Engineering Chirally Blind Protein Pseudocapsids into Antibacterial Persisters

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Supporting Information

ABSTRACT: Antimicrobial resistance stimulates the search for antimicrobial forms that may be less subject to acquired resistance. Here we report a conceptual design of protein pseudocapsids exhibiting a broad spectrum of antimicrobial activities. Unlike conventional antibiotics, these agents are effective against phenotypic bacterial variants, while clearing “superbugs” in vivo without toxicity. The design adopts an icosahedral architecture that is polymorphic in size, but not in shape, and that is available in both L and D epimeric forms. Using a combination of nanoscale and single-cell imaging we demonstrate that such pseudocapsids inflict rapid and irreparable damage to bacterial cells. In phospholipid membranes they rapidly convert into nanopores, which remain confined to the binding positions of individual pseudocapsids. This mechanism ensures precisely delivered influxes of high antimicrobial doses, rendering the design a versatile platform for engineering structurally diverse and functionally persistent antimicrobial agents.

KEYWORDS: protein design, antimicrobial resistance, artificial pseudocapsids, persister cells, superbugs, nanopores

With conventional antibiotics losing effectiveness at an alarming rate, antimicrobial resistance represents a major health threat. This challenge stimulates the search for more effective antimicrobial forms that may be different from traditional antibiotics altogether. Most antibiotics act by binding to individual intracellular targets in bacterial cells. A single genetic event is enough for bacteria to acquire resistance. As a consequence, bacteria develop into “superbugs”, strains that no longer respond to antibiotic treatments. The spread of these pathogens can be counteracted by antimicrobials tackling a bacterial cell as a whole, thus demonstrating potential to kill growing, persister, and dormant cells, the feat that is inaccessible to conventional antibiotics. The innate immune systems of multicellular organisms do not use antibiotics. Instead, they deploy host-defense effector molecules to the sites of infection. These molecules are standalone peptides or relatively small domains in globular proteins that recognize microbial surfaces. These molecules are diverse in structure and origin but share common physicochemical properties. Most of them are cationic and fold into amphipathic conformations upon binding to anionic microbial membranes. In membranes, these conformations assemble into higher-order oligomers that overcome a threshold of peptide concentration, beyond which antimicrobial effects become apparent. This is more characteristic of host-defense peptides than of effector domains of multifunctional proteins.

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that recognize pathogen surfaces without the need to self-oligomerize. Yet, in free forms these domains can oligomerize to induce bacteriostatic effects. This distinction presents a considerable opportunity for antimicrobial engineering. In principle, multiple copies of an effector domain can be arranged to fold into a discrete assembly, which upon contact with microbial membranes would instantaneously deliver peptide concentrations that significantly exceed those necessary to rupture microbial membranes. An ultimate benefit of such an agent is rapid and irreparable damage to a microbial cell without the need for the transition from unstructured monomers to membrane-active oligomers. As a result, this agent may be equally effective against susceptible, resistant, and persister cells, thus providing a foundation for antibacterial persisters: nanoscale agents whose activity is not subject to microbial membranes. An ultimate benefit of such an agent is rapid and irreparable damage to a microbial cell without the need for the transition from unstructured monomers to membrane-active oligomers. As a result, this agent may be equally effective against susceptible, resistant, and persister cells, thus providing a foundation for antibacterial persisters: nanoscale agents whose activity is not subject to bacterial phenotypes and acquired resistance. However, the success of this strategy relies on addressing the following principles.

Since bacteria are 0.2–1 μm in width, an assembly of tens of nanometers in diameter would inflict irreparable damage to a bacterial cell. To be discrete at these size ranges, the assembly is best confined to a platonic, symmetrical structure. The nature of the symmetry is of less importance as long as the assembly is locked into a three-dimensional form that is able to bind to microbial membranes. In this regard, viral capsids inspire a straightforward solution. These are self-assembled nanoscale protein cages or cages that do not kill bacteria on contact but provide suitable architectural templates for capsid-like assemblies of effector molecules. The antimicrobial function of the resulting structures permits structural polymorphism of the symmetry is of less importance as long as the assembly is locked into a three-dimensional form that is able to bind to microbial membranes. In this regard, viral capsids inspire a straightforward solution. These are self-assembled nanoscale protein cages or cages that do not kill bacteria on contact but provide suitable architectural templates for capsid-like assemblies of effector molecules. The antimicrobial function of the resulting structures permits structural polymorphism that is typical of aberrant and pseudocapsids. Furthermore, it also imposes no a priori constraints on the chirality of the effector domains. Polypeptide chains of reversed chirality, i.e., all-D peptides, are far more stable to proteolysis, fold in a similar manner to that of all-L peptides, and are likely to be nonimmunogenic. Antimicrobial D epimers are as effective as their L counterparts, which is consistent with the fact that host-defense peptides act by binding to the lipid components of bacterial membranes rather than docking to a specific protein. Herein we apply the outlined principles for the design of antimicrobial pseudocapsids or Ψ-capsids and demonstrate that such assemblies stand out among artificial and native particulate structures by being biologically persistent agents in vitro and in vivo, delivering nanoprecise antimicrobial effects that do not depend on chirality, with both their L- and D-forms exhibiting comparable activities.

RESULTS

Pseudocapsid Design. Our approach adapts a short host-defense motif from a multifunctional protein lactoferrin, a major component of the innate immune system. The antimicrobial properties of this protein are attributed to its N-terminal domain. The domain has a broad spectrum of targets including porins, DNA, and intracellular metabolites and can stimulate the immune system by neutralizing endotoxin. The host-defense motif of the domain is a hexapeptide RRWQWR, which has strong propensity for antiparallel β-sheet conformations. To render this motif self-complementary, the C-terminal arginine of the peptide was replaced with a glutamate. This modification facilitates interstrand Coulombic interactions with the N-terminal arginine of the opposite β-strand. The core of the motif is also homologous to a characteristic motif of tryptophan zippers, WTW, which folds with cross-strand tryptophan rings packed tightly against another. To capitalize on this analogy, the glutamine in the peptide was replaced with a threonine residue. This modification supports the formation of a tryptophanyl interface to cement a β-sheet bilayer with two cationic exteriors. In viruses, capsid proteins interface orthogonally with each other to network into pentagonal and cubic assemblies. Each of these units adopts a 3-fold rotational symmetry, which ensures their propagation into a closed, spheroid symmetry. To emulate the 3-fold symmetry of native cage-like subunits, the resulting monomeric peptide RRWTWE was converted into a triskel conjugate

Figure 1. Pseudocapsid design. (A) Molecular model of the triskel RRWTWE conjugate. (B) Snapshot of molecular dynamics simulations showing a pentagonal assembly unit formed by designed triskelions. (C) Two β-sheet arms (green and yellow) forming a bilayer interface via cross-strand packed tryptophans (gray). (D) Schematic representation of the designed triskelion as a monomer subunit in a truncated icosahedron shown as a 5-fold Schlegel orthographic projection. (E) Five-fold symmetry orthographic projections of two truncated icosahedra (black and red) forming a double-walled assembly. Note: for clarity only one of each of the pentagonal (green) and hexagonal (orange) units is highlighted in (D) and (E). (F) Single-walled triskelion assembly templated on a truncated icosahedron. (G) Double-walled triskelion assembly templated on a truncated icosahedron. Outer and inner layers in (F) and (G) are shown in green and yellow, respectively.
(Figures 1A and S1 in Supporting Information), with both L- and D-forms of this conjugate chemically synthesized (Figure S2). Each arm of this triskelion pairs in an antiparallel $\beta$-sheet with another arm of another triskelion. Each folded pair of two arms interfaces with another folded pair of two other conjugates (Figure 1B,C). The monomer has a trilateral symmetry, which enables it to assemble into penta- and hexagonal units forming $\beta$-sheet bilayer networks (Figure 1B).

Because $\beta$-strands are stabilized by interstrand interactions, it is physically impossible for the bilayers to have free, “sticky” edges. These have to close on one another. Triskelion structures have an intrinsic nonzero curvature.26,27 In propagating $\beta$-sheet networks, the curvature translates into a trans-sheet asymmetry, prompting the spontaneous closure of increasingly curved sheets into a minimum energy spheroid.11,28 This mode of assembly is analogous to that of viral particles, which follows an icosahedral symmetry, with a truncated icosahedron being one of the most common architectures.29,30 This polygon architecture offers an ideal template for the assembly of triskelions: it has only 3-fold vertices, each of which can host an individual triskelion (Figure 1D,E). Therefore, if triskelions fold cooperatively within the template, the propagating $\beta$-sheet networks should self-close into an equilibrated and discrete structure. The monolayer configuration of this structure is not stable, as the hydrophobic side chains of the tryptophanyl residues are oriented inward toward its water-filled core (Figure 1F). A $\beta$-sheet bilayer or a double-wall $\beta$-sheet, in which cationic arginyl residues furnish its exterior and interior surfaces, is deemed more stable (Figure 1C,G).31 In support of this conjecture, coarse grain molecular dynamics (MD) simulations32 showed that single-wall icosahedra assembled from the triskelion collapsed within the first 600 ns of simulations following an equilibration phase (Figure S3A). Under the same simulation conditions, double-
walled structures retained their initial configuration over 1 μs of coarse grain simulations, evolving into more compact shapes, for both L- and D-forms, which was also confirmed by 100 ns atomistic simulations (Figure S3B,C and Movie S1). Further simulations confirmed that the double bilayer maintained β-sheet networks intact, while allowing individual β-strand pairs to break and re-engage in the outer layer. This ability of β-sheets to rearrange without compromising the stability of the assembly renders changes in the networks cooperative and suggests that triskelions can indeed assemble cooperatively in the template, which should manifest in the formation of an equilibrated, discrete, and stable structure (Figure S4).

**Pseudocapsid Folding and Assembly.** Consistent with the simulations, circular dichroism (CD) spectra for both forms of the triskelion were characteristic of antiparallel β-sheet and β-turn conformations (Figure S5A). Fourier transform infrared (FT-IR) spectra revealed bands for the deconvoluted amide regions at 1650–1670 cm⁻¹ for β-turn structures and at 1630 and 1545 cm⁻¹ for β-sheets (Figure S5B). These results suggest that inter- and intramolecular hydrogen bonds in β-sheets and β-turns, respectively, support the cooperative folding of the triskelions into higher-order structures.

Indeed, transmission electron microscopy (TEM) and atomic force microscopy (AFM), performed in solution, revealed uniformly spherical capsids (Figure 2A–D). Some of these appeared as collapsed or open structures giving a double-walled or double-layered morphology (Figure 2B). Negatively and positively stained capsids gave darker and lighter interiors, respectively, due to stain accumulation. This is characteristic of synthetic virus-like and native virus structures (Figure S6). An enclosing boundary was also apparent, suggesting a space between the two layers (Figure 2B). Since most of the assemblies remained as dense spheroids (Figure 2A), we sought further evidence for the hollow nature of the assembled structures and probed their mechanical properties directly in solution by local nanoindentation with simultaneous topographic imaging (Figure S7). By mapping the effective elastic modulus of the capsids as a function of position on the assembled capsids, it was found that the capsids were seemingly compliant at their edges. This is expected since the capsids can move sideways in response to the vertically indenting AFM tip. More importantly, however, the capsids were appreciably more compliant to indentation at their centers, compared with surrounding areas. These results, which were consistent across all capsid sizes observed, are indicative of hollow spheroids (Figure S7). The diameters of the spheroids themselves were comparable by both width (TEM) and height (AFM) measurements and were dominated by a narrow size range of 20–40 nm (Figure 2E,F). An overlapping range of 10–20 nm was also evident, particularly by height measurements. Since these measurements are performed in solution and without staining, unlike TEM, the results exclude dehydration effects and contributions from stains, which may explain a lesser distinction between particle sizes observed by TEM (Figure 2E,F). The lower size range was half the size of the first one, suggesting that the triskelions may adopt an integer step size in assembly, generating structures that are polymorphic in size but not in shape. The assembled capsids remained in the same two diameter ranges at micro-to-millimolar peptide concentrations; that is, they were not concentration dependent (Figure S8A). The assembly was also complete in the first 2 h of incubation and did not deviate significantly from the size ranges over 15 h (Figure S8B). In good agreement with the simulation data (Figures 1 and S3), these results support a cooperatively assembled, stable structure. As expected, the monomeric peptide, which has no capacity to propagate, did not assemble even at high concentrations (Figure S9). This ability of the designed Ψ-capsids to accommodate different sizes is akin to that of viral capsids and synthetic virus-like assemblies that can adapt to repack into smaller and larger particles.37-39 This is also in marked contrast to mutant viral capsids and polymorphic virus-like particles, whose variations in morphology feature filamentous and aberrant structures with irregular serrations.13,14

The designed triskelions are asymmetric dendritic hubs with three peptide arms folding into equivalent β-strands (Figure S1). The asymmetry of each hub incorporates reciprocally into the trans-sheet asymmetry of propagating β-sheet networks. This contribution is additional to the intrinsic curvature of triskelions and helps them adjust to geometric variations in local environments. Turn structures that separate the peptide arms in hubs ensure the three-directional assembly of triskelions without sterically occluding the pairing of peptide arms. These features are complementary to one another and promote C₃ rotational symmetry, enhancing local symmetry interactions in a cooperative manner that is similar to that of native viruses40 and virus-derived systems.14 In particular, capsid proteins, which are asymmetric, form quasi 3-fold contacts to set up identical polygonal subunits that occupy different geometric environments.41 This is sufficient for relatively small (T < 4, where T is a triangulation number) viruses.42 Larger capsids are enabled by incorporating large or scaffolding proteins that serve as an inner core on which the capsid assembly can be templated.42 Scaffolding proteins for the designed pseudocapsids are not necessary, as the β-sheet networks arrange into bilayers stabilized by hydrophobic interfaces. These interfaces keep the assembly intact and rectify the impact of broken β-sheet contacts in the networks through cooperative rearrangements, i.e., acting as inner templates or cores. This agrees well with the results of MD simulations that showed that such rearrangements take place in the outer part of the bilayer. The hydrophobic face of the inner layer that seeds the interface appears to partially destabilize β-sheet networks in the outer layer, making them concomitant or “fluid”. In contrast, the inner layer itself remains intact in all of its constituent β-sheets (Figure S4). This property extends to the ability of the triskelions to adapt to size variations in the assembly. Indeed, course grained, optimized assembly simulations of double-wall networks confirmed that the triskelions can readily accommodate the size ranges observed experimentally (Figures 2 and S10).

Taken together the findings indicate that the designed triskelions propagate with the formation of thermodynamically stable Ψ-capsids exhibiting a degree of structural plasticity that helps accommodate size variations without compromising on morphological uniformity. Since the triskelions occupy the vertices of pentagonal and hexagonal faces in the truncated icosahedron, rather than tightly pack in the faces, the overall architecture of Ψ-capsids remains independent of size allowing for wide size variations. This property renders the assembly adaptable to morphological changes imposed by hierarchically complex and dynamic environments such as microbial membranes.
To test their behavior in membranes in sufficient detail, Ψ-capsids were introduced into reconstituted phospholipid bilayers that were assembled on mica substrates as described elsewhere. The resulting supported lipid bilayers (SLBs) provide suitable models for bacterial membranes, are flat (to within ∼0.1 nm) in their unperturbed state (Figure 3A,B), and allow for the accurate depth measurements of surface changes in solution and in real time by AFM. As gauged by these measurements, Ψ-capsids bound to the SLBs and disintegrated on them forming pore- and channel-like lesions (Figure 3A-C).

The conversion of individual assemblies into pores at their precise binding positions was complete within a few minutes of treatment: Ψ-capsids gradually sank in the lipid bilayers at the depths of a folded triskelion arm, ∼1.8 nm (0.3 nm translation per β-strand residues) (Figure 3A−D). Coarse grain simulations of the capsids in the lipid bilayers run over 10 μs revealed that the sinking effect occurred at the expense of pressing and displacing lipids deep into the bilayer interface (Figure 3E, Movie S2). In accord with this, atomistic simulations of a rudimentary pore showed that triskelion arms oriented toward the bilayer interface (Figures 3F and S11 and Movie S3). Such localized responses are consistent with a mechanism where triskelions reassemble in the bilayer and that forming pores do not expand and remain confined within the diameters of Ψ-capsids. This is important for three reasons.

Figure 3. Pseudocapsids porating phospholipid bilayers. (A) In liquid AFM topography of SLBs treated with Ψ-capsids. The images are taken at 4 min intervals. Individual Ψ-capsids are numbered (1−6) to highlight conversions into pores. Scale bars are 100 nm for the first image and 200 nm for the others. The height (color) bar is 10 nm. (B) Cross-sectional analyses of Ψ-capsids numbered as in (A) for each given time point. (C) Topography of a Ψ-capsid converting into a pore in 15 min, with the height profiles of the corresponding conversion area. Images are taken at 5 min intervals. Scale and height (color) bars are 50 and 10 nm, respectively. (D) Plot combining height profiles of three Ψ-capsids from (A) to show conversions as a function of time. (E) Snapshot of coarse-grain MD simulations of a Ψ-capsid binding to a phospholipid bilayer at 2 μs and simulated for an additional 8 μs. Key: outer and inner layers of the β-sheet bilayer are in green and yellow, respectively; golden beads denote phosphate groups linked to two types of polar groups (blue and magenta); aliphatic lipid tails are in cyan. (F) Snapshot of a rudimentary pore showing a triskelion arm stretching across the bilayer interface after 72 ns of a 100 ns atomistic simulation. Key: for clarity, only phosphate groups (golden beads) are shown for the bilayer.
First of all, the assemblies exhibit a larger structural plasticity than crystalline materials or viruses do, which allows them to rapidly rearrange into peptide−lipid oligomers at the sites of contact with phospholipid membranes. Second, these oligomers maintain the size of the resulting pores close to that of the landed capsids, thereby supporting precise and site-specific membrane disruption. Third, this behavior suggests that Ψ-capsids may support differential responses in cell environments favoring attack on microbial membranes. To gain a better insight into this, the biological properties of Ψ-capsids were assessed using a range of in vitro and in vivo assays.

**Biological Properties of Pseudocapsids.** Irrespective of chirality, Ψ-capsids were found to be antimicrobial and nonhemolytic (Table S1). The minimum inhibitory concentrations (MICs) of the capsids were comparable to those of antimicrobial agents including conventional antibiotics (Table S1). The monomeric peptide remained inactive at up to 100-fold MICs of the capsids (>100 μM) against all bacteria tested. However, the principal advantage of Ψ-capsids over antimicrobial compounds lies in their ability to exert rapid and irreparable damage to a bacterial cell, which makes them equally effective against susceptible cells and cells that are tolerant to conventional antibiotics. In liquid AFM experiments showed that the capsids can indeed disrupt membranes within minutes. MIC experiments cannot directly relate to the AFM results, as these are optical density measurements that do not consider changes at the cellular level. Therefore, we sought
complementary evidence from three series of experiments using planktonic and sessile bacterial culture of two of the most common pathogens, *E. coli* and *S. aureus*. In the first series, the antimicrobial activity of Ψ-capsids was assessed as a measure of total cell counts following capsid treatments. Negligible cell counts were observed for cultures treated with the capsids in comparison to the samples of untreated cells in which appreciable bacterial growth was observed (Figure S12). These findings provide end-point results of treated cell populations obtained over the same time scale as MIC measurements. To elucidate the antimicrobial kinetics of Ψ-capsids within the first hours and with a single-cell resolution, the second series of experiments was conducted. In these experiments thousands of individual *E. coli* cells were screened using a high-throughput microfluidic device comprising thousands of growth chambers. Each channel serves as a trap for an individual cell where the cell can grow through one of its distal poles, whereas the diameter of the channel matches that of the cell, thereby arresting its movement. The replicative age of this trapped, mother, cell increases by one finite number of generations. Such a “mother machine” then enables the single-cell monitoring of antimicrobial kinetics in situ and is able to reflect the responses of different cell phenotypes to antimicrobial agents. With this in mind, Ψ-capsids were introduced into *E. coli* cells trapped in the channels of the device (Figure 4A).

After 3 h of treatment under a constant flow the microfluidic environment was replaced by flowing the culture medium over 21 h to restore normal conditions for bacterial growth. Subsequent analyses revealed that Ψ-capsids killed all encountered cells (Figure 4). By contrast, persist cells and viable but not-culturable (VBNC) cells were found after ampicillin treatments (Figure 4A,B). These tolerant phenotypes represent common subpopulations in clonal bacterial cultures that persist antibiotic treatments even at high doses. Persister cells resume growth after the drug is removed from their environment, whereas the regrowth of VBNC cells often requires specific conditions. Both phenotypes contribute to infection relapses, prompting repetitive treatments, and can be linked to dormancy. With Ψ-capsids effectively reducing these phenotypes to susceptible cells, changes in cell morphology may shed light on the mechanism of action at the single-cell level. Indeed, the capsid-treated cells appeared shrunk and somewhat denser when compared to unaffected cells, suggesting that the capsids accumulated in the membranes. This is consistent with the AFM results (Figure 3), although the cells in the mother machine did not disintegrate and seemed intact (Figure 4B). Therefore, cells treated with capsids were microtomed and imaged by TEM (Figure 4C,D). Complete membrane destruction was evident (Figure 4D). Intriguingly, however, many cells appeared as empty and half-empty, having displaced and bulging membranes that tended to open up at distal poles (Figure 4D). This effect is not normally observed for bacteria damaged by host-defense peptides or membrane-active antibiotics, which porate bacterial membranes indiscriminately, causing cells to shrink and leak or, as polymyxins, aggregate with lipopolysaccharides into membrane-destabilizing blebs across the whole bacterial surface. The emphasis on distal poles is reminiscent of bacteriophages that preferentially target the poles of bacterial cells. Although, like polymyxins, Ψ-capsids favor anionic lipids, morphologically they are more of phage mimetics that may be attracted to outward membrane curvature or cell poles serving as DNA mobilization sites before cell division. The third series of experiments provided further insight into this. The attack of Ψ-capsids on *E. coli* cells was monitored by structured illumination microscopy (SIM), which was custom designed to image biomolecular and cellular processes in situ.

The capsids rapidly adhered to the cell surfaces, with adsorption at the distal poles being also apparent (Figure 5A). Within a doubling time (20 min), the capsids proved to come into direct contact with the cells accumulating in their
membranes and cytoplasm (Figure 5B,C). The affected cells then underwent sharp rupture accompanied by a burst of fluorescence intensity (Figure 5C and Movie S4). This effect was more profound in cocci cells (S. aureus). Although the distal poles of these cells are less defined, the transition from the initial contact with Ψ-capsids to the disintegration of their only membrane was somewhat sharper when compared to that for E. coli (Figure 5C and Movie S5). The killing for both bacteria was complete within 45 min (Figure 5D and Movies S4 and S5), while the time of cell disruption closely matched that needed for the conversion of the capsids into membrane-disrupting pores as gauged by AFM (Figure 3). Collectively, these findings suggest that Ψ-capsids need not disintegrate in the membranes, which they may effectively traverse reaching the protoplasm. Such a mode of action infers that the capsids may be able to circulate intact in cellular environments that are challenged by sustained and active bacterial growth.

To verify this in a biologically relevant model, Ψ-capsids were administered into G. mellonella larvae infected with a methicillin-resistant S. aureus (MRSA) strain susceptible to vancomycin.59,60 Over the first 48 h the treatments were as effective as those by high doses of vancomycin (Figure 6A). The survival rate of up to 80% proved to be steady for another 24 h for larvae treated with D/Ψ-capsids, whereas a 10% drop was observed for larvae treated with the l-form (Figure 6A). The resulting rate of 70% did not change over the total of 120 h of treatment for l/Ψ-capsids. In comparison, for the d-form it dropped to 50% on day 4, suggesting a depletion of the capsids in the larvae. The exact reason for this is unclear, although the decrease is likely to link to the fact that d/Ψ-capsids were more active during the first 72 h, possibly at the expense of more capsids being engaged with bacteria (Figure 6A).

The observed survival rates of the larvae proved to be superior over those reported for membrane-active antibiotics and bacteriocins, indicating that the capsids may be equally effective in killing bacterial cells from the outside and inside.60,61 These experiments also demonstrate the importance of preassembling effector monomers into the pseudocapsids. In particular, they provide evidence for the pseudocapsid assembly enabling prolonged antimicrobial effects. This is further emphasized by the failure of stand-alone host-defense peptides to exert any effects under the same conditions. For instance, the administration of strongly bactericidal human (LL37), insect (cecropin B), and animal (magainin 2) antimicrobial peptides into the infected larvae did not improve survival rates relative to those of mock injections with PBS (Figure 6A). Partly, this failure can be attributed to the enzymatic instability of the nonassembling peptides in vivo. However, both l- and d-forms of the pseudocapsids were similarly effective, which points out that it is the capsid assembly itself that determines the sustained antimicrobial activity. Furthermore, the capsids maintained high rates of bacterial clearance even when administered at

![Figure 6. Time−kill kinetics of pseudocapsids in vivo. (A) Survival of G. mellonella larvae infected with vancomycin-susceptible MRSA NCTC12493 strain when treated with Ψ-capsids and host-defense peptides. Vancomycin and phosphate-buffered saline (PBS) were used as positive and negative controls, respectively. Inoculations were done straight after the initiation of infection (first 2 h) without subsequent treatments. (B) Survival of MRSA-infected G. mellonella larvae treated with vancomycin, l/Ψ-capsids, and PBS, administered at 18 and 36 h after the initiation of infection. Survival rates for Ψ-capsids were significantly higher when compared to PBS control (Mantel−Cox test, p < 0.001). Gray arrows indicate inoculation time points. (C) Survival of G. mellonella larvae treated with Ψ-capsids. Inoculations were done straight after the initiation of infection (first 2 h) with two subsequent treatments at 24 and 48 h.](https://doi.org/10.1021/acsnano.9b06814)
significantly delayed injection times: the larvae treated with capsids at 18 and 36 h exhibited a 50% survival up to day 4 postinfection (Figure 6B), which is twice the time achieved by bacteriophage therapies. Finally, multiple injections of the capsids into uninfected larvae did not lead to appreciable cytotoxic effects (Figure 6C).

**CONCLUSIONS**

We have demonstrated that discrete pseudocapsids assembled from effector molecules deliver rapid and irreparable damage to bacterial cells. The damage results from a direct impact that such assemblies have on cells and ensues via multiple avenues starting with the rapid conversion of the pseudocapsids into membrane-disrupting pores. Pore formation is spatially confined to the binding position of a pseudocapsid, which ensures the rapid and highly localized influx of high antimicrobial doses at the site of contact. Such an impact is different from that of organic or inorganic nanoparticles that rupture membranes indiscriminately by a purely physical means, are unable to disintegrate, and are prone to agglomeration. In contrast, the protein pseudocapsids are cooperative ensembles of monomeric units that reassemble in membranes and integrate into the proteolipid. Thus, these ensembles are equilibrated, symmetry-driven nanoscale systems, which is a prerequisite if they are to remain autonomous within the set size range. The polygonal architecture of viral capsids proved to be a suitable framework for templating the assembly of monomeric units, which can be of virtually any chirality and topology. The framework is compatible with the double-layered arrangement of β-sheet networks that are stabilized by hydrophobic interfaces running tangentially to the capsid surface. Apart from the cases of scaffolding proteins, viral capsids employ single-layered β-pleated networks in this orientation (e.g., rhino- or poliovirus), which are typically built of closed β-barrel structures. To this end, in synthetic peptide and protein cages such networks have been viewed as single-layered structures.

An efficient strategy for the design is to repurpose host-defense effector molecules into structurally and functionally tunable motifs that are furnished with the ability to rapidly self-assemble. Individually, both monomeric units and the motifs may resemble membrane-active antibiotics, but their efficacy is no longer subject to the lag phase of reaching a critical threshold concentration on bacterial surfaces. Once assembled into a discrete pseudocapsid, the constituent monomers acquire a dual capacity of preconcentrated and stimulus-responsive antimicrobial doses. This preassembly of the monomers defines the ability of the capsids to differentiate between bacteria and erythrocytes. With the surfaces of red blood cells being weakly anionic, strong antimicrobial activities often carry an additional cost of hemolytic effects, which is common for toxin or venomous peptides such as bacterial or melittins (Table S1). The designed pseudocapsids are different in that they assemble from peptide monomers that individually are not antimicrobial but have an inherent ability to bind to microbial membranes. Therefore, the capsids themselves are antimicrobial agents that exert their activity through highly localized disruption of microbial membranes, accompanied by the conversion of the capsids into pores. Since the capsids did not lyse erythrocytes and were not cytotoxic in vivo, the mechanism supports the differential activity of the design. This biological responsiveness builds upon the structural plasticity of the capsids and, subsequently, requirements for nanoscale order are looser than those for viral subunits that tune their assembly to the size of encapsidated genomes. Like other paracrystals, which often exhibit periodic nanoscale patterns (e.g., striations or rings), the designed pseudocapsids display fine surface structure (Figure 2A–D). Although this did not feature readily recognizable patterns, the assemblies appeared to adopt a size integer and were monomorph in shape, lacking competing morphologies such as filaments. Combined, these properties represent a highly effective antimicrobial system that, unlike antibiotics, is not frustrated by antibiotic-tolerant phenotypes, such as persister or VBNC cells, or by “superbugs” such as MRSA, killing all. This outcome is notable in that the design may provide a useful tool to aid in a better understanding of how different bacteria phenotypes can be distinguished and selectively targeted.

A question remains as to how universal pseudocapsids are in overcoming different resistance mechanisms including cell surface fortifications, efflux pump blockages, or peptide antagonists that block access to cell membranes. As it has been long postulated and re-emphasized recently, host-defense polypeptides are evolutionarily conserved molecules, against which a widespread resistance cannot be developed easily. Resistance mechanisms against them exist but are not deemed systemic and are readily counteracted by relatively marginal alternations in peptide structure. In this light, the pseudocapsids introduced here may constitute a step change in the applied chemistry of host-defense effector molecules shifting the host-pathogen arms race in favor of more sustainable and adaptable antimicrobial treatments, serving an ultimate pursuit of reducing the spread of antimicrobial resistance.

**METHODS**

**Peptide Synthesis and Purification.** L- and D-Triskelions—(RRWTVWE)-β-A-K(RRWTVWE)-K(RRWTVWE)-am—were assembled on a Liberty microwave peptide synthesizer (CEM) using standard Fmoc/βBu solid-phase protocols with HBTU/DIPEA as coupling reagents on a Rink amide MBHA resin. Fmoc-K(Mtt)–OH was used to enable orthogonal conjugation via a trifunctional dendritic hub, β-AKK-am. Carboxyfluorescein-labeled triskelions were made on resin by coupling carboxyfluorescein to the N-terminus of β-A-RRWTVWE-β-AKK, with fully protected side chains, on the resin. The remaining two arms were then assembled on resin following Mtt removal. Magainin 2 and cecropin B were synthesized as peptide amides on a Rink amide MBHA resin, and LL37 was assembled as a peptide acid on an Fmoc-S(tBu)-Wang resin. After postsynthesis cleavage and deprotection (95% TFA, 2.5% TIS, 2.5% water) all peptides were purified by semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC). Peptide identities were confirmed by analytical RP-HPLC and MALDI-ToF.

MS [M + H]+: cecropin B, m/z 3834.6 (calc), 3836.0 (found); LL37, m/z 4493.2 (calc), 4495.8 (found); magainin 2, m/z 2465.9 (calc), 2467.0 (found); L-triskelion, m/z 3090.5 (calc), 3092.3 (found); D-triskelion, m/z 3090.5 (calc), 3092.8 (found); monomer, m/z 932 (calc), 932.6 (found); carboxyfluorescein-L-triskelion, m/z 3518.9 (calc), 3519.7 (found).

Analytical and semipreparative RP-HPLC was performed on a JASCO HPLC system (PU-980; Tokyo, Japan), using Vydac C18 analytical and semipreparative (both 5 μm) columns. Both analytical and semipreparative runs used a 10–70% B gradient over 30 min at 1 mL/min and 4.5 mL/min, respectively, with detection at 280 and 214 nm (buffer A, 5%, and buffer B, 95% aqueous CH3CN, 0.1% TFA).

**Capsid Assembly.** Triskelions were assembled overnight at the concentrations stated in the text (100–400 μM) in filtered (0.22 μm),
10 mM MOPS, phosphate, or PBS buffers, pH 7.4, at room temperature.

**Circular Dichroism Spectroscopy.** CD spectra were recorded on a JASCO J-810 spectropolarimeter fitted with a Peltier temperature controller. The measurements were taken in ellipticities in mdeg and converted to molar ellipticities (θ, deg cm² dmol⁻¹ res⁻¹) by normalizing for the concentration of peptide bonds. The data were collected with a 1 nm step, 1 s collection time per step, and are presented as the average of 4 scans.

**Fourier Transform Infrared Spectroscopy.** FT-IR spectra were recorded using a Tensor-27 series FTIR spectrometer equipped with a BioA TR II unit (Bruker Optics), as the sampling platform, and a photovoltaic mercury cadmium telluride (MCT) detector and a Bruker Optics workstation equipped with OPUS software. Low-volume (20 μL) capsid samples (100 μM) were placed in a circular sampling area of radius 2 mm with a path length of 6 μm. FT-IR spectra were recorded with resolution 4 cm⁻¹, scanner velocity 20 kHz, 256 scans, phase resolution 32, and zero filling factor 4. Spectra deconvolutions were performed by Gaussian peak fitting using the proprietary software.

**Molecular Dynamics Simulations.** MD simulations were performed using the GROMACS 2016 software with the GROMOS96 53A6 force field (atomic) and the MARTINI force field (coarse grain). An SPC water model was used for atomistic simulations and standard MARTINI water for coarse grain simulations. In all simulations, chloride and sodium ions were added up to a 150 mM concentration. Additional ions were placed if required for charge neutralization. The initial Ψ-capsid configuration was constructed geometrically using the editconf tool from GROMACS. The DLPC/DLPG (3:1) membrane was constructed with the PACKMOL software with dimensions of 12 × 12 nm (atomic) or 30 × 30 nm (coarse grain). Periodic boundary conditions were imposed, setting the minimal distances between the protein and the box boundaries to 3 nm. All simulations are run in the NPT ensemble maintaining the temperature at 303 K with the velocity rescale thermostat, and the pressure at 1 bar using the Parrinello–Rahman barostat, with either an isotropic or semi-isotropic coupling; simulations without and with membranes, respectively. For atomistic simulations of the Ψ-capsid in solution, multiple restrained equilibration runs (65 ns overall) precede 100 ns productions, which are run in three replicates. Similarly, for coarse grain simulations a 10 ns equilibration precedes 1 μs productions, which are run in three replicates. For simulations including membranes, the lipid patches were equilibrated for 500 ns (1 μs for coarse grain) before placing the peptide close in contact with the lipid surface (atomic) or at the minimal distance of 1 nm (coarse grain). Production was then carried out for 500 ns, and 10 μs for coarse grain. Atomistic simulations with membranes were run with an electric field of 130 mV/nm in the direction perpendicular to the membrane, with the higher potential region on the side of the bilayer where the peptide rests. Coarse grain MD modeling of double-walled Ψ-capsids was performed using the PACKMOL software: the capsids were constructed as two spherical cages, their radii determined by the number of triskelions packed into a single bilayer with a hydrophobic interface. The same, optimal packing of triskelions was used for all the sizes.

**Transmission Electron Microscopy.** Micrographs of the Ψ-capsids were recorded using a JEOL 1010 transmission electron microscope equipped with an Orius SC1000 (Gatan Inc.) CCD camera, operated at 80 kV. Droplets of capsid solution were placed on glow-discharged Cu finder grids coated with carbon film (mesh 200) or holey carbon film with graphene oxide (mesh 300), stained with uranyl acetate (aqueous 2%, w/v) for a few seconds, and buffer excess was removed by blotting paper. Micrographs of E. coli were recorded using a FEI Tecnai T12 transmission electron microscope equipped with a Morada G2 (Olympus Inc.) camera, operated at 80 kV. Samples were fixed with glutaraldehyde (aqueous 5%, w/v), treated with a postfixation agent osmium tetroxide (1%, w/v, 100 mM PIPES, pH 7.2), and stained with uranyl acetate (aqueous 2%, w/v). The resulting samples were embedded in a Spurr resin and were left to solidify at 60 °C, over 24 h. The resin was then microtomed to ultrathin sections, which were placed on Formvar carbon coated grids (mesh 200), poststained using lead citrate (aqueous 5%, w/v), and imaged.

Electron micrographs of MS2 bacteriophages were recorded using a JEOL JEM-4000 Plus transmission electron microscope equipped with a GatanView 4K (Gatan) camera operated at 120 kV. Droplets of phages in SM buffer (pH 7.5) were deposited on Formvar carbon coated copper grids (mesh 200), stained with uranyl acetate (aqueous 1%, w/v) for 2 min, and blotted with a filter paper. The phages were purchased from ATCC (Escherichia coli bacteriophage MS2, ATCC 15597-B1) and were cultured according to the proprietary protocols.

**In-Liquid AFM and Peak Force QNM Imaging.** Pseudocapsids were assembled overnight at 100 μM in 10 mM MOPS, pH 7.4, room temperature. A 50 μL sample of assembled solution was added to freshly cleaved mica, prehydrated with 50 μL of imaging buffer (120 mM NaCl, 20 mM MOPS, pH 7.4). Imaging was performed using PeakForce Tapping mode on a Multimode 8 AFM system (Bruker AXS, USA) using MSNL-E cantilevers (Bruker AFM probes, USA) and a 100 μL fluid cell (Bruker AXS, USA). Images were taken at a PeakForce frequency of 2 kHz, PeakForce amplitude of 20 nm, and PeakForce set-point of 10–30 mV (<100 pN). Both topography and DMT modulus channels were recorded. Images were processed using Gwyddion (http://gwyddion.net) for first-order line-by-line background subtraction (flattening), first-order plane fitting, and cross correlation averaging.

**Preparation of Unilamellar Phospholipid Vesicles for AFM Imaging.** 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC) with 1,2-dilauroyl-sn-glycero-3-phospho(-1'-rac-glycerol) (DLP) lipids used for vesicle construction were from Avanti Polar Lipids (Alabaster, AL, USA). DLPC was used as mammalian model membranes, and DLPC/DLPG (3:1, molar ratios) was used as bacterial model membranes. The lipids were weighed up, dissolved in chloroform–methanol (2:1, vol/vol), and dried under a nitrogen stream and then under vacuum to form a thin film. The film was hydrated in 10 mM phosphate buffer (pH 7.2) with shaking (1 hr, 220 rpm) and bath sonicated for 30 min. The obtained suspension was extruded using a hand-held extruder (Avanti Polar lipids) (29 times, polycarbonate filter, 0.05 μm) to give a clear solution of small unilamellar vesicles, the sizes of which (50 nm) were confirmed by photon correlation spectroscopy.

**In-Liquid AFM on Supported Lipid Bilayers.** Supported lipid bilayers were formed using a vesicle fusion method as described elsewhere. Freshly prepared vesicles (1.5 μL, 3 mg/mL) were added to cleaved mica prehydrated with buffer (70 μL, 120 mM NaCl, 20 mM MOPS, 20 mM MgCl₂). Following adsorption and incubation for 45 min, the samples were washed to remove unfused vesicles, and resulting SLBs were checked to confirm they were defect free. Peptides were then introduced into a 100 μL fluid cell (Bruker AXS, USA), where they were diluted to the final concentrations of 0.1–0.8 μM. All imaging was performed using PeakForce Tapping mode on a Multimode 8 AFM system (Bruker AXS, USA) using MSNL-E cantilevers (Bruker AFM probes, USA). Images were taken at a PeakForce frequency of 2 kHz, PeakForce amplitude of 10 nm, and PeakForce set-point of 10–30 mV (<100 pN). Images were processed using Nanoscope analysis software (Bruker AXS, USA) or Gwyddion (http://gwyddion.net) for first-order line-by-line background subtraction (flattening) and first-order plane fitting.

**Photon Correlation Spectroscopy.** Prepared phospholipid vesicles for AFM imaging were resuspended to a final concentration of 1 mg/mL and were analyzed on a Zetasizer Nano (ZEN3600; Malvern Instruments). Dynamic light scattering batch measurements were carried out in a low-volume disposable cuvette at 25 °C. Hydrodynamic radii were obtained through the fitting of autocorrelation data using the manufacturer’s Dispersion Technology Software (version 5.10).

**Minimum Inhibitory Concentrations Assay.** Minimum inhibitory concentrations were determined by broth microdilution on P. aeruginosa, E. coli, S. aureus, M. luteus, B. subtilis, S. typhimurium, and K. pneumoniae according to the Clinical and Laboratory Standards
inoculated into Mueller Hinton broth, grown overnight at 37 °C and 150 rpm, reincubated, and grown to an OD600 of 0.5–0.8. The cells were then pelleted by centrifugation (4000 rpm, 10 min) and, after removing the supernatant, were resuspended in 1 mL of PBS (10 mM) and transferred to an Eppendorf tube. FM4-64 (1 mg/mL, 5 μL) was added to each tube followed by rotation on a rotating disk for 5 min. The cells were then centrifuged (7000 rpm, 3 min), the supernatant was removed, and the cell pellet was resuspended in 1 mL of PBS. Bacteria was then diluted to an OD600 of 0.16 and 0.08 for E. coli and S. aureus, respectively. A 100 μL amount of each suspension was added to different wells of a chambered microscope coverslip (Ibidi μ-Slide 8 well) and left for 30 min to allow cells to settle onto the substrate. Immediately prior to each imaging experiment a 100 μL solution of Ψ-capsids assembled from carboxyfluorescein-labeled and unlabeled triskelions at 1:100 molar ratios was added to the well to a final concentration of 50 μM (total peptide). For control data sets, 100 μL of PBS was added prior to imaging. Time lapse imaging was performed at 5 min intervals using a custom-built structured illumination microscope. 46 Briefly, sinusoidal excitation patterns were generated by projecting a spatially filtered image of a spatial light modulator (SLM), configured to display a series of binary phase gratings, into the focal plane of the microscope objective lens (UPLSAPo 60x/1.3, Olympus). Sample images were acquired using a scientific CMOS camera (ORCA-Flash4.0, Hamamatsu Photonics), with the global exposure period of the camera’s rolling shutter synchronized to the pattern displayed on the SLM. At each time point FM4-64 and carboxyfluorescein-labeled capsids were imaged sequentially, using excitation at 561 nm with a 655 nm long-pass emission filter and excitation at 488 nm with a S25/50 bandpass emission filter, respectively. Images were reconstructed as described elsewhere, 57,58 and lateral drift and chromatic offsets in the reconstructed images were corrected using ImageJ. The FM4-64 color channel was then corrected for photobleaching using an exponential fit to the image intensity within a suitable region of interest. Manual background subtraction and color balancing were performed to optimize image contrast.

In Vivo MRSA Clearance Assay. The Galleria mellonella larvae assay was performed as described elsewhere. 57,58 Wax moth larvae were purchased in bulk from Livefood UK, stored at 4 °C upon arrival, and kept at 37 °C during the course of the assay. Ψ-Capsid samples were prepared from stock solutions in Milli-Q water to working concentrations in sterile PBS. Typically, a single bacterial colony was picked to inoculate 5 mL of tryptic soy broth (TSB), and the resulting culture was left to grow overnight (∼16 h) at 37 °C with shaking (200 rpm). The culture was then diluted (100X) into fresh TSB (5 mL) and incubated over 4 h at 37 °C with shaking (200 rpm). After centrifugation (2500g) for 10 min, pellets were obtained and resuspended in sterile PBS to an OD600 of 0.2, giving ∼1010 CFU/mL. The resuspended culture (10 μL aliquots; ∼1.2 × 106 CFU) was administered to larvae (seven groups; n = 10 in each group) behind the rear thoracic segments using a Tridak stepper pipette dispenser (Dymax, UK). The infected larvae were then treated by injection with 10 μL of Ψ-Ψ-capsid (12.5 μM), n/Ψ-capsid (12.5 μM), magainin 2 (12.5 μM), cecropin B (12.5 μM), L37 (12.5 μM), vancomycin (40 μM), or PBS at stated time points after inoculation. The toxicity of Ψ-capsid was assessed using two groups of larvae (n = 10 in each group). These were mock-infected with PBS and treated with 10 μL of Ψ or n/Ψ-capsid (25 μM) at stated time points after inoculation. For the delayed treatment assay, three groups of infected larvae (∼106 CFU) were treated with 10 μL of Ψ-Ψ-capsid (12.5 μM), vancomycin (40 μM), and fluoroacetamide (12.5 μM) at stated time points after inoculation. The toxicity of Ψ-Ψ-capsid was assessed using two groups of larvae (n = 10 in each group). These were mock-infected with PBS and treated with 10 μL of Ψ-Ψ-capsid (25 μM) at stated time points after inoculation. For the delayed treatment assay, three groups of infected larvae (∼106 CFU) were treated with 10 μL of Ψ-Ψ-capsid (12.5 μM), vancomycin (40 μM), and fluoroacetamide (12.5 μM) at stated time points after inoculation.
µM), or PBS at stated time points after inoculation. All experiments were carried out as blind studies, and the treatment identities were not revealed until each experiment was completed. Larvae were considered dead when they did not respond to touch to the head. Survival curves were generated and analyzed using GraphPad Prism 6 software.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.9b06814.

Biological activities of pseudocapsids and other anti-microbial agents used for comparison; RP-HPLC profiles and MALDI-ToF spectra for triskelions and peptides used in the study; MD simulations and molecular models for pseudocapsids; CD and FT-IR spectra for pseudocapsid folding; fluorescence microscopy and TEM images (PDF)

Movie as described in the text (MOV)

Movie as described in the text (MOG)

Movie as described in the text (MP)

Movie as described in the text (MP)

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Notes

The authors declare no competing financial interest.

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