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Peptoid efficacy against polymicrobial biofilms determined using propidium monoazide – modified quantitative PCR

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Biofilms containing *C. albicans* are responsible for a wide variety of clinical infections. The protective effects of the biofilm matrix, the low metabolic activity of microorganisms within a biofilm and their high mutation rate, significantly enhance the resistance of biofilms to conventional antimicrobial treatments. Peptoids are peptide-mimics that share many features of host defence antimicrobial peptides, but have increased resistance to proteases and therefore better stability *in vivo*. The activity of a library of peptoids was tested against both monospecies and polymicrobial bacterial/fungal biofilms. Selected peptoids showed significant bactericidal and fungicidal activity against the polymicrobial biofilms. This coupled with low cytotoxicity, suggests that peptoids could offer a new option for the treatment of clinically relevant polymicrobial infections in the future.

Introduction

It is well recognised that clinically relevant micro-organisms exist naturally as complex biofilm communities which differ substantially from their planktonic counterparts.[1,2] Biofilm organisms have a propensity for metabolic inactivity, and therefore antimicrobial agents showing promise in planktonic culture tend to be less efficacious against biofilms.[1] The biofilm mode of growth has therefore been proposed as a mechanism for the resistance of many chronic infections to antimicrobial agents.[3]

The majority of *in vitro* treatment studies on biofilms focus on single species biofilms, whereas it is recognised that biofilms *in vivo* are naturally polymicrobial, consisting of several species which may include members of both the bacterial and fungal genera. In polymicrobial infections *in vivo*, micro-organisms have been proposed to influence each other either by exchange of molecules (sensing and signaling) or by physical contact (biofilm architecture) which may ultimately facilitate competitive, synergistic or neutral relationships.[4,5] In addition to a growing interest in multispecies bacterial biofilms[6] there is also an emerging interest in the study of polymicrobial fungal-bacterial biofilms. Cross-kingdom biofilms containing *Candida albicans*[7] have been shown to be associated with clinical infections of both biotic and abiotic surfaces, ranging from the cornea[8] to endotracheal tubes[9] respectively.[10] Although the role of *C. albicans* in polymicrobial biofilm formation is likely to be a complex one, recent works suggest that *C. albicans* dramatically modifies the physical environment and 3D architecture of polymicrobial biofilms[11] as well as influencing interspecies protein expression[12] and eDNA release.[13]

Of particular concern in the current climate of antimicrobial resistance are the findings, from drug susceptibility studies, that fungal cells can modulate the action of antibiotics and that bacteria can influence antifungal activity.[14] Given the importance of polymicrobial biofilms *in vivo*[10] and the increased bacterial resistance to antibiotics observed in polymicrobial biofilms with *C. albicans*[15], new treatments for cross-kingdom biofilms are urgently required. The efficacy of the innate immune system's host defence peptides (HDPs) in providing the first line of defence against infection, and their broad spectrum action against both bacteria and fungi, has prompted our research group and others to investigate HDPs as templates for the design of innovative therapeutics.[16-19] There is particular need to identify new strategies for managing infection that spare conventional systemic antimicrobial drugs. Despite promising *in vitro* antimicrobial activities against a range of bacterial and fungal pathogens, HDPs have been shown to be susceptible to degradation by proteinases at wound and inflammatory sites[20,21], potentially limiting their application as anti-infectives *in vivo*. Despite the fact that the clinical success of such peptides has been somewhat limited[22], continuing advances in the design of peptide mimics and the unmet clinical need for novel antimicrobials have reinvigorated this research field.

Oligo *N*-substituted glycines (peptoids) are peptide analogs that combine many of the features of HDPs, with the added advantage that they are resistant to proteinases[23] and therefore offer a new avenue for antimicrobial therapeutics. Peptoids are structural isomers of peptides, where the side-chain functionality is attached to the nitrogen atom of the amide backbone, rather than the α-carbon of a peptide sequence (see Figure 1).

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# These authors contributed equally to this work

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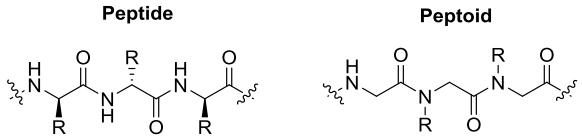


Figure 1. Representative structure of an α-peptide and an α-peptoid.

Peptoids have previously been shown to be efficacious against planktonic micro-organisms[24,25] and against bacterial biofilms[26] but to date their activity against fungal biofilms and, in particular, polymicrobial bacterial-fungal biofilms remains to be determined.

In this study we initially screened the efficacy of a linear peptoid library against *C. albicans, Staphylococcus aureus* and *Escherichia coli* monospecies biofilms and then determined selected peptoid efficacy against polymicrobial bacterial-fungal biofilms consisting of *C. albicans – S. aureus* or *C. albicans – E. coli*. We used a crystal violet assay for initial screening purposes and adapted a novel quantitative PCR (qPCR), approach employing propidium monoazide (PMA)[27], for determining viable organism numbers within the polymicrobial biofilms. The PMA-qPCR assay is highly advantageous over traditional colony forming unit (CFU) assays in that it allows quantification of viable but non-culturable (VBNC) micro-organisms. The PMA-qPCR assay is therefore particularly relevant for quantification of micro-organism within biofilms, which by their very nature are likely to contain VBNC micro-organisms. Additionally, the crystal violet assay cannot distinguish between different species within a polymicrobial biofilm. However, qPCR can determine viable cell counts for individual specific pathogens within polymicrobial biofilms and therefore it offers a significant advantage in studies of this kind. To the best of our knowledge, this is also the first time that peptoids have been proven to be efficacious against fungal biofilms.

Results and Discussion

Peptoid design and synthesis

To assess the anti-biofilm activity of peptoids, a library of 18 linear peptoids was synthesized. The sequences were designed around a *NxNyNy* subunit (Table 1), repeated two, three or four times (6-, 9- and 12 residue peptoids respectively), where *Nx* is a positively charged lysine-type amine with various side chain lengths (*N*ah *N*-(6-aminohexyl) glycine, *N*Lys *N*-(4-aminobutyl) glycine and *N*ae *N*-(2-aminoethyl) glycine) and *Ny* is either the chiral aromatic building block *N*spe *N*-(*S*-phenylethyl) glycine or the achiral *N*phe *N*-benzylglycine. Peptoids were synthesised with a repeat unit of three residues in order to induce an amphipathic structure and included the bulky *N*phe and *N*spe monomers as these are reported to encourage a helical structure that can lead to an improved antimicrobial effect. [28-31] The 18 peptoids were classified into 3 families (peptoids **1-6**; peptoids **7-12** and peptoids **13-18**) based on their positively charged building blocks (*N*ah, *N*Lys or *N*ae). All peptoids were synthesised manually on resin using the sub-monomer method[32] using a shaker platform at room temperature (acylation steps 15 mins and displacement steps 15 mins). The library was purified using RP-HPLC to >95% purity (see Supplementary Material for peptoid synthesis and characterisation).

Peptoid efficacy against single species biofilms

For initial screening purposes, the entire peptoid library was tested against single species biofilms of *C. albicans*, *S. aureus* and *E. coli*, using a crystal violet assay (Figure 2).

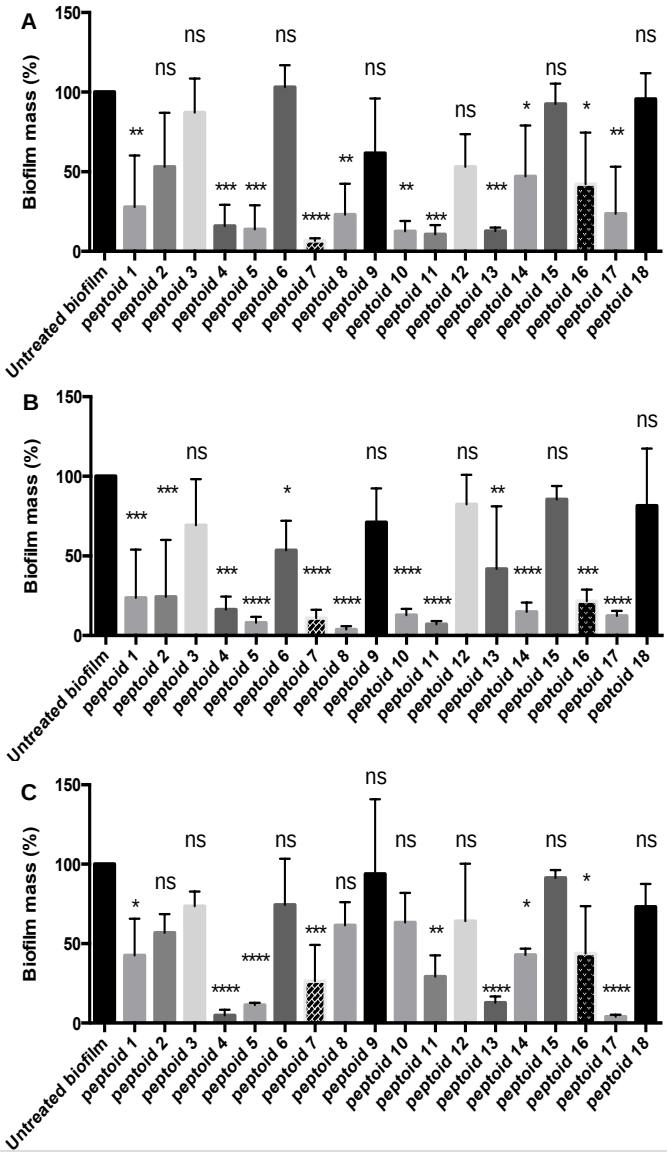
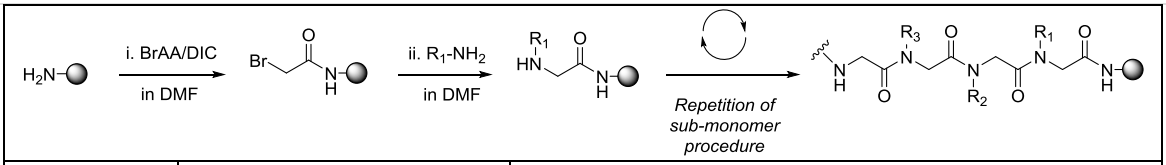
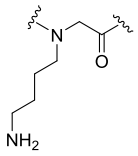
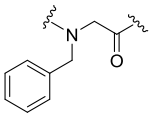
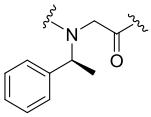
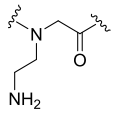
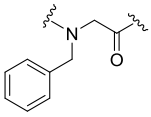
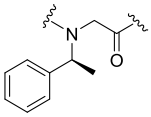
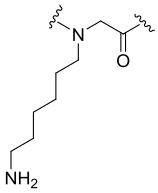
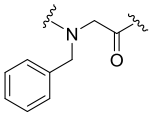
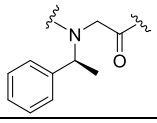


Figure 2. Efficacy of a family of 18 peptoids against *C. albicans* (A), *S. aureus* (B) *and E. coli* (C) monospecies biofilms determined by crystal violet assay. Biofilms were treated with peptoids (100 μM) and compared with untreated, control biofilms. The results (average of 3 independent experiments) were plotted as percent biomass of controls. Statistical analysis was determined by one-way ANOVA followed by Tukey's post hoc correction for multiple comparisons (ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

Table 1. Peptoid library synthesised on solid phase using the sub-monomer method: i. acylation step (bromoacetic acid and DIC in DMF, 15 mins, 50 ˚C); ii. displacement step (primary amine in DMF, 15 mins, 50 ˚C). Peptoids were split into 3 families (peptoids 1-6; peptoids 7-12 and peptoids 13-18) on the basis of their building blocks.



Peptoid	Sequence	N_x	N_y
1	(*N*Lys*N*phe*N*phe)$_4$	***N*Lys**	***N*phe**
2	(*N*Lys*N*phe*N*phe)$_3$		
3	(*N*Lys*N*phe*N*phe)$_2$		
4	(*N*Lys*N*spe*N*spe)$_4$		***N*spe**
5	(*N*Lys*N*spe*N*spe)$_3$		
6	(*N*Lys*N*spe*N*spe)$_2$		
7	(*N*ae*N*phe*N*phe)$_4$	***N*ae**	***N*phe**
8	(*N*ae*N*phe*N*phe)$_3$		
9	(*N*ae*N*phe*N*phe)$_2$		
10	(*N*ae*N*spe*N*spe)$_4$		***N*spe**
11	(*N*ae*N*spe*N*spe)$_3$		
12	(*N*ae*N*spe*N*spe)$_2$		
13	(*N*ah*N*phe*N*phe)$_4$	***N*ah**	***N*phe**
14	(*N*ah*N*phe*N*phe)$_3$		
15	(*N*ah*N*phe*N*phe)$_2$		
16	(*N*ah*N*spe*N*spe)$_4$		***N*spe**
17	(*N*ah*N*spe*N*spe)$_3$		
18	(*N*ah*N*spe*N*spe)$_2$		

The 18 peptoids tested had variable antifungal and antibacterial activities when screened for inhibitory activity against these single species biofilms Peptoids **5**, **7** and **17** were amongst the most efficacious peptoids from each of the three peptoid families across all three single species biofilms studied.

The peptoid library tested in this study contained peptoids of three different lengths: 6, 9 and 12 residue peptoid analogues. In agreement with previous studies[24,30-31], the longest 12 residue peptoids tended to be most efficacious. The 12-mer peptoids were more active against *C. albicans* than 9 residue peptoids which had an intermediate activity and the 6 residue sequences were mostly inactive; for example, the comparison between peptoids **7** (12-mer), **8** (9-mer) and **9** (6-mer). For *S. aureus*, the longer 12-mer sequences also had good activity, but interestingly in most cases the comparable shorter 9 residue analogues caused a greater reduction in biofilm mass. As with *C. albicans*, the shortest 6 monomer peptoids were inactive. For *E. coli*, the longest peptoids were the most active, except for those containing the *N*ah monomer, where the intermediate length 9 residue peptoids were the most active (i.e. peptoids **13** and **17**). Direct comparisons of sequence length effects with previously published literature[28,29] are complicated by the differing side chains used and the resulting differences in charge: length ratios.

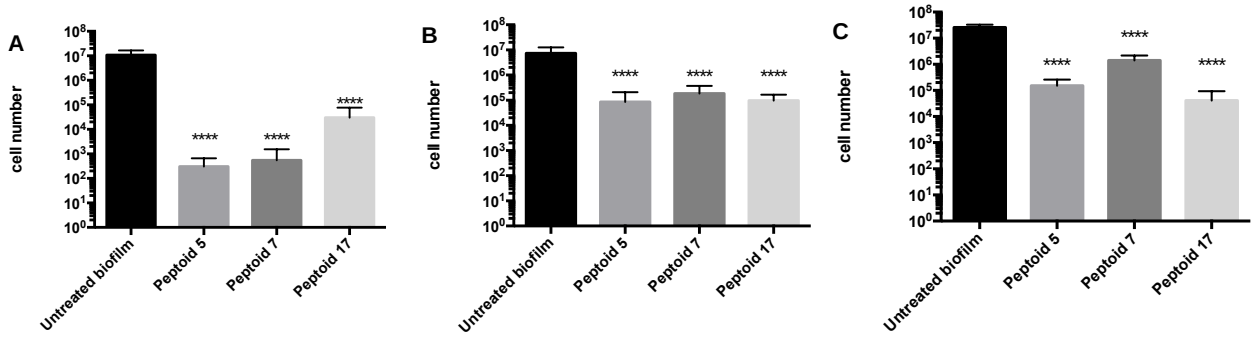


Figure 3. PMA-qPCR quantification of cell number following peptoid treatment of monospecies biofilms. Cell numbers of *C. albicans* (A), *S. aureus* (B) *and E. coli* (C) treated with 100 μM peptoids 5, 7 and 17 (****: $p < 0.0001$. One-way ANOVA followed by Tukey's post hoc correction for multiple comparisons (average of 3 independent experiments).

The success of many AMPs is attributed to their overall positive charge, which helps them to target prokaryotic cell membranes over mammalian cells[16]. Therefore, the effect of different cationic monomers was investigated across the peptoid library. When comparing peptoid sequences of the same overall length (i.e. 12 residues), peptoids containing the shortest *N*ae monomer tended to be more active than the longer *N*Lys monomer (e.g. compare peptoid **1** and peptoid **7**) for all species. On the whole, peptoids containing the *N*ah monomer were less active than those containing either *N*ae or *N*Lys[30], although peptoid **17** is an exception. Potentially the reduced activity of the *N*ah*N*y*N*y peptoids could stem from the greater flexibility of the longer aminohexane chain.

The peptoid library was designed to include sequences containing either the chiral *N*spe or the achiral *N*phe monomer to determine the effect of sequence chirality on the anti-biofilm activities of the compounds. Therefore, a range of chiral and achiral peptoid analogues were tested. Overall, sequences containing *N*spe were more efficacious than their achiral analogues (e.g. compare achiral peptoid **1** and chiral peptoid **4**) across all three biofilm species tested. However, for peptoids containing the shortest *N*ae monomer, there was little difference in activity between the chiral and achiral peptoids (i.e. peptoids **10-12** and **7-9** respectively) against *S. aureus* or *C. albicans*. The differences in activity for chiral and achiral members of the entire peptoid library were even less pronounced against *E. coli*.

The entire peptoid library was also screened against two representative mammalian cell lines, HaCaT and HepG2, to determine if the sequences were selective for the bacteria and fungi tested (data provided in Supplementary Material). The majority of the peptoids had ED_{50} values > 100 μM against both, indicating that they had minimal cytotoxic effects on model human endothelial and keratinocyte cell lines. The only sequences that showed toxicity were the longest 12 residue peptoids containing the chiral *N*spe monomer, peptoids **4**, **10** and **16**.

Although it is generally acknowledged that peptoids are inherently resistant towards proteolysis[23,33], we compared the tryptic digestion profile of one selected peptoid, peptoid **7**, against the naturally occurring alpha helical peptide LL-37. Peptoid **7** showed no degradation following treatment with trypsin for 24 hours whereas LL-37 was completely degraded to peptide fragments during the same time period (see Supplementary Material).

Peptoid efficacy against polymicrobial biofilms

Although widely used in biofilm assays, the crystal violet assay detects both live and dead organisms, in addition to matrix components. In this study, we developed a novel PCR method to selectively and quantitatively determine fungicidal and bactericidal activity against both monospeices and polymicrobial biofilms.

Conventional CFU assays have been reported to underestimate live cell numbers because they cannot quantify VBNC cells.[34,35] Furthermore, micro-organisms have been shown to enter the VBNC sate when exposed to antibiotic treatment.[36] Therefore the use of non-cultivation-based assays to evaluate the efficacy of novel antimicrobials is particularly appealing for studying micro-organisms in biofilm form.

PMA is a photo-reactive dye with a high affinity for DNA, with which it forms a covalent linkage upon exposure to intense visible light. The use of PMA allows qPCR quantification of DNA from living cells only, because PMA binds covalently to DNA which lacks the protection of the cell membrane in a viable micro-organism.[27,] Since PMA discriminates between live and dead cells on the basis of their membrane integrity[37], its addition to the qPCR protocol is particularly suitable for quantifying the efficacy of membrane-targeting agents such as peptoids.

Three peptoids [**5** (*N*Lys*N*spe*N*spe)$_3$, **7** (*N*ae*N*phe*N*phe)$_4$, and **17** (*N*ah*N*spe*N*spe)$_3$] were chosen for further study, as they represented some of the most active peptoids in the single species crystal violet assays and showed negligible toxicity to the two mammalian cell lines tested (see Supplementary Material). In addition all monospecies biofilms treated with these peptoids showed significant reduction in cell numbers, indicating strong bactericidal and fungicidal activity (Figure 3). The three peptoids had a similar efficacy against *S. aureus*, reducing cell

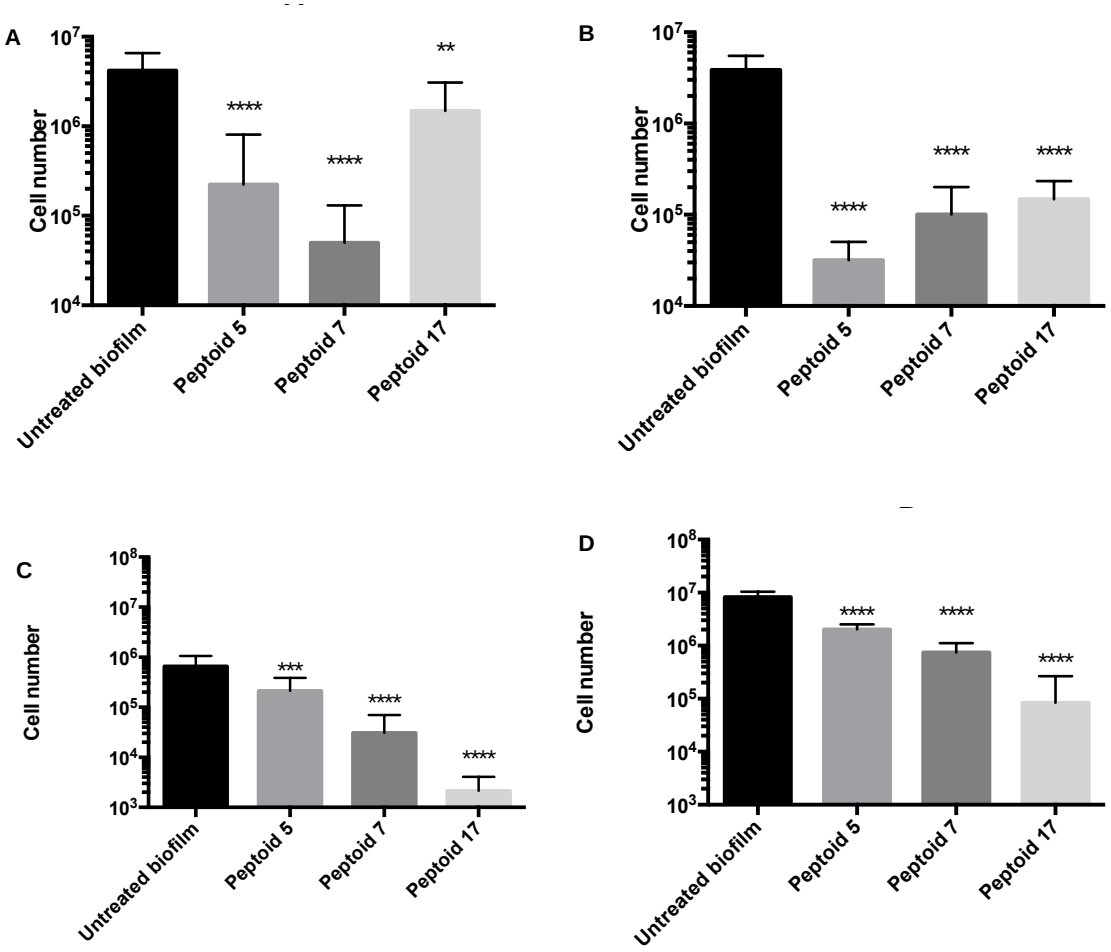


Figure 4. PMA-qPCR quantification of cell numbers following peptoid treatment of polymicrobial biofilms consisting of *C. albicans* and either *S. aureus* or *E. coli*. Cell numbers of *C. albicans* (A) and *S. aureus* (B) within the same polymicrobial biofilm, following treatment with 100 μM peptoids 5, 7 and 17. Cell numbers of *C. albicans* (C) and *E. coli* (D) within the same polymicrobial biofilm, following treatment with 100 μM peptoids 5, 7 and 17 (**: $p < 0.01$; ****: $p < 0.0001$. One-way ANOVA followed by Tukey's post hoc correction for multiple comparisons (average of 3 independent experiments).

numbers by approximately two orders of magnitude, with peptoids **5** and **7** showing even better effects (reduction in cell number by over 4 orders of magnitude against this microorganism). In agreement with the crystal violet assay results, the *N*ah containing peptoid **17** did not perform quite as well against *C. albicans*, although it still caused a significant reduction in the fungal biofilm. Conversely, in the *E. coli* monospecies biofilms, peptoid **17** was the most effective.

Peptoids **5**, **7** and **17** were also tested against mixed species biofilms containing *C. albicans* and either *S. aureus* or *E. coli*. (Figure 4 shows treatment at 100 μM). At this treatment concentration the mixed species biofilm containing *C. albicans* and *E. coli*, the biofilm cell numbers were reduced more for *C. albicans* than for *E. coli*. In this particular polymicrobial biofilm, the *N*ah containing peptoid (**17**) was the most active. In the mixed species biofilm containing *C. albicans* and *S. aureus,* cell numbers were reduced more for bacteria than the fungi. Peptoid **5** was better able to reduce the cell count of *S. aureus* when the biofilm was treated at 100 μM and peptoid **7** showed the greatest reduction in *C. albicans*. It was noted that *C. albicans* appeared to be less susceptible to peptoid **17** when it was present in a biofilm with *S. aureus*, but this did not appear to be the case in the presence of *E. coli*. Since there has been very limited work on peptoid efficacy in polymicrobial biofilms it remains to be determined if this is a unique phenomenon.

To look in more detail at the activity of peptoids **5**, **7** and **17** cross-kingdom biofilms were treated with these compounds at concentrations ranging from 10 – 100 μM (PMA-qPCR method used to determine cell viability as before – see details in Supplementary Information). In the mixed species biofilms investigated, all three peptoids (**5**, **7** and **17**) were found to significantly reduced the cell numbers of *S. aureus* by approximately 100%, even when treated at 10 μM. Reduction in cell viability for *E. coli* or *C. albicans* was less efficacious at 10 μM, but considerable reductions in cell numbers were seen at 25 μM and 50 μM. For all three species present in the cross-kingdom biofilms (i.e. *C. albicans, S. aureus* or *E. coli*), peptoid **17** showed the greatest reduction in cell numbers.

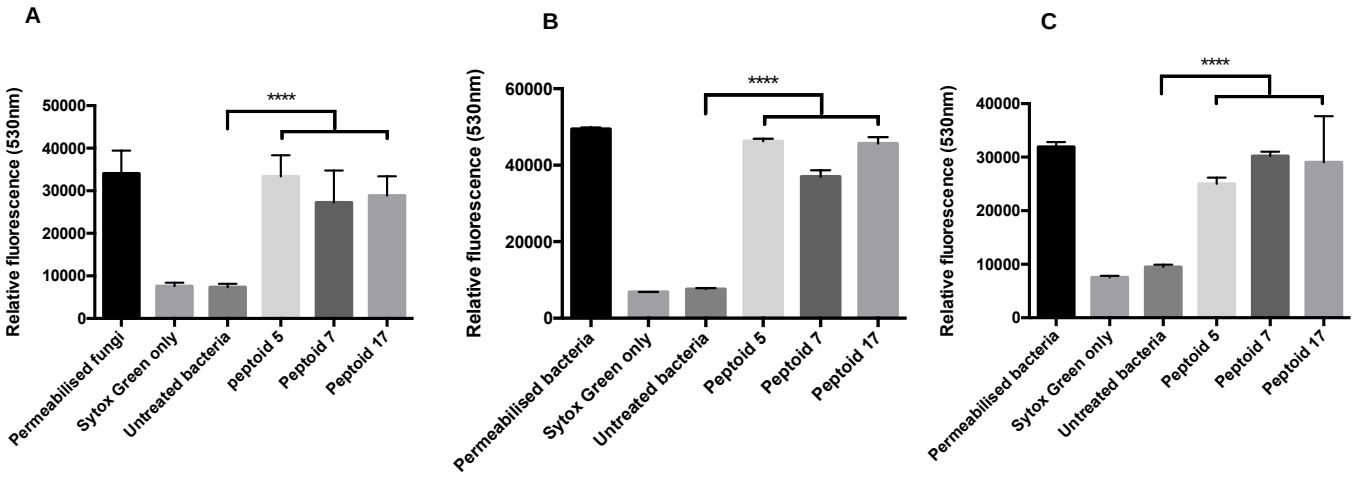


Figure 5. **Propensity for peptoids 5, 7 and 17 to permeabilise the microbial membranes of (a) *C. albicans*, (b) *E. coli* and (c) *S. aureus,* as determined by the SYTOX Green assay.** The efficacy of membrane permeabilisation by peptoids was compared with untreated controls and the results (average of 3 independent experiments) were analysed by ANOVA followed by Tukey's post hoc correction for multiple comparisons (****: $p < 0.0001$). Cells permeabilised by heat treatment were used as positive controls.

Effect of peptoids on microbial cell membranes

The majority of linear antimicrobial peptides (AMPs) have been shown to exert their antimicrobial effects via disruption of pathogen cellular membranes. It is thought that as linear antimicrobial peptoids (such as those studied herein) are structurally very similar to this class of AMP that they may also exert their biological mode of action via cell membrane disruption. However, at present there are only a few studies reported in the literature that provide experimental evidence to support this hypothesis.[38-42]

To help elucidate the mode of action of the peptoids studied in the qPCR experiments and to verify the use of the PMA-qPCR assay (PMA depends upon the integrity of the cell membrane), a membrane permeabilisation assay was performed with peptoids **5**, **7** and **17** at 100 µM against *C. albicans*, *S. aureus* and *E. coli.* The assay carried out uses the dye, SYTOX® Green, which is able to bind nucleic acids but is impermeable to living eukaryotic and prokaryotic cells. Therefore, this dye is routinely used to assess the integrity of cell membranes. If a microbial cell membrane has been compromised, for example by treatment with a peptoid, SYTOX® Green can bind to the cellular nucleic acids. This association event increases the fluorescence of the dye and renders cells with compromised membranes as brightly green fluorescent.[43,44]

The propensity for the peptoids (**5**, **7** and **17**) to permeabilise cell membranes was assessed using SYTOX® Green (Figure 5). The data obtained clearly showed that upon peptoid treatment a large increase in fluorescence was recorded signifying that all of the peptoids assayed caused cell membrane permeabilisation in *C. albicans* (Figure 5A), *E. coli* (Figure 5B) and *S. aureus* (Figure 5C).

The results show that the peptoids that have the greatest antimicrobial effect against single species biofilms (Figure 3) also cause the greatest membrane permeabilisation via the SYTOX® Green assay (Figure 5). For example, against *C. albicans* peptoid **5** causes the greatest reduction in cell number and causes the greatest increase in fluorescence. The aforementioned results strongly support that the microbial action of peptoids **5**, **7** and **17** is due to membrane disruption, but additional action via a secondary intracellular target cannot be completely ruled out.

Conclusions

In conclusion, peptoids, an emerging class of peptidomimetics that have previously been tested principally for their activity against bacteria in planktonic and biofilm form[26,28,29,45,46] were shown for the first time, to be efficacious against *C. albicans* biofilms and against cross-kingdom polymicrobial biofilms. We have shown the *in vitro* effectiveness of a peptoid library against both single species *C. albicans, S. aureus* and *E. coli* biofilms and we also report their excellent fungicidal and bactericidal activity in polymicrobial biofilms using PMA-modified qPCR. In addition SYTOX® Green membrane leakage assays carried out identified that the cell membranes of *C. albicans*, *S. aureus* and *E. coli* are the likely targets through which the peptoids screened exert their antimicrobial mode of action.

The peptoid library studied showed similar activity patterns across the three species tested by crystal violet assay, i.e. peptoids that were active at some level against one species also tended to be active against the others. However, it was noted that these peptoids generally caused the greatest reduction in biofilm mass against *S. aureus*, followed by *C. albicans,* with *E. coli* biofilms showing the least reduction in biofilm mass following peptoid treatment. Planktonic *E. coli* has also proven more difficult to treat than Gram positive bacteria in previous studies on peptoid efficacy.[24, 25]

From the qPCR data peptoid **17** (*N*ah*N*spe*N*spe)$_3$ was identified as the most promising candidate within the library. This peptoid has low toxicity, significant activity against *S. aureus* in a mixed species biofilm with *C. albicans* at 10 μM and it also shows good activity against both species within the *C. albicans* and *E. coli* cross-kingdom biofilm when treated at higher concentrations.

Finally, peptoids potentially have an advantage over conventional antifungals and antibacterials in that their proposed biological mode of action, through the disruption of cellular membranes, limits the emergence of resistance and would require the targeted fungi and bacteria to undertake the arduous task of altering cellular membrane composition. Furthermore, their enhanced stability towards proteolytic degradation means that peptoids, rather than peptides, may represent a more realistic class of molecule for the development of new therapeutics.

Experimental Section

Peptoid synthesis

Detailed synthesis procedure can be found in the Supplementary Information. Fmoc-protected Rink Amide resin (normally 100 mg, 0.1 mmol, typical loading between 0.6–0.8 mmol g^{-1}) was swollen in DMF (at least 1 hour at room temperature, overnight preferred) in a 20 mL polypropylene syringe fitted with two polyethylene frits (Crawford Scientific). The resin was deprotected with piperidine (20% in DMF *v/v*, 2 x 20 min) and washed with DMF (3 x 2mL). The resin was treated with bromoacetic acid (8 eq. with respect to the resin, 2M in DMF) and DIC (8 eq., 2M in DMF) for 15 minutes at 50 ˚C on a heated shaker at 400 rpm. The resin was washed with DMF (3 x 2 mL), before the desired amine sub-monomer was added (4 eq., 1M in DMF) and allowed to react for 15 minutes at 50 ˚C on the shaker. The resin was again washed with DMF (3 x 2 mL) and the bromoacetylation and amine displacement steps were repeated until the final sub-monomer had been added and the desired peptoid sequence had been obtained. The resin was shrunk in diethyl ether (3 mL) and final cleavage from resin was achieved using a TFA cleavage cocktail (4 mL, TFA:TIPS:H_2O, 95:2.5:2.5) on the shaker at 400 rpm for 60 minutes. The resin was removed by filtration and the cleavage cocktail removed *in vacuo*. The crude product was precipitated in diethyl ether (30 mL) and the precipitate retrieved by centrifuge for 15 min at 5,000 rpm. The ether phase was decanted and the crude product dissolved in a mixture of acidified H_2O and MeCN and lyophilised before purification by RP-HPLC and subsequent characterisation (see Supplementary Information for equipment, procedures and data).

Peptoid cytotoxicity studies

All 18 peptoids were tested for their potential cytotoxicity against Hep G2 epithelial and HaCaT keratinocyte cell lines. Cytotoxicity analyses were performed in 96-well plates (Costar, Fisher Scientific) using alamarBlue® (Invitrogen) for cell viability detection as described by the manufacturer. The HepG2 cells were grown at 37 °C, 5% CO_2 in DMEM high glucose supplemented with heat-inactivated foetal bovine sera (FBS, 10%; Biosera Ltd) and penicillin/streptomycin (P/S, 1%). HaCaT cells were subcultured at 37 °C, 5% CO_2 in DMEM high glucose supplemented with heat-inactivated foetal bovine sera (FBS, 10%; Biosera Ltd) and penicillin/streptomycin (P/S, 1%). Cells were counted using a Neubauer Improved Haemocytometer. HepG2 or HaCaT cells were seeded 24 hours prior to treatment in 96 well plates at a concentration of 2x10^5 cells/mL in 100 μL of medium (2x10^4 cells/well). After 24 hours, cells were incubated with the compounds in a dilution series in triplicate from 2 – 100 μM (5 mM stock solutions in DMSO) in 50 μL of the media for 1 hour. Afterwards, 40 μL were removed from each well before the addition of 90 μL of the media, followed by incubation for 24 hours at 37 °C, 5% CO_2. Then, 10 μL of alamarBlue® (Invitrogen) was added to each well before incubation (2 hours for Hep G2 cells or 1 hour for HaCaT cells) prior to assessing cell viability using a fluorescent plate reader (Biotek; Ex 560 nm / Em 600 nm). All data was measured in triplicate on a minimum of two occasions to ensure a robust data set was collected.

Proteolytic stability studies

Peptoids are considered to be inherently stable to proteolysis[23], however peptoid stability was confirmed by comparing the tryptic digestion profile of one selected, peptoid 7 with the naturally occurring host defence peptide, LL-37 (Innovagen AB, Lund, Sweden). Substrate stocks (5 mg/ml) of Peptoid 7 and LL-37 were prepared and 7.5μL of each substrate stock was incubated with 5μl trypsin and 37.5μL of incubation buffer (50mM tris-HCl pH 7.8, containing 9 mM $CaCl_2$). After 0 hours (for mass spectrometric verification prior to tryptic digestion) or 24 hours treatment with trypsin the reaction mixtures were acidified by addition of an equal volume (50μL) of 10% trifluroacetic acid to stop further enzyme activity. The resulting samples were air-dried and then reconstituted in 50μl acetonitrile/water/TFA (40/59.5/0.5 %, *v/v/v*) and 1 μL was carefully placed onto a stainless steel MALDI target. Samples were covered immediately with 1 μl of matrix (53 mM α-cyano-4-hydroxycinnamic acid in acetonitrile/water/TFA, 70/29.98/0.02 %, v/v/v) solution. MALDI-TOF MS was performed using a linear time-of-flight Voyager DE-mass spectrometer (Perseptive Biosystems, UK). The samples were analysed in positive detection mode, and internal mass calibration with known standards established the mass accuracy as ±0.1 %. Fifty laser scans were averaged for each sample and variable laser intensities were used to ensure the most representative mass spectra for the wells were produced.

Micro-organism strains and growth conditions

C. albicans (NCTC 3179) was subcultured aerobically on Sabouraud agar plates and propagated in yeast peptone dextrose broth. *E.coli* (ATCC 29522) and *S.aureus* (NCTC 6571) were grown on blood agar plates and propagated in brain heart infusion (BHI) broth.

Preparation and treatment of single species biofilms

Overnight cultures of *C. albicans* were washed and resuspended in a modified RPMI-1640 (Sigma-Aldrich, St Louis, USA) medium to yield an inoculum of 1.0 × 10^6 cells/mL. Overnight cultures of *S. aureus* or *E. coli* were washed and resuspended in brain heart infusion broth (Oxoid, Basingstoke, UK) to yield an inoculum of 5.0 × 10^6 cells/mL. A total volume of 100 μL of each inoculum was added to microtitre plate wells (Thermo Fisher Scientific, Roskilde, Denmark). An initial biofilm was allowed to form for 4 hours. Wells were washed three times with 200 μL PBS to facilitate removal of planktonic cells and the biofilms were then treated with 100 μM of peptoids 1-18 in the appropriate broth. Plates were incubated for a further 24 hours to allow biofilm maturation. After removal of planktonic cells by washing, biofilms were quantified by the crystal violet assay or by PMA-qPCR.

Preparation and treatment of polymicrobial biofilms

Overnight cultures of *C. albicans* were prepared in microtitre plates as outlined above and allowed to adhere for 4hrs to facilitate initial biofilm formation. Planktonic *C. albicans* cells were then removed as outlined above before the addition of 100µl of *S. aureus* or *E. coli* (5.0×10^6 cells/mL). Bacteria were allowed to adhere to the *C. albicans* biofilms for 4hrs to facilitate polymicrobial biofilm formation. Following a washing step, the biofilms were then treated with peptoids (10-100 µM), and incubated for a further 24 hours to allow biofilm maturation. Wells were washed as previously outlined and the polymicrobial biofilms were quantified by PMA-modified qPCR.

Biofilm quantification by crystal violet assay

Washed biofilms were fixed with 100 µl methanol for 10 minutes. Following removal of methanol, the wells were air dried and stained with crystal violet solution (Clin-Tech Ltd, Guildford, UK) for 20 minutes at room temperature. Excess stain was removed by washing, the plate was then air dried and bound crystal violet was re-solubilised in 160 µL 33% acetic acid prior to reading at 57 0nm in a microtitre plate reader (Tecan GENios, Zürich, Switzerland).

Biofilm quantification by PMA-modified qPCR

To determine the bactericidal and fungicidal activity of peptoids against both single species and polymicrobial biofilms, the biofilms were detached from the microtitre plate wells prior to quantification. Wells were washed as previously described before 100 µL of BHI broth was added and the plate was sealed. Biofilm detachment was achieved by sonication for 5mins in an ultrasonic bath (Dawe, Middlesex, UK). The remaining cells were then collected in 80 µL BHI. Twenty µL of PMA (Biotium Inc., California, USA) (2mM in broth[47]) was added to the biofilm suspensions (180µl) and incubated at 37°C (5 min) prior to photoactivation with a broad-spectrum LED flood light placed at 15cm from the tubes, (which were mixed by inversion during the 20min photoactivation step)[48]. DNA was extracted using the microLYSIS®-Plus kit (Microzone, Haywards Heath, UK) as per the manufacturer's instructions and qPCR was performed in a Mx3005P qPCR System (Agilent Technologies, California USA) as detailed in Supplementary tables.[49-51]

Generation of standard curves for PMA-qPCR

To allow quantification of the numbers of *C. albicans, S. aureus* and *E. coli* within both single species and polymicrobial biofilms, DNA standards were prepared by extraction of DNA from planktonic organisms using the microLYSIS®-Plus kit and purified using the DNeasy kit (Qiagen, Manchester, UK). DNA standards corresponding to cell numbers from 10^1 to 10^6 were used in PMA-qPCR assays to generate standard curves from which the numbers of living organisms within the biofilms could be determined.

SYTOX® Green assay

Briefly, mid log phase microorganism cultures were adjusted to the appropriate concentration (OD 0.7 at 600nm for S. aureus and E. coli, OD 2.0 at 600nm for C. albicans). A 50 µL of microorganism suspension in Mueller Hinton broth (MHB) was added to each well of a 96 well black flat bottomed plate. 50 µL of each peptoid to be tested at final concentration of 100 µM were added to the wells. SYTOX® Green was added to each well to a final concentration of 5 µM. The plate was covered, protected from light and incubated for 2 hours at 37 ˚C. Microorganisms which had been heat treated at 99 ˚C for 10 minutes to permeabilise their membranes served as positive controls. Bacteria without any form of peptoid treatment acted as negative controls. Additional control wells containing SYTOX® Green only were included to ensure there was no background fluorescence. Wells containing SYTOX® Green and peptoid only were included to ensure there were no interactions between the SYTOX® Green and peptoids that could lead to non-specific fluorescence measurements. The plate was read on a fluorimeter (SpectraMax Gemini X fluorimeter; λex 480 nm, λem 530 nm).

Statistical analysis

The susceptibility of *C. albicans*, *S.aureus* and *E.coli* in both single species and polymicrobial biofilms to novel peptoids was determined by biofilm inhibition assays. All data represent an average of 3 independent experiments and were subject to statistical analysis by One-way ANOVA followed by Tukey's post hoc correction for multiple comparisons, where ns: p>0.5; * p< 0.5; **p<0.05, ***p< 0.001; **** p< 0.0001.

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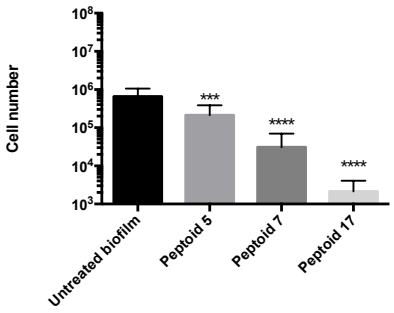
Entry for the Table of Contents

FULL PAPER

Peptoids have been shown to reduce the cell numbers of *C. albicans*, *S. aureus* and *E. coli* in polymicrobial biofilms, as quantified by a novel PMA-qPCR assay.



Yu Luo, Hannah L. Bolt, Gabriela A. Eggimann, Danny F. McAuley, Ronan McMullan, Tanya Curran, Mei Zhou, Colin A. B. Jahoda, Steven L. Cobb and Fionnuala T. Lundy**

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Peptoid efficacy against polymicrobial biofilms determined using propidium monoazide – modified quantitative PCR