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Star-like poly(peptoid)s with selective antibacterial activity

Pedro Salas-Ambrosio,^a Antoine Tronnet,^{b,c} Mostafa Badreldin,^a Luzangel Reyes,^a Marc Since,^d Sandra Bourgeade-Delmas,^e Bruno Dupuy,^c Pierre Verhaeghe,^{b,f} Colin Bonduelle*^a

We developed new macromolecular engineering approaches enabling the preparation of star-like polypeptoids by ring-opening polymerization. Parallely to the evaluation of their cytotoxicity of the HepG2 human cell line, their screening toward a wide variety of Gram-positive and Gram-negative bacteria highlighted several compounds showing not only good but also selective antimicrobial activity.

Introduction,

Antimicrobial peptides (or AMPs) are specific macromolecules made of amino acids that are mainly produced by microorganisms to fight against other competing microorganisms. 1–3 As selective biocides, AMPs are potentially active against all types of microorganisms, thanks to their ability to destabilize cell membranes. 4–7 This particular mechanism of action is due to various physicochemical properties including their amphiphilic character. 6,8 So far, the pharmaceutical development of AMPs remains limited for various reasons including elevated cost of production, scalability issues and their sensitivity to proteases, preventing for instance their oral administration. 9–12 This limitation is a tremendous drawback to treat several infections such as the ones caused by *Clostridioides difficile*, a very important nosocomial pathogen infecting the intestinal tract (an environment full of proteases). 13,14 Synthetic macromolecules are certainly the best candidates to overcome the limitations posed by the production and use of AMPs. 15,16 In this direction, access to large amounts of material is an important asset of using polymer chemistry. Like AMPs, polymers are macromolecules, but unlike AMPs, they are polydisperse in size and constituted of monomer units whose sequence is not controlled. 17,18 Among all polymeric analogs, those made of amino acids called polypeptide polymers are showing great potential by providing one of the best biomimetic and bioactive structures for further biomaterials science applications. 15 Although polypeptide polymers demonstrated very efficient antimicrobial activity (including against drug resistance bacteria), they are susceptible to protease degradation. In this context, “polypeptoids”, also called poly(N-substituted glycines) are N-alkylated analogs of synthetic polypeptide that can provide enhanced resistance to proteolysis. 19,20 The simple preparation of polypeptoids involves the use of building blocks called N-alkylated-N-carboxyanhydrides or NNCAs that are polymerized through the ring-opening polymerization (ROP) using nucleophiles as initiators. 21,22 This facile route of synthesis enables the preparation of well define copolymers whose chemical composition can easily be adjusted. 23

A very interesting point is that ring-opening polymerization reaction paves the way to various topologies (cyclic, branched, conjugates, etc.) that would be difficult to implement using other preparation methods (biotechnology, solid-phase synthesis, etc.). 15 For instance, star polymers are macromolecules with elongated polymeric arms using a core constituted of more than 3 arms and they are known to have the lowest entanglement as compared to cyclic and linear polymers. 24,25 In infectiology, star-like polypeptides have shown impressive antimicrobial activities. They greatly enhanced antimicrobial property as compared to linear polymers, meanwhile they decreased cell toxicity. 26–29

Scheme 1. Preparation of star-like polypeptoids from NNCA by ring-opening polymerization. Herein, the goal was to extend the use of polypeptoids copolymers obtained by ring-opening polymerization to these new star-like architectures. We based our design on copolypeptoids merging cationic (ZLys-NNCA) and hydrophobic (Phe-NNCA) monomers and used dendritic macroinitiators (poly(amidoamine) (PAMAM)). The goal of this study was to evaluate if this macromolecular engineering approach enhanced or modulated the antimicrobial properties of their linear copolymer analogues (Scheme 1).

Results and discussion

Synthesis and characterization of star-like poly(sarcosine).

Several methodologies are well established to design star-like polymers such as atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain-transfer (RAFT) polymerization, living anionic polymerization, ring-opening metathesis polymerization and ring-opening polymerization (ROP).²⁴ Usually, a dendritic core with “n” functional groups is used to promote polymerization growth.²⁴ For instance, star-like polypeptides (from NCAs)²⁶ were successfully developed with PAMAM dendrimers that are displaying primary amines as terminal end groups. With N-alkylated NCA, an early study already reported the preparation of polysarcosine from poly(trimethyleneimine) dendrimers with 64 primary amine groups.³⁰ Here, we decided to employ PAMAM dendrimers macroinitiators to perform various copolymerizations of NNCA monomers. In a first step, we optimized the ROP from PAMAM-(NH₂)₁₆ with Sar-NCA (as a model NNCA) targeting a DP = 30 (for each -NH₂ terminal group). According to already published procedures, we set up three different methodologies (table 1): Method A) Direct addition of initiator: we prepared a 0.4 M Sar-NCA (480 equiv.) solution in anhydrous DMF and we added an aliquot of PAMAM-(NH₂)₁₆ (1 equiv., solution in DMF at 0.01 M), Method B) Direct addition of monomer:²⁶ we prepared a solution of Sar-NCA (480 equiv.) that we added to a solution with PAMAM-(NH₂)₁₆ (1 equiv.) targeting a final concentration of 0.37 M in DMF (the volume ratio of DMF was 16:1 for monomer:initiator), Method C) Reduced concentration: we prepared a solution of Sar-NCA (480 equiv.) that we added to a solution with PAMAM-(NH₂)₁₆ (1 equiv.) targeting a final concentration of 0.1 M in DMF (the volume ratio of DMF was 1:1 for monomer:initiator). The polymerizations were followed by FTIR monitoring the NNCA peak at 1850 cm⁻¹. Upon full conversion, the s-(P(Sar)₃₀)₁₆ were precipitated in Et₂O, dried under vacuum and isolated in good yields 78-87%. Then, we characterized the polymers obtained from each method by SEC and ¹H-NMR (see table 1 and figure 1). From the SEC analyses in DMF, we characterized polymers with numbered-molar masses (M_n) similar to the theoretical values (M/I theoretical, table 1). Careful considerations of the MW values and of dispersity made us conclude that the best methodology was the use of a lower concentration providing a dispersity value Đ_M = 1.11 and also the highest yield (87%). With this method, the ROP from different PAMAM (PAMAM with 4, 8, 16 -NH₂ terminal groups) further showed that conversions were not significantly influenced by the number of arms and that the efficiency of the polymerization of each arm was approximately the same (data not shown). We then characterized the s-(P(Sar)₃₀)₁₆ by ¹H-NMR in DMSO-d₆. We observed the characteristic peaks corresponding to poly(sarcosine) signals a and b (3.80-4.42 and 2.50-3.10 ppm) but we were not able to determine the DP_{NMR} because i) the signals of the dendrimer initiator were overlapped by the signals of poly(sarcosine)³¹ and ii) the signals of the dendrimer initiator were very weak due to the low proportion of the PAMAM protons, as compared to the protons belonging to the copolymer arms (1:480). In addition to NMR, SEC

analyses provided clear evidence that we successfully prepared star-like polypeptoids in quantitative yields (see esi figure S1).

Figure 1. Star-like polysarcosine. A) The preparation was achieved by ROP of NNCA from PAMAMs. B) ¹H NMR in DMSO-d₆ of the s-(P(Sar)₃₀)₁₆ obtained with 3 different methods (A: direct addition of initiator; B: direct addition of monomers; C: reduced concentration). Table 1. Comparison of SEC characterizations and yields obtained by analyzing s-(P(Sar)₃₀)₁₆ from three methodologies studied

M/I _{theo} (g/mol)	M _n (g/mol) ^a	M _w (g/mol) ^a	Đ _M	Yield (%)		
Method A	30	3.7x10 ³	4.69x10 ³	5.29x10 ³		
1.13	86					
Method B	30	3.7x10 ³	3.68x10 ³	4.12 x10 ³	1.12	78
Method C	30	3.7x10 ³	3.5x10 ³	3.90 x10 ³	1.11	87

^a SEC performed in DMF. ^b Calculated from the ¹H-NMR spectrum in DMSO-d₆

Synthesis and characterization of star-like poly(peptoid)s copolymers.

In infectiology, early studies involving branched copolymers constituted of methylmethacrylate and dimethyl-aminoethyl-methacrylate demonstrated strong inhibition growth of *Escherichia coli* and *Staphylococcus aureus* in diffusion agar plates.³² Since then, similar works were developed to apply this macromolecular engineering approach with other polymers including antimicrobial polypeptides.^{32–35} Overall, star-like polypeptide were shown to enhance the antimicrobial efficacy: to access such star-shaped copolymers means preparing macromolecules incorporating of cationic and hydrophobic side chains. So far, no one has extended the use of this design to antibacterial polypeptoids. Using methodologies previously developed for cyclic polypeptoids,²⁰ we prepared copolymers by mixing N-protected-lysine-like NNCA (ZLys-NNCA) and phenylalanine-like NNCA (Phe-NNCA) using PAMAM dendrimers initiators. Monomers were prepared through an optimized three-step synthesis method (see esi). We then performed the ROP from PAMAM with different numbers of arms (PAMAM with 4, 8, 16 -NH₂ terminal groups) at M/I = 30. We mixed ZLys-NNCA and Phe-NNCA in equal stoichiometry (to target of 50% of hydrophobic content) in DMF and under argon atmosphere (0.1 M at RT, step 1, Scheme 2)

Scheme 2. Synthesis of star-like copolypeptoids from ZLys NNCA and Phe-NNCA varying the number of arms.

We monitored the NNCA disappearance of the N-COO signal at 1850 cm⁻¹ by FTIR. Then we isolated the star-protected copolymers S-P(ZNLys-NPhe) 1-3 by precipitation, drying under high vacuum in 40-45% yields and characterized them by SEC and NMR analyses (table 2, figure 2 and figure S2, S3).[‡] First, we characterized the copolymers 1-3 by ¹H-NMR in DMSO-d₆ (figure 2A and figure S3-S7). We found signals attributable to the polymer backbone (CH₂, peak a,a'), signals attributable to the N-Cbz-aminobutyl-glycine (ZNLys) moiety (peaks

b-h) and signals attributable to N-benzyl-glycine (NPhe) moiety (peaks i,j). At 20°C, the determination of the hydrophobic content from the integrations of these signals gave us values far from what we expected (figures 2A, S4A, S5A). An ¹H NMR analysis performed at higher temperatures further showed that this deviation comes from a poor solubility of the hydrophobic monomer units in the organic solvent (peak j+h and i, figure 2B). On another hand, we could not determine the DPNMR due to i) the high monomer/initiator ratio (120:1 for PAMAM with 4 arms and even higher for PAMAM with 16 arms) and ii) to the PAMAM signals overlapped with the copolymer (peaks b, e, a, a'). Second, we characterized the copolymers 1-3 by SEC in DMF. These analyses allowed to calculate a 2-fold lower weighted-molar mass (M_w) for the different synthesized star copolymers. This result was not surprising as star polymers are known to elute at lower volumes, compared to their linear counterparts, this being due to a difference on the hydrodynamic radius.³⁶ As other factors such as solvation can also give rise to differences, the decrease in hydrodynamic radius was better evidenced by plotting the double logarithm of mean square radius (RMS radius) versus molar masses (MW) between linear and star-like polymers (figure 2D).³⁷ Indeed, comparing linear copolymer P(ZNLys-Nphe) initiated with allylamine at M/I = 30 with the star-like copolypeptoid S-P(ZNLys-NPhe) 1, the difference in slope values was in agreement with the difference expected for branched polymers (values of slope < 0.5).³⁸ This SEC characterizations also evidenced that we prepared star-shaped copolymers with 2 and 3 (see figure S3). It is to note that we obtained a polymer dispersity that increased with the number of arms (ĐM = 1.18 for 4 arms and up to 1.40 for 16 arms), a feature already reported with star-like polypeptides.²⁸

Table 2. Star-[P(Nlys-Nphe)] copolymers: molar masses from SEC, hydrophobic content from ¹H-NMR and yields.

Arms (g/mol)	M/I Mn (g/mol)	H/C (%) a	Mw (g/mol)	MW _{theo} ĐM	Yield before deprotection (%)‡	Yield upon deprotection (%)‡	H/C
S-P(ZNLys-NPhe) 1	4	30	50	2.78x10 ⁴	1.20x10 ⁴	1.42x10 ⁴	1.18
45	52	21					
S-P(ZNLys-NPhe) 2	8	30	50	5.24x10 ⁴	1.75x10 ⁴	2.38x10 ⁴	1.36
43	55	41					
S-P(ZNLys-NPhe) 3	16	30	50	1.02x10 ⁵	3.59x10 ⁴	5.05x10 ⁴	1.40
40	53	62					
S-P(ZNLys-NPhe) 4	16	30	30	1.13x10 ⁵	3.62x10 ⁴	5.11x10 ⁴	1.41
39	35	55					
S-P(ZNLys-NPhe) 5	16	30	10	1.24x10 ⁵	4.05x10 ⁴	5.28x10 ⁴	1.30
45	12	42					

a SEC performed in DMF. b Hydrophobic content calculated from the ¹H-NMR spectrum in DMSO-d₆. c Calculated from the ¹H-NMR spectrum in D₂O

Figure 2. Representative characterizations of star-[P(ZNLys-NPhe)]: copolymer 1 with 4 arms. 1H-NMR spectra A) in DMSO-d₆ before deprotection at 20 °C; B) in DMSO-d₆ before deprotection at 70 °C; C) in D₂O upon deprotection. D) Double logarithm plot of the root means square radius plot versus molar masses of a linear copolypeptoid M/I = 30 and the star-like copolymer bearing 4 arms of P(ZNLys-NPhe) 1 M/I = 30. The slope for linear polymer was 0.8±0.04 and for star polymer slope 0.4±0.04.

Following a methodology we previously developed,²⁰ we then performed the deprotection of the Z-Lys side chains in TFA by adding 2 equivalents of HBr (relative to cationic side chains in TFA, RT). After 3 h, the copolymers (S-P(NLys-NPhe)) were isolated by precipitation in diethyl ether, dialyzed (MWCO = 10 kDa), and lyophilized (yields 21-62%, table 2, figure 2C).[‡] To verify the chemical composition in monomer units, we analyzed the samples by 1H-NMR in D₂O (figure 2C). We found signals attributable to the polymer backbone (CH₂, peak a,a'), signals attributable to the N-aminobutyl-glycine (NLys) moiety (peaks b-e) and signals attributable to NPhe moiety (peaks i,j). We calculated the hydrophobic content by carefully comparing the integration of NLys (peaks c,d) and NPhe (peak j). In marked contrast to hydrophobic contents before deprotection, these contents were in agreement with the theoretical ratio given by the stoichiometry of NNCA monomers (48-54%, table 2). Once again, we could not determine the DPNMR due to the high monomer/initiator ratio (120:1 for PAMAM with 4 arms and even higher for PAMAM with 16 arms) and due to the PAMAM signals overlapped with the copolymer (peaks b, e, a, a').

Antimicrobial potency: number of arms

A key work reported in 2016 by G. Qiao et al. was that star-like polypeptides showed good in vitro and in vivo activities against multidrug-resistance bacteria.²⁶ Composed of several polymeric arms (either 16 or 32 with a polymerization degree of 30), containing lysine and valine (hydrophobic content 30%), these star-like polypeptides were active in killing Gram-negative and Gram-positive bacteria, including multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The antibacterial structure-activity relationship studies revealed that increasing arm numbers (16) and length (30) enhanced antimicrobial activity but this increase was associated with an increase in cytotoxicity.²⁸ More recently, I. Kim et al. prepared copolymers made of TFA-lysine and Cbz-glutamate monomer units by grafting from tris-(2-aminoethyl)amine initiators, using ring-opening polymerization. Copolymers bearing 3 arms, having a polymerization degree of 30 and containing 20% of hydrophobic content were shown to be very good antimicrobial activity meanwhile keeping low hemolytic effects.³⁹ Even more recently, J. Cai et al. reported the preparation of a star polymer with 8 arms attached to polyethyleneimine dendrimers and constituted of mixed cationic side chains (lysine or ornithine with diaminoheptylic acid).²⁹ The best properties were found with ornithine at M/I = 20 with improved activity against Gram-positive methicillin-resistant *S. aureus* and *S. epidermidis*, and *P. aeruginosa*.

In this work, copolymers 1-3 were used to evaluate the antimicrobial potency towards representative pathogens: Gram-positive (*C. difficile*, Methicillin-sensitive- and -resistant *S. aureus*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Streptococcus pneumoniae*), Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *A. baumannii*), and anaerobic or microaerophilic bacteria (*Bacteroides fragilis* and *Helicobacter pylori*). The results are presented in figure 3 (and table S1): we determined the MIC from the microdilution method

bacteria (see experimental section, approximately 105 CFU/mL) for 24-48 h with increasing concentrations of polypeptoids.

We found that S-P(Nlys-Nphe) 1 showed interesting activity against most of Gram-positive and Gram-negative bacteria: the MIC was found below 62.5 µg/mL except for *S. pneumoniae* (MIC above 250 µg/mL). The best antimicrobial activities were obtained against *B. fragilis* (anaerobic Gram-negative bacteria) with a MIC value of 7.8 µg/mL and against *L. monocytogenes* (Gram-positive bacteria) with a MIC value of 15.6 µg/mL in comparison with the positive control amoxicillin that was less active: 15.6 µg/mL for *B. fragilis* and 250-500 µg/mL for *L. monocytogenes* (see ESI).

S-P(Nlys-Nphe) 2 (figure 3 and table S1) was comparatively less active against the different Gram-positive and Gram-negative bacteria. However, as compared to copolymer 1, we observed a similar activity against *L. monocytogenes* (MIC value of 15.6-31.25 µg/mL) and an enhanced activity against *B. fragilis* (MIC value of 7.8 µg/mL). Finally, the antimicrobial potency of S-P(Nlys-Nphe) 3 was very similar to the one of 2: the copolymer was moderately active against most of the different Gram-positive and Gram-negative bacteria tested with several MIC values found below 250 µg/mL (figure 3). Copolymer 3 was more selective with an optimized antimicrobial efficacy found against *L. monocytogenes* and *B. fragilis*.

Interestingly, 3 showed decreased anti-*B. fragilis* activity reflected in 4-fold increase of the inhibitory concentration (MIC = 31.25 µg/mL) value as compared to 2.

In line with previous work on polypeptides,²⁶⁻²⁹ antimicrobial activities evaluated on a single arm (linear copolymer, Figure S8 and Table S4 in esi) showed that the star-like architecture optimized the anti-infective potency, especially for *S. aureus*, *E. faecalis*, *P. aeruginosa* and *B. fragilis*. Overall, these first studies established that star-like polypeptoids can promote antibacterial properties with a significantly broad spectrum of action although some antibacterial activities were more selective. Also in agreement with the literature, the number of arms was an important parameter to tune the activity:^{27,28} MIC values against *B. fragilis* were for instance optimized with the star copolymer 2 bearing 8 arms.

Figure 3. Minimum inhibitory concentrations (MIC) of star-like polypeptoids (S-P(Nlys-Nphe) 1-3 against representative pathogens. *MRSA = Methicilline resistant *S. aureus*.

Antimicrobial potency: varying the hydrophobic content

To better understand the structure/antimicrobial activity relationship, we also modified the cationic/hydrophobic ratio of each arm of the star-like topology to see how this parameter could also influence the antimicrobial efficacy. Indeed, the hydrophobic content is an important parameter when the antibacterial activity involves membrane destabilization.^{23,40,41} In this new study, we prepared a small series of star-P(ZNlys-Nphe) using a PANAM dendrimer having 16 (-NH₂) terminal functions because this topology was expected to present the lowest cytotoxicity.²⁶ We performed the polymerization using ZLys-NNCA (1, 1.4 and 1.8 equiv.; 50, 70, 90 hydrophobic content) and Phe-NNCA (1, 0.6, 0.2 equiv.; 50, 30, 10% hydrophobic content) using PAMAM initiator at M/I = 30 (4.16x10⁻³ equiv.) in DMF 0.1 M at RT under argon atmosphere (scheme 3).

We followed the conversion of the ROP by monitoring FTIR until reaction completion (NNCA stretching at 1850 cm⁻¹). Purification of the copolymers was achieved by precipitation in Et₂O and drying under high vacuum. Star like copolymers were then isolated in 39-45% yields and characterized by 1H-NMR in DMSO-d₆ (figure S5-S7). We found signals attributable to the polymer backbone (CH₂, peak a,a'), signals attributable to the ZNlys

moiety (peaks b-h) and signals attributable to Nphe moiety (peaks i,j). Once again, i) the determination of the hydrophobic content from the integrations of these signals gave us values far from what we expected (figures S4-S7); ii) we could not determine the DPNMR due to the high monomer/initiator ratio (120:1 for PAMAM with 4 arms and even higher for PAMAM with 16 arms) and due to the PAMAM signals overlapped with the copolymer (peaks b, e, a, a').

Scheme 3. Synthesis of star-like copolypeptoids from ZLys NNCA and Phe-NNCA varying the hydrophobic content.

We also characterized the copolymers by SEC in DMF (see esi figure S2, S3). These analyses allowed calculating a 2-fold lower weighted-molar mass (M_w) for the different star copolymers synthesized 3-5, similarly to polymers 1 and 2. Moreover, we observed a decrease in hydrodynamic radius by plotting the double logarithm of mean square radius (RMS radius) versus molar masses (MW) between linear and star-like polymers (figure S3)³⁷ demonstrating that we successfully prepared star-shaped copolymers (dispersities $\Delta M = 1.3-1.4$).

Then, we performed Z-Lys side chains deprotection in TFA by adding 2 equivalents of HBr (relative to cationic side chains in TFA, RT). After 3 h, the copolymers were purified by precipitation in diethyl ether, dialyzed, and lyophilized (yields 42-62%, see esi). To determine the DPNMR and the chemical composition in monomer units, we analyzed the samples by ¹H-NMR in D₂O (see figure S5-S7). We calculated the hydrophobic content by carefully comparing the integration of signals belonging to NLys side chains (peak c,d) and NPhe side chains (peak j) and we found values that were in agreement with the expected ones (table 2). Moreover, NMR characterization confirmed that the copolymers did not suffer from chemical modifications during the deprotection step and that cationic copolymers containing hydrophobic units were prepared.

This new series of copolymers were then studied in biology to evaluate the antimicrobial activity. The results are presented in table S2. First, we observed that copolymer 4 with a hydrophobic content of 30%, was very active against *L. monocytogenes* with a MIC value of 15.6 $\mu\text{g/mL}$ and *B. fragilis* with a MIC value of 31.25 $\mu\text{g/mL}$. However, other MIC values obtained with other Gram-negative and Gram-positive bacteria tests were found higher (MIC = 62.5 $\mu\text{g/mL}$ and above) as compared to the copolymer 3 composed of higher amount of hydrophobic units. With even lower hydrophobic content, star-like copolymer 5 provided a good MIC value of 15.6-31.25 $\mu\text{g/mL}$ against *L. monocytogenes*. Surprisingly, this activity was more selective than for copolymer 4 because other MIC values were found above 62.5 $\mu\text{g/mL}$. Overall, we observed that star-like copolymer 4 with a 30% of hydrophobic content presented a broader and better antimicrobial activity (MIC < 62.5 $\mu\text{g/mL}$) as compared to copolymers with 10 and 50% or linear counterpart (figure S8 and table S4). This study demonstrated that modulating hydrophobic content in star-like polypeptoids modify the activity against bacteria as we also previously observed for cyclic polypeptoids.²⁰

Table 3. Cytotoxicity over CaCO-2 cells and selectivity indices (SI) of star-like polypeptoids. Minimum inhibitory concentration (MIC) determined on *B. fragilis* and *L. monocytogenes*.

Polym	CaCO-2 CC50 ($\mu\text{g/mL}$)	MIC	<i>B. fragilis</i> ($\mu\text{g/mL}$)	SI
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	(CC50/MIC)	MIC			
L. monocyt.	($\mu\text{g}/\text{mL}$)	SI			
	(CC50/MIC)				
1	79 ± 3	7.8	10	15.6	5
2	90 ± 9	7.8	11	15.6	6
3	138 ± 5		31.25	4	31.25
4	47 ± 2	31.25	3	15.6	3.0
5	42 ± 3	250	<1	15.6	3
Doxoa	8 ± 0.1	-	-	-	-
Amoxb		15.6		250-500	

a Doxo: doxorubicin, bAmox: amoxicillin

Later, we also assessed the cytotoxicity of the copolymers (1-5) using the human hepatic CaCO-2 cell line (table 3). We observed moderated cytotoxicity for all the star-like copolypeptoids and contrary to star-like polypeptides, no significant change in cytotoxicity values was observed neither by modifying the amount of arms nor by changing the hydrophobic content.² Thus, the values of the selectivity index indicated that some star-like copolypeptoids could be interesting starting points for the conception of further antimicrobial compounds against *B. fragilis* or against *L. monocytogenes* (table 3).

Membrane disruption and protease resistance.

It is expected that the antimicrobial activity of our polypeptoid structures (made of cationic and hydrophilic side chains) was explained by bacterial membrane destabilization. We verified in vitro this possible mechanism by using two different liposome models containing carboxyfluorescein (CF): i) neutral liposome membranes constituted of soybean phosphatidylcholine and cholesterol (SPC:Chol, 8:2) with a zeta potential of $\zeta = -3.9 \pm 0.9$ mV and ii) negatively charged liposome membranes constituted of 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (PG) and cholesterol (PG:Chol, 8:2) with a zeta potential of $\zeta = -33.3 \pm 2.2$ mV. The liposomes were first loaded with the CF fluorophore. Then, the star-like copolymers P(NLys-NPhe) 1-5 were prepared at a concentration of $90 \mu\text{g}/\text{mL}$ and mixed with the liposomes. Upon incubation (1.5 h), the concentration of released of carboxyfluorescein was measured by fluorimetry ($\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 528$ nm) and reported in percentage of release (%CF release). As depicted in figure 4, we clearly observed that copolymers having a hydrophobic content of 50% were better in destabilizing both negatively and neutral charged liposome models (80-90% leakage, figure 4). This result was verified, whatever the number of arms. In marked contrast, changing the hydrophobic content was strongly influencing the membrane destabilization of the liposomes: the lower the hydrophobic content in the 16-arm polypeptoid was the lower ability to destabilize the liposomes we observed (5 afforded 0% and 3% of CF release).

Figure 4. Membrane destabilization by active cyclic copolypeptoids using two different liposome models. SPC:Chol (8:2); PG:Chol (8:2).

The results obtained with this model of membrane destabilization was particularly in agreement with the MIC results obtained against *B. fragilis* and could explain why 5 had the lowest MIC value ($250 \mu\text{g}/\text{mL}$) against this pathogen. However, the antibacterial tests presented in table S2 also showed that copolymer 4 (hydrophobic content of 30%) exhibited

a broader spectrum of action and some higher antibacterial activities than copolymers 3 and 5 (with hydrophobic content of 10 and 50%). We attributed this difference to the fact that bacterial membranes are much more complex than a simple mixture of phospholipids. Overall, by using liposomes, we verified that the star-like polypeptoids could destabilize the bacterial cell membrane with a significant influence of the hydrophobic content on this destabilization.

Finally, peptoids are known to resist proteolytic degradation mediated by proteases.^{19,20} This characteristic made them as an attractive backbone to develop therapeutic agents that could be administered orally. However, proteolysis of cationic polypeptoids is scarcely documented.²⁰ In this work, we carried out a proteolysis experiment using trypsin (one of the main proteases presented in the digestive tube), we measured the proteolytic activity implementing a methodology developed for small peptoids, that consisted in reacting 4-Fluoro-7-nitrobenzofurazan (NBDF) with secondary amines (SNAr reaction) resulting from the proteolysis of the amide bonds upon digestion of the polymer (0.1 mM in NaHCO₃ buffer 50 mM pH = 7.8) at 37 °C (see esi). The proteolysis was performed for all the star-like copolymers P(NLys-NPhe) (1-5) and compared with a linear polypeptide (poly(lysine-phenylalanine) M/I = 200 having 10% hydrophobic content (see esi). We followed the progression of the reaction by taking aliquots at a certain time and upon NBDF treatment we measured the fluorescence ($\lambda_{em} = 535$ nm, figure S9). These NBDF experiments clearly indicated that all the star-like copolymers were not degraded by trypsin within 50 min, while the linear polypeptide was rapidly degraded in the first 20 min. In agreement with a previous study,²⁰ these results indicated that star-like copolypeptoids could be an interesting approach for further oral administration development.

Conclusions

In summary, by using grafting-from dendrimers, ring-opening copolymerization of NNCAs was shown to afford antimicrobial structures. This methodology allowed accessing star-like topologies with fine-tuning over the hydrophobic content and the number of arms. The star-shaped polypeptoids were containing cationic side chains and a preliminary antibacterial structure-activity relationship study allowed establishing that these copolymers were having significant activity. In general, star-like polypeptoids were better at killing Gram-positive germs, particularly *L. monocytogenes* with MIC values of 15.6-32.5 $\mu\text{g}/\text{mL}$ and star-like copolypeptoids with 8 arms enhanced the antibacterial activity against the anaerobic Gram-negative *B. fragilis* (MIC = 7.8 $\mu\text{g}/\text{mL}$). All these antimicrobial structures were able to destabilize bacterial membrane at a high hydrophobic content of 50% and they were able to resist trypsin proteolysis. We believe that these dendritic polypeptoids could bring interesting solutions in health applications that merge materials science and infectiology.

Experimental

Materials. All the chemicals and solvents in this work were purchased from Sigma Aldrich, Fluorochem, Acros, TCI, Strem and, unless otherwise described, were used without any purification. Dimethylformamide (DMF), tetrahydrofuran (THF) were obtained from a solvent system purificator (PureSolv, Innovative Technology), kept under argon atmosphere and freshly used. MilliQ water was obtained from a (Purelab Prima, ELGA). The monomers synthesized were stored at 4 °C under argon atmosphere and weighted in the glove box Jacomex GP13 No. 2675 at the Laboratoire de Chimie des Polymères Organique (LCPO, Bordeaux, France). Studies using *Clostridioides difficile* 630 Δ erm were performed at LPBA Pasteur Institute, Paris, France; studies using *Staphylococcus aureus* CIP 4.83, *Staphylococcus aureus* ATCC 33591 (MRSA or methicilline resistant strain), *Enterococcus*

faecalis CIP 103015T, Streptococcus pneumoniae CIP 104.471, Listeria monocytogenes CIP 82.110T, Escherichia coli CIP 53.126, Acinetobacter baumannii CIP 70.34T, Pseudomonas aeruginosa CIP 82.118, Bacteroides fragilis CIP 77.16 and Helicobacter pylori were performed at FONDEREPHAR, Toulouse, France.

Methods. The IR spectra were recorded using the FTIR spectrometer (Vertex 70, Bruker), and the samples were measured with the ATR (GladiATR, Pike Technologies) from Fisher technologies performing 32 scans at the LCPO (Bordeaux, France). The raw data were obtained with the Opus7.5 software and processed using the Originlab 2016 software. The NMR spectra were recorded using a Bruker Avance III 400 spectrometer (LCPO, Bordeaux, France). The spectra were analyzed at 295 K and data was analyzed using Mestrenova 14.1.2 software. The chemical shifts of the signals are given in ppm. The spectra obtained were calibrated using the residual solvent signals (CHCl₃ 7.26 ppm, H₂O 4.79 ppm, DMSO-d₆ 2.50 ppm). The signals were categorized as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br). Polymer molar masses were determined by Size Exclusion Chromatography (SEC) using: a) Dimethylformamide (DMF + lithium bromide LiBr 1g/L) as the eluent (LCPO, Bordeaux, France). Measurements in DMF were performed on an Ultimate 3000 system from Thermoscientific equipped with diode array detector DAD. The system also included a multi-angle light scattering detector MALS and differential refractive index detector dRI from Wyatt technology. Polymers were separated on two Shodex Asahipack gel columns GF310 and GF510 (300 x 7.5 mm) (exclusion limits from 500 Da to 300 000 Da) at a flow rate of 0.5 mL/min. Columns temperature were held at 50°C. The chromatograms were recorded with the Chromeleon 7.2 software and Astra 7.1.0 software and analyzed using the Originlab 2016 software. The dn/dc were determined experimentally. b) Hexafluoro-2-propanol (HFIP+ 0,05% KTFA) as the eluent (LCPO, Bordeaux, France). Measurements in HFIP were performed on an Ultimate 3000 system from Thermoscientific equipped with diode array detector DAD. The system also included a multi-angle light scattering detector MALS and differential refractive index detector dRI from Wyatt technology. Polymers were separated on PL HFIP gel column (300 x 7.5 mm) (exclusion limit from 100 Da to 150 000 Da) at a flow rate of 0.8 mL/min. Column temperature were held at 40°C. The chromatograms were recorded with the Chromeleon 7.2 software and Astra 7.1.0 software and analyzed using the Originlab 2016 software. Either the molar mass was calculated using a calibration curve or the dn/dc value. The calibration curve was performed with polystyrene standards with molar masses in the range 0.9-364 kg/mol. The dn/dc were determined experimentally. MALDI-MS spectra were performed at CESAMO facility (Bordeaux, France) on an Autoflex maX TOF mass spectrometer equipped with a frequency tripled Nd:YAG laser emitting at 355 nm. Spectra were recorded in the positive-ion mode using the reflectron and with an accelerating voltage of 19 kV. For MALDI-MS analyses, polypeptoids deposits were prepared according to the two following recipes: 1) Polypeptoids and the cationic agent (NaI) were dissolved in methanol at 10 mg/mL. The α -CHCA matrix (α -cyano-4-hydroxycinnamic acid) solution was prepared by dissolving 10 mg in 1 mL of methanol and the solutions were combined in a 10:1:1 volume ratio (matrix: sample: salt). One microliter of this solution was deposited onto the grid and vacuum-dried before analysis. 2) the polymer and the cationic agent (NaI) were dissolved in methanol at 10 mg/mL. The dithranol matrix solution was prepared by dissolving 10 mg in 1 mL of dichloromethane and the solutions were combined in a 10:1:1 volume ratio (matrix: sample: salt). One microliter of this solution was deposited onto the grid and vacuum-dried before analysis.

Synthesis of monomers and aminoacid precursors. The preparation is described in ESI.

Synthesis of star-poly(N-methyl)glycine S-P(Sar). N-Methyl-NCA monomer (Sar-NCA, 0.1 g, 8.7×10^{-4} mol, 480 equiv.) was weighed in a glovebox under pure argon, introduced in a flame-dried Schlenck vessel, and dissolved with 4 mL of anhydrous DMF. In another Schlenck vessel PAMAM-(NH₂)₁₆ 29.5 mg (20% w/t in MeOH, 1.81×10^{-6} mol, 1 equiv.) was dried and dissolved with 4.7 mL of anhydrous DMF. The monomer solution was added to the initiator solution with vigorous stirring avoiding cloudiness during the addition. The mixture was stirred at room temperature under argon until completion (20 days) and confirmed by FTIR. The polymer was then recovered by precipitation in diethyl ether and dried under high vacuum. Yield: 87% (0.059 g, white solid). Molar mass (SEC in DMF, dn/dc = 0.0942): Mn = 3.50×10^4 g/mol ĐM = 1.11. ¹H-NMR DMSO-d₆ 400MHz δ (ppm): 2.50-3.10 (m, 3H, CH₃), 3.80-4.42 (m, 2H, CH₂).

Star-poly[(N-aminobutylglycine)-(N-benzylglycine)] [S-P(Nlys-Nphe)] 4 arms. N-(Cbz-(2-aminobutyl))-NCA (ZLys-NNCA, 0.2 g, 6.53×10^{-4} mol, 60 equiv.) and N-benzyl-NCA (Phe-NNCA, 124.8 mg, 6.53×10^{-4} mol, 60 equiv.) were weighed in a glovebox under pure argon, introduced in a flame-dried Schlenck vessel, and dissolved with 6 mL of anhydrous DMF. In another Schlenck vessel PAMAM-(NH₂)₄ 28.1 mg (20% w/w in MeOH, 1.09×10^{-5} mol, 1 equiv.) was dried and dissolved with 7 mL of anhydrous DMF. The monomer solution was added to the initiator solution with vigorous stirring avoiding cloudiness during the addition. The mixture was stirred at room temperature under argon until completion and confirmed by FTIR. The polymer was then recovered by precipitation in diethyl ether and dried under high vacuum. Yield: 45% (0.122 g, yellowish solid).

SEC (DMF, dn/dc = 0.0816): 12000 g/mol, ĐM = 1.18

¹H-NMR DMSO-d₆ 400MHz δ (ppm): 1.12-1.65 (m, 4H, 2CH₂), 2.86-3.02 (m, 2H, CH₂), 3.07-3.27 (m, 2H, CH₂), 3.75-4.48 (m, 2H, CH₂), 4.87-5.09 (m, 2H, CH₂), 6.94-7.47 (m, 5.84H, Ar+NHCO). We could not observe the CH₂ of the benzyl group approx. 4.5 ppm.

Deprotection method to prepare Star-poly[(N-aminobutylglycine)-(N-benzylglycine)] Polym 1. In a test tube, 100 mg of S-P(Nlys-Nphe)] 4 arms (1.9×10^{-4} mol, MW = 262.32 g/mol monomer unit and taking into account as 50% mol) were completely dissolved with 1 mL of trifluoroacetic acid. To the polymer solution, 68.5 μL of HBr 33% (3.8×10^{-4} mol) was added and the solution was stirred at 20 °C for 3 hours. Then, the polymer was precipitated on diethyl ether, centrifuged at 4000 rpm the supernatant was removed, the solid was resuspended in 1 mL of water and the pH was adjusted to pH = 7 with a NaHCO₃ saturated solution. The solutions were dialyzed using regenerated cellulose membranes (MWCO 10 kDa), the first time with Milli-Q water, followed by phosphates buffer pH = 7 (0.05 M) and two replacements with Milli-Q water. The solutions were freeze-dried (BenchTop Pro, SP Scientific). Upon freeze-drying, the copolymer was recovered as a yellowish powder in 21% yield (0.0185 g).

Calculated hydrophobic content by comparison of 2CH₂ (1.37-1.92 ppm) and Ar (6.93-7.39 ppm) according to:

$$H/C\% = \frac{[XH]}{Ar} \times 50$$

$$H/C\% = 52\%$$

¹H-NMR D₂O 400 MHz δ (ppm): 1.34-1.92 (m, 4H, 2CH₂), 2.75-3.04 (m, 2H, CH₂), 3.09-3.35 (m, 2H, CH₂), 3.373-4.63 (m, 4H, 2CH₂), 6.93-7.39 (m, 5.2H, Ar).

Star-poly[(Z-N-aminobutylglycine)-(N-benzylglycine)] [S-P(ZNlys-Nphe)] 8 arms. The protected polymer was obtained according to the 4 arm copolymer through ROP of Phe-NNCA and ZLys-NNCA. Yield: 43% (0.118 g, yellowish solid). SEC (DMF, dn/dc = 0.0816): 17500 g/mol, ĐM = 1.36. Hydrophobic content calculation by comparison of Ar (6.94-7.47 ppm) and

2CH₂ (1.12-1.65 ppm) = 51%. ¹H-NMR DMSO-d₆ 400 MHz δ (ppm): 1.12-1.65 (m, 4H, 2CH₂), 2.86-3.02 (m, 2H, CH₂), 3.07- 3.27 (m, 2H, CH₂), 3.75-4.48 (m, 2H, CH₂), 4.87-5.09 (m, 2H, CH₂), 6.94-7.47 (m, 6.15H, Ar+NHCO). We could not observe the CH₂ of the benzyl group approx. 4.5 ppm.

Star-poly[(N-aminobutyglycine)-(N-benzylglycine)] [S-P(Nlys-Nphe)] 8 arms (polym 2). The deprotection was carried out as polym 1. Yield: 41% (0.036 g, yellowish solid). Calculated hydrophobic content by comparison of Ar (1.37-1.92 ppm) and 2CH₂ (6.93-7.39 ppm) = 55%. ¹H-NMR D₂O 400 MHz δ (ppm): 1.34-1.92 (m, 4H, 2CH₂), 2.75-3.04 (m, 2H, CH₂), 3.09-3.35 (m, 2H, CH₂), 3.373-4.63 (m, 4H, 2CH₂), 6.93-7.39 (m, 5.48H, Ar).

Star-poly[(Z-N-aminobutyglycine)-(N-benzylglycine)] [S-P(ZNlys-Nphe)] 16 arms. The protected polymer was obtained according to the 4 arm copolymer through ROP of Phe-NNCA and ZLys-NNCA. Yield: 40% (0.11 g, yellowish solid). SEC (DMF, dn/dc = 0.0816): 35900 g/mol, ΔM = 1.40. Hydrophobic content calculation by comparison of Ar (6.94-7.47 ppm) and 2CH₂ (1.12-1.65 ppm) = 52%. ¹H-NMR DMSO-d₆ 400 MHz δ (ppm): 1.12-1.65 (m, 4H, 2CH₂), 2.86-3.02 (m, 2H, CH₂), 3.07- 3.27 (m, 2H, CH₂), 3.75-4.48 (m, 2H, CH₂), 4.87-5.09 (m, 2H, CH₂), 6.94-7.47 (m, 6.42H, Ar+NHCO). We could not observe the CH₂ of the benzyl group approx. 4.5 ppm.

Star-poly[(N-aminobutyglycine)-(N-benzylglycine)] [S-P(Nlys-Nphe)] 16 arms (polym 3). The deprotection was carried out as polym 1. Yield: 62% (0.054 g, yellowish solid). Calculated hydrophobic content by comparison of Ar (1.37-1.92 ppm) and 2CH₂ (6.93-7.39 ppm) = 53%. ¹H-NMR D₂O 400 MHz δ (ppm): 1.34-1.92 (m, 4H, 2CH₂), 2.75-3.04 (m, 2H, CH₂), 3.09-3.35 (m, 2H, CH₂), 3.373-4.63 (m, 4H, 2CH₂), 6.93-7.39 (m, 5.32H, Ar).

Star-poly[(Z-N-aminobutyglycine)-(N-benzylglycine)] [S-P(ZNlys-Nphe)] 16 arms and 30% H/C. The protected polymer was obtained according to the 4 arm copolymer through ROP of Phe-NNCA and ZLys-NNCA. Yield: 39% (0.12 g, yellowish solid) SEC (DMF, dn/dc = 0.0816): 36200 g/mol, ΔM = 1.41. Hydrophobic content calculation by comparison of Ar (6.94-7.47 ppm) and 2CH₂ (1.12-1.65 ppm) = 51%. ¹H-NMR DMSO-d₆ 400 MHz δ (ppm): 1.12-1.65 (m, 4H, 2CH₂), 2.86-3.02 (m, 2H, CH₂), 3.07- 3.27 (m, 2H, CH₂), 3.75-4.48 (m, 2H, CH₂), 4.87-5.09 (m, 2H, CH₂), 6.94-7.47 (m, 6.13H, Ar+NHCO). We could not observe the CH₂ of the benzyl group approx. 4.5 ppm.

Star-poly[(N-aminobutyglycine)-(N-benzylglycine)] [S-P(Nlys-Nphe)] 16 arms (polym 4). The deprotection was carried out as polym 1. Yield: 55% (0.0486 g, yellowish solid). Calculated hydrophobic content by comparison of Ar (1.37-1.92 ppm) and 2CH₂ (6.93-7.39 ppm) = 35%. ¹H-NMR D₂O 400 MHz δ (ppm): 1.34-1.92 (m, 4H, 2CH₂), 2.75-3.04 (m, 2H, CH₂), 3.09-3.35 (m, 2H, CH₂), 3.373-4.63 (m, 4H, 2CH₂), 6.93-7.39 (m, 3.47H, Ar).

Star-poly[(Z-N-aminobutyglycine)-(N-benzylglycine)] [S-P(ZNlys-Nphe)] 16 arms and 10% H/C. The protected polymer was obtained according to the 4 arm copolymer through ROP of Phe-NNCA and ZLys-NNCA. Yield: 45% (0.148 g, yellowish solid). SEC (DMF, dn/dc = 0.0816): 40500 g/mol, ΔM = 1.30. Hydrophobic content calculation by comparison of Ar (6.94-7.47 ppm) and 2CH₂ (1.12-1.65 ppm) = 50%. ¹H-NMR DMSO-d₆ 400 MHz δ (ppm): 1.12-1.65 (m, 4H, 2CH₂), 2.86-3.02 (m, 2H, CH₂), 3.07- 3.27 (m, 2H, CH₂), 3.75-4.48 (m, 2H, CH₂), 4.87-5.09 (m, 2H, CH₂), 6.94-7.47 (m, 5.97H, Ar+NHCO). We could not observe the CH₂ of the benzyl group approx. 4.5 ppm.

Star-poly[(N-aminobutyglycine)-(N-benzylglycine)] [S-P(Nlys-Nphe)] 16 arms (polym 5). The deprotection was carried out as polym 1. Yield: 42% (0.0431 g, yellowish solid)

Calculated hydrophobic content by comparison of Ar (1.37-1.92 ppm) and 2CH₂ (6.93-7.39 ppm) = 12%. ¹H-NMR D₂O 400 MHz δ (ppm): 1.34-1.92 (m, 4H, 2CH₂), 2.75-3.04 (m, 2H, CH₂), 3.09-3.35 (m, 2H, CH₂), 3.373-4.63 (m, 4H, 2CH₂), 6.93-7.39 (m, 1.19H, Ar).

Antimicrobial assays:

To test the activity against bacteria the methodology was as follows: mother solutions of the polypeptoids under assay were prepared by adding 1 mL of sterile distilled water (SDW) directly in the Eppendorf containing the product, the solution was transferred in a sterile tube. Eppendorf was rinsed 3 times (SDW) and the resulting solutions were added to the tube. The final volume was adjusted to a concentration of 500 μ g/mL with SDW. Shaking (4 times/h) during 1 h. Mother solutions were homogenized and appear clear expected.

Storage at -20°C.

Culture maintaining: *S. aureus*, *E. faecalis*, *L. monocytogenes*, *E. coli*, *A. baumannii*, *P.*

aeruginosa: Muller Hinton agar, incubation 24 h at 36 \pm 1°C under aerobiosis. *S.*

pneumoniae: Muller Hinton agar, incubation 24 h at 36 \pm 1°C under anaerobiosis. *B. fragilis*

and *H. pylori*: Columbia agar + 5% sheep red cells, incubation 24 – 48 h at 36 \pm 1 °C under anaerobiosis for *B. fragilis* (and under microaerophilic conditions for *H. pylori*).

Assays: *S. aureus*, *E. faecalis*, *L. monocytogenes*, *E. coli*, *A. baumannii*, *P. aeruginosa*: Muller Hinton broth, and Muller Hinton agar, incubation 24 h at 36 \pm 1°C under aerobiosis. *S.*

pneumoniae: Muller Hinton broth, supplemented with 10% SVF and Muller Hinton agar,

incubation 24 h at 36 \pm 1°C under anaerobiosis. *B. fragilis*: Muller Hinton broth and agar,

incubation 24 h at 36 \pm 1°C under anaerobiosis. *H. pylori*: Muller Hinton broth supplemented with 10% SVF and Muller Hinton agar supplemented with 10% horse blood, incubation 24 h at 36 \pm 1°C under microaerophilic conditions.

MIC (Minimal Inhibitory Concentration): Strains were maintained on agar specified above. A fresh suspension was prepared just before each assay in Tryptone salt and adjusted to 10⁸ CFU/mL. Each well of a 96-well microtiter plate was filled with 100 μ L of specify broth. Then, 100 μ L of a mother solution were added to the first well of two lines of the microtiter plate and 2 fold dilutions were performed from well 1 to well 10. All the wells (except column 11: sterility control) were inoculated with 1 to 3 μ L of the tested suspension (multi inoculator Denley). Microplates were then incubated under conditions described above. MIC was determined as the lowest concentration without any visible growth versus positive control.

C. difficile strain was cultured in an anaerobic chamber with an atmosphere of 5% H₂, 5% CO₂, 90% N₂. Cells were cultured on a Brain-Hearth Infusion (BHI) medium at 37 °C. The Minimal Inhibitory Concentration (MIC) of tested polymers was performed by the micro dilution method. A twofold serial dilution of tested polymers and control drugs were prepared in highly purified water to give a final concentration range from 1000 to 8 μ g/mL and 20 μ L of each concentration was added to a 96-well plate. Then, an overnight bacterial solution of *C. difficile* having an OD₆₀₀ between 0.45 and 0.60 (10⁷-10⁸ CFU/mL) was diluted in BHI at 10⁵ CFU/mL, each well was inoculated anaerobically with 180 μ L and incubated at 37°C. MIC reported is the minimal drug concentration that totally suppressed the growth of bacteria after 24 h at 37 °C, by visual determination. The experiments were performed by triplicates. The uninoculated medium was used as a negative control to test for contamination of the growth medium. The positive control consisted of a well inoculated with *C. difficile* but no antimicrobial compound was added.

Membrane destabilization studies:

The analyses were performed at the Centre d'Etudes et de Recherche sur le Médicament de Normandie (CERMN, Caen Normandie, France). The procedure is described as follows. Liposome mixtures of SPC:cholesterol (SPC:Chol) or DOPG:cholesterol (PG:Chol) in a molar ratio of 8:2 were formulated. Liposomes were formulated according to the adapted method of the thin lipid film hydration.⁵⁸ Lipid solutions in chloroform/methanol (4:1) were evaporated under nitrogen flow and left under vacuum for 2-3 h to form a lipid film. Carboxyfluoresceine (CF) was dissolved in phosphate buffer Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) to reach 70 mM, pH was adjusted with concentrated NaOH to 7.4. This thin lipid film was then hydrated in the CF solution and vortexed 1h. At RT the yielded multilamellar vesicles (MLVs) were then extruded 13 times with a mini-extruder (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA) through polycarbonate membranes with a pore diameter of 100 nm (Avanti Polar Lipids, Inc.). The obtained LUVs were separated from possible unincorporated CF by passage through a Sepharose[®] CL-4B loaded (Sigma-Aldrich) column, using PBS buffer as eluent. The LUVs size was assessed by DLS (NanoZS[®], Malvern Instruments, Worcestershire, UK) after a 1:100 dilutions in the PBS buffer. Liposomes were stable within 4 month: SPC:Chol having a Z-average 153.1±0.5 nm, PDI=0.095 ±0.02 and Z-potential -3.9±0.9; PG:Chol having a Z-average 138.1±1.7 nm with a PDI=0.093 ±0.04 and Z-potential -33.3±2.2. The entrapment of CF was measured by the dequenching of fluorescence after the addition of 2 µL 20% (v/v) Triton X-100 measured with a Synergy 2 microplate reader (Biotek, Colmar, France) equipped with the appropriate filters (λ_{ex} = 485/20 nm and λ_{em} = 528/20 nm). CF release assay was performed in a final volume of 100 µL, using 10 µM LUVs in PBS buffer. Polypeptoids solubilized in PBS buffer were then added to the solution to reach a final concentration of 90 µg/mL. The fluorescence was recorded immediately (F₀) and for 1.5 h at 25 °C. It was compared with that measured at the end of the experiment after the addition of 2 µL of 20% Triton X-100 solution to achieve complete liposome leakage (F_{max}). The percentage of CF release was calculated according to the following equation:⁵⁹

$$\left[\% \text{ CF leakage} \right]_{(t)} = \frac{(F_t - F_0)}{(F_{\text{max}} - F_0)} \times 100$$

where F_t was the fluorescence intensity at time t, F₀ the initial fluorescence intensity, and F_{max} the final fluorescence intensity after adding Triton X-100. Fluorescences of PBS and polypeptoids alone at the same concentration were measured as negative controls.

Cytotoxicity assays:

The evaluation of the tested peptides cytotoxicity by MTT assay on the Caco-2 cell line (human epithelial cell line) was done according to Mosmann with slight modifications. The experiments were performed at PHARMA-DEV in Toulouse, France. Briefly, cells (1.10⁵ cells/mL) in 100 µL of the complete medium [DMEM High Glucose supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) and 1X NEAA] were seeded into each well of 96-well plates and incubated at 37 °C in a humidified 5% CO₂ with 95% air atmosphere. After a 24 h incubation, various concentrations of peptides and appropriate controls were then added (100 µL) and the plates were incubated for 72 h at 37 °C. Each well was then microscope-examined for detecting possible precipitate formation before aspiration of the medium. MTT solution (0.5 mg/mL in complete DMEM High Glucose, 100 µL) was then added to each well. Cells were incubated for 2 h at 37 °C. The MTT solution was then removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. Plates were shaken vigorously (300 rpm) for 5 min. The absorbance was measured at 570 nm with a microplate spectrophotometer

(Eon Bio Tek). Water was used as blank and doxorubicin (Sigma Aldrich) as a positive control. CC50 were calculated by non-linear regression analysis processed on dose-response curves, using TableCurve 2D V5 software. CC50 values represent the mean value calculated from three independent experiments.

Protease assay: The protocol was adapted from a known method for peptoid analysis.¹⁹ A polymer solution was prepared (0.1 mM) in 1 mL of NaHCO₃ buffer 50 mM pH = 7.8 equilibrated at 37 °C for 10 min and 47 µL of trypsin 42 µM (1 mg/mL) was added. At a certain time, 2.3 µL of the reaction mixture were taken, placed in an Eppendorf tube, quenched with 200 µL acetonitrile and 200 µL sodium borate buffer 0.1 M and kept in an ice bath. 4-Fluoro-7-nitro-benzofurazan (NBDF) (10 µL, acetonitrile solution 10 mM) was added, immediately the solution was incubated at 60 °C for 3 min and 500 rpm in the Eppendorf thermomixer R. The sample was placed on ice and 20 µL of 3 N HCl were added to stabilize the 4-amino-7-nitro-benzofurazan product. The fluorescence was measured in a Spectra max M2 (Molecular devices) with $\lambda_{ex} = 470$ nm and $\lambda_{em} = 540$ nm. The values were adjusted using the appropriate blank. The raw data was obtained from SoftMaxPro V5 and analyzed in Origin2016 software

‡ The yields with the copolymers are lower than the yields obtained with polysarcosine: we attribute this observation to the difference in solubility that exists between these two structures whereas they were purified by the same process (precipitation in diethyl ether).

Conflicts of interest

“There are no conflicts to declare”.

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