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Increasing Heterogeneity in Antimicrobial Peptide Combinations Enhances Their Synergistic Activities

Thao N. Nguyen, Hamid Teimouri, and Anatoly B. Kolomeisky*

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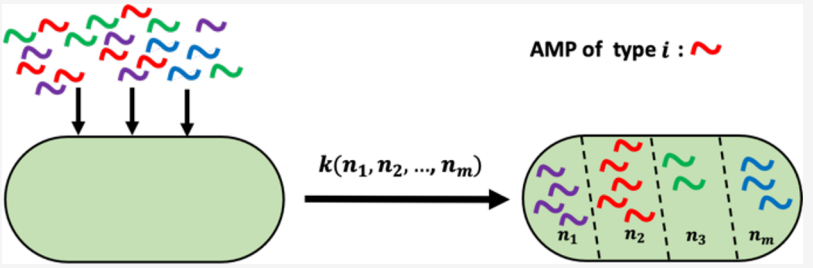

ABSTRACT: Antimicrobial peptides (AMPs) are short biopolymers produced by living organisms as an immune system defense against infections. They have been considered as potential alternatives to conventional antibiotics. Experiments suggest that combining several types of different AMPs might enhance their antimicrobial activity more effectively than using single-component AMPs. However, a clear understanding of the underlying microscopic mechanisms is still lacking. We present a theoretical investigation of antibacterial cooperativity mechanisms involving several types of AMPs. It is argued that synergy results from intermolecular interactions when the presence of one type of AMP stimulates the association of another type of AMP to bacteria. It is found that increasing the number of different AMPs in the mixtures increases the number of such interactions, making them more efficient in eliminating infections. Our theoretical framework provides valuable insights into the mechanisms of antimicrobial action.

Pathogenic bacteria remain a serious health threat because of their capability of developing mechanisms to resist antibiotics.[1−6] It has been argued that bacterial fast multiplication rates allow them to evolve rapidly, either through spontaneous mutations or via the so-called horizontal transfer of resistant genes, quickly producing antibiotic-resistant strains.[7−9] While conventional antibiotics are still considered as the main therapeutic strategy, the lack of new antimicrobial compounds severely limits the treatment of infections caused by multidrug-resistant bacteria. For these reasons, the development of alternatives to antibiotics is crucial.[10−12] In recent years, antimicrobial peptides (AMPs) have gained significant attention due to their broad-spectrum activity, rapid action, and potential to overcome bacterial resistance.[13−19] Antimicrobial peptides, also known as host defense peptides, are a diverse group of short positively charged amino acid oligomers produced by multicellular organisms as part of their innate immune systems. AMPs have been also observed to be efficient not only against bacteria but also in eliminating viruses, fungi, and even cancer.[14,20−27]

Importantly, the selective toxicity of AMPs allows them to inhibit only bacterial growth while being relatively safe for their hosts. In addition, it has been found that they are much less susceptible to developing resistance as compared to antibiotics.[13,18,19,28−30] These characteristics stimulated strong interest in studying AMPs as an alternative to conventional antibiotics. Other industrial applications of AMPs, such as in agriculture, cosmetics, and food science, have been considered as well.[19,31]

However, an important consideration for the application of AMPs as pharmaceutical drugs is minimizing possible negative side effects while optimizing their efficacy to address the evolving threat of antimicrobial resistance. Despite being reasonably safe to their hosts, long-term use of AMPs has been associated with several toxic side effects as well as with an increased likelihood of bacterial resistance.[32] Certain broad-spectrum AMPs are also known to exhibit hemolytic activities (destruction of red blood cells), preventing them from having clinical applications.[15] To overcome these disadvantages, it has been proposed to combine multiple types of AMPs. Specific "cocktails", typically containing two or more types of different AMPs, have been observed to exhibit antibacterial synergy

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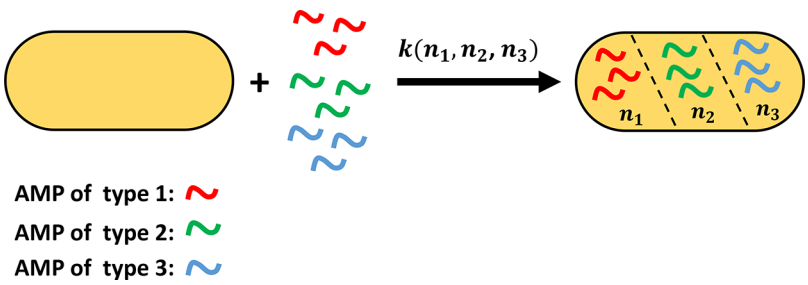

Figure 1. Schematic view of the association of 3 types of AMPs to the bacterial cell wall. Rate constant $k(n_1, n_2, n_3)$ describes cooperativity between three types of AMPs. See the text for more details.

which lowers the minimal effective dosage compared to the application of single-type AMPs.[29,33,34]

More interesting, however, is the observation that increasing the heterogeneity of AMP combinations, i.e., adding more different components to such mixtures, increases the degree of synergy. One example is the comparison of the minimal inhibitory concentration (MIC) between the application of 1, 2, and 3-AMP combinations taken from the 6 types of commercially available AMPs: cecropin A (insect), apidaecin (insect), melittin (insect), LL 19-27 (mammal), indolicidin (mammal), and pexiganan (synthetic and analogous to magainin) against *E. coli* MG1655. It was found that synergy between AMPs is a common phenomenon and that 3-AMP combinations produced more synergistic effects than 2-AMP combinations, which is quantified by a decline in MIC for each component.[35] What is even more surprising is that the total concentrations of AMPs in 1-component, 2-component, and 3-component systems were similar, but more heterogeneous mixtures exhibited much stronger cooperativity. Similar observations have been made when resistance evolution was investigated between treatments using a single type of AMP, AMP pairs, and a random AMP library consisting of more than 1 million peptides. Results obtained from the combinatorial peptide mixture achieved lower levels of resistance compared to pairs,[36] which suggests that multiple AMPs can be more effective in hindering resistance evolution. At the same time, the microscopic mechanisms of how increasing heterogeneity might lead to stronger cooperativity between AMPs remain not understood.

In this Letter, we present a theoretical study on the role of heterogeneity of AMPs combinations in stimulating antibacterial cooperativity. Our idea is to extend the recently proposed theoretical framework[37] to quantify the increasing synergy with the number of different AMPs in mixtures. In this chemical-kinetic approach, it was argued that stronger cooperativity is a result of faster associations of AMPs to bacteria which are due to stronger interactions between different complementary peptide species.[37] We show that increasing the number of AMPs corresponds to a larger number of intermolecular interactions, leading to faster binding to bacteria that eventually kills them faster. While our theoretical analysis is developed for an arbitrary number of peptide components, specific calculations to illustrate the method are performed for 3-AMP combinations. This allows us to provide a possible microscopic explanation of the role of heterogeneity in stimulating antibacterial cooperativity.

Let us consider a process of bacterial elimination by the mixtures of AMPs as schematically shown in Figure 1. The peptides must first attach to the bacterial membrane to destroy the bacterial cell. We hypothesize that the combined effect of different types of peptides is a result of intermolecular interactions that occur on the bacterial membrane. It is assumed that due to these interactions, AMPs associate with membranes faster, allowing them to kill bacteria sooner. It is hypothesized that this is the origin of the synergy in the mixtures of AMPs. We recently presented a theoretical investigation of the cooperativity for 2-AMP mixtures. Our idea here is to extend this chemical-kinetic approach to general mixtures of m different peptide components. To better explain our theoretical method, we concentrate first on 3-AMP systems ($m = 3$).

To start our analysis, let us consider a scenario in which bacteria are subjected to a mixture of three distinct types of antimicrobial peptides (AMPs). We define $C_1(t)$, $C_2(t)$, and $C_3(t)$ as the corresponding concentrations of AMPs of type 1, 2, and 3, respectively, at time t. Assuming that to kill a single bacterium, we always need N AMP molecules of any type to be associated with the bacterial membrane, one can present the following chemical-kinetic equations for processes in this system

$$\frac{dB(t)}{dt} = \lambda B(t) - \sum_{n_1,n_2,n_3} \frac{N!}{n_1!n_2!n_3!} k(n_1, n_2, n_3)[C_1(t)]^{n_1} [C_2(t)]^{n_2}[C_3(t)]^{n_3}B(t) \quad (1)$$

$$\frac{dC_1(t)}{dt} = -\sum_{n_1,n_2,n_3} n_1 \frac{N!}{n_1!n_2!n_3!} k(n_1, n_2, n_3)[C_1(t)]^{n_1}[C_2(t)]^{n_2} [C_3(t)]^{n_3}B(t) \quad (2)$$

$$\frac{dC_2(t)}{dt} = -\sum_{n_1,n_2,n_3} n_2 \frac{N!}{n_1!n_2!n_3!} k(n_1, n_2, n_3)[C_1(t)]^{n_1} [C_2(t)]^{n_2}[C_3(t)]^{n_3}B(t) \quad (3)$$

$$\frac{dC_3(t)}{dt} = -\sum_{n_1,n_2,n_3} n_3 \frac{N!}{n_1!n_2!n_3!} k(n_1, n_2, n_3)[C_1(t)]^{n_1} [C_2(t)]^{n_2}[C_3(t)]^{n_3}B(t) \quad (4)$$

In these equations, n_1, n_2, and n_3 are the numbers of AMPs of type 1, 2, and 3, respectively, associated with the bacterial membrane, and we also have a condition that $N = n_1 + n_2 + n_3$. In addition, $k(n_1, n_2, n_3)$ are effective association rates for binding exactly n_1 AMP molecules of type 1, n_2 AMP molecules of type 2, and n_3 AMP molecules of type 3.

The physical meaning of eqs 1−4 is the following. The concentration of bacteria in the system, $B(t)$, increases with the rate constant λ due to bacterial cell divisions, as described by the first term in eq 1. However, AMPs can bind to bacteria and

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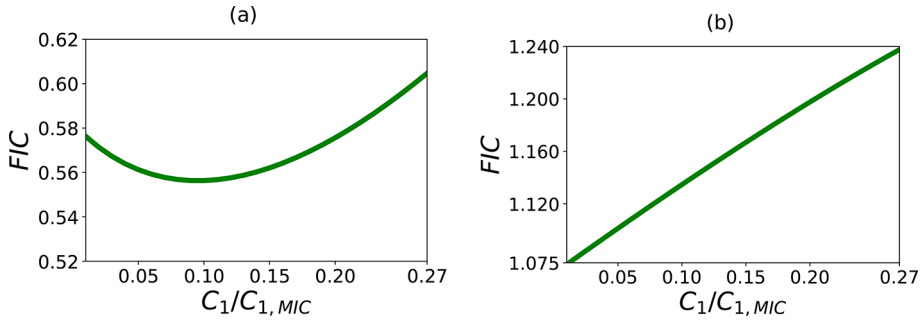

Figure 2. Fractional inhibitory concentration as a function of the normalized concentration $C_1/C_{1,MIC}$ of AMP of type 1. (a) $\Delta E = 1.5k_BT$. (b) $\Delta E = -1.5k_BT$. In these calculations, the following parameters have been used: $N = 10$, $\lambda = 1/20$ min^{-1}, $k_1 = 2\lambda$, $k_2 = 5\lambda$, and $k_3 = 10\lambda$.

kill them, decreasing their concentration, as described by the second term in eq 1. It is assumed that a total of N AMP molecules of any type are needed to kill the bacteria, but the exact location of different AMPs does not matter. This means that there are $\frac{N!}{n_1!\,n_2!\,n_3!}$ different possibilities for the association of exactly n_1 AMP molecules of type 1, n_2 AMP molecules of type 2, and n_3 AMP molecules of type 3 to the bacterial membrane. This explains the factorial multiplier and the summation over different association pathways. Simultaneously, each process of bacterial eradication removes n_1 peptides of type 1, n_2 peptides of type 2, and n_3 peptides of type 3. This is the reason for coefficients n_1, n_2, and n_3 in eqs 2, 3, and 4, respectively.

An important parameter in our theoretical approach is the effective rate constant $k(n_1, n_2, n_3)$. It reflects all possible intermolecular interactions between different AMPs that can lead to cooperativity. More specifically, it can be written as

$$k(n_1, n_2, n_3) = k_1^{n_1/N} k_2^{n_2/N} k_3^{n_3/N} \exp\left(\frac{n_1}{N}\frac{n_2}{N}\frac{N\Delta E_{12}}{k_BT} + \frac{n_1}{N}\frac{n_3}{N}\frac{N\Delta E_{13}}{k_BT} + \frac{n_2}{N}\frac{n_3}{N}\frac{N\Delta E_{23}}{k_BT} \right) \quad (5)$$

where ΔE_{ij} is the interaction energy between AMPs of type i and j ($i \neq j$ and $i, j = 1, 2, 3$) per one bound AMP molecule. It is important to note here that the effective rate constant must depend on the barriers and not the free-energy difference due to association of AMPs. However, as frequently observed for chemical reactions, we assume here that barriers and free-energy difference correlate between each other, allowing us to use ΔE_{ij} in eq 5. For simplicity, we also assume that all interactions are of the same magnitude, i.e., $\Delta E_{ij} = \Delta E$.

To quantify the existence of synergy in the system, we utilize a so-called fractional inhibition coefficient, FIC,[37,38] defined as $FIC = FIC_1 + FIC_2 + FIC_3$ where we also have

$$FIC_1 = \frac{MIC_{(1\text{ in combination})}}{MIC_{(1\text{ alone})}} = \frac{C_1}{C_{1,MIC}}$$
$$FIC_2 = \frac{MIC_{(2\text{ in combination})}}{MIC_{(2\text{ alone})}} = \frac{C_2}{C_{2,MIC}}$$
$$FIC_3 = \frac{MIC_{(3\text{ in combination})}}{MIC_{(3\text{ alone})}} = \frac{C_3}{C_{3,MIC}} \quad (6)$$

In these expressions, the MIC is a minimal inhibitory concentration at which the bacterial growth stops, i.e., when $dB/dt = 0$ in eq 1. AMP combinations with FIC < 1 exhibit a synergy and positive antibacterial cooperativity, FIC = 1 corresponds to additivity, and FIC > 1 describes antagonistic (negative cooperativity) AMP combinations.

Thus, our procedure to quantify the degree of synergy in the system is the following. Using eq 1 when $dB/dt = 0$, we estimate MICs for AMP components of the mixture and for the single-component AMPs. This will allow us to explicitly evaluate the FIC parameters. As an illustration, let us consider the simplest case when there is no intermolecular interaction, $\Delta E = 0$. Then, the effective rate constant is given by

$$k(n_1, n_2, n_3) = k_1^{n_1/N} k_2^{n_2/N} k_3^{n_3/N} \quad (7)$$

that after substitution into eq 1 for $dB/dt = 0$ leads to

$$\lambda = (k_1^{1/N} C_1 + k_2^{1/N} C_2 + k_3^{1/N} C_3)^N \quad (8)$$

while for single AMP systems, it can be shown that

$$\lambda = k_1 C_{1,MIC}^N = k_2 C_{2,MIC}^N = k_3 C_{3,MIC}^N \quad (9)$$

Combining eqs 8 and 9, we derive that

$$FIC(\Delta E = 0) = \frac{C_1}{C_{1,MIC}} + \frac{C_2}{C_{2,MIC}} + \frac{C_3}{C_{3,MIC}} = 1 \quad (10)$$

This result is expected since in the absence of intermolecular interactions no synergy or antagonism should be observed.

For the general case of AMP combinations with intermolecular interactions between different species, numerical calculations as described above can be performed for any set of parameters. The results of specific calculations for different 3-component AMP mixtures are presented in Figure 2. It shows FIC as a function of the normalized MIC concentration for component 1 ($C_1/C_{1,MIC}$) for different interaction energies with fixed values of $C_3/C_{3,MIC} = 1/3$. For attractive intermolecular interactions (Figure 2a, $\Delta E > 0$), FIC values are always less than 1, indicating positive cooperative antibacterial behavior. However, for repulsive interactions (Figure 2b, $\Delta E < 0$), we always observe FIC > 1, suggesting a negative cooperative behavior.

To understand better the microscopic picture of antibacterial action in our theoretical model, it is convenient to consider a simpler limiting case of $N \gg 1$ that allows for explicit analytical calculations of the properties of the system. It is also a realistic situation since experiments indicate a large number of AMPs are needed to kill a single bacterial cell, $N \simeq 10^4$–10^8.[14,39]

One can see that although there are many possible combinations (n_1, n_2, n_3) of AMPs that can kill the bacteria,

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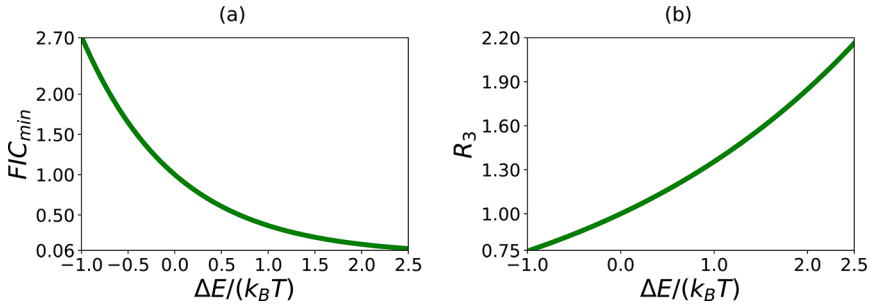

Figure 3. (a) Minimal FIC as a function of the intermolecular interaction energy. (b) The kinetic acceleration parameter R_3 as a function of the intermolecular interaction energy. Calculations are performed for the limiting case of $N \gg 1$.

for very large N, the bacterial eradication is dominated by the processes when equal amounts of each type of peptides are associated with the bacterial membrane, i.e., for $n_1 \simeq n_2 \simeq n_3 \simeq N/3$. In this case, the effective rate constant can be well approximated as

$$k(n_1 = n_2 = n_3 = N/3) \simeq k_1^{1/3} k_2^{1/3} k_3^{1/3} \exp\left[\frac{1}{3}\frac{N\Delta E}{k_B T}\right] = k_1^{1/3} k_2^{1/3} k_3^{1/3} x^{3N} \tag{11}$$

where we defined a dimensionless energetic parameter $x = \exp\left[\frac{\Delta E}{9k_B T}\right]$. Then, because for large N $\frac{N!}{\left(\frac{N}{3}\right)!\left(\frac{N}{3}\right)!\left(\frac{N}{3}\right)!} \simeq 3^N$, from eq 1, one can derive the expression from which MICs can be explicitly estimated:

$$\lambda \simeq 3^N k_1^{1/3} k_2^{1/3} k_3^{1/3} C_1^{N/3} C_2^{N/3} C_3^{N/3} x^{3N} \tag{12}$$

Comparing this equation with similar expressions for the single-component AMPs

$$\lambda = k_1 C_{1,\mathrm{MIC}}^N = k_2 C_{2,\mathrm{MIC}}^N = k_3 C_{3,\mathrm{MIC}}^N \tag{13}$$

yields the relation between the intermolecular interactions and the degree of lowering MICs in the 3-component mixture

$$x^9 = \left(\frac{C_{1,\mathrm{MIC}}}{3C_1}\right)\left(\frac{C_{2,\mathrm{MIC}}}{3C_2}\right)\left(\frac{C_{3,\mathrm{MIC}}}{3C_3}\right) \tag{14}$$

From Figure 2a, one can see that there is a minimal FIC value that can be achieved for any given interaction energy. It can be argued that for large N it can be approximated when $\frac{C_{1,\mathrm{MIC}}}{3C_1} \approx \frac{C_{2,\mathrm{MIC}}}{3C_2} \approx \frac{C_{3,\mathrm{MIC}}}{3C_3}$. This can be associated with the highest degree of synergy, and using eq 14 it can be shown that

$$\mathrm{FIC}_{\mathrm{minimal}} \simeq \frac{1}{x^3} = \exp\left[-\frac{\Delta E}{3k_B T}\right] \tag{15}$$

This parameter is explicitly evaluated in Figure 3a for the $N \gg 1$ limiting case. It shows that increasing attractive intermolecular interactions leads to stronger cooperativity as quantified by lowering the minimal FIC values.

There is another way to quantify cooperativity in three-component AMP combinations by defining a dimensionless kinetic parameter R_3

$$R_3 = \left(\frac{\sum_{n_1,n_2,n_3} \frac{N!}{n_1!\,n_2!\,n_3!} k(n_1, n_2, n_3)\left[\frac{C_1}{3}\right]^{n_1}\left[\frac{C_2}{3}\right]^{n_2}\left[\frac{C_3}{3}\right]^{n_3}}{k_1 C_1^N}\right)^{1/N} \tag{16}$$

It specifies how much faster a single AMP molecule binds to the bacterial membrane from the 3-component solution in comparison with the association from the single-AMP solution. The coefficient 1/3 in the concentrations appears because the AMP combination is obtained by mixing equal volumes of the solutions with AMPs of type 1, 2, and 3, as typically done in experiments.[33,37] The idea of this approach is that synergy is observed for $R_3 > 1$, while $R_3 = 1$ corresponds to the additivity and $R_3 < 1$ is for the antagonistic AMP mixtures. In other words, in synergistic systems, AMPs associate quicker with the bacterial membranes, allowing them to eliminate infections faster. Again considering the limit of $N \gg 1$, the kinetic parameter can be analytically evaluated, yielding

$$R_3 \simeq x^3 = \exp\left[\frac{\Delta E}{3k_B T}\right] \tag{17}$$

Figure 3b shows the results of specific calculations for the kinetic parameter R_3 for different interaction energies. AMPs associate faster with the bacterial membrane for larger attractive interactions ($\Delta E > 0$), while for negative interactions ($\Delta E < 0$) the associations are slower. One can clearly see the correlations between the degree of cooperation and the kinetic association rates for AMPs, in agreement with our theoretical hypothesis that synergy is governed by the speed of AMPs' associations to the bacterial membranes.

We can now generalize our theoretical approach for mixtures with m AMP components. In this case, the chemical-kinetic equation to describe the temporal evolution of bacterial concentration is given by

$$\frac{dB(t)}{dt} = \lambda B(t) - \sum_{n_1,n_2,\ldots,n_m} \frac{N!}{\prod_{j=1}^m n_j!} k(n_1, n_2, \ldots, n_m) \prod_{j=1}^m C_j^{n_j}(t) B(t) \tag{18}$$

with the condition that $n_1 + n_2 + \cdots + n_m = N$. The effective rate constant, which contains all the information about intermolecular interactions, can be written as

$$k(n_1, n_2, \ldots, n_m) = \left(\prod_{i=1}^m k_i^{n_i/N}\right) \varepsilon(n_1, n_2, \ldots, n_m) \tag{19}$$

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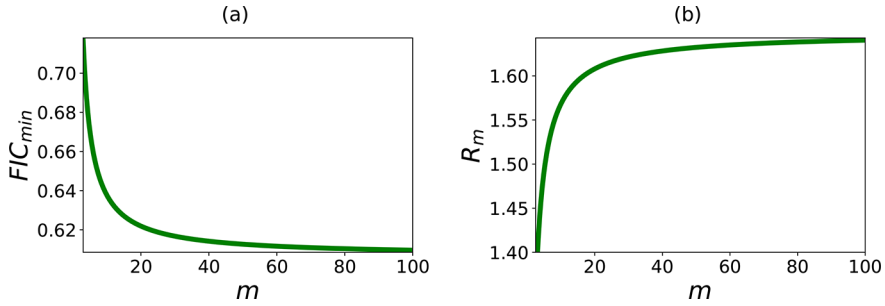

Figure 4. (a) Minimal FIC as a function of the number of components m in the combination of AMPs. (b) Kinetic parameter R_m as a function of number of components m in the combination of AMPs.

where we again assume equal interaction energies for all different AMP pairs ($\Delta E_{ij} = \Delta E$), and the energetic factor ε is equal to

$$\varepsilon(n_1, n_2, ..., n_m) = \exp\left[\frac{N\Delta E}{k_B T}\sum_{i<j}^{m}\frac{n_i}{N}\frac{n_j}{N}\right] \tag{20}$$

The degree of antibacterial specificity for the m-component system is now specified as

$$\mathrm{FIC} = \sum_{j=1}^{m}\frac{C_j}{C_{j,\mathrm{MIC}}} \tag{21}$$

where C_j is MIC for the AMPs of type j in the m-component mixture, while $C_{j,\mathrm{MIC}}$ is MIC for a single-component solution of AMPs of type j.

Again, for understanding the microscopic picture of antibacterial cooperativity, it is more convenient to consider the limit of $N \gg 1$. This allows us to obtain analytical expressions to quantify the synergy between AMPs. In this case, the dominating bacterial elimination process is taking place for $n_1 \simeq n_2 \simeq \cdots \simeq n_m \simeq N/m$. We define a dimensionless energetic parameter

$$x_m = \exp\left[\frac{\Delta E}{m^2 k_B T}\right] \tag{22}$$

and the effective rate constant can now be written as

$$k(n_1 = n_2 = ... = n_m = N/m) \simeq \left(\prod_{j=1}^{m} k_j^{1/m}\right)\exp\left[\frac{(m-1)}{2m}\frac{N\Delta E}{k_B T}\right] = \left(\prod_{j=1}^{m} k_j^{1/m}\right)x^{Nm(m-1)/2} \tag{23}$$

Then, MICs for the m-component AMP mixture can be found from eq 18 by taking $dB/dt = 0$ and accounting for

$$\frac{N!}{\left(\frac{N}{m}\right)!\left(\frac{N}{m}\right)!...\left(\frac{N}{m}\right)!} \approx m^N \tag{24}$$

which leads to

$$\lambda \simeq m^N\left(\prod_{j=1}^{m} k_j^{1/m}\right)x_m^{Nm(m-1)/2}\left(\prod_{j=1}^{m} C_j^{N/m}\right) \tag{25}$$

At the same time, for pure AMP components, it can be shown that

$$\lambda = k_j C_{j,\mathrm{MIC}}^N, \quad \text{for } j = 1, \ldots, m \tag{26}$$

Combining eqs 25 and 26, we obtain the expression that relates intermolecular interactions and MICs of the AMP components

$$x_m^{m^2(m-1)/2} = \prod_{j=1}^{m}\left(\frac{C_{j,\mathrm{MIC}}}{mC_j}\right) \tag{27}$$

One can see that for $m = 3$ it reduces to eq 14 that was derived for three-component AMP mixtures.

As a measure of the degree of synergy, we can now estimate the minimal FIC that can be achieved in m-component systems. In this case, all $C_j/C_{j,\mathrm{MIC}}$ are approximately the same, and using eq 27 we obtain

$$\mathrm{FIC}_{\mathrm{minimal}} \simeq \frac{1}{x^{m(m-1)/2}} = \exp\left[-\frac{(m-1)}{2m}\frac{\Delta E}{k_B T}\right] \tag{28}$$

For $m = 2$, it reproduces the result obtained earlier in ref 37, while for $m = 3$ we recover, as expected, eq 15.

For the m-component systems, we can also define the kinetic acceleration parameter

$$R_m = \left[\frac{\sum_{n_1,n_2,...,n_m}\frac{N!}{\prod_{j=1}^{m} n_j!}k(n_1, n_2, ..., n_m)\prod_{j=1}^{m}\left[\frac{C_j}{m}\right]^{n_j}}{k_1 C_1^N}\right]^{1/N} \tag{29}$$

Considering this quantity in the limit of very large N allows us to obtain a simple expression

$$R_m \simeq x_m^{m(m-1)/2} = \exp\left[\frac{(m-1)}{2m}\frac{\Delta E}{k_B T}\right] \tag{30}$$

Figure 4 illustrates our specific calculations for minimal FIC values and for the kinetic acceleration parameter R_m. One can see that increasing the number of components (Figure 4a), i.e., increasing heterogeneity of AMP mixtures, lowers FIC, which is a clear sign of increasing synergy between the AMPs fighting bacteria. At the same time, more heterogeneous systems (Figure 4b) associate faster with the bacterial membranes, allowing them to do their work faster, which is another sign of increased cooperativity.

One of the advantages of our theoretical approach is that it can not only quantify the correlations between heterogeneity and synergy but also explain why increasing the number of AMP components leads to stronger antibacterial cooperativity. One can argue that intermolecular interactions between different peptides stimulate faster associations to the bacterial membrane, allowing for the more efficient elimination of

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infection. This means that the larger the number of such interactions, the stronger the synergy that is expected. We can explicitly estimate the number of such intermolecular interactions for different m-component AMP combinations. It is reasonable to expect that the largest number of such interactions will occur when approximately equal amounts of AMPs are mixed together, i.e., $n_j \simeq N/m$ for all $j = 1, ..., m$. One AMP molecule of the given type interacts with $(N - N/m)$ molecules of other types, and there are m types of such peptides. Then one can estimate the maximal number of contacts (MNC) as

$$\mathrm{MNC} = \frac{m(N/m)(N - N/m)}{2} = \frac{N^2(m-1)}{2m} \tag{31}$$

The coefficient 2 reflects the fact that in our procedure, we counted every interaction twice. This is the quantity that increases from $N^2/4$ for $m = 2$ to $N^2/2$ for very large m. Thus, one can see that increasing the number of AMP components m will increase the number of interactions, and this should accelerate the association of peptides with the bacterial membrane. These results fully agree with available experimental observations.[33] They also provide a microscopic explanation of why stronger heterogeneity in AMP mixtures might stimulate stronger synergy in removing bacterial infections.

In this Letter, we presented a theoretical investigation of the role of heterogeneity of AMP combinations in developing antibacterial synergy. Stimulated by experimental observations that AMP combinations with a larger number of components are more efficient antibacterial systems, a chemical-kinetic description of the processes of association of AMPs with bacterial membranes is developed. By explicitly calculating concentrations at which bacterial growth stops, it is shown that synergy is observed for attractive intermolecular interactions between different AMP species. It is also found that cooperativity correlates with kinetic rates of association, supporting our theoretical arguments that faster binding of AMP molecules governs synergy. In addition, our calculations show that increasing the number of AMP components in the system, as a measure of heterogeneity, increases the efficiency of the antibacterial action. Furthermore, microscopic arguments to explain these observations are proposed. It is argued that there are more intermolecular interactions in more heterogeneous systems, stimulating faster associations to the bacterial membranes that eventually kill bacteria.

While the proposed theoretical method provides a simple and clear physical picture of how heterogeneity is correlated to antibacterial cooperativity in AMP systems, it is also crucial to discuss the limitations of this approach. To simplify the calculations, the interaction energies between all different pairs of peptides are assumed to be the same in our theoretical model. But in real systems, they definitely differ from each other, and because energies are utilized in exponential terms, the errors due to this approximation might be significant. At the same time, we believe that while quantitative results will be modified, the main physical predictions will not change. In addition, only pairwise interactions between AMPs in the bacterial membranes are assumed, while more than two-body interactions might also play a role. But the weakest approximation of our theoretical method is the assumption that the rate-limiting step in bacterial elimination is the association with the membranes. It is very much possible that other downstream biochemical processes after AMPs bind to bacteria might govern the process of bacterial killing.[40,41] However, despite these limitations, the main advantage of our theoretical approach is the ability to explain all available experiments and to explicitly calculate some properties of the antibacterial action of AMPs that can be tested in experiments. This should advance our understanding of the molecular mechanisms of these complex biological processes.

AUTHOR INFORMATION

Corresponding Author

Anatoly B. Kolomeisky − *Department of Chemistry, Rice University, Houston, Texas 77005, United States; Center for Theoretical Biological Physics, Department of Chemical and Biomolecular Engineering, and Department of Physics and Astronomy, Rice University, Houston, Texas 77005, United States;* orcid.org/0000-0001-5677-6690; Email: tolya@rice.edu

Authors

Thao N. Nguyen − *Department of Chemistry, Rice University, Houston, Texas 77005, United States; Center for Theoretical Biological Physics and Applied Physics Graduate Program, Smalley-Curl Institute, Rice University, Houston, Texas 77005, United States*

Hamid Teimouri − *Department of Chemistry, Rice University, Houston, Texas 77005, United States; Center for Theoretical Biological Physics, Rice University, Houston, Texas 77005, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jpclett.3c02216

Notes

The authors declare no competing financial interest.

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