

Using Diphenylphosphoryl Azide (DPPA) for the Facile Synthesis of Biodegradable  
Antiseptic Random Copolypeptides

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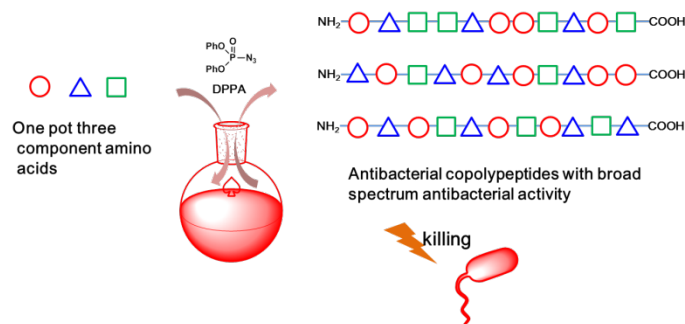
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## Abstract

We have developed a facile method for the large-scale synthesis of random copolypeptides composed of multiple (i.e. cationic, hydrophobic, and hydrophilic) amino acids and have optimized their relative ratios for broad-spectrum antibacterial effect. The copolypeptides obtained have measured compositions close to the design ratios in spite of the differing reactivities of the different amino acids. An optimised random copolypeptide of lysine, leucine and serine (denoted as KLS-3) mimicking the composition of LL-37 host defense peptide gives broad spectrum antibacterial activity against clinically relevant Gram-negative and Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (PAO1) with minimum inhibitory concentrations (MICs) of 32-64  $\mu\text{g/mL}$ , as well as good MICs against multi-drug resistant Gram-negative bacteria of *E. coli* EC 958 (64  $\mu\text{g/mL}$ ) and *Klebsellia pneumonia* PTR3 (128  $\mu\text{g/mL}$ ). This method can be applied to the facile large-scale copolymerization of multiple amino acids, including unnatural amino acids, to make effective antibacterial copolypeptides.



## 1. Introduction

The alarming rise in bacterial resistance to the myriad plethora of small molecules used to annihilate them, including common disinfectants to even “later” generation antibiotics, necessitates alternative strategies to kill the evolving multi-drug resistant (MDR) superbugs.<sup>[1-2]</sup> Recently, the United States Food and Drug Administration issued the ban of 19 chemicals from use in hand soap and wash and off-the-counter consumer antiseptic products.<sup>[3]</sup> The active antiseptic chemicals used till now are often triclosan or triclocarban which may lead to bacterial resistance with continued use.<sup>[4-5]</sup>

On the other hand, amphiphilic cationic macromolecules such as cationic polymers or peptides have been shown to be less likely to evoke resistance.<sup>[6-7]</sup> Small molecule antiseptic chemicals or antibiotics typically kill bacteria by targeting specific enzymes<sup>[8]</sup>, proteins<sup>[9]</sup>, nucleic acids<sup>[10]</sup> or folic acid<sup>[11]</sup> so that bacterial resistance evolves with these specific targets. The amphiphilic cationic macromolecules typically kill bacteria by a physical electrostatic process instead which involves the electrostatic binding of the cationic polymer/peptide to the anionic cell membrane followed by insertion of the hydrophobic domain of the amphiphiles into the bacterial cytoplasmic membrane;<sup>[12-13]</sup> this physical killing process without specific enzyme or nuclei acid targeting leads to reduced resistance evolution<sup>[14]</sup>. For example, one recently reported antimicrobial peptide (AMP), Teixobactin, elicited no drug-resistance after 25-day culture.<sup>[15]</sup> For

many consumer applications, peptides offer the advantages of biodegradability, and biocompatibility.

However, there are several hurdles to applying AMPs as disinfective agents, including cost, efficacy, bioavailability, etc.<sup>[17]</sup> Although many host defense peptides have defined amino acid sequences and secondary structures (Figure 1a)<sup>[18]</sup>, it has been shown that random copolypeptides can also act effectively to kill bacteria<sup>[14, 19]</sup>. The random polymers/polypeptides may assume globally amphiphilic conformation only when in contact with bacterial membrane to effect good antibacterial activity (Figure 1b).<sup>[16]</sup> Peptides with precise sequences are usually synthesised via solid phase peptide synthesis (SPPS), but this method is highly expensive and not viable for the large-scale synthesis of peptides for consumer products. On the other hand, amphiphilic macromolecules which do not have precise sequences nor any secondary structures in solution can also have good antimicrobial effects.<sup>[16, 19-22]</sup> To achieve good bactericidal property, the compositions of these macromolecules which may be polymers, polypeptides or polysaccharides, may be tuned.<sup>[23-24]</sup> We have previously reported chitosan-graft-peptide copolymers that have good antimicrobial activity by a polymerization technique involving N-carboxyl anhydride (NCA).<sup>[20, 25]</sup> Random/graft copolymers may show antibacterial activities comparable to natural AMPs which have highly precise primary amino acid sequences, but are generally much easier or cheaper to synthesize. Further, it is generally thought that mixtures of random

copolypeptides having similar compositions but different sequences are more difficult for bacteria to evolve resistance to.<sup>[17]</sup>

There are numerous techniques such as ATRP (atom transfer radical polymerization) or RAFT (reversible addition-fragmentation chain-transfer polymerization) for synthesizing vinyl-based copolymers but they cannot make polypeptides and the resulting copolymers are usually not biodegradable/biocompatible. However, there are only few polymerization techniques for synthesizing polypeptides.<sup>[26-28]</sup> For  $\alpha$ -peptide synthesis via the polymerization route, only the N-carboxyanhydride (NCA) polymerization technique has been widely used. Polypeptides are much more acceptable environmentally for disinfection or for personal care applications such as cosmetics. Further, the NCA polymerization requires stringent reaction conditions (such as strict absence of water and nucleophiles) for the synthesis of the highly pure NCA monomer starting material. Large amounts of anhydrous solvents are usually involved so that this method is also rather uneconomical and environmentally unfriendly. In addition, the requirement of anhydrous conditions entails use of a glove box which further complicates the process. Also, it is usually difficult to sequentially prepare several  $\alpha$ -amino acid NCA monomers because their shelf lives are rather short.

Diphenylphosphoryl azide (DPPA) is a coupling agent used for the synthesis of peptides in the presence of a base via the modified Curtius reaction.<sup>[29-30]</sup>

Early in 1991, Nishi et al synthesized homopeptides by the DPPA method but

the composition is limited to one amino acid, or one diaminoacid (Figure 1c).<sup>[31-</sup>  
<sup>32]</sup> In this method,  $\alpha$ -amino acids with unprotected  $\alpha$ -amine and carboxylic acid groups were used as monomers, and DPPA was the catalyst to polymerize the amino acids in the presence of organic base (triethylamine). Unlike NCA method, there is no need to prepare highly pure monomer precursor and the condensation polymerization is not highly sensitive to moisture or oxygen or competing nucleophiles. Amino acids with side chain protected were used directly for polymerization unlike in NCA whereby the cyclized monomer must be first made; also, the purification of peptides can be realized by simple washing. Hence, this method is relatively simple and easy, economical (with less solvent consumption) and environmentally friendly. Further, this method avoids racemization during the synthesis process.<sup>[31-32]</sup> However, prior to this report, this method had been applied to polymerization of only one type of amino acid (Figure 1c) and has not been applied to peptides composed of multiple amino acids which have differing reactivity ratios. The host defense peptides (HDPs) secreted by multicellular organisms typically contain various types of amino acids, including at least cationic amino acids (to confer the peptide with an overall cationic charge) and hydrophobic amino acid residues with no less than 30% content.<sup>[12, 18, 33]</sup>

In this work, we apply the simple and powerful DPPA method to prepare random polypeptides composed of multiple types of amino acid in the feed, and demonstrate the applicability of these random polypeptides as antimicrobial

agents (Figure 1d). We have chosen to mimic the compositions of AMP LL-37 and lipopeptide polymyxin B which are respectively a human host defense peptide and an FDA-approved drug.

## 2. Results and Discussion

We first chose the natural host-defense peptide LL-37 as a model peptide (amino acid sequence in Figure S1a) for DPPA-based synthesis. LL-37 is a 37-amino-acid peptide expressed in humans that has broad-spectrum antibacterial activity against various types of pathogens such as bacteria, fungi, and viruses<sup>[34-35]</sup> and also has antibiofilm activity.<sup>[36]</sup> It is the only member in the cathelicidin AMP family expressed in humans. Its derivative IR-12 was also taken into consideration (Figure S1a) in the design. After analysis of the amino acid compositions of these two peptides, we found that the ratios of cationic, hydrophobic, and hydrophilic (that are not cationic) amino acids were about 40%:40%:20% (Table S1). For simplicity, L-lysine (K), leucine (L), and serine (S) were chosen as the cationic, hydrophobic, and hydrophilic amino acids, respectively (Figure 2a) because they are amongst the most frequently occurring amino acids in AMPs.<sup>[37-38]</sup> The molar feeding ratios of the cationic lysine was set at 40% to be the same as that in LL-37 and IR-12 but the feeding molar ratios of leucine and serine were varied from 30-50% and 30-10% respectively; the obtained peptides were denoted KLS\* (\* means protected peptides). The respective side protection groups of lysine and serine were *tert*-butylcarbonate (Boc) and *tert*-butyl (*t*Bu), which are easily removed by trifluoroacetic acid (TFA) to obtain the respective deprotected KLS peptides.

By dispersing the amino acids in anhydrous DMSO (600 mg amino acid/mL DMSO) and triethylamine, DPPA was then added. After 2 d reaction at room temperature, the solution was precipitated in excess water and washed with ethanol and ether. The whole process for polymerization and workup was easy to handle and only relatively small amounts of organic solvents were needed even for a fairly large scale (here we achieved 500 mg final products) reaction. The gel permeation chromatography (GPC) curves of these protected peptides indicate that peptides obtained by this method had very narrow distributions (PDIs around 1.22, Figure 3A). The  $^1\text{H}$  NMR spectra further demonstrated that all three amino acids were incorporated into peptides (Figure S2A). The peaks at 1.4 and 1.3 ppm are attributed to the Boc and *t*Bu group of lysine and serine, respectively. The peak at 0.9 ppm is attributed to the methyl proton in leucine. After deprotection by TFA, the actual ratios of these three amino acids in the deprotected peptides were calculated from  $^1\text{H}$  NMR spectra in TFA-*d* (Figure S2B); the ratios are summarized in Table 1. Amongst the 3 amino acids, serine showed higher content in the actual peptides than the designed content, which suggests that the activity of serine was higher in this reaction. In contrast, leucine showed the poorest incorporation (i.e. polymerization reactivity), although there was a general increase of the leucine content from KLS-1 to 3.

The molecular weights of these deprotected peptides were determined by GPC in acetic acid buffer mobile phase. The peptides showed a number average molecular weight of 2000 Da and the PDIs were around 1.2 (Table 1 and Figure S4A). Circular dichroism (CD) results demonstrated that no racemization occurred after either

polymerization or deprotection, and the peptides mainly adopt a random-coil conformation in aqueous solution (PBS pH 7.4, Figure 4). The peptides self-assembled into nanoparticles in aqueous solution and the sizes and surface charges of nanoparticles were tested by dynamic light scattering (DLS) and zeta potential measurement (Table 2). The KLS series showed an average hydrodynamic diameter of 98-145 nm. The zeta potentials were in the range 24-31 mV.

The antibacterial activities of KLS series were then tested by the broth dilution method. Minimum inhibitory concentrations (MICs) were measured against both Gram-positive and Gram-negative bacteria. *E. coli* and *P. aeruginosa* PAO1 were chosen as representative Gram-negative bacteria and *S. aureus* and methicillin-resistant *S. aureus* (MRSA) were chosen as representative Gram-positive bacteria. The MICs of LL-37 were also tested as a control; the results are summarized in Table 1. The control natural peptide LL-37 showed good antibacterial activity against the Gram-negative *E. coli* strain (MIC 32-64  $\mu\text{g/mL}$ ) but poor antibacterial activity against the two Gram-positive SA and MRSA strains (MICs more than 512  $\mu\text{g/mL}$ ). On the other hand, our polypeptides mimicking LL-37 (KLS-1 to 3) showed good antibacterial activity against both Gram-positive and Gram-negative strains. From KLS-1 to 3, the antibacterial activity slightly improved with increasing leucine component. KLS-3 with the highest (hydrophobic) leucine content showed MIC values against *E. coli*, PAO1, SA and MRSA in the range of 32-64  $\mu\text{g/mL}$ . We also tested these synthetic mimics against other clinically relevant bacteria species (Table S3). Further, KLS-3 has a MIC of 64  $\mu\text{g/mL}$  and 128  $\mu\text{g/mL}$  against the multi-drug resistant (MDR) Gram-negative *E. coli*

EC958 and the Multi-Drug Resistant (MDR) Gram-negative *Klebsellia pneumonia* PTR3 respectively (Table S3). Hence, KLS-3 which is a random copolypeptide which is also a compositional analogue of LL-37 has been successfully synthesized by the DPPA method and it shows good broad spectrum activity against clinically relevant MDR bacteria.

We also synthesized random copolypeptides mimicking polymyxin B (chemical structure in Figure S1b), denoted  $\text{DLT}$  for the feed components, L-diaminobutyric acid ( $\text{D}$ ), leucine (L) and threonine (T). L-diaminobutyric acid is an unnatural amino acid. For simplicity, the cyclic structure of polymyxin B was not mimicked and hydrophobic tail (7-8 carbon atoms) was calculated as two hydrophobic leucine molecules. The protected  $\text{DLT}^*$  series showed a higher molecular weight than the  $\text{KLS}^*$  series, with number average molecular weight around 6500-7000 Da (Table S1). But both series showed narrow distributions (PDI=1.2-1.3) and similar molecular weights for each series (Figure 3a and b). The amino acid compositions were verified with  $^1\text{H}$  NMR spectrum in  $\text{DMSO-}d_6$  (Figure S3A). The characteristic peak at 5.0 ppm is due to the methylene protons ( $\text{CH}_2$ ) on the group of lysine. After deprotection by HBr and TFA, the carboxybenzyl groups at  $\sim 7.5$  ppm disappeared from the  $^1\text{H}$  NMR spectrum (Figure S3B). The amino acid compositions were also calculated by  $^1\text{H}$  NMR in  $\text{TFA-}d$ ; the results are summarized in Table 1. After deprotection, the molecular weights of  $\text{DLT}$  series were around 2200 Da (Table 1 and Figure S4B), which are similar to those of  $\text{KLS}$  series, suggesting that the DPPA method permits control of molecular weight regardless of the types of feed amino acids. The results show that diaminobutyric acid

was the easiest component to introduce into the peptide chains and leucine was the hardest. The CD results show that  $\Delta$ LT displayed a random coil conformation in PBS and the L-configuration of amino acids was preserved (Figure 4). The hydrodynamic diameter of  $\Delta$ LT series nanoparticles in water was around 44-59 nm, smaller than KLS series (Table 2). The zeta potentials of these nanoparticles were in the range 15-19 mV.

The antibacterial activities of  $\Delta$ LT series were then studied. polymyxin B sulfate was tested as a control and it showed high antibacterial activity only against Gram-negative strains (Table 1). The MICs of polymyxin B against *E. coli* and *P. aeruginosa* were low as 1-2  $\mu\text{g/mL}$ , while the MICs against Gram-positive strains *S. aureus* and MRSA were 32-64  $\mu\text{g/mL}$ . Different from polymyxin B, the  $\Delta$ LT polypeptides showed antibacterial activities against both Gram-positive and Gram-negative strains. From  $\Delta$ LT-1 to 3, as the  $\Delta$  component increased, the antibacterial activity decreased (the MICs against *E. coli* and *P. aeruginosa* increased from 16-32  $\mu\text{g/mL}$  to 64-128  $\mu\text{g/mL}$ ). Notably, the  $\Delta$ LT series showed good antibacterial activities (and better than that of polymyxin B) against MRSA, with MICs in the range 16-32  $\mu\text{g/mL}$ .  $\Delta$ LT-1 exhibits the best range of MIC against various multi-drug resistant Gram-negative bacteria, including *E. coli* EC958 and *P. aeruginosa* D25 with MICs of 64  $\mu\text{g/mL}$  and 32  $\mu\text{g/mL}$  respectively (Table S3).

Compared with traditional SPPS and NCA polymerization, the DPPA method use less solvent and is a much simpler procedure (Table S4). These peptides can be used as mimics of antimicrobial and host defense peptides. The

peptides obtained showed a narrow distribution and conformation integrity. What is more, this method can be applied to diversity of amino acid types, such as  $\alpha$ -L-amino acid,  $\beta$ , or  $\gamma$ -amino acid, D-conformation  $\alpha$ -amino acid, and even some sugar based amino acid. The D-conformation peptides could increase proteinase resistance and avoid rapid clearance in body.<sup>[39-40]</sup>

### **3. Conclusions**

We have developed a simple and versatile DPPA copolymerization technique to synthesize antibacterial copolypeptides composed of multiple types of amino acid, including cationic, hydrophobic and hydrophilic amino acids, and natural and unnatural amino acids. Studying from nature, we developed two polypeptides with average compositions mimicking those of the host defense peptide LL-37 and lipopeptide antibiotic polymyxin B. The synthesized copolypeptides have narrow molecular weight distributions. The optimised polypeptide (KLS-3) has good broad-spectrum MICs (32-64  $\mu\text{g/mL}$ ) against both gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli* and *P. aeruginosa*), and also effectively kill the multi-drug resistant strains (MRSA, MDR *E. coli* EC958 and MDR *K. pneumoniae*). Although for simplicity, only three amino acids were tested here, our approach could be used with a larger range of feed types to increase the diversity of antibacterial peptides which can be synthesized in large-scale.

### **Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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[1] Fischbach, M. A.; Walsh, C. T., *Science* **2009**, *325*, 1089.

[2] Neu, H. C., *Science* **1992**, *257*, 1064.

[3]<http://www.npr.org/sections/health-shots/2016/09/02/492394717/fda-bans-19-chemicals-used-in-antibacterial-soaps>.

[4] Chuanchuen, R.; Beinlich, K.; Hoang, T. T.; Becher, A.; Karkhoff-Schweizer, R. R.; Schweizer, H. P., *Antimicrob. Agents Chemother.* **2001**, *45*, 428.

[5] Yazdankhah, S. P.; Scheie, A. A.; Høiby, E. A.; Lunestad, B.-T.; Heir, E.; Fotland, T. Ø.; Naterstad, K.; Kruse, H., *Microb. Drug Resist.* **2006**, *12*, 83.

[6] Guilhelmelli, F.; Vilela, N.; Albuquerque, P.; Derengowski, L. d. S.; Silva-Pereira, I.; Kyaw, C. M., *New edge of antibiotic development: antimicrobial peptides and corresponding resistance* **2014**, 63.

[7] Peschel, A.; Sahl, H.-G., *Nat. Rev. Microbiol.* **2006**, *4*, 529.

[8] Kelly, J. A.; Moews, P. C.; Knox, J. R.; Frère, J.-M.; Ghuysen, J.-M., *Science* **1982**, *218*, 479.

[9] Cocito, C.; Di Giambattista, M.; Nyssen, E.; Vannuffel, P., *J. Antimicrob. Chemother.* **1997**, *39*, 7.

[10] Kersten, H.; Kersten, W., *Inhibitors of nucleic acid synthesis: biophysical and biochemical aspects*. Springer Science & Business Media: 2013; Vol. 18.

- [11] Bermingham, A.; Derrick, J. P., *Bioessays* **2002**, *24*, 637.
- [12] Zasloff, M., *Nature* **2002**, *415*, 389.
- [13] Brogden, K. A., *Nat. Rev. Microbiol.* **2005**, *3*, 238.
- [14] Findlay, B.; Zhanel, G. G.; Schweizer, F., *Antimicrob. Agents Chemother.* **2010**, *54*, 4049.
- [15] Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schäberle, T. F.; Hughes, D. E.; Epstein, S., *Nature* **2015**, *517*, 455.
- [16] Mowery, B. P.; Lindner, A. H.; Weisblum, B.; Stahl, S. S.; Gellman, S. H., *J. Am. Chem. Soc.* **2009**, *131*, 9735.
- [17] Marr, A. K.; Gooderham, W. J.; Hancock, R. E., *Curr. Opin. Pharm.* **2006**, *6*, 468.
- [18] Hancock, R. E.; Sahl, H.-G., *Nat. Biotechnol.* **2006**, *24*, 1551.
- [19] Zhou, C.; Qi, X.; Li, P.; Chen, W. N.; Mouad, L.; Chang, M. W.; Leong, S. S. J.; Chan-Park, M. B., *Biomacromolecules* **2010**, *11*, 60.
- [20] Li, P.; Zhou, C.; Rayatpisheh, S.; Ye, K.; Poon, Y. F.; Hammond, P. T.; Duan, H.; Chan - Park, M. B., *Adv. Mater.* **2012**, *24*, 4130.
- [21] Yuan, W.; Wei, J.; Lu, H.; Fan, L.; Du, J., *Chem. Commun.* **2012**, *48*, 6857.
- [22] Chen, J.; Wang, F.; Liu, Q.; Du, J., *Chem. Commun.* **2014**, *50*, 14482.
- [23] Liu, R.; Chen, X.; Chakraborty, S.; Lemke, J. J.; Hayouka, Z.; Chow, C.; Welch, R. A.; Weisblum, B.; Masters, K. S.; Gellman, S. H., *J. Am. Chem. Soc.* **2014**, *136*, 4410.
- [24] Tsai, C.-W.; Hu, W.-W.; Liu, C.-I.; Ruaan, R.-C.; Tsai, B.-C.; Jin, S.-L. C.; Chang, Y.; Chen, W.-Y., *Int. J. Pharm.* **2015**, *494*, 498.

- [25] Li, P.; Poon, Y. F.; Li, W.; Zhu, H.-Y.; Yeap, S. H.; Cao, Y.; Qi, X.; Zhou, C.; Lamrani, M.; Beuerman, R. W., *Nat. Mater.* **2011**, *10*, 149.
- [26] Kricheldorf, H. R., *Angew. Chem. Int. Ed.* **2006**, *45*, 5752.
- [27] Hadjichristidis, N.; Iatrou, H.; Pitsikalis, M.; Sakellariou, G., *Chem. Rev.* **2009**, *109*, 5528.
- [28] Huang, J.; Heise, A., *Chem. Soc. Rev.* **2013**, *42*, 7373.
- [29] Shioiri, T.; Ninomiya, K.; Yamada, S., *J. Am. Chem. Soc.* **1972**, *94*, 6203.
- [30] Joullié, M. M.; Lassen, K. M., *Arkivoc* **2010**, *8*, 189.
- [31] Nishi, N.; Tsunemi, M.; Hayasaka, H.; Nakajima, B. i.; Tokura, S., *Die Makromolekulare Chemie* **1991**, *192*, 1789.
- [32] Nishi, N.; Naruse, T.; Hagiwara, K.; Nakajima, B. i.; Tokura, S., *Die Makromolekulare Chemie* **1991**, *192*, 1799.
- [33] Powers, J.-P. S.; Hancock, R. E., *Peptides* **2003**, *24*, 1681.
- [34] Frohm, M.; Agerberth, B.; Ahangari, G.; Stähle-Bäckdahl, M.; Lidén, S.; Wigzell, H.; Gudmundsson, G. H., *J. Biol. Chem.* **1997**, *272*, 15258.
- [35] Gordon, Y. J.; Huang, L. C.; Romanowski, E. G.; Yates, K. A.; Proske, R. J.; McDermott, A. M., *Curr. Eye Res.* **2005**, *30*, 385.
- [36] de la Fuente-Nunez, C.; Cardoso, M. H.; Candido, E. D.; Franco, O. L.; Hancock, R. E. W., *Biochim. Biophys. Acta-Biomembr.* **2016**, *1858*, 1061.
- [37] Wang, G.; Li, X.; Wang, Z., *Nucleic Acids Res.* **2015**, gkv1278.
- [38] Mishra, B.; Wang, G., *J. Am. Chem. Soc.* **2012**, *134*, 12426.
- [39] Fernandez-Lopez, S.; Kim, H.-S.; Choi, E. C.; Delgado, M.; Granja, J. R.;

Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxon, K. M., *Nature*

**2001**, *412*, 452.

[40] Stach, M.; Siriwardena, T. N.; Kohler, T.; van Delden, C.; Darbre, T.; Reymond,

J. L., *Angew. Chem. Int. Ed.* **2014**, *53*, 12827.

*Figure 1.* (a) Host-defense and antimicrobial peptides adopt an  $\alpha$ -helix conformation in solution; and (b) random peptides or other amphiphilic cationic polymers adopt a globally amphiphilic conformation when subjected to biomembrane surfaces. (Blue ball shows hydrophobic domain and red circle means cationic domain. Hydrophilic domain was omitted for clarity) <sup>[16]</sup>. (c) The homopeptides of using one amino acid or a diaminoacid as building blocks by DPPA method (The inset was the chemical structure of DPPA). (d) A three-component synthetic antibacterial peptides with random sequences and compositions by DPPA method (Dots, triangles, and squares were three different amino acids).

*Figure 2.* Synthetic routes of short peptides (a) KLS and (b)  $\text{\textcircled{D}LT}$  which mimic LL-37 and polymyxin B, respectively.

*Figure 3.* GPC curves of KLS\* (a) and  $\text{\textcircled{D}LT}$ \* (b) series.

*Figure 4.* Typical CD spectra of KLS-1 and  $\text{\textcircled{D}LT}$ -1 in PBS.

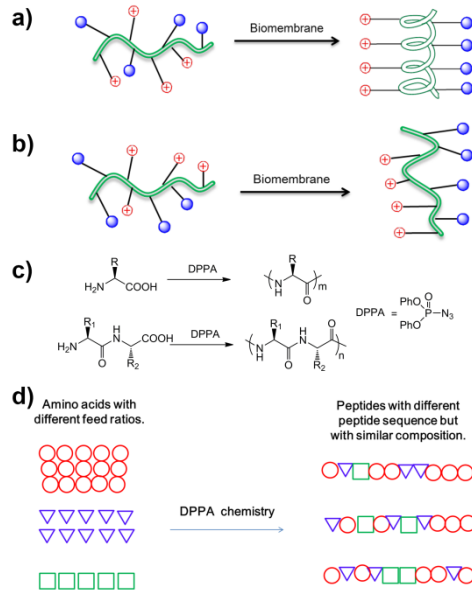
*Table 1.* Compositions, molecular weights, MICs and haemolytic activity of short peptides synthesized by DPPA method.

copolypeptides	<i>F</i> (A1:A2:A3) <sup>a</sup>	<i>f</i> (A1:A2:A3) <sup>b</sup>	<i>M<sub>n</sub></i> <sup>c</sup>	PDI <sup>c</sup>	MIC (μg/mL) <sup>d</sup>			
					Gram negative		Gram positive	
					<i>E. c</i>	<i>P. a</i>	<i>S. a</i>	MRSA
LL-37	-	30%:43%:27%	4493	1.00	>512	64	>512	>512
KLS-1	40%:30%:30%	45%: <b>20%</b> :35%	1852	1.22	64	128	128	64
KLS-2	40%:40%:20%	47%: <b>23%</b> :30%	2292	1.08	64	64	64	128
KLS-3	40%:50%:10%	41%: <b>37%</b> :22%	2003	1.26	32	64	64	64
polymyxin B	-	45%;36%:18%	1302	1.00	2	1	32	64
ΔLT-1	45%:25%:30%	<b>52%</b> :19%:29%	2261	1.26	32	32	128	32
ΔLT-2	45%:35%:20%	<b>56%</b> :22%:22%	2285	1.25	64	128	16	32
ΔLT-3	45%:45%:10%	<b>61%</b> :24%:15%	2297	1.27	64	128	32	16

a). Feed molar ratio of amino acids A1, A2 and A3 (A1, A2, and A3 refer respectively cationic, hydrophobic, and hydrophilic amino acids. For KLS, A1, A2, and A3 are respectively <sub>L</sub>-Lysine (K), Leucine (L), and Serine (S). For ΔLT, A1, A2, and A3 are respectively <sub>L</sub>-diaminobutyric acid (Δ), Leucine (L), and threonine (T)). b). Actual molar ratio of amino acids A1, A2, and A3 by <sup>1</sup>H NMR calculation for KLS and ΔLT series. c). Obtained by water phase GPC in which 0.5 M HAc-NaAc was used as mobile phase (40 °C). d). *E. c*: *E. coli* ATCC 8739, *P. a*: *P. aeruginosa* PAO1, *S. a*: *S. aureus* 29213, and MRSA: MRSA BAA40.

Table 2. Size and zeta potential of KLS and DLT series by DLS.

<b>copolypeptides</b>	<b>Size(nm)</b>	<b>Zeta potential (mV)</b>
KLS-1	145	+ 23.8
KLS-2	98.5	+ 25.2
KLS-3	106	+ 30.8
DLT-1	58.8	+ 18.6
DLT-2	43.8	+ 14.8
DLT-3	56.1	+ 16.3



*Figure 1.* (a) Host-defense and antimicrobial peptides adopt an  $\alpha$ -helix conformation in solution; and (b) random peptides or other amphiphilic cationic polymers adopt a globally amphiphilic conformation when subjected to biomembrane surfaces. (Blue ball shows hydrophobic domain and red circle means cationic domain. Hydrophilic domain was omitted for clarity) <sup>[16]</sup>. (c) The homopeptides of using one amino acid or a diaminoacid as building blocks by DPPA method (The inset was the chemical structure of DPPA). (d) A three-component synthetic antibacterial peptides with random sequences and compositions by DPPA method (Dots, triangles, and squares were three different amino acids).

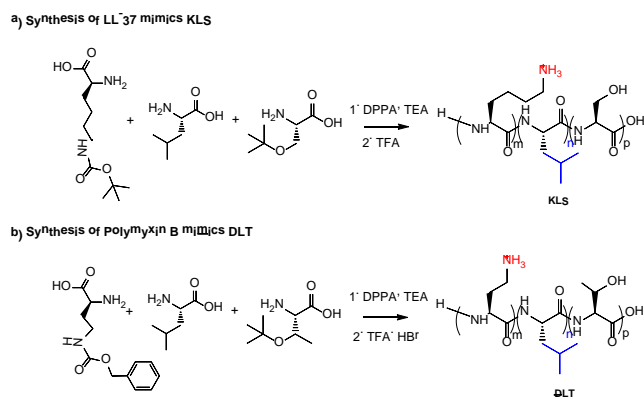


Figure 2. Synthetic routes of short peptides (a) KLS and (b) DLT which mimic LL-37 and polymyxin B, respectively.

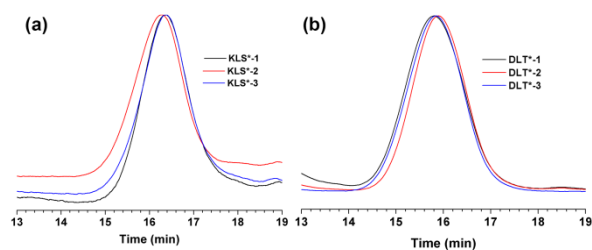


Figure 3. GPC curves of KLS\* (a) and DLT\* (b) series.

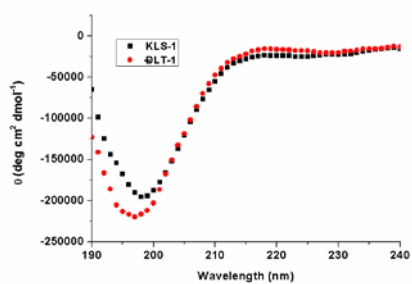
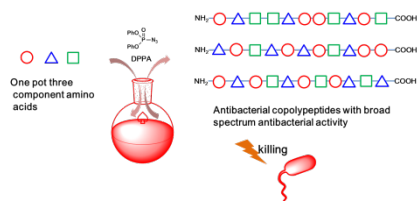


Figure 4. Typical CD spectra of KLS-1 and DLT-1 in PBS.

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A facile method for the large-scale synthesis of random polypeptides composed of multiple (i.e. cationic, hydrophobic, and hydrophilic) amino acids was proposed by using diphenylphosphoryl azide. The copolypeptides mimicking hose defense peptide LL-37 and lipopeptide antibiotic polymyxin B showed broad-spectrum antibacterial effects against both Gram-positive, Gram-negative strains and even multi-drug resistant clinically relevant strains.

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Supporting information of

**Using Diphenylphosphoryl Azide (DPPA) for the  
Facile Synthesis of Biodegradable Antiseptic Random  
Copolypeptides**

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Duan\*, Mary B. Chan-Park\*

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## 1. Materials and synthesis

### 1.1 Materials.

All amino acids and dipeptides were purchased from Shanghai GL company, China. Anhydrous triethylamine, trifluoroacetic acid, diphenylphosphoryl azide, 33% HBr in acetic acid, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. DMSO were distilled with CaH<sub>2</sub> and stored with 4A molecular sieves. Bacteria,,, MHB broth, xx were purchased from xx. 3T3 cells.

### 1.2 Synthesis of protected copolypeptides by DPPA method.

Taking KLS\*-1 as an example, H-Lys(Boc)-OH (1.00 g, 4.1 mmol), H-Leu-OH (399 mg, 3.04 mmol), and H-Ser(tBu) (491 mg, 3.04 mmol) were dispersed in anhydrous DMSO (3.2 mL) under N<sub>2</sub>. TEA (4.07 mL, 23.3 mmol) and DPPA (2.84 mL, 13.2 mmol) were added. The mixture was then stirred at room temperature for 2 d. The solution was then washed with DI water (50 mL x3), ethanol (50 mL x3), and ether (50 mL x3). The residue was then dried under vacuum at 50 °C. Yield 500 mg.

### 1.3 Synthesis of KLS.

KLS\* (250 mg) was dissolved in 5 mL TFA under N<sub>2</sub> and stirred at room temperature for 1 h. Most of the solvent was removed under vacuum and cold ether (50 mL) was added and washed trice. The residue was then dissolved in DI water and

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the pH was adjusted to 7. After dialysis for three days against DI water in a dialysis tube (MWCO 500-1000 D), the product was obtained by freeze-drying.

#### *1.4 Synthesis of DLT.*

DLT\* (250 mg) was dissolved in 5 mL TFA under N<sub>2</sub>. HBr in acetic acid (1.5 mL) was added dropwise at an ice bath. The solution was then stirred at room temperature for 1 h. Ether (50 mL) was added and washed three times. The residue was then dissolved in DI water and the pH was adjusted to 7. After dialysis for three days against DI water in a dialysis tube (MWCO 500-1000 D), DLT was obtained by freeze-drying.

## **2. Spectroscopy Characterization.**

<sup>1</sup>H NMR spectra were recorded with a Bruker Avance DPX 300 instrument at 298 K and the solvents were DMSO-*d*<sub>6</sub>, TFA-*d*, and D<sub>2</sub>O.

Organic phase GPC for those peptides with protection groups was performed on an Agilent 1100 Series equipped with GPC-SEC (size exclusion chromatography) data analysis software. Peptides were dissolved in 0.1 M LiBr in DMF and the GPC eluent was kept at 40 °C. Polystyrene was used as standard.

The molecular weights and polydispersities of peptides after deprotection were measured using a Waters' gel permeation chromatography (GPC) system equipped with a 2410 refractive index detector (RID), using two untrahydrogel column and sodium acetate buffer (0.5 M of NaAc and 0.5 M of HAc, pH ~4.5) as mobile phase at

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40°C with a flow rate of 1.0 mL min<sup>-1</sup>. Monodisperse pullulan was used as the standard to generate the calibration curve.

Circular dichroism was tested by using a circular dichroism spectrometer (Chirascan<sup>TM</sup>, Applied Photophysics, UK). Peptides were dissolved in 20 mM sodium phosphate buffer (pH 7.4). A quartz cell (Hellma 106-QS Demountable Cells) with a path length of 0.1 mm was used. Spectra were generated from 190 to 240 nm wavelengths at 0.1 nm intervals, 50 nm/min speed, 0.5 s response time, and 0.5 nm bandwidth. Each spectrum is the average of 10 scans and the spectrum was expressed in molar ellipticity after background subtraction.

### **3. Minimum inhibitory concentrations (MICs).**

Bacteria cells were grown overnight at 37 °C in MHB broth to a mid-log phase and diluted to 10<sup>5</sup> CFU mL<sup>-1</sup> in MHB broth. A two-fold dilution series of 100 µL product solution in the broth was made on 96-well microplate, followed by the addition of 100 µL bacterial suspension (10<sup>5</sup> CFU mL<sup>-1</sup>). The plates were incubated at 37 °C for 18-24 h, and the absorbance at 600 nm was measured with a microplate reader (BIO-RAD Benchmark Plus, US). Positive control was without product, and negative control was without bacteria inoculum. MICs were determined as the lowest concentration that inhibited cell growth by more than 90%.

(a)

5 10 15 20 25 30 35  
LL-37 LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES  
KR-12

(b)

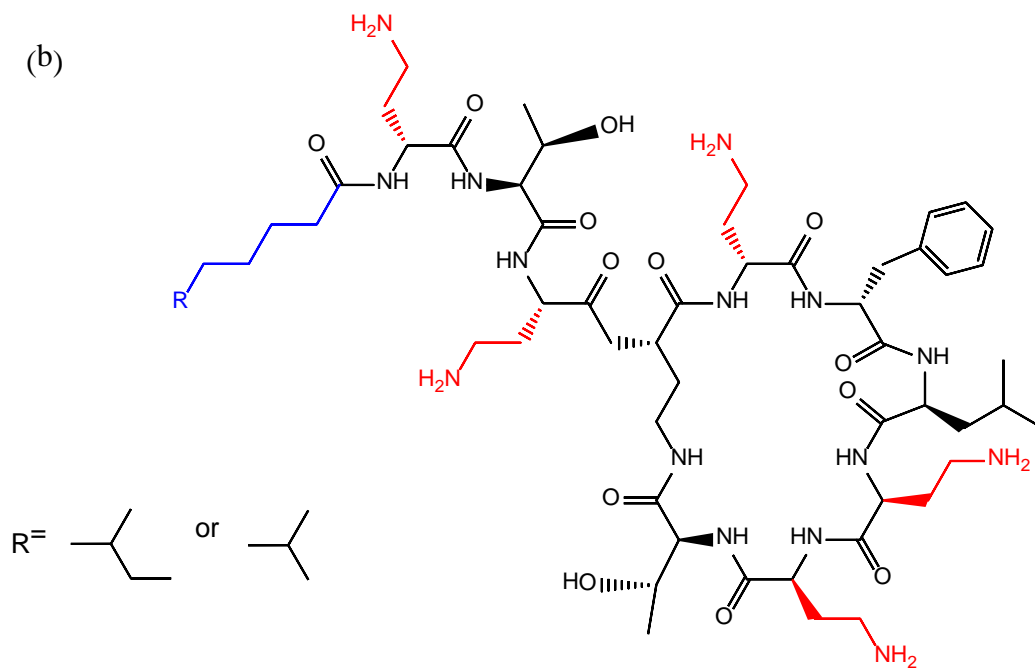


Figure S1. (a) Peptide sequences of host-defense peptide LL-37 and its smaller antimicrobial peptide KR-12<sup>1</sup>; (b) chemical structure of antibiotic polymyxin B.

Table S1. Amino acid composition analysis of AMPs LL-37, KR-12, and polymyxin B.

AMPs or antibiotic	Cationic	Hydrophobic	Hydrophilic
LL-37	RKKKKKRRKRR 11(6K5R), 30% <sup>a</sup>	LLGFFIGFIVIFLLVP 16(4F4L2G2I2V1G1P), 43%	DSEEQDNTES 10(3E2D2S1N1Q1T), 27%
KR-12	KRRKR 5(3R2K), 42%	IVIFL 5(2I1F1L1V), 42%	QD 2(1D1Q), 16%
polymyxin B	DDDDDD, 45%	LFLL <sup>b</sup> , 36%	TT, 18%

a. Capitalized letter represents the abbreviation of amino acid (R: arginine; K: lysine; there are 6 lysine and 5 arginine residues in each LL-37 peptide; the cationic amino acid composition is calculated to be 30%). b. The hydrophobic tail in polymyxin B (7 or 8 carbon atoms, blue color in Figure S1b) was calculated as two leucine molecules (L).

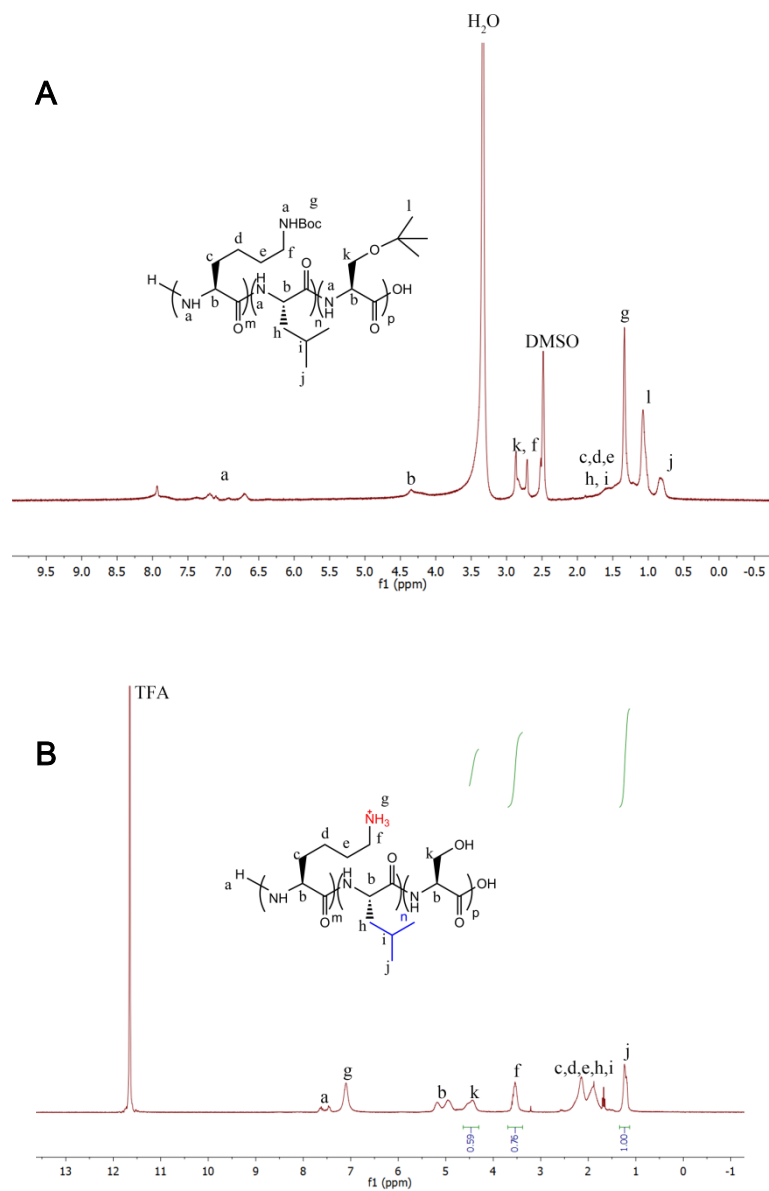


Figure S2.  $^1\text{H}$  NMR spectra of KLS\*-1(A) and KLS-1 (B) in  $\text{DMSO-}d_6$  and  $\text{TFA-}d$ , respectively.

### Typical calculation of amino acid ratio of KLS by $^1\text{H}$ NMR in $\text{TFA-}d$ .

Taking KLS-1 as an example,  $A_f$ ,  $A_j$ , and  $A_k$  were the integration area of protons f, j, and k. The actual ratio of amino acids K, L, and S,  $f(\text{K:L:S})$ , were calculated as followed.

$$\begin{aligned}
 f(\text{K:L:S}) &= n(\text{Lys}):n(\text{Leu}):n(\text{Ser}) \\
 &= (A_f/2):(A_j/6):(A_k/2) = (0.76/2):(1.00/6):(0.59/2) \\
 &= 45\%:20\%:35\%
 \end{aligned}$$

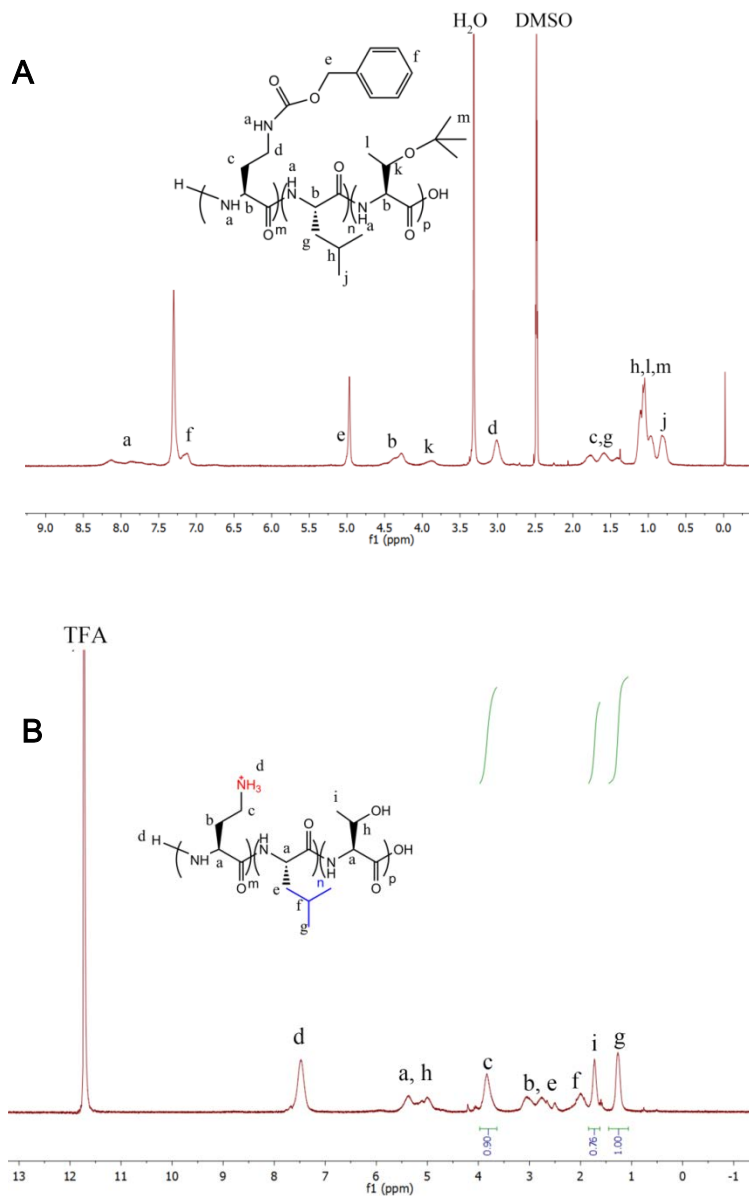


Figure S3.  $^1\text{H}$  NMR of protected polymers  $\text{DLT}^*-1$  (A) and  $\text{DLT}-1$  (B) in  $\text{DMSO}-d_6$  and  $\text{TFA}-d$ , respectively.

### Typical calculation of amino acid ratio of $\text{DLT}$ by $^1\text{H}$ NMR in $\text{TFA}-d$ .

Taking  $\text{DLT}-1$  as an example,  $A_d$ ,  $A_j$ , and  $A_l$  were the integration area of protons d, g, and I, respectively. The actual ratio of amino acids  $\text{D}$ , L, and T,  $f(\text{D:L:T})$ , were calculated as followed.

$$\begin{aligned}
 f(\text{D:L:T}) &= n(\text{Dab}):n(\text{Leu}):n(\text{Thr}) \\
 &= (A_d/2):(A_g/6):(A_i/3) = (0.90/2):(1.00/6):(0.76/3) \\
 &= 52\%:19\%:29\%
 \end{aligned}$$

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*Table S2.* GPC results of protected peptides in DMF with LiBr (1 g/L).

<b>Polymers</b>	<b><math>M_n</math></b>	<b><math>M_w/M_n</math></b>
KLS*-1	4319	1.22
KLS*-2	5090	1.25
KLS*-3	4416	1.25
DLT* -1	6978	1.32
DLT* -2	6540	1.28
DLT*-3	6983	1.32

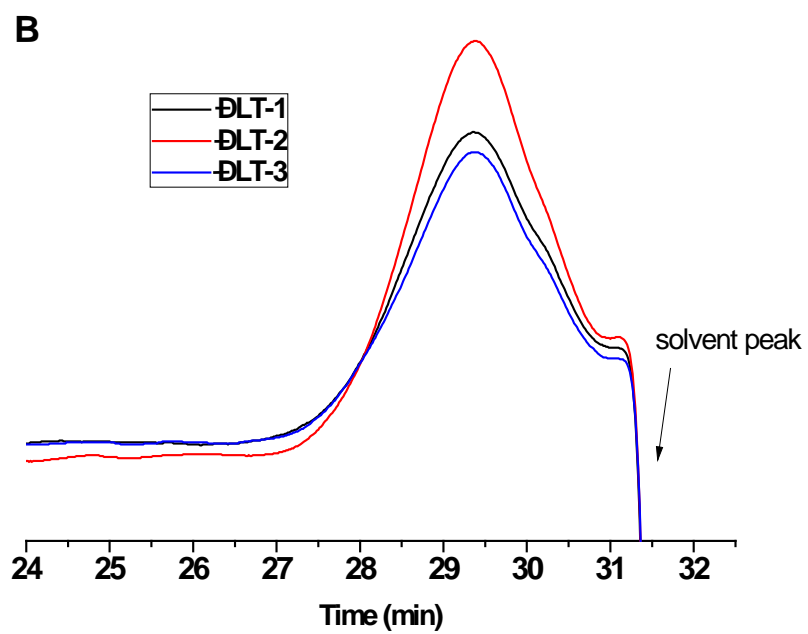
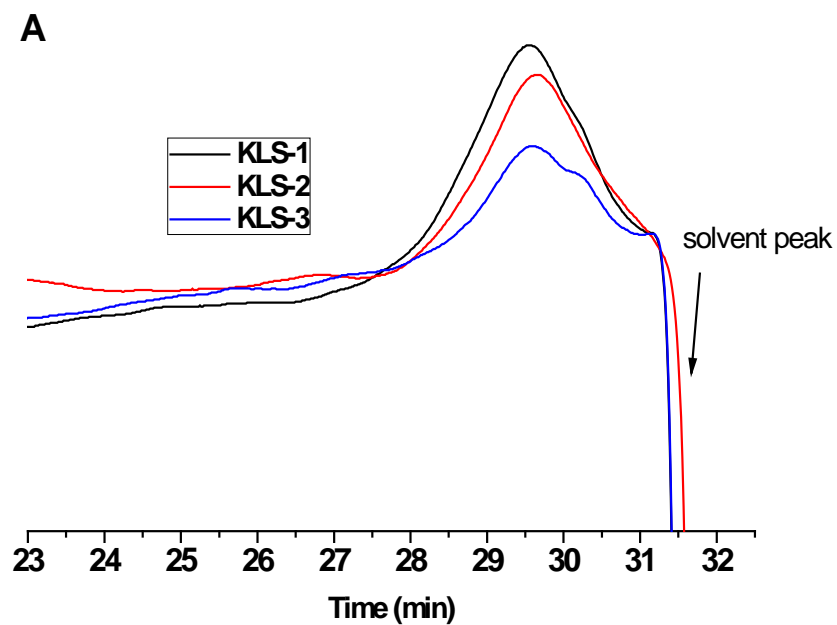


Figure S4. GPC curves of KLS (A) and DLT (B) series.

Table S3. MICs of KLS and DLT against some clinical strains.

Polymers	MIC ( $\mu\text{g/mL}$ )			
	Gram negative			Gram positive
	<i>E. coli</i> 958	<i>K. pneumonia</i> PTR3	<i>P. aeruginosa</i> D25	<i>S.aureus</i> USA-300 (ORSA)
KLS-1	512	256	256	256
KLS-2	64	256	256	128
KLS-3	64	128	256	128
DLT-1	64	128	32	128
DLT-2	64	>512	512	64
DLT-3	256	>512	256	64

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*Table S4.* Comparison of different peptide synthesis methods.

Methods	Accurate peptide length and sequence	Large scale synthesis	Solvent consumption <sup>a</sup>	Time consumption <sup>a</sup>
SPPS	YES	NO	0.8 L	1 day
NCA	NO	YES	1.2 L	3-4 days
DPPA	NO	YES	0.2 L	1-1.5 days

- a. Taking KLS an example and supposing the peptide length was 20 for the SPPS method.

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## References

1. Wang, G., *J. Biol. Chem.* 2008, 283, 32637.