In addition, recent experimental studies suggest that combining two or more different types of AMPs is more effective in eliminating bacteria than a single AMP type. However, the underlying microscopic picture of such synergy remains not well understood. This paper investigates the oligomerization of different types of AMPs and their impact on antimicrobial activity using effective chemical-kinetic models. We specifically analysed the reversible formation of hetero-oligomers from two types of AMPs using two different criteria to measure the degree of cooperativity. It is found that considering only the concentrations of initial AMP components always leads to an apparent stronger synergistic effect due to increasing the overall heterogeneity of the system. However, considering the method that also explicitly accounts for the presence of oligomers suggests that these reactions make positively-cooperating systems more synergistic and negatively-cooperating systems less synergistic. Physical-chemical arguments to explain these observations are presented. Our findings provide new insights into the role of AMP oligomerization in the antimicrobial activity of AMPs and can be explored in the development of novel antimicrobial strategies.



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1. Introduction

Antimicrobial peptides (AMPs) correspond to several classes of biological molecules produced by the immune systems of most prokaryotic and eukaryotic organisms that have been considered as potential alternatives to antibiotics [1,2]. AMPs are polymers consisting of ∼10–50 amino acid subunits, and they are predominantly positively charged, which facilitates binding to the negatively charged membranes of a wide variety of bacterial cells while discouraging association to healthy human cells [1–3]. Accordingly, AMPs show strong selectivity for attacking pathogens, including bacteria, and exhibit low toxicity to humans [4,5]. The unique properties of antimicrobial peptides stimulated significant research efforts aimed at developing AMPs into new classes of antibiotics [5]. However, these activities are frequently slowed down by our limited understanding of the molecular picture of antimicrobial action.

One of the proposed mechanisms for AMPs' action against bacteria is the formation of pores in bacterial

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membranes after binding to them, leading to leakage of cell contents, disruption of proper cell functioning, and ultimately cell death [6–8]. This mechanism is associated with a lower risk of AMPs inducing resistance due to the reduced likelihood of bacteria developing mutations in the cell membranes [3,9]. Regardless of the specific mechanisms of action, it is generally believed that the first step in AMPs' antimicrobial activity, is always the association to the bacterial cell membrane [10]. Despite the advantages of AMPs, instances of bacterial resistance to AMPs and increased AMP toxicity over time have been observed [5,8,11–20]. To alleviate this danger, it was proposed to utilise combinations of AMPs instead of pure single AMP compounds [2,3,10,21–30]. Several experimental studies demonstrated that mixtures of two or more different AMPs can be more effective at inhibiting bacterial growth, reflecting synergistic antimicrobial activity, with an even lower risk of inducing bacterial resistance [31–42].

To explain the observations of synergistic antimicrobial activities of AMP combinations, we recently proposed a new quantitative theoretical framework [43,44]. The main idea of this approach is that AMPs can disrupt bacterial cells more efficiently if they associate faster with the membranes, and the inter-molecular interactions between different peptide species might accelerate the association dynamics. This allowed us to develop an effective chemical-kinetic approach that could explicitly estimate the degree of cooperativity between different groups of AMPs [43,44]. This method has been successfully utilised to quantitatively analyze several experimental AMP combinations where synergy has been observed. We were able to explain why increasing the heterogeneity of AMP combinations leads to stronger synergy in the removal of bacterial infections. However, a better understanding of the mechanisms of cooperativity is needed to develop AMP combinations as potential antibiotics. This is especially important in light of experimental studies that observed the chemical association of AMPs to form new hetero-oligomers species (from different AMP types) in solution before binding to the cellular membranes [2,10,45–53]. These observations raised a question of how the oligomerization process can influence the antimicrobial properties since these species might also participate in the binding to the cellular membranes. It has been suggested that hetero-oligomerization might be an important factor in supporting the synergistic antimicrobial activities [11,12,25,30,54–63].

This study aims to elucidate the role of hetero-oligomer formation in promoting the synergy between different AMP species. We extend the original theoretical framework [43,44] to account for the reversible formation of oligomer species that can also bind to the membrane, potentially enhancing the overall efficiency of removing the infection. In this work, cooperativity refers to how combinations of AMPs interact to achieve antibacterial effects greater than (synergy), equal to (additive), or less than (antagonism) the sum of their individual component effects. Two different methods are used to evaluate the effect of oligomerization on synergistic activities of AMPs.

It is found that considering only the concentrations of original AMP species predicts that oligomers will always apparently move the systems in the direction of stronger synergy. At the same time, a more comprehensive method that explicitly accounts for oligomers suggests that oligomers make the systems with already positive cooperativity more synergistic and the systems with negative cooperativity more antagonistic. It is argued that the presence of oligomers effectively modifies the original two-component mixtures into new three-component mixtures, increasing the overall heterogeneity and allowing AMPs to associate faster with the membrane for positive inter-molecular interactions or more slowly for negative inter-molecular interactions. Our theoretical analysis provides specific suggestions about how AMP combinations with the ability to make oligomers before binding to membranes might be explored to make more efficient antibiotics.

2. Theoretical method

Let us consider a system of two types of AMP molecules that can reversibly associate into a new oligomeric (hetero-oligomer) AMP species, as illustrated in Figure 1. The AMPs of type 1 and type 2 can bind together with the rate constant k_{on}, while the hetero-oligomers (labeled as species H) can dissociate with the rate constant k_{off}. All three types of AMP species are active against bacteria. They can bind to the bacterial membrane with the effective rate constant $k(n_1, n_2, n_H)$ where n_1, n_2 and n_H are the numbers of AMPs of type 1, type 2 and type H, respectively, that can simultaneously associate to the membrane. This is similar to the situation when bacteria are subjected to a mixture of three distinct types of antimicrobial peptides, which has been recently analysed by using an effective chemical-kinetic approach [43]. However, our system is different since all three AMP species are not independent from each other, and their concentrations are related via a reversible oligomerization process. Our goal is to account for the effect of reversible oligomerization by extending the original chemical-kinetic method.

To analyze the bacterial elimination process by the system of two types of AMPs that can also produce hetero-oligomer species, we assume that the concentration of

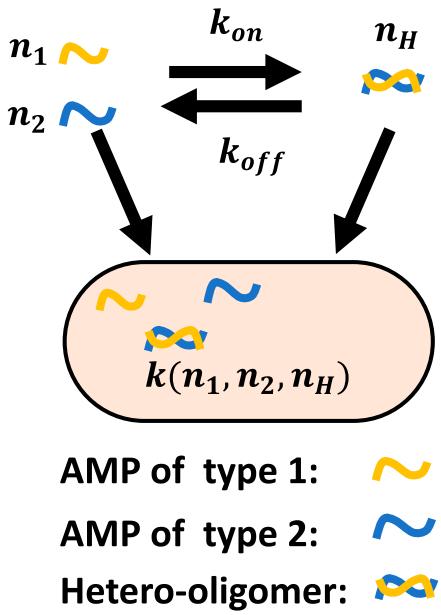

Figure 1. Schematic view of the system of two different types of AMPs (type 1 and type 2) that can also bind together in the solution to form a hetero-oligomer particle. All three species can associate to the cellular membranes. The corresponding association and dissociation rate constants for the hetero-oligomerization are k_{on} and k_{off}. n_1 is the number of AMP molecules of type 1, n_2 is the number of AMP molecules of type 2, and n_H is the number of AMP molecules of type 3 (hetero-oligomer). The rate constant $k(n_1, n_2, n_H)$ describes the binding of the three types of AMPs to the membrane.

bacteria at time t is given by $B(t)$, and it increases due to cell division with a rate constant λ. One can define the concentrations of AMP species as $C_1(t)$ (type 1), $C_2(t)$ (type 2), and $H(t)$ (hetero-oligomer). We also assume that to kill a single bacterium, N AMP molecules (in any combination) are always required [43,44].

In our formulation, we postulate that N AMP molecules (in any combination of AMP types) are required to kill a single bacterium. While this is an approximation, it reflects dominating views on the molecular mechanisms of how AMPs kill bacteria. According to this picture, AMPs must cover the membrane as a carpet before they can aggregate and stimulate the production of pores in the membrane, eventually killing the bacteria. This means that, independently of the efficiencies of different AMPs, for the given bacterial cell, the same overall threshold should be achieved before the bacterial cell can be eliminated. Assuming that n_1, n_2 and n_H are the number of AMP molecules of type 1, type 2 and type H, respectively, that can simultaneously bind to the bacterial membrane, we must have $N = n_1 + n_2 + n_H$. Then the following effective chemical-kinetic equations describe the processes in the system,

$$\frac{dB(t)}{dt} = \lambda B(t) - \sum_{n_1,n_2,n_H} k_{n_1,n_2,n_H} C_{n_1,n_2,n_H} \tag{1a}$$

$$\frac{dC_1(t)}{dt} = -\sum_{n_1,n_2,n_H} n_1 k_{n_1,n_2,n_H} C_{n_1,n_2,n_H} - k_{on} C_1(t) C_2(t) + k_{off} H(t) \tag{1b}$$

$$\frac{dC_2(t)}{dt} = -\sum_{n_1,n_2,n_H} n_2 k_{n_1,n_2,n_H} C_{n_1,n_2,n_H} - k_{on} C_1(t) C_2(t) + k_{off} H(t) \tag{1c}$$

$$\frac{dH(t)}{dt} = -\sum_{n_1,n_2,n_H} n_H k_{n_1,n_2,n_H} C_{n_1,n_2,n_H} + k_{on} C_1(t) C_2(t) - k_{off} H(t). \tag{1d}$$

where $k_{n_1,n_2,n_H} = \frac{N!}{n_1! n_2! n_H!} k(n_1, n_2, n_H)$, representing the rate at which a combination of AMPs $((n_1, n_2, n_H)$ leads to bacterial cell death, $N = n_1 + n_2 + n_H$ is the total number of AMP molecules, and $C_{n_1,n_2,n_H} = [C_1(t)]^{n_1} [C_2(t)]^{n_2} [H(t)]^{n_H} B(t)$.

These expressions can be understood using the following arguments. One can view the binding of different types of AMPs to the bacteria as an association chemical reaction. The concentration of bacteria increases due to cell division with the rate constant λ and decreases due to the combined action of AMP species of type 1, type 2, and H that kills bacteria. The concentrations of AMPs of type 1 and type 2 decrease due to the association with the cell membranes and the oligomerization, while they increase because of oligomers breaking apart back into monomeric AMP species. Similarly, the concentration of oligomers decreases due to the binding to the membrane and the oligomer breaking apart, while it increases because of the formation of oligomers from the original AMP monomers. The combinatorial factor $\frac{N!}{n_1! n_2! n_H!}$ gives the number of possible different association pathways when n_1 molecules of type 1, n_2 molecules of type 2 and n_H molecules of type H bind to the membrane, assuming that $N = n_1 + n_2 + n_H$ is always the same total number of AMPs molecules needed to kill the bacteria. Each such association simultaneously eliminates n_1 AMPs of type 1, n_2 AMPs of type 2, and n_H of oligomeric AMPs from the pool of available peptides.

In our analysis, we also assume that the chemical equilibrium in the oligomerization process is always quickly established,

$$C_1 + C_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} H, \tag{2}$$

leading to

$$K_c = \frac{k_{on}}{k_{off}} = \frac{[H(t)]}{[C_1(t)][C_2(t)]}, \tag{3}$$

where K_c is the equilibrium constant for this process. This is a reasonable approximation since the time scales for oligomerization chemical reactions are typically much shorter than the time scales for the processes of elimination of bacterial infection. Equation (3) is an important relation because it explicitly couples the concentrations of different types of AMPs in the system.

A central part of our theoretical method is an assumption that more efficient elimination of bacteria correlates with faster association of AMPs to the membrane [43,44]. This emphasises the importance of the effective association rate constant $k(n_1, n_2, n_H)$ that should reflect the inter-molecular interactions during binding to the membrane. It can be written as [43,44]

$$k(n_1, n_2, n_H) = k_1^{\frac{n_1}{N}} k_2^{\frac{n_2}{N}} k_H^{\frac{n_H}{N}} \exp\left(\frac{n_1}{N}\frac{n_2}{N}\frac{N\Delta E_{12}}{k_B T} + \frac{n_1}{N}\frac{n_H}{N}\frac{N\Delta E_{1H}}{k_B T} + \frac{n_2}{N}\frac{n_H}{N}\frac{N\Delta E_{2H}}{k_B T}\right), \tag{4}$$

where the terms $\frac{n_1}{N}$, $\frac{n_2}{N}$, and $\frac{n_H}{N}$ give the fractions of each type of AMP species associated with the membrane. The parameters ΔE_{ij} specify the interaction energy between AMPs of type i and j estimated per one-bound AMP molecule. They are responsible for the existence of cooperativity in the anti-bacterial action of AMP molecules. Specifically, the parameter ΔE represents the interaction energy between AMP molecules that influences whether they associate slower ($\Delta E < 0$), with the same rate, ($\Delta E = 0$), or faster ($\Delta E > 0$) to the cellular membrane in comparison to associations of pure individual components.

To simplify our analysis, we also assume that all interactions between different AMP molecules are the same, $\Delta E_{ij} = \Delta E$, which leads to a simpler expression for the association rate constant,

$$k(n_1, n_2, n_H) = k_1^{\frac{n_1}{N}} k_2^{\frac{n_2}{N}} k_H^{\frac{n_H}{N}} \exp\left(\frac{N\Delta E}{k_B T} A\right), \tag{5}$$

with

$$A = \left(\frac{n_1}{N}\frac{n_2}{N} + \frac{n_1}{N}\frac{n_H}{N} + \frac{n_2}{N}\frac{n_H}{N}\right). \tag{6}$$

One should also notice the exponential dependence of the equilibrium constant on the energy parameter ΔE, $K_c \simeq \exp(\frac{N\Delta E}{k_B T} A)$.

To proceed, we need to derive a general expression for the Minimal Inhibitory Concentration (MIC), which is the smallest concentration of AMPs required to stop the bacterial growth. In our chemical-kinetic language, this corresponds to the condition $dB(t)/dt = 0$ in Equation (1a).

$$\lambda = \sum_{n_1,n_2,n_H} \frac{N!}{n_1! n_2! n_H!} k(n_1, n_2, n_H)[C_1]^{n_1}[C_2]^{n_2}[H]^{n_H}; \tag{7}$$

where $k(n_1, n_2, n_H)$ is given by Equations (5) and (6). We can further simplify this expression by using the equilibrium condition, $H = K_c C_1 C_2$,

$$\begin{aligned}\lambda = \sum_{n_1,n_2} & \frac{N!}{n_1! n_2! (N - n_2 - n_1)!} \\ & \times k(n_1, n_2, n_H = N - n_2 - n_1)\left[\frac{C_1}{C_{1,MIC}}\right]^{n_1} \\ & \times \left[\frac{C_2}{C_{2,MIC}}\right]^{n_2} \\ & \times \left[K_c \frac{C_1}{C_{1,MIC}}\frac{C_2}{C_{2,MIC}}\right]^{N-n_2-n_1} [C_{1,MIC}]^{N-n_2} \\ & \times [C_{2,MIC}]^{N-n_1};\end{aligned} \tag{8}$$

where the individual MIC for the AMP molecules of type 1 and type 2 are defined as

$$C_{1,MIC} = \left(\frac{\lambda}{k_1}\right)^{\frac{1}{N}}, \quad C_{2,MIC} = \left(\frac{\lambda}{k_2}\right)^{\frac{1}{N}}. \tag{9}$$

There are different ways to estimate the degree of cooperativity for the mixture of AMP molecules, and in our analysis, beyond considering the concentrations of original AMPs, we also explore a quantity known as a fractional inhibition coefficient (FIC) defined as [44],

$$FIC = FIC_1 + FIC_2 + FIC_H; \tag{10a}$$

$$FIC_1 = \frac{MIC_{(1\text{ in combination})}}{MIC_{(1\text{alone})}} = \frac{C_1}{C_{1,MIC}}; \tag{10b}$$

$$FIC_2 = \frac{MIC_{(1\text{ in combination})}}{MIC_{(1\text{alone})}} = \frac{C_1}{C_{1,MIC}}; \tag{10c}$$

$$FIC_H = \frac{MIC_{(\text{H in combination})}}{MIC_{(\text{Halone})}} = \frac{H}{H_{MIC}}. \tag{10d}$$

When the FIC parameter is less than one, the AMPs show stronger antimicrobial activity in the mixture than separately as individual species, whereas when it is larger than one, the antimicrobial activity is weaker in the mixture than when the bacteria are exposed to each AMP separately. For $FIC = 1$, the AMPs are equally effective against the bacteria in the mixture and separately.

Thus, $FIC < 1$ reflects synergy and positive cooperativity, $FIC > 1$ corresponds to the antagonistic antimicrobial activity and negative cooperativity, while $FIC = 1$ describes the additive antimicrobial activity. It is important to note that FIC parameters explicitly account for all types of AMP molecules that participate in the elimination of bacterial infection.

To illustrate our theoretical method of evaluating the effect of oligomerization, it is convenient to start with the simplest situation of zero inter-molecular interactions, $\Delta E = 0$. In this case, the association rate constant simplifies into

$$k(n_1, n_2, n_H) = k_1^{\frac{n_1}{N}} k_2^{\frac{n_2}{N}} k_H^{\frac{n_H}{N}}, \tag{11}$$

which after substitution into Equation (1a) when $\mathrm{d}B(t)/\mathrm{d}t = 0$ (zero bacterial growth rate) leads to

$$\lambda = (k_1^{\frac{1}{N}} C_1 + k_2^{\frac{1}{N}} C_2 + k_H^{\frac{1}{N}} H)^N. \tag{12}$$

At the same time, if bacteria were exposed separately to single AMP components, Equation (1a) would produce

$$\lambda = k_1 C_{1,MIC}^N = k_2 C_{2,MIC}^N = k_H H_{MIC}^N. \tag{13}$$

Combining Equations (12) and (13), yields an estimate for the FIC parameter,

$$FIC(\Delta E = 0) = \frac{C_1}{C_{1,MIC}} + \frac{C_2}{C_{2,MIC}} + \frac{H}{H_{MIC}} = 1. \tag{14}$$

One should also notice here that generally the FIC parameter can be rewritten as

$$FIC(\Delta E) = \frac{C_1}{C_{1,MIC}} + \frac{C_2}{C_{2,MIC}} + K_c \frac{C_1}{C_{1,MIC}} \frac{C_2}{C_{2,MIC}}, \tag{15}$$

for any value of ΔE after the application of the equilibrium condition.

Also, in this case ($\Delta E = 0$) one can explicitly estimate the relation between the concentrations C_1 and C_2 at which the bacterial growth stops. Substituting the chemical equilibrium condition from Equation (3) into Equation (12) leads to

$$\lambda^{\frac{1}{N}} = k_1^{\frac{1}{N}} C_1 + k_2^{\frac{1}{N}} C_2 + k_H^{\frac{1}{N}} K_c C_1 C_2, \tag{16}$$

which after using Equation (13) produces a compact relation,

$$\left(\frac{C_1}{C_{1,MIC}}\right) = \frac{1 - \left(\frac{C_2}{C_{2,MIC}}\right)}{1 + K_c \left(\frac{k_H \lambda}{k_1 k_2}\right)^{\frac{1}{N}} \left(\frac{C_2}{C_{2,MIC}}\right)}. \tag{17}$$

For realistic values of the parameter $N \gg 1$ ($N \sim 10^4 - 10^8$ [11,12,40]), this expression further simplifies into

$$\left(\frac{C_1}{C_{1,MIC}}\right) = \frac{1 - \left(\frac{C_2}{C_{2,MIC}}\right)}{1 + K_c \left(\frac{C_2}{C_{2,MIC}}\right)}. \tag{18}$$

One can see that without oligomerization ($K_c = 0$) the combination of two AMPs is always additive without inter-molecular interactions [43,44]. However, the presence of hetero-oligomers ($K_c \neq 0$) effectively modifies the anti-bacterial behaviour of the mixture of AMPs. Thus, using the method of estimating only the concentrations of C_1 and C_2 that stop the bacterial growth leads to different predictions that using the FIC parameters.

Another situation for which analytical results can be obtained is when the killing rate is strongly antagonistic ($\Delta E \to -\infty$). In this case, there are only three possible association scenarios when only molecules of type 1, only molecules of type 2, or only hetero-oligomers can bind to the membrane, producing from Equation (1a) for $\mathrm{d}B/\mathrm{d}t = 0$,

$$\lambda = k_1 C_1^N + k_2 C_2^N + k_H (K_c C_1 C_2)^N, \tag{19}$$

which after using Equation (12) leads to

$$\left(\frac{C_1}{C_{1,MIC}}\right)^N = \frac{1 - \left(\frac{C_2}{C_{2,MIC}}\right)^N}{1 + \left(\frac{k_H \lambda}{k_1 k_2}\right) K_c^N \left(\frac{C_2}{C_{2,MIC}}\right)^N}. \tag{20}$$

This suggests that for the strong antagonistic killing effects without oligomerization ($K_c = 0$) the combinations of AMP molecules are strongly antagonistic, while the oligomerization ($K_c \neq 0$) changes this behaviour. The FIC parameters can be also estimated in this limit.

For general values of the inter-molecular interactions ($\Delta E \neq 0$), one can always solve numerically exactly Equation (1a), leading to Equations (8) and (15), allowing us to estimate the concentrations C_1, C_2 and the FIC parameters.

3. Results and discussion

Figure 2 presents the results of our calculations for normalised concentrations of AMPs of type 1 and type 2, respectively, for different killing effects. For synergistic killing effects, $\Delta E > 0$ (Figure 2(a)), the antibacterial behaviour is always synergistic, and increasing the equilibrium constant K_c further enhances the cooperative behaviour. One can see this by noting that for larger K_c the bacterial growth will stop at smaller concentrations C_1 and C_2. When there are no inter-molecular interactions (Figure 2(b)), the system exhibits additive antibacterial behaviour without oligomerization ($K_c = 0$), but

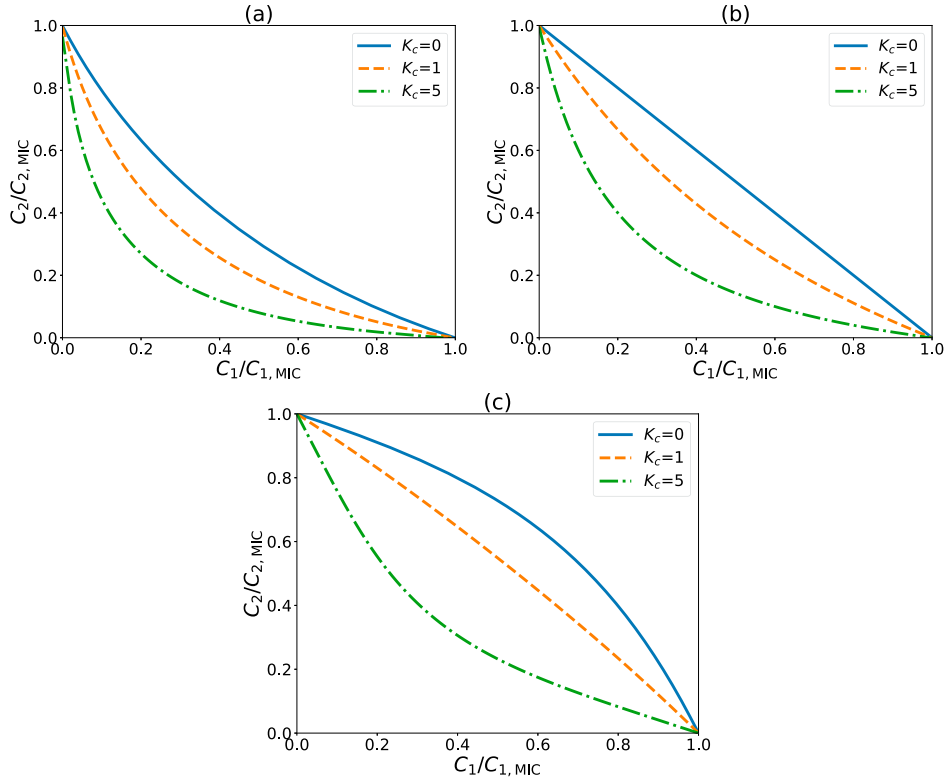

Figure 2. Normalized concentrations of AMPs of type 1 and type 2 at which the bacterial growth stops for different inter-molecular interactions (in units of k_BT): (a) $\Delta E = 10$, (b) $\Delta E = 0$, (c) $\Delta E = -10$. In calculations, the following parameters have been used: $N = 10$, $\lambda = 1/20$ min^{-1}, $k_1 = 2\lambda$, $k_2 = 5\lambda$, and $k_3 = 10\lambda$, as in [43].

allowing for the reversible formation of hetero-oligomers always makes the system to look more positively cooperative. This is because H species also participate in association with the membrane, requiring fewer AMP molecules of type 1 and type 2 to stop the bacterial growth. This leads to the effective synergy between AMPs of type 1 and type 2. Figure 2(c) illustrates the case with antagonistic killing effects. While without oligomerization ($K_c = 0$, solid blue curve) the behaviour is always antagonistic, this method predicts that allowing for the oligomerization reaction makes the system less antagonistic ($K_c = 1$, dashed orange curve), and for larger equilibrium constants the system might even become effectively synergistic ($K_c = 5$, dotted green curve).

However, the extreme synergistic effect is artificially inflated here because the effect of hetero-oligomers is not explicitly considered. Adding hetero-oligomers to the AMP mixture always requires smaller amounts of AMPs of type 1 and type 2 to remove the infection. If one follows only the concentrations C_1 and C_2 that stop the bacterial growth in evaluating the degree of cooperativity, this would always predict that oligomers move all systems, regardless of cooperativity, in the direction of stronger synergy.

The results in Figure 3 show the normalised concentrations of AMP components of type 1 and type 2 for different degrees of oligomerization. When the oligomerization reaction is absent (Figure 3(a), $K_c = 0$), the anti-bacterial behaviour of the system correlates with the sign of the inter-molecular interactions. It is antagonistic for $\Delta E < 0$ (dotted green curve), additive for $\Delta E = 0$ (dashed orange curve), and synergistic for $\Delta E > 0$ (solid blue curve). On the other hand, turning on the oligomerization reaction ($K_c > 0$) apparently changes the anti-bacterial behaviour (Figures 3(b,c)). The antagonistic systems (dotted green curves) start to look first less antagonistic (Figure 3(b)) and for larger equilibrium constants (Figure 3(c)) the system might even become synergistic. At the same time, the originally additive system (dashed orange lines) becomes more apparently positively cooperative with increasing the amplitude of the equilibrium constant. The originally synergistic system (solid blue curves) becomes more cooperative with increasing the degree of oligomerization. We also note that increasing K_c makes the different curves for different inter-molecular interactions closer to each other. All these observations can be explained by the appearance of hetero-oligomers that also participate in killing the

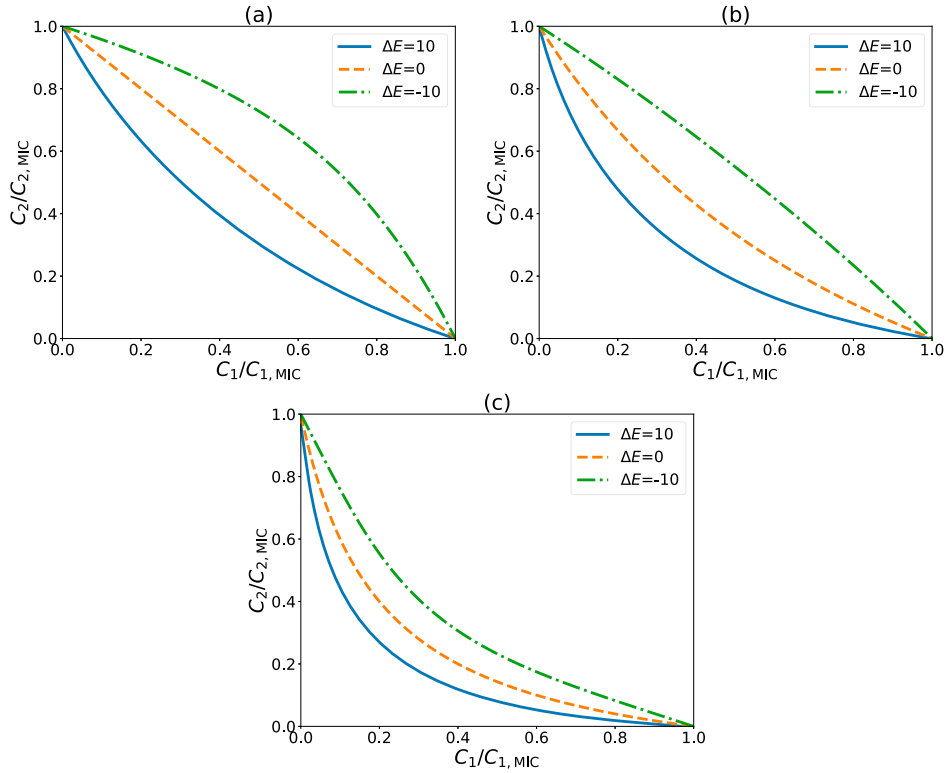

Figure 3. Normalized concentrations of AMPs of type 1 at which the bacterial growth stops for different equilibrium constants: (a) $K_c = 0$, (b) $K_c = 1$, (c) $K_c = 5$. In calculations, the following parameters have been used: $N = 10$, $\lambda = 1/20$ min^{-1}, $k_1 = 2\lambda$, $k_2 = 5\lambda$, and $k_3 = 10\lambda$, as in [43].

bacteria, effectively reducing the amounts of the AMP components of type 1 and type 2 in the process. In addition, for larger K_c the anti-bacterial dynamics become dominated by hetero-oligomers, which makes the system less dependent on the amplitude of the inter-molecular interactions. But again, this method of evaluation of degree of synergy produces artificial inflation of results, suggesting that oligomers always move the systems into more synergistic directions.

To eliminate the possible artifacts in evaluating the degree of cooperativity in AMP combinations with reversible oligomerization, it is important to consider the FIC parameters. It is a better approach since it comprehensively accounts for the presence of all AMP species. As was already shown (Equation (13)), for the systems without inter-molecular interactions we always have $FIC(\Delta E = 0) = 1$, and this corresponds to the additive behaviour. Figure 4 shows the results of our exact numerical calculations for the FIC parameters as a function of the normalised concentrations of one of the AMP components for different inter-molecular interaction energies. For synergistic killing effects (Figure 4(a)), increasing the degree of oligomerization makes the synergy stronger. However, the effect can be seen only for relatively small concentrations of AMPs, reaching the largest degree of cooperativity for $(\frac{C_1}{C_{1,MIC}}) \simeq 0.2$ when $K_c = 5$. For large concentrations, the oligomerization does not make any difference.

Surprisingly, the results for antagonistic killing effects (Figure 4(b)) for the FIC parameter differ from our analysis that only considered the concentrations of AMP compounds of type 1 and type 2 (see Figure 2(c)) that stop bacterial growth. Here, we predict that increasing the degree of oligomerization (larger K_c) makes the system more antagonistic, although the effect again can be seen only for relatively small concentrations, producing the largest degree of negative cooperativity for $(\frac{C_1}{C_{1,MIC}}) \simeq 0.2$ when $K_c = 5$. For larger concentrations, the presence of oligomers does not affect the antibacterial behaviour.

Our theoretical predictions in Figure 4 can be explained using the following arguments. For relatively small concentrations C_1 and C_2 the oligomerization reaction creates H species with comparable concentrations. This is the situation that leads to the largest heterogeneity [43], yielding the strongest positive cooperativity for $\Delta E > 0$, or the strongest negative cooperativity for $\Delta E < 0$. At the same time, for larger C_1 and C_2 concentrations larger amounts of hetero-oligomers are

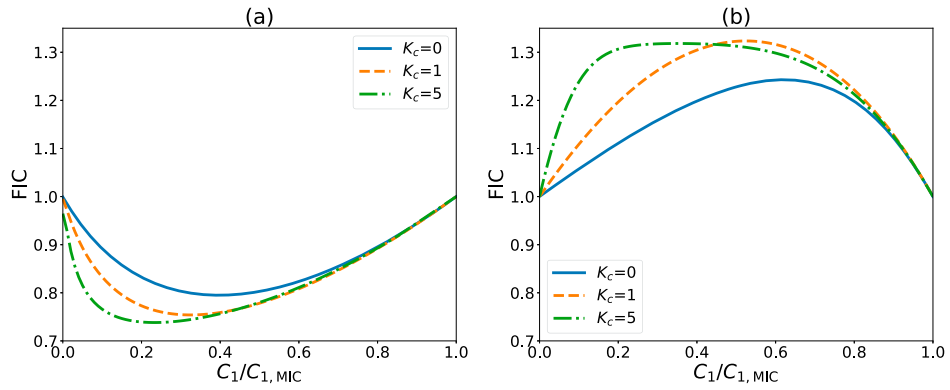

Figure 4. FIC parameters as a function of the normalised concentration of AMPs of type 1 for different inter-molecular interactions: (a) $\Delta E = 10$, and (b) $\Delta E = -10$. In calculations, the following parameters have been used: $N = 10$, $\lambda = 1/20$ min^{-1}, $k_1 = 2\lambda$, $k_2 = 5\lambda$, and $k_3 = 10\lambda$, as in [43].

produced for $K_c \gg 1$, and these species dominate the anti-bacterial behaviour, lowering the heterogeneity and producing smaller degrees of positive or negative cooperativity. This is exactly the behaviour observed in Figure 4.

To make these arguments more quantitative, we notice that the maximal heterogeneity is achieved when $C_1 \sim C_2 \sim H$ [43], or taking into account the equilibrium and normalisation,

$$\left(\frac{C_1}{C_{1,MIC}}\right) \sim \left(\frac{C_2}{C_{2,MIC}}\right) \sim K_c \left(\frac{C_1}{C_{1,MIC}}\right)\left(\frac{C_2}{C_{2,MIC}}\right), \tag{21}$$

which leads to the estimate of the condition of the strongest positive or negative cooperativity,

$$\left(\frac{C_1}{C_{1,MIC}}\right)_{strong} \sim \frac{1}{K_c}. \tag{22}$$

This estimate explains well the observations in Figure 4: for $K_c = 5$ it gives $(\frac{C_1}{C_{1,MIC}})_{strong} \sim 0.2$. Thus, the oligomerization has the strongest effect when it leads to comparable concentrations of all AMP components in the system because it corresponds to the largest heterogeneity in the system.

Figure 5 shows the result of our theoretical calculations for the FIC parameters for different degrees of oligomerization. We found that for antagonistic killing effects ($\Delta E < 0$, dotted green curves), the anti-bacterial behaviour is always antagonistic, and increasing K_c makes the negative cooperativity even stronger. Similarly, for synergistic killing effects ($\Delta E > 0$, solid blue curves), the anti-bacterial behaviour is always synergistic, and increasing K_c makes the positive cooperativity even stronger. Without inter-molecular interactions ($\Delta E - 0$, dashed orange curves), the FIC is always equal to unity, which corresponds to the additive behaviour. In this case, varying the degree of oligomerization does not affect the anti-bacterial behaviour of the system that always remains additive.

We can also explicitly estimate the concentrations of AMP components at which the bacterial growth will stop, as illustrated in Figure 6 for different interactions parameters ΔE and different bacterial cell division rates λ. In all situations, with faster bacterial growth, larger concentrations of AMP components 1 and 2 are required, as expected. However, synergistic bacterial killing ($\Delta E > 0$, Figure 6(a)) usually requires much lower numbers of antimicrobial peptides than for the antagonistic cases ($\Delta E < 0$, Figure 6(c)).

Our theoretical analysis suggests that it is critically important to utilise a proper method of evaluating the degree of cooperativity in the AMPs systems with reversible oligomerization. When considering only the concentrations of monomeric AMP species, one might erroneously predict that the reversible formation of AMP oligomers always moves the systems in the direction of more positive cooperativity, independently of the inter-molecular interactions. However, this is an artifact of neglecting the effect of H species. Applying a more comprehensive method of estimating the FIC parameters that account for the antibacterial action of all participating AMP molecules, clarifies the microscopic picture.

The obtained results also demonstrate that increasing the equilibrium constant K_c first enhances oligomerization, producing more hetero-oligomers (H). This increases the heterogeneity of the system, allowing for more efficient bacterial killing. However, stronger oligomerization ($K_c \gg 1$) eventually leads to a decrease in the amount of free AMP species, which lowers heterogeneity and slows the bacterial killing. This interplay suggests that an optimal balance exists where sufficient

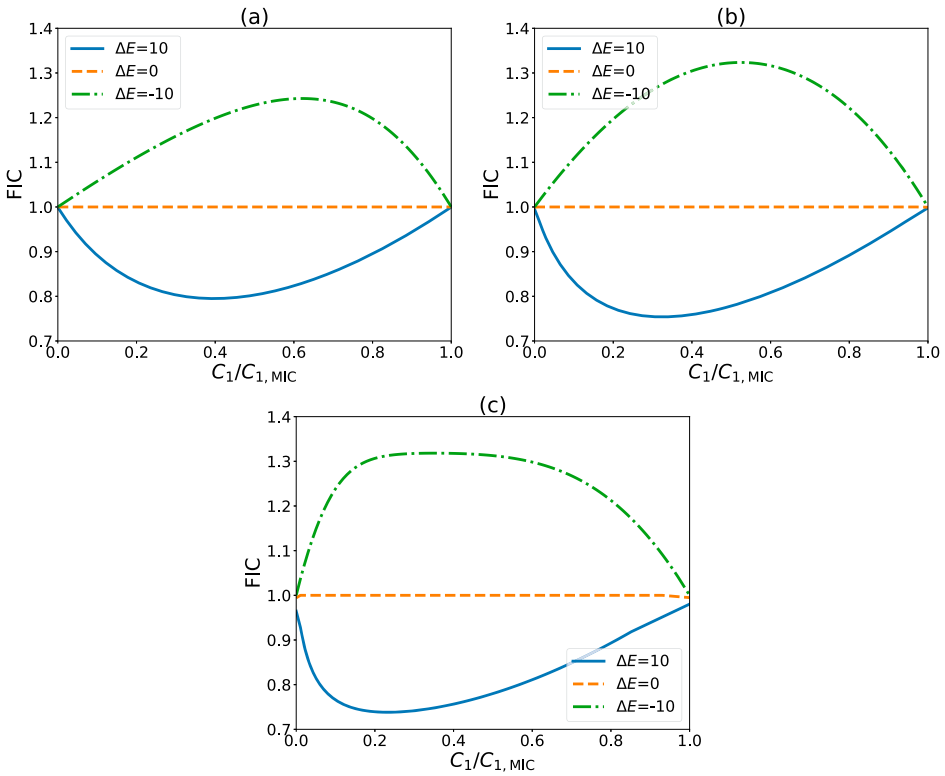

Figure 5. FIC parameters as a function of the normalised concentrations of AMPs of type 1 for different degrees of oligomerization: (a) $K_c = 0$, (b) $K_c = 1$, (c) $K_c = 5$. In calculations, the following parameters have been used: $N = 10$, $\lambda = 1/20$ min^{-1}, $k_1 = 2\lambda$, $k_2 = 5\lambda$, and $k_3 = 10\lambda$, as in [43].

oligomerization occurs to enhance bacterial inhibition without overly depleting free AMP species, as reflected in the overall cooperativity.

4. Summary and conclusions

We investigated the role of reversible oligomerization of AMP species in eliminating bacterial infections by extending the original chemical-kinetic approach to account for the activities of hetero-oligomer species. It is found that, surprisingly, the evaluation of the degree of synergy in these systems strongly depends on the method. If one only follows the concentrations of monomeric AMP molecules, then the predictions are that oligomerization will always make the systems more positively cooperating, independently of the sign of inter-molecular interactions. However, it is argued that this result is an artifact of neglecting the anti-bacterial activities of hetero-oligomers. By using a more comprehensive method that estimates the FIC parameters, which take into consideration all AMP species, it is found that the effect of oligomerization depends on the inter-molecular interactions. We predict that oligomers make originally positively cooperating systems even more synergistic, while originally negatively cooperating systems become more antagonistic, while no effect is observed for originally additive systems.

The model highlights the critical balance between two competing factors: oligomerization and heterogeneity. While increasing oligomerization first makes the system more heterogeneous and thus more efficient in killing the bacterial cells, further increase in the equilibrium constant lowers the amount of free AMP components, slowing the elimination of bacteria. Our theoretical approach provides a microscopic picture to explain these observations. It is argued that the presence of oligomers increases the overall heterogeneity of the system which influences its antibacterial efficiency. But when the equilibrium is shifted too strongly in the direction of oligomers, the positive effects of oligomerization disappear. Our chemical-kinetic method also allows us to obtain quantitative estimates of changes in the degrees of synergy for different AMP systems.

It is also crucial to discuss the limitations of our theoretical approach. First, the same inter-molecular energies are assumed for interactions of different AMP species, while different interactions are in realistic systems. At the same time, it is expected that the physical picture of underlying processes will not change. Second, only the oligomerization process has been considered, while

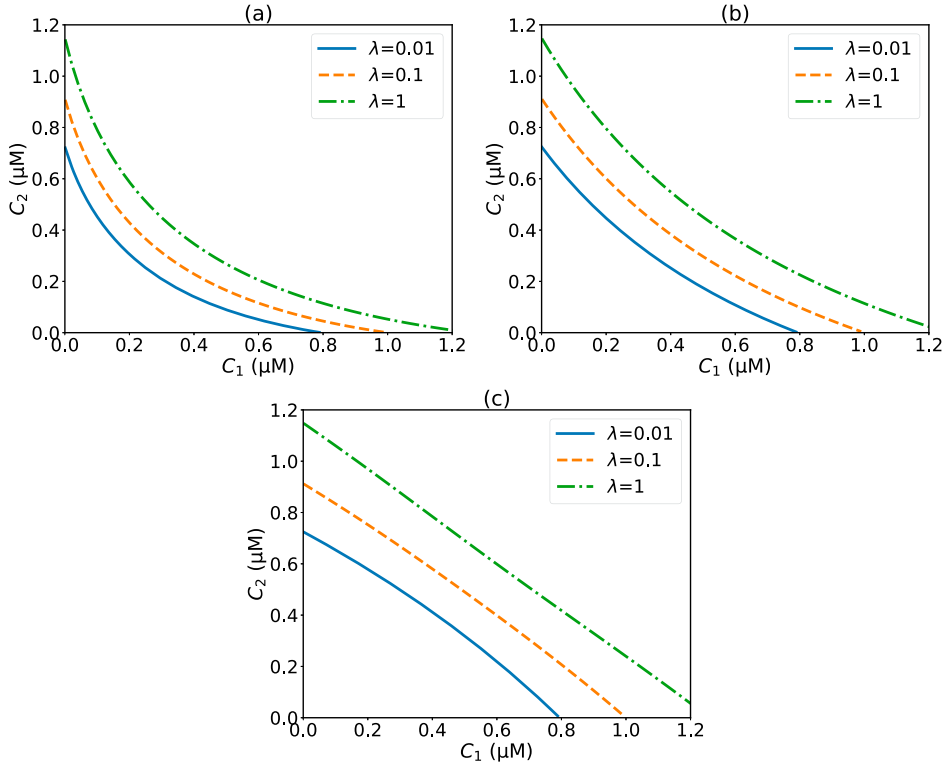

Figure 6. Concentrations of AMPs of type 1 and 2, in μM, required to stop the bacterial growth for different values of growth rates λ (in units of min^{-1}) and for different interaction parameters ΔE (in units of k_BT). (a) $\Delta E = 10$, (b) $\Delta E = 0$, (c) $\Delta E = -10$. In calculations, the following parameters have been used: $N = 10, k_1 = 2/20\ \text{min}^{-1}, k_2 = 5/20\ \text{min}^{-1}$, and $k_3 = 10/20\ \text{min}^{-1}$. It is also assumed that MICs for pure AMP components are $C_{1,MIC} = C_{2,MIC} = 1\ \mu$M, which is a typical value for antimicrobial peptides.

more complex chemical processes might take place, complicating the overall anti-bacterial activities. Third, the main assumption of our theoretical framework is that the faster association to the cellular membranes is the rate-limiting step in breaking the membranes. However, other biochemical processes might be more relevant in some systems.

In this work, we focus on dimerisation as the primary mode of AMP oligomerization, recognising that higher-order oligomerization might also occur in real biological systems. The choice of dimerisation allows for a simplified and tractable model that clarifies the molecular mechanism of these complex processes. The inclusion of higher-order oligomerization would increase the complexity of the model and add some quantitative modifications. However, it will not change our main conclusions that oligomerization increases the heterogeneity of the system, thus increasing the bacterial killing abilities of AMP systems.

Despite these issues, our theoretical approach provides a consistent physical-chemical analysis of the role of AMP oligomerization that might be used in developing more efficient antibiotic systems.

Specifically, one can improve AMP-based antibiotics by first considering whether the AMP combinations show positive or negative cooperativity. For AMPs with positive cooperativity, the tendency to create hetero-oligomers should be stimulated, but the equilibrium should not be shifted too much in the direction of the oligomers. For AMPs with negative cooperativity, hetero-oligomerization should be discouraged, since it increases antagonistic killing effects in the system, leading to enhanced antagonistic antimicrobial activity. Future theoretical studies can investigate the physical-chemical properties associated with AMPs that form hetero-oligomers in a pair vs what properties are associated with a lack of hetero-oligomerization, so that the optimal AMP combinations can be selected. Future experimental studies can also develop methods to stimulate or inhibit the hetero-oligomer formation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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