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Towards Sequence-Controlled Antimicrobial Polymers: Effect of Polymer Block Order on Antimicrobial Activity

Peter R. Judzewitsch, Thuy-Khanh Nguyen, Sivaprakash Shanmugam, Edgar H. H. Wong and Cyrille Boyer**

Abstract: Synthetic polymers have shown promise in combating the rise in infectious disease caused by multidrug-resistant bacteria. However, the biological effects of sequence control in synthetic antimicrobial polymers are currently not well understood. As such, we investigate the antimicrobial effects of monomer distribution within linear high-order quasi-block copolymers consisting of aminoethyl, phenylethyl, and hydroxyethyl acrylamides made in a one-pot synthesis approach via photoinduced electron transfer-reversible addition-fragmentation chain transfer polymerisation (PET-RAFT). Through different combinations of monomer/polymer block order, antimicrobial and haemolytic activities are tuneable in a manner comparable to antimicrobial peptides.

Antimicrobial resistance has recently been classified as a pressing healthcare issue by the World Health Organization.[1] The development of novel antibiotic agents is urgently required to combat this global challenge. Antimicrobial peptides (AMPs) have been recognised as a promising class of antimicrobial agent for combating multidrug-resistant bacteria due to their mode of action, namely bacterial cell wall disruption.[2] AMPs are generally described as amphiphilic chains consisting of 12-50 amino acid residues,[3] 30-50% of which are hydrophobic,[2b, 3-4] and with a variable charge of +1 to +10 from cationic residues.[5] Cationic residues allow for semi-selective binding to bacterial cells, while hydrophobic residues cause insertion into and disruption of the phospholipid cell membrane.[2b, 6] AMP activity is dependent on the precise sequence of amino acids and their secondary structures.[7] Composition and spatial arrangement of functionalities have been shown to impact selectivity in terms of bacterial vs. mammalian cell selectivity,[6a, 8] while the addition of segregated domains has allowed for bacterial genus specificity.[9] Selectivity of bacterial cells over mammalian cells is vital, while bacterial genus specificity could provide an avenue to prevent indiscriminate decimation of commensal microflora. Despite this promise, there are limitations with the use of AMPs. Precise sequence control makes production and purification of AMPs highly laborious, and troubleshooting for product discovery extremely time-consuming. As a result, AMPs are expensive to develop and produce in large quantities, and are also subject to proteolysis, which reduces their long-term stability in biological environments.[2b, 10]

Recently, synthetic polymers mimicking membrane disruptive actions of AMPs have emerged as novel antimicrobial candidates. In contrast to AMPs, complex synthetic polymers have greater potential for economic large scale manufacturing using automated processes[11] and are less susceptibile to proteolysis. Advances in polymerisation techniques have enabled the production of more complex polymer structures,[12] some of which have been investigated as synthetic antimicrobial polymers.[13] Thus far, studies have focused upon tailoring global composition of random copolymer systems, where chemical functionalities are statistically distributed over the length of a polymer chain.[14] Few studies have been performed on block copolymer arrangements, and only performed using a two monomer system, i.e. hydrophobic and cationic.[15] High order multiblock copolymers are attractive for antimicrobial applications as one can manipulate localised domain concentration within a polymer chain, potentially mimicking the functional group spatial segregation endowed by the precise monomer sequence and secondary structures in AMPs.

Polymer block order manipulation via a facile one-pot synthesis approach hence represents a step towards understanding the importance of localised monomer domain concentration in synthetic polymers.[15h, 15i, 16] Therefore, in this study, we strategically prepared a library of novel multiblock copolymers, made via a highly efficient polymerisation technique termed photoinduced electron transfer-reversible addition-fragmentation chain transfer (PET-RAFT), and screened against a range of bacteria. This study offers new insights in how bacteria specificity may be tuned using synthetic polymer chemistry.

We decided to use a ternary monomer system where the composition of polymers made was predominantly set at 50:30:20 molar ratio of cationic: hydrophobic: hydrophilic groups. Our group and others have shown that the incorporation of a hydrophilic functionality can ameliorate mammalian cell cytotoxicity without inhibiting antibacterial activities.[13j, 17] Specifically, an all acrylamide system was used with monomers *tert*-butyl (2-acrylamidoethyl) carbamate (Boc-AEAm, monomer A), 2-phenylethyl acrylamide (PEAm, monomer B) and 2-hydroxyethyl acrylamide (HEAm, monomer C) chosen to mimic the cationic, hydrophobic and hydrophilic functionalities of the amino acids lysine, phenylalanine, and serine, respectively (**SI, Figure S1**). Noteworthy, the *tert*-butyloxycarbonyl groups were removed after polymerisation to yield cationic primary amine groups.[13g, 13j] HEAm allows for the ability to modulate charge and/or hydrophobicity independently.[18] However, this benefit means there are a significant greater number of potential combinations and permutations. As such, the singular composition above was mainly used to determine the effect of monomer/polymer block placement within polymer chains.

Polymerisations were performed using a one-pot PET-RAFT technique, proceeding under environmental friendly conditions using visible light.[15k, 19] Significantly, PET-RAFT allows for facile temporal control of polymerisation, permitting easy addition of monomer as well as greater oxygen tolerance. We have prepared polymers with 3 different degrees of polymerisation (DP_n) (i.e. 20, 40 and 100). The monomer conversions and overall compositions of the polymers were estimated by NMR

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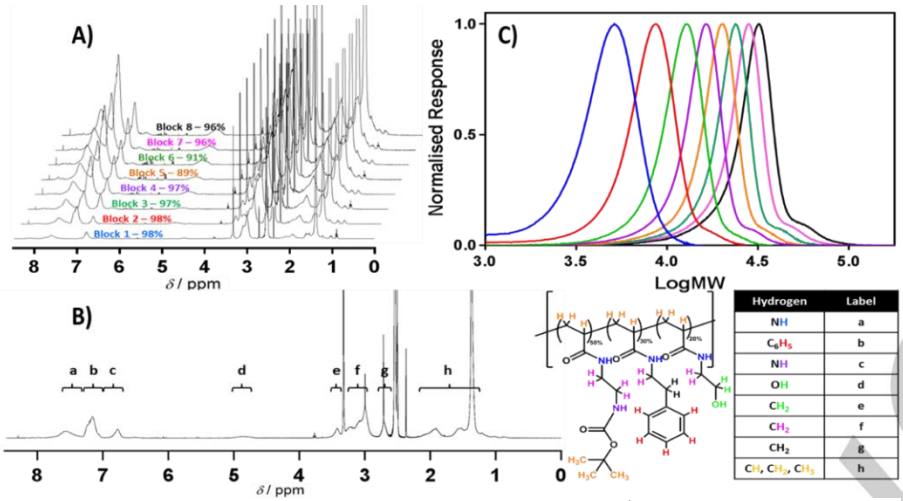

Figure 1. Synthetic evolution of quasi-octablock P21 as evidenced by (a) ^{1}H NMR spectra for each chain extension step. (b) ^{1}H NMR spectrum of the octa-polymers and attribution of signals for the different chemical groups. (c) molecular weight distribution determined by GPC.

analysis (see **Figure 1a-b** and **SI, Figure S2-58, Table S1**). The compositions calculated for the final polymers are in good agreement with the feed ratios during synthesis. To determine copolymer structures, we have also monitored monomer conversions for model co/terpolymerisation by NMR. Using the feed compositions and monomer mixtures employed for the synthesis of our antimicrobial polymers, we observed even monomer consumption during polymerisation, which confirmed that all the monomers are statistically distributed within the polymers (**SI, Figure S2-22)**.

Quasi-block copolymers were prepared without intermediate purifications and successive monomer additions were performed with a targeted monomer conversion of at least 90%. Monomer conversions at each step have been calculated via NMR (**SI, Figure S23-58 and Table S1**). Most reactions which preceded further chain extensions had conversions greater than 95%, except in a few instances. The presence of residual monomer in sequential monomer addition results in a discrepancy of less than 1 monomer unit per chain for most polymers (**SI, Table S1)**. However, considering the inherent error in radical polymerisation techniques as demonstrated by Harrison et al.,[20] we consider this to have minimal effect. As previous one-pot multiblock polymers with incomplete monomer conversions were described as quasi-block copolymers,[11d-f, 15h, 15i, 16e, 21] we decided to use this terminology in the text to reflect the slight imperfection in the polymer chains.

All polymers had dispersity (*Đ*) values in the range of 1.09-1.24 as determined by GPC analysis. These $Đ$ values are in the range reported for previous multiblock copolymers synthesised using acrylamide monomers.[15c, 15g, 15h] Furthermore, a good correlation between experimental and theoretical number-averaged molecular weight (M_n) confirmed good control over the polymerisation reactions (**SI, Table S2**). The synthesis protocol was employed to produce multiblock copolymers with a high degree of livingness as shown through discrete shifts of molecular weight in **Figure 1c,** albeit there were instances of low molecular weight tailing in multi-block formulations. We estimated the livingness of the final block copolymers using techniques described previously in the literature (**SI, Table S3, Figure S59-60**).[15h, 19c, 22] The livingness ranges from 75-90% in mol %, with one exception being at 61% for a quasi-octablock copolymer (**SI, Table S3**).

Polymers have been grouped into various families dependent on structure and monomer distributions (**Figure 2**)**.** Polymers P1-3, P7-12, and P15-30 are terpolymers with static molar composition of 50% cationic monomer A, 30% hydrophobic monomer B and 20% hydrophilic monomer C. P4-6 and P13-14 are bipolymers where the hydrophilic group has been replaced with an equivalence of cationic monomer to maintain the hydrophilic: hydrophobic molar ratio, resulting in 70% A, 30% B and 0% C. **Figure 2** shows a heat map representation of the minimum inhibitory concentrations (MICs) of polymers against three different gram-negative bacteria, including *Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumannii* and one gram-positive bacterium, i.e. *Staphylococcus aureus*. Surprisingly, despite maintaining global composition, variations in distribution of monomers within polymer chains has a significant effect on the MICs (**Table S6**).

Initially, statistical copolymers (P1-6) were made as a basis to compare any increase or decrease in efficacy brought about by variations in monomer order. The MICs of these polymers in **Figure 2** are comparable to those achieved previously in our group.[13j] Statistical ternary polymers (P1-3) with a DP_n ranging from 20 to 100 have a similar MIC against Gram-negative species (32-64 μg/mL), although activity is reduced vs. *A. baumannii* (128-256 μg/mL). Activity against *S. aureus* was poor (256 μg/mL), but improved when the DP_n is lowered to 20 (128 μg/mL). In the case of P4-6, containing no hydrophilic group, there is a marked reduction in antibacterial efficacy at 100 DP_n. For example, P4 was inactive against almost all bacteria tested (> 256 μg/mL). However, with decreasing chain length to 20 units, some activity was restored (64-128 μg/mL), including against *S. aureus* (64 μg/mL). This clearly reaffirms the importance of the hydrophilic group in synthetic antimicrobial polymers. We have previously shown that the hydrophilic group reduces unwanted protein complexation, preserving antimicrobial activity of polymers.[13j]

Next, polymers separating hydrophilic/cationic from hydrophobic domains were studied (P7-14). Previous works[15a, 15b] have investigated diblock copolymers with a binary monomer combination, however, no other studies based on ternary monomer combination and higher order multiblock structures have been reported for antimicrobial applications. The rationale was that this segregation would result in localised domains with increased activity in membrane disruption due to greater hydrophobic concentrations. Likewise, clustering cationic residues onto discrete sections was hypothesised to potentiate a greater attraction to bacterial membranes. However, **Figure 2** (P7-14) indicates that the segregation of hydrophobic domains from hydrophilic/cationic residues in both ternary and binary systems completely removed the antimicrobical activity (> 256 μg/mL). Furthermore, the reduction in polymer chain length was

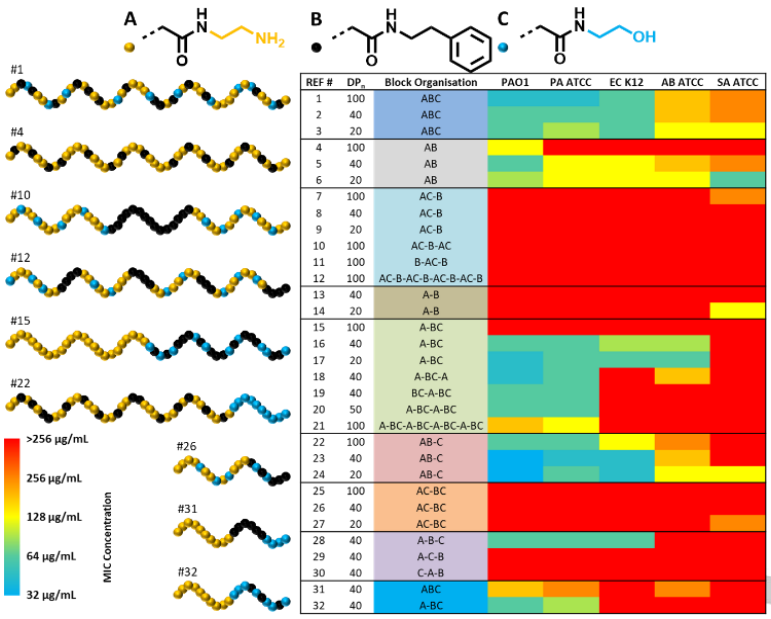

Figure 2. Heat map of MICs for all polymers synthesised in this study where the selection of polymer structures from each family is shown. The bacterial strains are *Pseudomonas aeruginosa* PAO1 and ATCC 27853 strains, *Escherichia coli* K12 strain, *Acinetobacter baumannii* ATCC 19606 strain, and *Staphylococcus aureus* ATCC 29213 strain.

unable to yield any form of antibacterial efficacy. This was attributed to the formation of stable micelles that are unable to allow hydrophobic interactions with the bacterial cell in the case of di- and tri-block copolymers. Dynamic light scattering readings (SI, **Table S4**) of P7-8 & 10-11 confirm the formation of micelles, however P9 & 12-14 do not appear to form stable structures, indicating a dependency on DP_n of hydrophobic block as well as number of blocks. In AMP studies, high hydrophobicity can result in a predisposition for self-interactions and reduce antimicrobial activity.[6e, 23] Also, in some antimicrobial polymer studies, the self-assembly of polymers in solution removes the antimicrobial activities.[14a, 24] In previous work,[15a-c] less hydrophobic monomers were used allowing block structures to maintain antimicrobial efficacy. This suggests that the type of hydrophobic monomers is an important consideration for antimicrobial polymers with clustered/block formations, especially with highly hydrophobic functional groups such as phenylethyl.[25]

As a consequence, we decided to incorporate hydrophilic groups within the hydrophobic block, whilst maintaining a separate cationic block (P15-21). This was to determine the effect of an amphiphilic domain on the MIC, while maintaining complete separation of cationic and hydrophobic groups. Results indicate that antimicrobial efficacy was dependent on chain length with the 100 DP_n P15 & 21 having no or reduced activity, respectively. P16-17 maintained high efficacy against Gram-negative bacteria, including an increased activity vs. *A. baumannii* (64-128 and 64 µg/mL) compared to their random equivalents P2-3 (128 and 128-256 µg/mL). We decided to increase the number of blocks moving from diblocks to triblocks and 4-blocks (P18-20). Interestingly, P18-20 showed a reduction in activity against *E. coli* and *A. baumannii* whilst maintaining activity vs. *P. aeruginosa* strains (64 µg/mL). This bacterial specificity towards both *P. aeruginosa* strains is surprising for synthetic polymers as genus specific antimicrobial activity has previously been reported within AMP studies[9] but never for synthetic polymers. The quasi-octablock P21 also showed a level of bacterial specificity; however, the overall action was reduced (128-256 µg/mL). This could be attributed to the DP_n rather than the level of organisation as 40 and 20 DP_n polymers tend to consistently have the most activity. Noteworthy, P15-21 have no activity toward *S. aureus*.

Next, we decided to copolymerise hydrophobic and cationic functionalities in one block, with the hydrophilic monomer segregated as a separate entity (P22-24). This combination revealed activity against Gram-negative species comparable to P1-3 (32-64 µg/mL for *P. aeruginosa* and *E. coli*), including the activity against *A. baumannii* for shorter chain polymers (128-256 µg/mL). Once again, however, there is no activity against *S. aureus* (>256 µg/mL).

We then decided to distribute the hydrophilic monomer amongst separate cationic and hydrophobic blocks (P25-27). Hydrophilic monomer was copolymerised in each block, aliquoted according to the overall cationic to hydrophobic ratio. Interestingly, no antimicrobial activity was observed despite having a block comprised of both hydrophobic and hydrophilic monomers analogous to P16-21. Here it is postulated that hydrophobic interactions were not disrupted sufficiently which will limit the polymers' ability to interact with cell membranes.

The final tests using the preselected polymer composition focused on complete separation of all 3 monomers into separate segments (P28-30). For this, a single DP_n of 40 was selected to ensure the best chance of antimicrobial capability as short polymers present higher activity based on our earlier results. In this instance, only P28 showed any antimicrobial effect, which was comparable to other polymers against *P. aeruginosa* and *E. coli* only (64 µg/mL). This polymer had one long cationic end and one short hydrophilic end sandwiching a completely hydrophobic section. This seems to provide adequate disorder of hydrophobic interactions to prevent stable micelle formation, and allow for membrane interaction and disruption.

Based on the bacterial specificity which eventuated when hydrophobic monomers were halved and copolymerised with hydrophilic monomers in P19-21, composition was varied to halve the overall hydrophobic monomer (P31-32) and targeting 40 DP_n. The difference was made up using hydrophilic monomer such that cationic mol% would remain constant at 50 mol%. The rationale is that it would determine if halving the hydrophobic content in a statistical configuration would maintain similar activity and/or specificity. When divided into blocks containing a separate cationic section and an amphiphilic section similar to P16-21, this would simultaneously satisfy a localised 70:30 hydrophilic to hydrophobic ratio in the amphiphilic quasi-block. P31 had widespread reduced MIC activity, indicating that the global reduction in hydrophobic functionalities was not enough to impart selectivity or specificity and instead reduced antimicrobial activity. The diblock P32, however, maintains activity vs. *P. aeruginosa* (64-128 µg/mL) and reduced activity against other species, comparable to P19-20. This is attributed to the reduction in hydrophobicity of this block while maintaining a

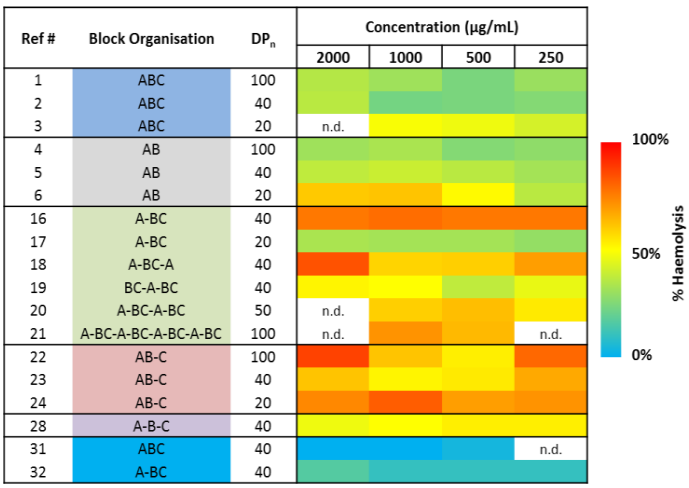

Figure 3. Haemolysis heatmap of polymers showing antimicrobial activity.

localised 70:30 ratio of hydrophilic to hydrophobic groups and a high concentration of cationic functionalities in the second block.

In addition to antimicrobial tests, haemolysis was performed to ascertain the mammalian cell compatibility of polymers with low MIC values using sheep red blood cells (RBCs). For those with antimicrobial activity, haemolysis was tested at polymer concentrations varying from 250 to 2000 μg/mL. **Figure 3** and **SI, Table S5** show the average haemolysis %, high values indicate low biocompatibility. Typically, the HC_{50} level (defined as the sample concentration at which 50% of RBCs are lysed) is used as a marker for biocompatibility.[26] Agglutination was determined visually post haemolysis when the RBC pellet was resuspended. Once again, polymers tested have been grouped according to the predefined families in **Figure 2**.

Initially, P1-3 & 4-6 were tested to determine a baseline haemolysis of the composition used. In the case of statistical P2-3, haemolysis was seen to increase (from 27% to 52% at 1000 μg/mL) with a reduction in DP_n (from 40 to 20), as shown in **Figure 3**. For 100 and 40 DP_n P1-2, haemolysis was comparable and under the HC_{50} level for all concentrations. Likewise, RBCs could be resuspended easily after tesing, indicating a lack of agglutination and good biocompatibility. However, P4-6 had haemolysis increasing (from 35% to 41% to 63% at 2000 μg/mL) as DP_n was reduced (from 100 to 40 to 20, respectively). In addition, at higher concentrations of P4-6, RBCs could not be easily resuspended (**SI, Table S5**) indicating agglutination of cells, attributed to the absence of hydrophilic groups.

For P16-21, where the cationic block was separated from hydrophilic/hydrophobic block, almost all resulted in increased haemolysis in contrast to P1-3. Copolymerising into a hydrophobic/hydrophilic block results in a localised composition of 60 mol% hydrophobic and 40 mol% hydrophilic monomers, despite maintaining a global polymer composition. Such an increase in hydrophobic content has previously been shown to be haemolytic,[14b] although this was on a global composition scale. This family of polymer also had widespread agglutination (SI, **Table S5**) probably due to high local concentration of cationic species, which is consistent with previous report.[15c] As an exception, P17 shows reduced haemolysis (33-37%) when compared to its random equivalent (P3, 45-52%). In this case, the hydrophobic/hydrophilic block is only 10 DP_n, which is possibly too short to sufficiently disrupt RBC membranes. However, agglutination still occurred as the RBC pellet was difficult to resuspend, once again likely due to a large cationic block. Despite the general increase in haemolysis, there is evidence of variation within these polymers. P19 showed less haemolysis (41-55%) compared with other 40 DP_n blocks of similar compositions (57-85%). P19 has telomeric hydrophobic/hydrophilic block ends surrounding a large cationic centre. Halving and separating the membranolytic segments may provide greater modulation of hydrophobicity and thus lessen haemolytic activity. In contrast, P18 was the opposite, with a long hydrophobic/hydrophilic centre block and two short cationic tags resulting in greater haemolysis (61-85%) than P19.

The final family of active antimicrobial P22-24 were above the HC_{50} level for all DP_n ranges as shown in **Figure 3** (55-88%). Here, copolymerising hydrophobic and cationic monomers has created a localised cationic and hydrophobic block with a 50:30 molar ratio, as opposed to the 20:30 split of P16-21. This should provide greater modulation of hydrophobicity. Instead, combined with the hydrophilic block on one end, a further increase in haemolysis was observed. This indicates that modulation of hydrophobicity is better achieved through neutral hydrophilic monomers rather than cationic species.

Lastly, a reduction in haemolysis was shown for P31-32 with modulated composition as described previously, namely with a 70:30 local hydrophilic to hydrophobic ratio in one block. Reducing hydrophobic content is known to minimise haemolytic activity and no haemagglutination was observed. When viewed with the highly specific action of P32 against *P. aeruginosa,* this indicates that localised block composition can be targeted for bacterial genus specificity and reduced haemolysis. This highlights the benefits of adding a third neutral hydrophilic monomer species to decouple hydrophobicity from cationicity such that localised compositions may be tailored appropriately.

In summary, PET-RAFT was successfully employed to make a library of 32 well-defined multiblock copolymers comprised of three key components (i.e., cationic, hydrophobic and hydrophilic groups) in a one-pot approach. This study has shown that bacteria genus specificity can be tuned simply via the order of polymer blocks and to some extent via the combined modulation of polymer chain length. Manipulating blocks to contain localised cationic segments coupled with an amphiphilic hydrophilic-*stat*-hydrophobic section showed specific action vs. *P. aeruginosa*. Furthermore, antimicrobial activity and haemolytic activity are dependent on distribution of monomers within blocks. Indeed, the localised ratio of hydrophobic to hydrophilic functional groups within amphiphilic sections appears to be a critical factor to influence biocompatibility as well as antimicrobial activity. This shows that tailoring individual block structures rather than global composition may yield more specific biological outcomes akin to those induced by the precise monomer sequence in AMPs.

Experimental Section

Experimental details may be found in the Supporting Information

Keywords: antimicrobial, multiblock, quasi-block, sequence-controlled, polymers, RAFT polymerisation

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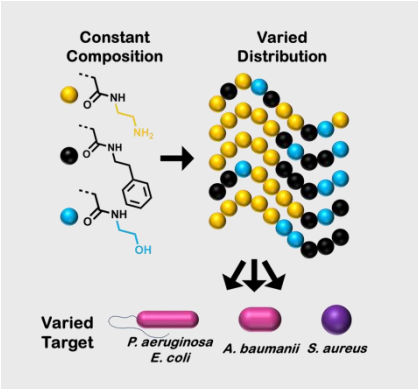

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