Multivalent Antimicrobial Peptides from a Reactive Polymer Scaffold

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Abstract: We report an application of the principle of multivalency to create new antimicrobial agents using the reactive polymaleic anhydride (PMA) chain to link antimicrobial tetrapeptides to afford multivalent variants containing ∼40 monomer units. Relative to the free peptides, the product shows a 10-fold improvement in IC_{50} without provoking more severe hemolysis of red blood cells. Thus, multivalency or polyvalency may offer a route to enhance the activity of antimicrobial peptides.

As multidrug-resistant bacterial strains emerge in increasing numbers, the need to identify different kinds of antibiotics is growing. For many years, a wide variety of antimicrobial peptides (AMPs) secreted by multicellular organisms in response to infection by foreign viruses, bacteria, or fungi[1,2] have been considered as a potential source of new antibiotics but with limited practical success. As prospective antibiotics, AMPs offer a broad-spectrum effect that is indifferent to much of the drug resistance provoked by repeated exposure to standard antibiotics such as penicillins.[3,4] However, their relatively high inhibitory concentrations, sensitivity to salts, and cytotoxic effects have limited their utility to topical applications.[5] Different strategies have been pursued in efforts to increase the effectiveness of AMPs. Sequence changes in natural peptides can notably reduce hemolysis while preserving activity.[6] Inserting unnatural D-amino acids or β-amino acids into peptide sequences, combinatorial designs based on linear or cyclic sequences,[7−10] synthetic chemical mimetics,[11−14] and dendrimeric constructs of short peptides[15,16] are other alternatives.

Our approach for designing AMPs is based on their proposed mechanism of action. Several lines of evidence suggest that AMPs act on bacterial membranes.[2,17−24] Cells become permeable to large solutes following exposure to peptides, and their membrane potential is lost.[25] While the actual target and mode of action of AMPs are incompletely understood, current models emphasize the need to coat a significant fraction of the membrane surface to produce a lethal effect (Figure 1A). In some models, several peptide monomers associate to form a complex that inserts itself into the bilayer to create a transmembrane pore.[17,26,27] In others, peptide monomers coat the target membrane surface extensively before sections of the membrane split off as vesicles and destroy the integrity of the membrane.[28] Either model accounts for relatively high local density and synergy of monomeric peptides (Figure 1B). Initial attachment of AMPs to bacterial surfaces or membranes seems to be mediated by weak electrostatic interactions.[22] General thermodynamic considerations suggest that tethering numbers of weakly interacting ligands can enhance overall avidity for a target such as a cell surface.[29−32]

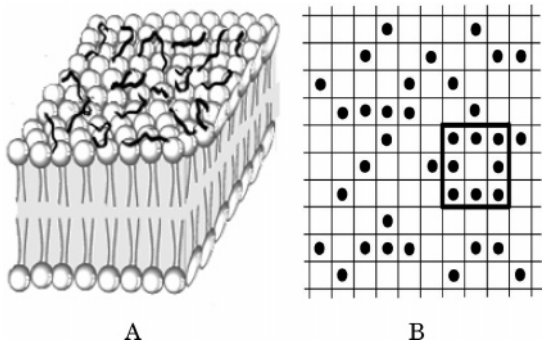

Figure 1. (A) Highly schematic view of initial binding of AMPs to a bacterial membrane. (B) Lattice model of surface coverage by AMPs showing relatively high local density and the formation of a critical sized cluster.

One argument in favor of multivalent designs for antimicrobial peptides seems straightforward: if a critical nucleus is essential for killing bacteria, then it might be possible to overcome the threshold requirement by prenucleating a number of appropriately spaced and oriented monomeric peptides. Conversely if extended but delocalized coating of target membranes is necessary, no effects of tethering at all need result, regardless of size or topology. Given the uncertainty regarding the target, mode of binding, and lack of structural or sequence relationship among antimicrobial peptides, it seems worth investigating basic aspects of multivalency in the mode of action of these molecules.

Multivalent strategies offer the potential of reducing the concentration of AMPs if the avidity of the complex can be increased relative to that of the monomeric peptide. In many cases the overall killing concentration of AMPs is close to that for inflicting damage on host cells or tissues, as detected by hemolysis assays, for example.[33] The question we address here is whether a significant gain in killing concentration can be achieved by covalently linking large numbers of monomeric antimicrobial peptides without compromising hemolytic values. An appropriate measure of effectiveness of a peptide or construct is the hemolytic index HI (also referred to as the membranolytic selectivity index), defined as the ratio of the concentration that produces 50% hemolysis (HD_{50}) to the concentration that inhibits bacterial growth by 50% (IC_{50}): $HI = HD_{50}/IC_{50}$.[34]

Here, we explore designs of multivalency for enhancing the activity of AMPs by covalently linking them on linear polymer scaffolds. This strategy has been used previously in multivalent designs.[30] In this report, we use a reactive polymaleic anhydride (PMA) chain to link highly truncated antimicrobial peptides, RRWW−NH_2 and RWRW−NH_2, to form multivalent complexes. Known AMPs[35] cover a wide range of size, sequence, and structure, sharing only amphipathicity and positive charge.[1,3] Tryptophan and arginine are frequent among known AMPs;[36] and even short R,W rich peptides have been found to have antimicrobial activity.[7,36−38] Recently R- and W-containing AMPs and even dipeptides with modified W side chain have been reported to be active antibacterial agents.[39,40] The monomers used in this work are tetrameric peptides RWRW−NH_2 and RRWW−NH_2, which have moderate activity and were designed with a balanced content of charged and bulky/lipophilic groups with an amidated C termini.

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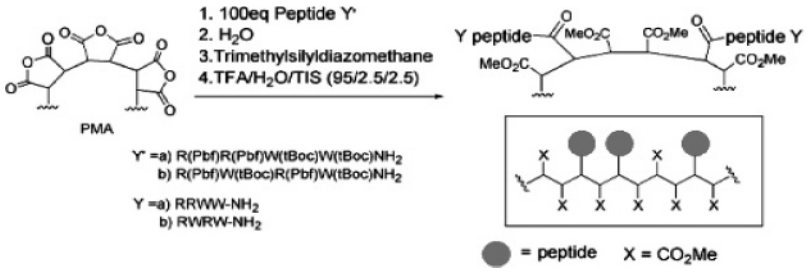
Scheme 1. Synthetic Scheme for Constructing Multivalent AMPs Based on Arg-Trp Tetramers and a Reactive Polymer, PMA[a]



[a] Unreacted groups on the polymer are capped by carboxymethylation. PMA control was generated by the addition of $MeNH_2$ instead of peptides without taking step 4.

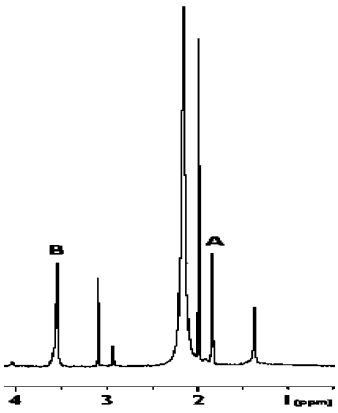


Figure 2. Composition analysis of multivalent AMPs by ^{1}H NMR (Bruker 400 MHz, acetonitrile-d_3). The chemical shifts used for composition analysis were peak A at 1.80 ppm (assigned to be β-CH_2 of Arg residue in peptides) and peak B at 3.50−3.70 ppm (assigned to be CO_2Me).

The polymer used here is PMA; a copolymer of polyethylene and maleic anhydride (PEMA) has been used successfully to display sugars for recognition by a cell surface protein, the lectin concanavalin A.[41]

Peptide monomers were synthesized using standard FMOC solid-phase chemistry, and the N termini were capped for comparison with the polymeric species. PMA was supplied with an average molecular weight of 5000, or 50 maleic anhydride units per chain (according to manufacturer specifications). The multivalent AMPs were derived by conjugation of the maleic anhydride moieties with the free N termini of monomeric peptides in which all side chains were protected to avoid cross-linking. After the reaction was quenched with H_2O, the resulting carboxylate groups from unreacted or reacted anhydride were methylated by excess trimethylsilyldiazomethane (the stepwise reaction is summarized in Scheme 1). The final polymer product has a series of branches that are peptide or carboxymethyl groups (free carboxylate groups were not detected), confirmed by ^{1}H NMR analysis as described in Supporting Information. As a control for the polymer backbone itself, a sample of the polymer was fully substituted by addition of methylamine instead of peptide.

The monomer ratio in each polymer was calculated by integration of ^{1}H NMR resonances: peak A at 1.80 ppm (assigned to β-CH_2 of Arg side chains in peptides) and peak B at 3.50−3.70 ppm (assigned to CO_2Me) (Figure 2). The ratio of these two peaks was used to determine the extent of peptide substitution, giving the calculated molecular weight and monomer content shown in Table 1.

The results showed that an average of 40 sites per PMA chain were coupled to peptides. The molecular weight distribution in each of the polypeptides was determined using gel permeation chromatography. The peak average molecular weight (M_p) of the polymeric peptides relative to standards is also listed in Table 1.

Table 1. Molecular Weight and Monomer Content in PMA

	molecular weight (Da)		
compd	calcd	obsd[a]	monomer content
PMA-RRWW	33 940	31 000	40
PMA-RWRW	33 254	30 500	39

[a] The peak average molecular weight (M_p).

Table 2. Summary of Bioassay Results with Multivalent AMPs and Monomeric Peptides

	IC_{50},[a] μg/mL			hemolytic index HI	
antimicrobial agents	*E. coli*	*B. subtilis*	HD_{50}, μg/mL red blood cell	*E. coli*	*B. subtilis*
Ac-RWRW-NH_2	510	142	1850	3.6	13.0
Ac-RRWW-NH_2	586	131	2280	3.9	17.4
PMA-RWRW	33	10	128	3.9	12.8
PMA-RRWW	39	12	135	3.5	11.2
PMA control	NA[b]	NA[b]	>1000	NA	NA

[a] The results are the mean of three independent experiments each performed in parallel. [b] No killing was detected.

Table 2 compares data on monomeric and polymeric peptides. The MIC values of the tetrapeptides in these experiments confirm that RWRW and RRWW are active antimicrobials despite their size, in agreement with data from Svendsen's group.[39] We find significant enhancement of antibacterial effect relative to the free peptides in polymers against the Gram-negative bacteria *E.coli* and the Gram-positive bacteria *B.subtilis*, roughly a 10-fold improvement. There is a concomitant increase in hemolytic activity on fresh red blood cells, compensated by the reduction in IC_{50} so that the ratio, expressed as the hemolytic index HI, remains roughly constant. The PMA control showed no activity against bacteria and low hemolytic activity. RWRW appears to be slightly more effective in polymers than RRWW. A QSAR analysis of the antimicrobial activity of R and W peptides suggests that charge and multiple W side chains are major characteristics while sequence is less important.[6,37] Biomimetic antimicrobial polymers of a different design have been previously reported by Tew and his colleagues.[12,42]

It has also been previously shown that connecting several weakly interacting ligands on cell surfaces can lead to large increases in avidity.[29,30] Dendrimeric AMPs constructs that contain a maximum of eight monomer units showed improved solubility, salt resistance, and stability to proteolysis.[34] A concern with multivalent strategies with respect to AMPs is to avoid exacerbating cytotoxic effects that can easily outweigh the positive effects on killing bacteria.[43] We show here that it is possible to decrease the IC_{50} value of a moderately active

tetrapeptide by roughly 1 order of magnitude using multivalent constructs while preserving HI values. The antibacterial polymeric peptides of this study increase in overall cationic charge as the number of monomeric peptides increases, while variations in their hydrophobicity depend on the conformation. Current mechanisms postulate that membrane disruption by AMPs involves cooperativity, in the process of assembling intermediates or overcoming repulsion of neighboring bound peptides.[1,18,21] Dendrimeric or polymeric mimics offer the potential to increase the local concentration of monomeric units while decreasing the entropy of self-assembly.[34] On the other hand, if peptide assembly is required to prenucleate local structures that induce membrane lysis, then killing by free peptides should be stimulated by subcritical concentrations of polymer. To test this prediction, we added varying amounts of monomeric RWRW to 0.3× of the MIC of the polymeric peptide. Interestingly, we detected no influence of the polymer on killing by the monomeric peptides in these assays (data not shown). Thus, monomers do not appear to interact with the polymers and the two may act via different pathways. In any case, membrane target models for action by AMPs are likely to be oversimplified; interaction with negatively charged polymers of the bacterial cell wall has been implicated in some AMPs.[44,45] The mechanism of multivalent AMPs and their activities in high salt and proteolysis conditions are currently under study.

In this work, we show that PMA affords a practical reactive scaffold for the assembly of multivalent AMPs that are more effective on a weight basis than the individual monomers. The monomers are small and relatively inexpensive to synthesize and could be derived from de novo design or fragments of natural AMPs in future work. In both sequences we tested (RWRW and RRWW), we detect enhanced antimicrobial activity, with an increase of roughly 10-fold in potency against Gram-negative and Gram-positive strains. At the same time the hemolytic activity of the polymers increases such that the hemolytic index remains roughly constant. Thus, a multivalent strategy based on PMA or related reactive polymers may offer a strategy for producing effective antibacterial agents in large quantities. Even if these are restricted to topical applications, they may prove to have practical utility as microbicides.

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Supporting Information Available: Experimental details of the synthesis and characterization of antimicrobial agents, antibacterial assays, and hemolysis assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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