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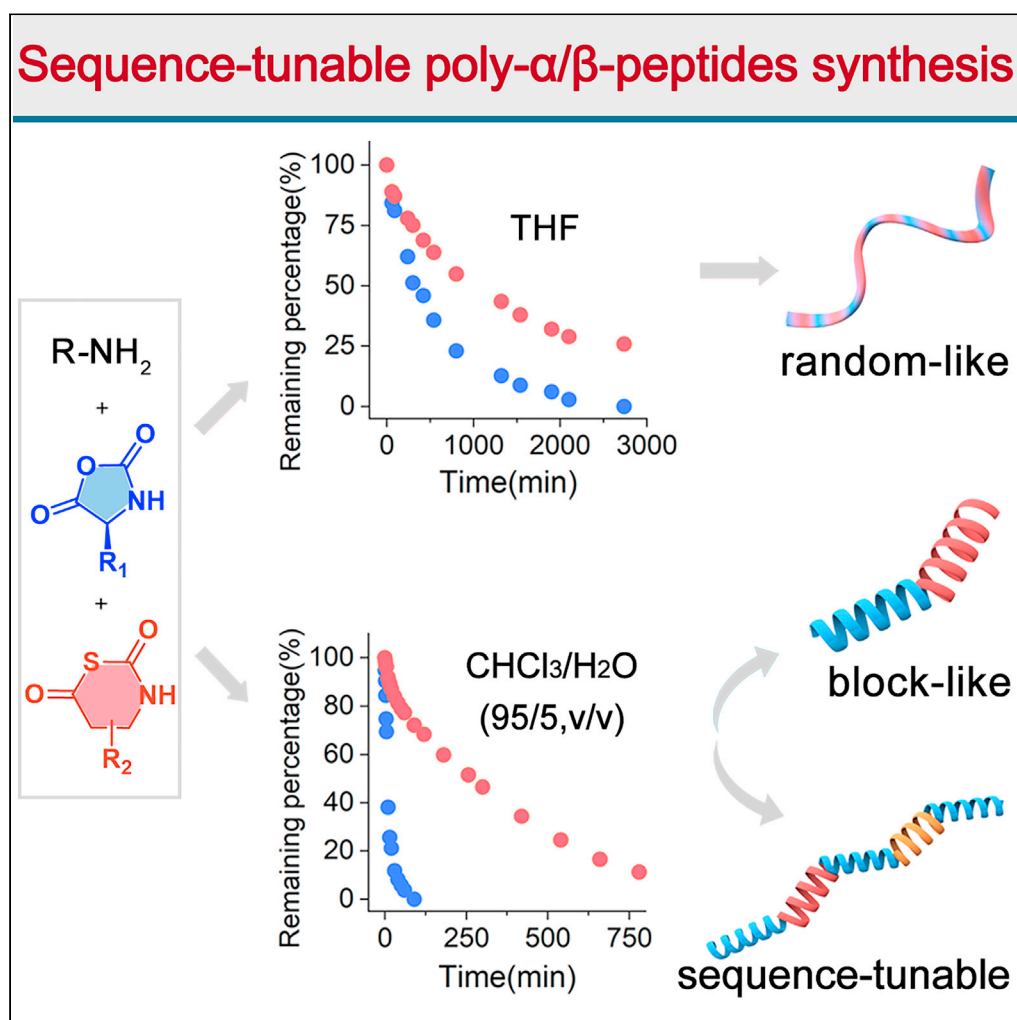**Article**

Synthesis of poly-α/β-peptides with tunable sequence via the copolymerization on *N*-carboxyanhydride and *N*-thiocarboxyanhydride

Sequence-tunable poly-α/β-peptides synthesis

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Highlights

Realizing controlled
synthesis of poly-α/
β-peptides via one-pot
polymerization

Sequence-tunable
copolymerization via
solvent-dependent
polymerization kinetics

Adjustable proteolytic
stability and antibacterial
activity of poly-α/
β-peptides

Tunable self-assembly
behavior of poly-α/
β-peptides via one-pot
polymerization

Article

Synthesis of poly-α/β-peptides with tunable sequence via the copolymerization on N-carboxyanhydride and N-thiocarboxyanhydride

Min Zhou,[1] Jingcheng Zou,[2] Longqiang Liu,[2] Ximian Xiao,[2] Shuai Deng,[2] Yueming Wu,[2] Jiayang Xie,[2] Zihao Cong,[2] Zhemin Ji,[2] and Runhui Liu[1,2,3,*]

SUMMARY

The fascinating functions of proteins and peptides in biological systems have attracted intense interest to explore their mimics using polymers, including polypeptides synthesized from polymerization. The folding, structures and functions of proteins and polypeptides are largely dependent on their sequence. However, sequence-tunable polymerization for polypeptide synthesis is a long-lasting challenge. The application of polypeptides is also greatly hindered by their susceptibility to enzymatic degradation. Although poly-α/β-peptide has proven to be an effective strategy to address the stability issue, the synthesis of poly-α/β-peptide from polymerization is not available yet. Hereby, we demonstrate a living and controlled copolymerization on α-NCA and β-NTA to prepare sequence-tunable poly-α/β-peptides. This polymerization strategy shows a prominent solvent-driven characteristic, providing random-like copolymers of poly-α/β-peptides in THF and block-like copolymers of poly-α/β-peptides in a mixed solvent of CHCl$_3$/H$_2$O (95/5, v/v), and opens new avenues for sequence-tunable polymerization and enables facile synthesis of proteolysis tunable poly-α/β-peptides for diverse applications.

INTRODUCTION

Proteins and peptides play important roles in biological systems and have been actively explored for both fundamental and applicational studies (Hancock and Sahl, 2006; Liu and Yin, 2020; Schmidt et al., 2015). However, the application of proteins and peptides is hindered by their susceptibility to enzymatic degradation. In addition, it is important to have a deep understanding on folding of proteins and peptides, and to explore new folding designs and structures (Hamuro et al., 1999; Horne and Gellman, 2008; Sang et al., 2020). These drive the development of α/β-peptides, such as α/β-peptide foldamers, for exploration and application in diverse fields such as drug discovery, protein mimicking and antimicrobial study (Girvin et al., 2019; Girvin and Gellman, 2018; Goodman et al., 2007; Pilsl and Reiser, 2011). α/β-peptides have demonstrated great potential in biological functions owing to their increased stability upon enzymatic degradation and controlled sequence in designing folding and functions (Schmitt et al., 2004, 2007).

Currently, α/β-peptides can only be synthesized via the solid-phase peptide synthesis that is time consuming and difficult for large-scale synthesis, especially for long polypeptides and incorporation of unnatural amino acids (Figure 1A) (Chan-Seng and Lutz, 2014). Although poly-α-peptides can be synthesized via the ring-opening polymerization of α-amino acid N-carboxyanhydrides (α-NCAs) (Cheng and Deming, 2012; Deming, 1997; Kricheldorf, 2006; Rasines Mazo et al., 2020; Song et al., 2019; Wu et al., 2018, 2020), no reliable polymerization strategy is available for synthesis of poly-α/β-peptides. In addition, the folded, self-assembled structures and functions of proteins and polypeptides are largely dependent on their defined sequence along the backbone (Girvin et al., 2019; Goodman et al., 2007; Schmitt et al., 2007). However, the synthesis of sequence-controlled polypeptides via polymerization is a long-lasting challenge (Jones, 2008; Lutz, 2010; Lutz et al., 2013). Therefore, a breakthrough in sequence-controlled poly-α/β-peptide synthesis is highly desired, though sequence-controlled polymerizations are reported recently on the synthesis of polyacrylates, polynorbornene, poly(styrene-b-maleimide) and polyesters (Jia et al., 2021; McGraw et al., 2020; Pfeifer and Lutz, 2007; Yasir et al., 2020).

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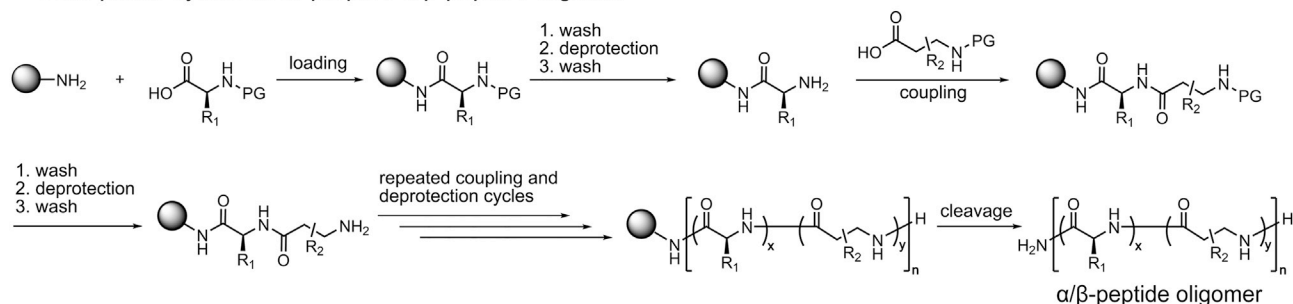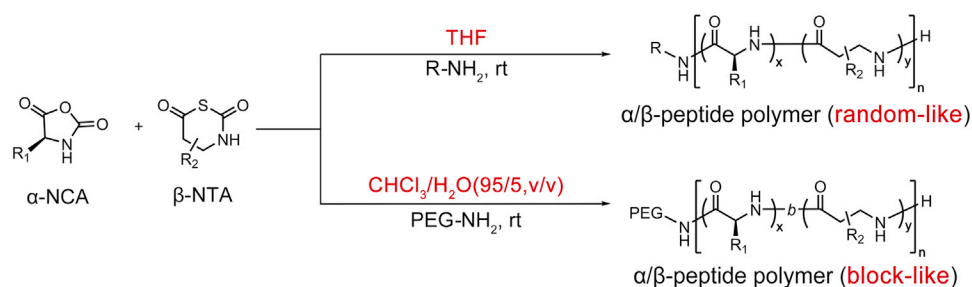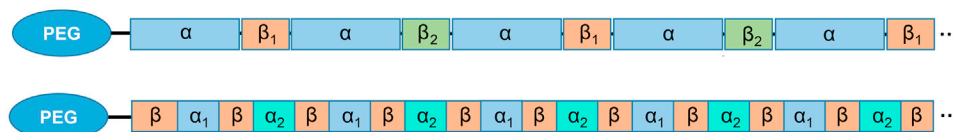
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A   Solid phase synthesis to prepare α/β-peptide oligomer

B   Solvent-tuning copolymerization to prepare random-like or block-like α/β-peptide polymer

C   Sequence-tunable copolymerization to prepare sequence-tunable poly-α/β-peptide

Figure 1. Strategies to prepare poly-α/β-peptides

(A) Solid-phase synthesis to prepare α/β-peptide oligomers.

(B) The solvent-tuning copolymerization on α-NCAs and β-NTAs to prepare random-like poly-α/β-peptides in THF and block-like poly-α/β-peptides in CHCl₃/H₂O (95/5, v/v).

(C) Sequence-tunable polymerization on α-NCAs and β-NTAs to prepare sequence-tunable poly-α/β-peptides in CHCl₃/H₂O (95/5, v/v).

Hereby, we report a facile and controlled synthesis of sequence-tunable poly-α/β-peptides from the copolymerization on α-NCAs and β-amino acid thiocarboxyanhydrides (β-NTAs) under mild conditions using primary amine as the initiator (Figure 1B). The reaction rate of α-NCAs and β-NTAs in the copolymerization on a mixture of α-NCAs/β-NTAs can be tuned with solvents to give either poly-α/β-peptides with random-like distribution of two types of amino acid subunits or poly-α/β-peptides with block-like sequence (Figure 1B). Moreover, this α-NCA/β-NTA copolymerization strategy enables the sequence-tunable synthesis of poly-α/β-peptides for functional studies (Figure 1C).

RESULTS AND DISCUSSION

Using N^{ε}-tert-butyloxycarbonyl-L-lysine NCA (Boc-α-L-Lys NCA) and β^{3}-benzyl-L-carboxyhomoglycine NTA (Bn-β^{3}-LCHG NTA) as representative monomers (Figures S1–S15), we found that 4-tert-butylbenzyl-amine (tBuBnNH₂) can initiate the copolymerization on a mixture of α-NCA and β-NTA at room temperature in variable solvents (DMF, CHCl₃, DMSO, Toluene, CH₃CN, DCM, and THF) to give expected poly-α/β-peptides, poly-(Boc-α-L-Lys/Bn-β^{3}-LCHG), with narrow dispersities ($Đ$ = 1.13–1.23) (Table 1, entry 1–7). We performed the following polymerization studies in THF that is a widely used solvent for α-NCA polymerization. The polymerization on the mixture of Boc-α-L-Lys NCA and Bn-β^{3}-LCHG NTA at variable monomer:initiator ratios preceeded successfully using tBuBnNH₂ as the initiator to give corresponding poly-α/β-peptides with expected chain lengths and narrow dispersities ($Đ$ = 1.19–1.31) (Table 1, entry 7–11). The structural diversity of poly-α/β-peptides can be obtained easily via the copolymerization on a mixture

Table 1. Copolymerization on α-NCAs and β-NTAs[a]
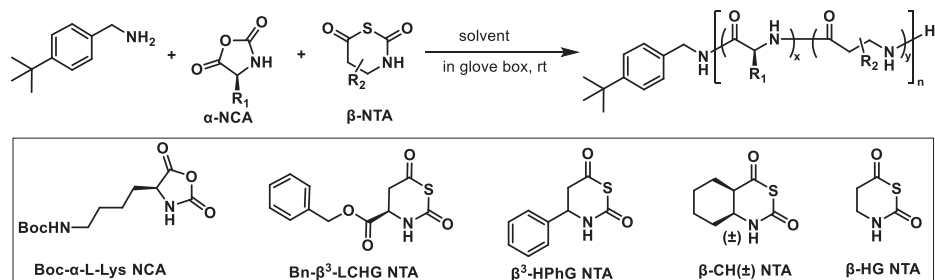
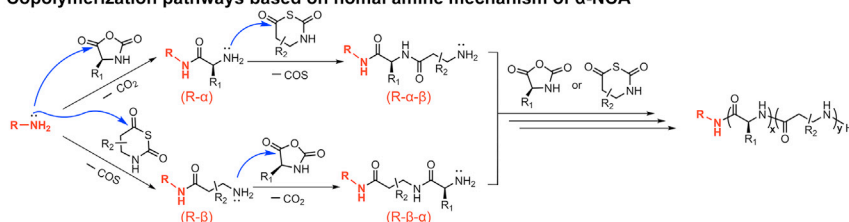| Entry	Monomers	$[M^{\alpha}]_0:[M^{\beta}]_0:[I]_0$	Solvent	$M_{n, th}$ (kDa)	$M_{n, GPC}$ (kDa)	Đ
1	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	15:15:1	DMF	6.7	6.1	1.17
2	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	15:15:1	CHCl₃	6.7	5.7	1.16
3	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	15:15:1	DMSO	6.7	4.4	1.17
4	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	15:15:1	Toluene	6.7	6.3	1.23
5	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	15:15:1	MeCN	6.7	4.8	1.13
6	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	15:15:1	DCM	6.7	5.5	1.16
7	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	15:15:1	THF	6.7	7.2	1.19
8	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	30:15:1	THF	10.1	9.1	1.19
9	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	45:15:1	THF	13.5	13.6	1.25
10	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	60:15:1	THF	16.9	16.1	1.26
11	Boc-α-L-Lys NCA, Bn-β³-LCHG NTA	60:30:1	THF	20.1	19.0	1.31
12	Boc-α-L-Lys NCA, β³-HPhG NTA	15:15:1	THF	5.8	5.5	1.13
13	Boc-α-L-Lys NCA, β-HG NTA	15:15:1	THF	4.6	5.2	1.12
14	Boc-α-L-Lys NCA, β²,³-CH(±) NTA	15:15:1	THF	5.5	4.7	1.14

[a]Copolymerizations on Boc-α-L-Lys NCA and Bn-β³-LCHG NTA were operated in the nitrogen-filled glove box at room temperature to prepare poly-α/β-peptides using different solvents (entry 1–7), and with variable chain lengths (entry 7–11, using THF as the solvent); Copolymerizations on α-NCAs and β-NTAs with different combinations at room temperature (entry 12–14, using THF as the solvent). $M_{n,GPC}$ is the obtained number of average molecular weight from GPC; Đ means the dispersity index; DP is the obtained degree of copolymerization.

of α-NCA and β-NTA bearing different sidechain substitution groups, as demonstrated by the copolymerizaiton on a mixture of Boc-α-L-Lys NCA/β³-HPhG NTA, Boc-α-L-Lys NCA/β-HG NTA, and Boc-α-L-Lys NCA/β²,³-CH(±) NTA to give corresponding poly-α/β-peptides with narrow dispersities (Đ = 1.12–1.19) (Table 1, entry 12–14).

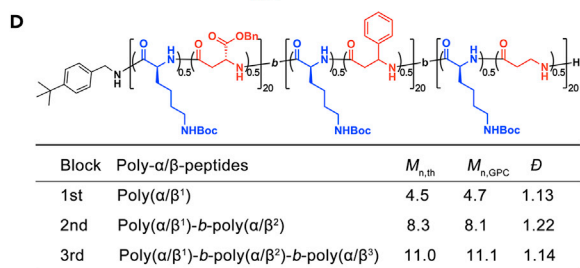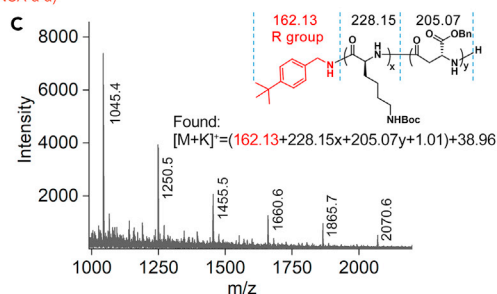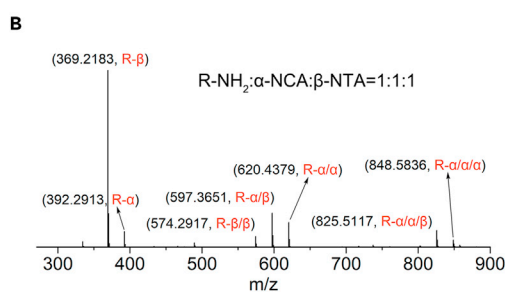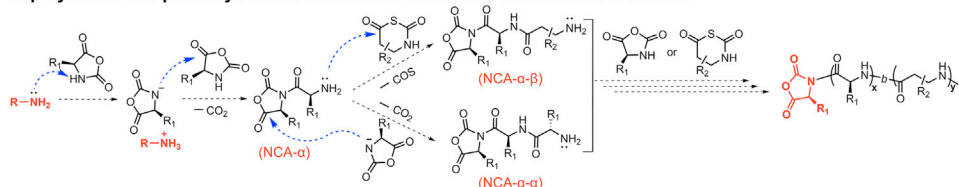Previous study in our group indicated that primary amine-initiated ring-opening polymerization on β-NTAs follows a normal amine mechanism (NAM), utilizing terminal amine as the reactive center for chain propagation (Zhou et al., 2020). The ring-opening polymerization on α-NCAs is proposed to follow either a NAM or an activated monomer mechanism (AMM) (Cheng and Deming, 2012; Hadjichristidis et al., 2009). Therefore, primary amine-initiated ring-opening polymerization on a mixture of α-NCA/β-NTA could follow either a NAM or an AMM mechanism (Figure 2A). To figure out the mechanism we performed ESI-MS characterization on a 1:1:1 mixture (molar ratio) of tBuBnNH₂, Boc-α-L-Lys NCA and Bn-β³-LCHG NTA and found multiple peaks, bearing the terminal group from the initiator tBuBnNH₂, corresponding to possible compounds in the NAM pathway (Figures 2A and 2B); in sharp contrast, we found no observable ESI-MS peaks, bearing a terminal NCA ring, corresponding to any possible compound in the AMM pathway (Figures 2A and 2B). The MALDI-TOF-MS characterization on tBuBnNH₂-initiated copolymerization of Boc-α-L-Lys NCA/Bn-β³-LCHG NTA revealed the existence of expected poly-α/β-peptides all with an C-terminal tBuBnNH group, indicating the NAM route of copolymerization on α-NCA/β-NTA (Figure 2C).

The NAM mechanism indicates that primary amine-initiated polymerization on α-NCA/β-NTA is a living polymerization that is capable to prepare poly-α/β-peptides with multiple blocks and with defined

Figure 2. Mechanism study of primary amine-initiated copolymerization on α-NCA and β-NTA

(A) Two possible initiation and chain propagation pathways based on normal amine mechanism and activated monomer mechanism of α-NCA.

(B) HRESI-MS analysis on mixing $tBuBnNH_2$, Boc-α-L-lys NCA and Bn-β^3-LCHG NTA at 1:1:1 molar ratio for polymerization initiation study (using THF as the solvent at 25°C, $[M]_0 = 0.2$ M).

(C) MALDI-TOF-MS characterization of $tBuBnNH_2$-initiated copolymerization on Boc-α-L-lys NCA and Bn-β^3-LCHG NTA ($[M^\alpha]_0:[M^\beta]_0:[I]_0 = 15:15:1$, using THF as the solvent at 25°C, $[M]_0 = 0.2$ M).

(D) $tBuBnNH_2$-initiated copolymerization on α-NCA and β-NTA to prepare poly-α/β-peptide triblock copolymer with each block having different α/β-amino acid composition. GPC characterization was conducted using DMF as the mobile phase at a flow rate of 1 mL/min.

(E) GPC traces of the poly-α/β-peptide triblock copolymer.

C-terminal groups from the primary amine initiators. Using a 1:1 molar ratio mixture of an α-NCA and a β-NTA, we successfully synthesized the triblock copolymer poly(Boc-α-L-Lys$_{0.5}$/Bn-β^3-LCHG$_{0.5}$)$_{20}$-*b*-poly(Boc-α-L-Lys$_{0.5}$/β^3-HPhG$_{0.5}$)$_{20}$-*b*-poly(Boc-α-L-Lys$_{0.5}$/β^{2,3}-CH$_{0.5}$)$_{20}$ with a narrow dispersity of 1.14 (Figures 2D and 2E). A primary amine with a morpholino-naphthalimide fluorophore was able to initiate the copolymerization on a mixture of Boc-α-L-Lys NCA and β-HG NTA to give a C-terminal fluorophore-labelled poly-α/β-peptide that displays strong fluorescence (Figures 2F and 2G). This poly-α/β-peptide can be further functionalized with an N-terminal maleimide to give a terminal dual-functionalized poly-α/β-peptide that has broad application in protein conjugation, imaging and mechanism study (Figures S16–S18) (Baumann et al., 2020; Feng et al., 2010). These demonstrations on block copolymer synthesis and C-terminal functionalization confirmed living polymerization property and polymerization initiation via the nucleophilic addition of primary amine initiator.

When THF was used as the solvent for copolymerization on a mixture of α-NCA and β-NTA, using Boc-α-L-Lys NCA and Bn-β^3-LCHG NTA as the model, we found no significant gradient separation in compositional profile of two amino acid subunits within the poly-α/β-peptide chain, though Boc-α-L-Lys NCA have a slightly higher reactivity than Bn-β^3-LCHG NTA according to the kinetic plot of residual monomer (Figures 3A–3C) and instantaneous copolymer composition $F_{\alpha\text{-NCA}}$ (Figures 3C and S19). (Gleede et al., 2019) This copolymerization proceeded close to "ideal copolymerization" ($r_{\alpha\text{-NCA}} \times r_{\beta\text{-NTA}}$ = 0.93, $r_{\alpha\text{-NCA}} \times r_{\beta\text{-NTA}}$ = 1 means ideal copolymerization) (Yasir et al., 2020), which in combination with the instantaneous copolymer composition $F_{\alpha\text{-NCA}}$ revealed that the resulting poly-α/β-peptide was a random-like copolymer and the β-amino acid residue has a nearly even distribution along poly-α/β-peptide chain (Figure 3C) (Yasir et al., 2020). The obtained number average molecular weight (M_n) of poly-α/β-peptide from different feeding ratio of monomer/initiator [M$^\alpha$ + M$^\beta$]$_0$/[I]$_0$ matched the theoretical values of M_n well and displayed a linear correlation with [M$^\alpha$ + M$^\beta$]$_0$/[I]$_0$, which in combination with the low dispersity (Đ = 1.19–1.26) indicating a controllable polymerization (Figure 3D).

Poly-α/β-peptides have proven to be important mimics and modifications of nature polypeptides, such as host defense peptide (HDP), to effectively improve the stability upon enzymatic degradation and therapeutic potential (Konai et al., 2018; Schmitt et al., 2004, 2007). The copolymerization on a mixture of α-NCA and β-NTA enabled facile synthesis of poly-α/β-peptides for functional studies, as our proof-of-concept demonstration to mimic HDP using Boc-α-L-Lys NCA and β^{2,3}-CH(±) NTA (Figures 3E and 3F, S22–S25). The obtained cationic/hydrophobic amphiphilic poly-α/β-peptides had 30%, 50% or 70% of the β-amino acid residues and displayed potent and broad-spectrum activities against a series of multi-drug resistant Gram positive and Gram negative bacteria, including nine clinically isolated pathogens of *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (Figure 3F). It's noteworthy that incorporation of β-amino acid residues substantially increased polypeptides' stability against enzymatic degradation, with the observation that poly(α-L-Lys$_{0.5}$/β^{2,3}-CH$_{0.5}$)$_{20}$ displayed two-fold and three-fold increase of the half-life than poly(α-L-Lys)$_{20}$ in the presence of trypsin and proteinase K23, respectively (Figures 3G and 3H, S26–S29).

Surprisingly, the copolymerization on α-NCA and β-NTA displayed a solvent-dependent tuning of the reaction and resulting poly-α/β-peptides, giving random-like copolymers of poly-α/β-peptides using THF as the polymerization solvent as demonstrated above, and block-like copolymers of poly-α/β-peptides using a mixed solvent of CHCl$_3$/H$_2$O (95/5, v/v) as demonstrated by the PEG-NH$_2$ initiated copolymerization on a mixture of Boc-α-L-Lys NCA and Bn-β^3-LCHG NTA (Figure 4A). Meanwhile, MALDI-TOF-MS analysis on PEG-NH$_2$ initiated copolymerization of Boc-α-L-Lys NCA/Bn-β^3-LCHG NTA in the mixed solvent of CHCl$_3$/H$_2$O showed the molecular weight of copolymerization product matching theoretical values of poly-α/β-peptides with an C-terminal PEG-NH group, indicating NAM route of the copolymerization as well in the mixed solvent of CHCl$_3$/H$_2$O (Figures 2A and S30). The block-like copolymerization in CHCl$_3$/H$_2$O can be explained by the strikingly enlarged difference in polymerization kinetics between Boc-L-Lys α-NCA and β^3-LCHG NTA in this mixed solvent than in THF (Figure 4B), which is also reflected from the
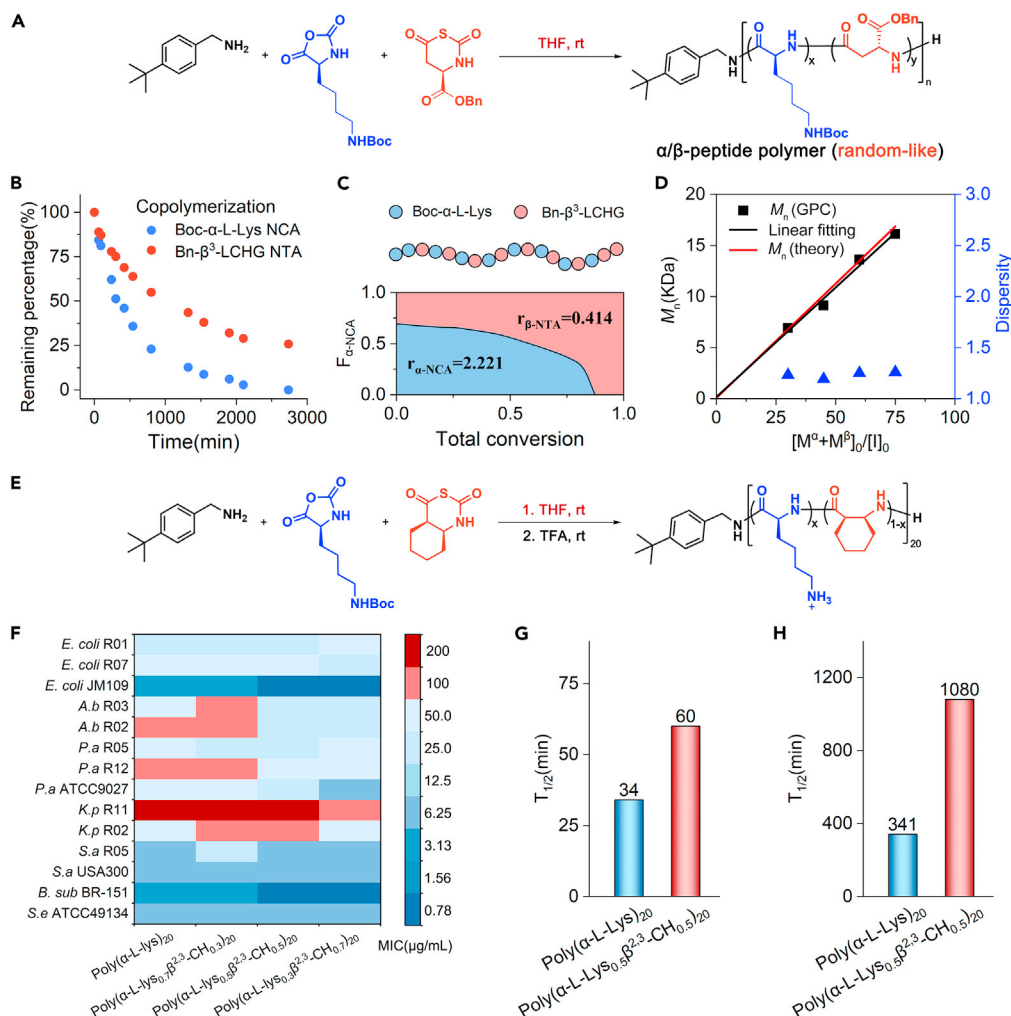
Figure 3. Primary amine-initiated polymerization on α-NCA and β-NTA in THF

(A) tBuBnNH$_2$-initiated copolymerization on Boc-α-L-Lys NCA and Bn-β^3-LCHG NTA in THF gives poly-α/β-peptides as random-like polymers.

(B) Conversion of Boc-α-L-Lys NCA and Bn-β^3-LCHG NTA in tBuBnNH$_2$-initiated copolymerization using THF as the solvent ([M^α]$_0$:[M^β]$_0$:[I]$_0$ = 15:15:1, rt, [M]$_0$ = 0.2 M).

(C) The instantaneous copolymer composition of α-amino acid in the poly-α/β-peptides chains (F) versus total conversion of monomers based on the reactivity ratios of Boc-α-L-Lys NCA (blue) and Bn-β^3-LCHG NTA (red).

(D) The relationship between M_n or dispersity (Đ) value (Table 1, entry 7–10) of poly-α/β-peptide prepared via α-NCA and β-NTA copolymerization and the initial molar ratio of the α- and β-amino acid monomers:initiator ([M^α + M^β]$_0$/[I]$_0$) ratio.
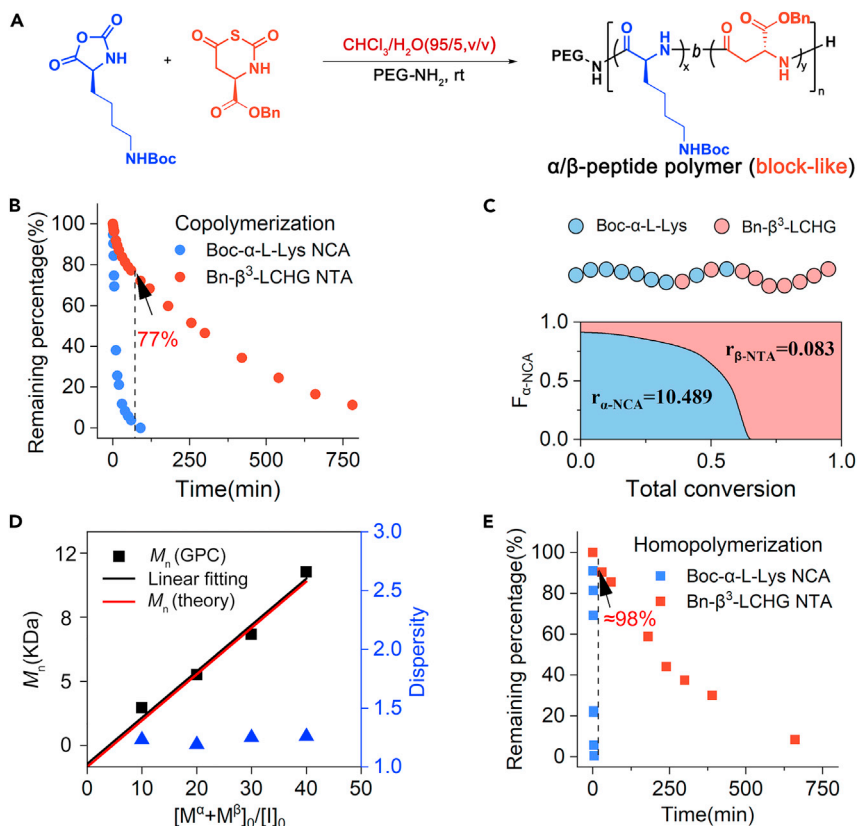
(E) tBuBnNH$_2$-initiated copolymerization on Boc-α-L-Lys NCA and β^{2,3}-CH(±) NTA for synthesis of HDP-mimicking amphiphilic poly-α/β-peptides.

(F) Antimicrobial activities of poly-α/β-peptides against multiple clinically isolated drug-resistant bacteria, evaluating with the minimum inhibitory concentration (MIC). The poly-α/β-peptides have incrementally increased the ratio of hydrophobic β^{2,3}-CH subunits from 30% to 70%.

(G) The half-life of poly(α-L-Lys)$_{20}$ and Poly(α-L-Lys$_{0.5}$β^{2,3}-CH$_{0.5}$)$_{20}$ against trypsin (trypsin/polymer = 1/40, w/w) at 25°C in PBS buffer.

(H) The half-life of poly(α-L-Lys)$_{20}$ and Poly(α-L-Lys$_{0.5}$β^{2,3}-CH$_{0.5}$)$_{20}$ against proteinase K23 (proteinase K23/polymer = 1/10, w/w) at 25°C in PBS buffer.

24-fold increase on the ratio of monomer reactivity ($r_{α\text{-NCA}}/r_{β\text{-NTA}}$ = 126.4 in CHCl$_3$/H$_2$O $vs.$ $r_{α\text{-NCA}}/r_{β\text{-NTA}}$ = 5.36 in THF, Figures 4C and S20). The instantaneous copolymer composition F$_{α\text{-NCA}}$ revealed a block-like copolymer of poly-α/β-peptides with α-amino acid residues and β-amino acid residues enriching in the first block and the second block, respectively (Figure 4C). The obtained M_n of poly-α/β-peptide from different

Figure 4. Primary amine-initiated polymerization on α-NCA and β-NTA in a mixed solvent CHCl₃/H₂O (95/5, v/v)

(A) PEG-NH₂ initiated Boc-L-Lys α-NCA and Bn-β³-LCHG NTA copolymerization in the mixed solvent of CHCl₃/H₂O (95/5, v/v) gives poly-α/β-peptides as block-like copolymers.

(B) Corresponding conversion of Boc-L-Lys NCA and Bn-β³-LCHG NTA ([M^α]$_0$:[M^β]$_0$:[I]$_0$ = 15:15:1, rt, [M]$_0$ = 0.2 M) in the mixed solvent of CHCl₃/H₂O (95/5, v/v).

(C) The instantaneous copolymer composition of α-amino acid in the poly-α/β-peptide chains (F) versus total conversion based on the reactivity ratios of Boc-L-Lys NCA (blue) and Bn-β³-LCHG NTA (red).

(D) The relationship between M_n or Đ value of poly-α/β-peptide, prepared via PEG-NH₂ initiated copolymerization on α-NCA and β-NTA in the mixed solvent of CHCl₃/H₂O (95/5, v/v), and the initial molar ratio of total α-NCA and β-NTA vs. initiator, ([M^α + M^β]$_0$/[I]$_0$).

(E) Conversion of Boc-L-Lys NCA and Bn-β³-LCHG NTA, respectively, in tBuBnNH₂-initiated homopolymerization in the mixed solvent of CHCl₃/H₂O (95/5, v/v) ([M]$_0$:[I]$_0$ = 15:1, rt, [M]$_0$ = 0.2 M).

feeding ratios of monomer/initiator [M^α + M^β]$_0$/[I]$_0$ matched the theoretical values of M_n well and displayed a linear correlation with [M^α + M^β]$_0$/[I]$_0$, which in combination with the narrow dispersity (Đ = 1.12–1.23) indicating a controllable polymerization in CHCl₃/H₂O (Figures 4D and S31). This result was echoed by the observation on the kinetics in homopolymerization that Boc-α-L-Lys NCA was consumed within 5 min, whereas about 98% percent of β³-LCHG NTA was left unreacted during the same time window (Figure 4E).

Sequence-controlled polymerization for polypeptide synthesis was a long-standing challenge (Jones, 2008; Lutz, 2010; Lutz et al., 2013). We found with surprise that our strategy for copolymerization on α-NCA and β-NTA in a mixed solvent CHCl₃/H₂O (95/5, v/v) provided a perfect solution for sequence-tunable copolymerization to prepare polypeptides based on the obvious difference in homopolymerization on α-NCA and β-NTA (Figures 4E and S21). Using PEG-NH₂ as the polymerization initiator, repeated addition of a Boc-α-L-Lys/Bn-β³-LCHG mixture into the reaction afforded sequence-tunable polypeptide with alternate Boc-α-L-Lys blocks and Bn-β³-LCHG blocks for total 10 blocks of peptides (Figure 5A). The living copolymerization property and successful growing of each new blocks were confirmed by the progressive shift of GPC traces to a larger M_n and high monomer conversion ratio (>99%, HPLC analysis) in the polymerization reaction between the iterative addition (Figure 5B). Moreover, the obtained M_n of
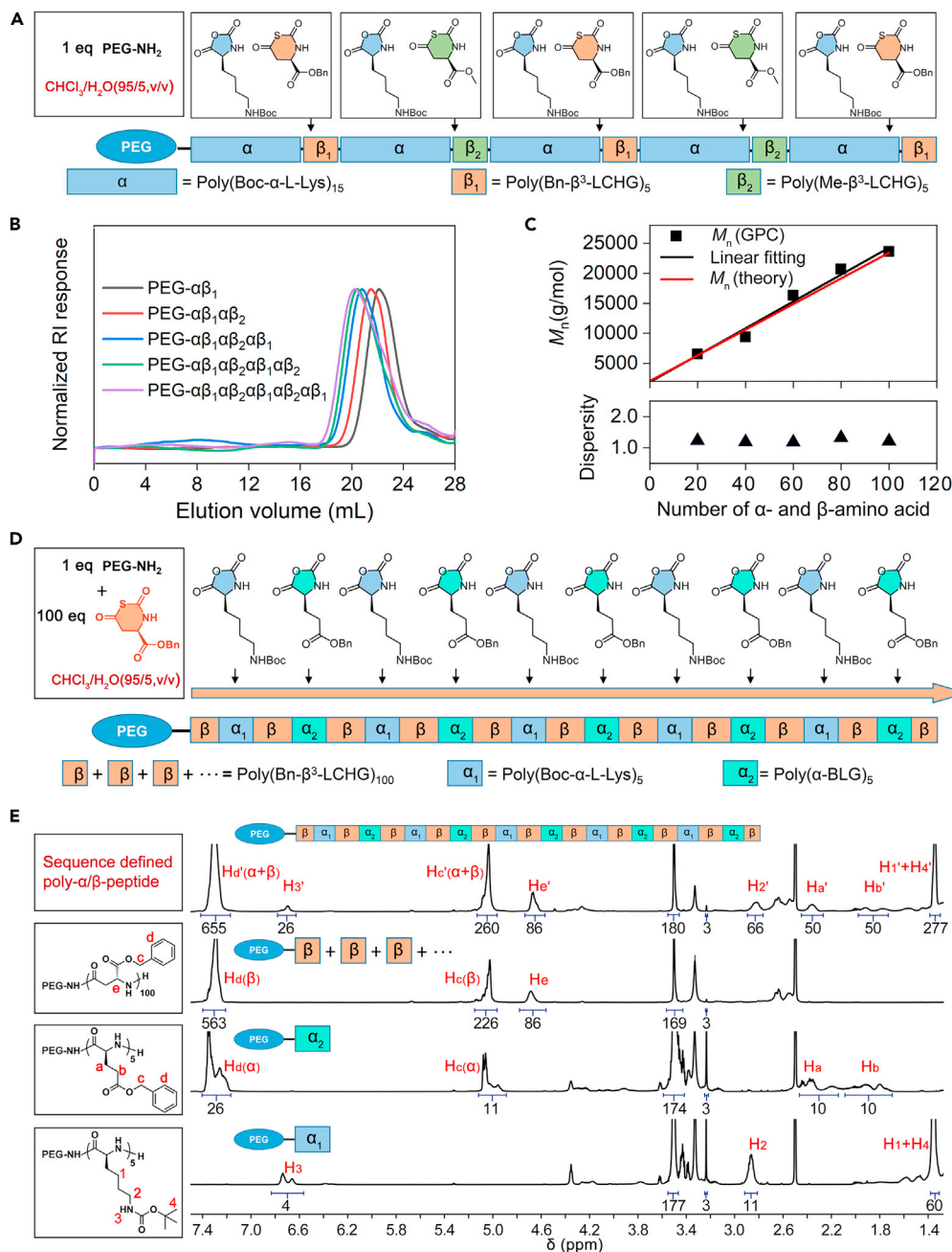
Figure 5. Sequence-tunable copolymerization on α-NCA and β-NTA in a mixed solvent CHCl₃/H₂O (95/5, v/v)

(A) PEG-NH₂ initiated copolymerization on α-NCA and β-NTA to prepare sequence-tunable decablock poly-α/β-peptide, with each block in the form of (poly-α-peptide)-*b*-(poly-β-peptide). A mixture of α-NCA:β-NTA 15:5, relative to 1 equiv. of the initiator PEG-NH₂, was added to the reaction each time, using Boc-α-L-Lys NCA, Bn-β³-LCHG NTA, and Me-β³-LCHG NTA as monomers.

(B) GPC traces of reaction solutions for PEG-terminated multi-block poly-α/β-peptides, from diblock to decablock.

(C) The controlled polymerization in (a) gave the decablock poly-α/β-peptide with a narrow dispersion and an excellent match between the obtained and calculated M_n.

(D) 21-block poly-α/β-peptide with alternate β-peptide block and α-peptide block, by adding 5 mer of Boc-α-L-Lys NCA or α-BLG NCA repeatedly at intervals into the ongoing polymerization reaction of β-NTA (Bn-β³-LCHG NTA), using an initial molar ratio of Bn-β³-LCHG NTA:PEG-NH₂ at 100:1.

(E) ¹H NMR spectrum of PEG-terminated 21-block poly-α/β-peptide that contains total 25 Boc-L-Lysine residues and 25 BLG residues. ¹H NMR spectra of PEG-terminated poly-(Boc-α-L-Lys)₅, poly-(α-BLG)₅ and poly-(Bn-β³-LCHG)₁₀₀ were used for comparison. All NMR spectra were collected using DMSO-d₆ as the solvent.
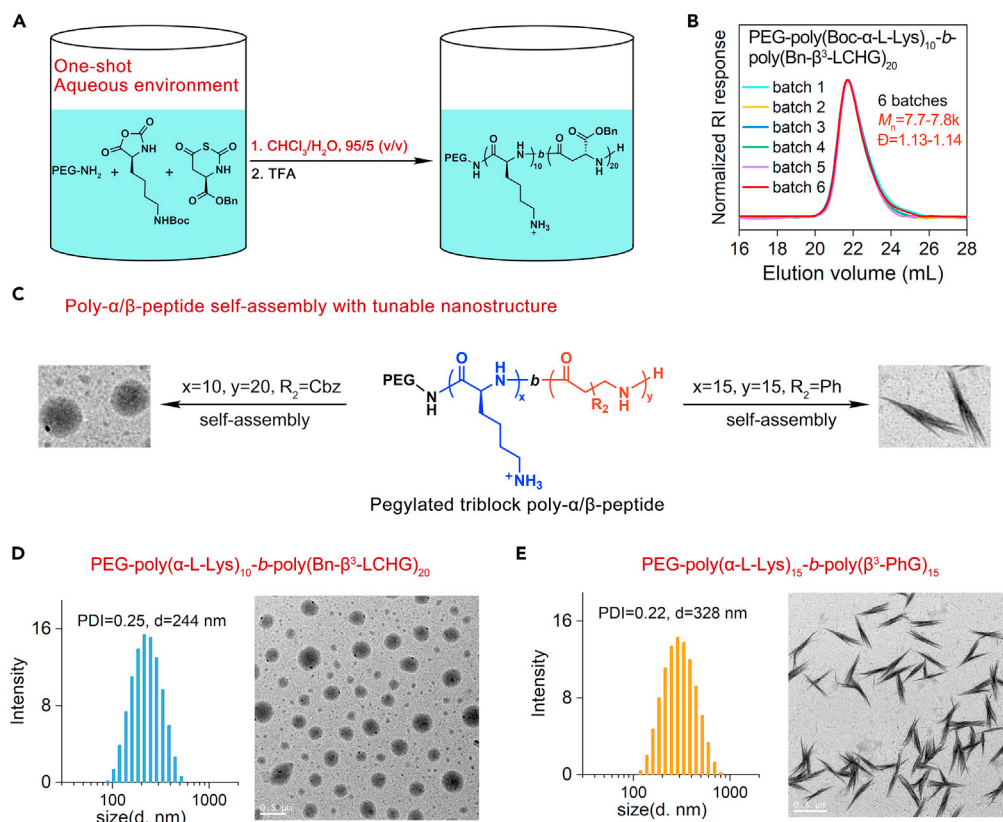
Figure 6. Self-assembly study of PEG-terminated triblock poly-α/β-peptide prepared via one-shot copolymerization on α-NCA and β-NTA

(A) One-shot preparation of PEG-functionalized triblock poly-α/β-peptide PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ via open-vessel copolymerization in an aqueous environment of CHCl$_3$/H$_2$O (95/5, v/v).

(B) GPC traces of PEG-poly(Boc-α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ from six batches.

(C) The different self-assembly behavior of PEG-functionalized triblock poly-α/β-peptides with different components of β-amino acid.

(D) DLS analysis on size distributions and TEM images of self-assembled PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ (c = 0.87 mg/mL) in a mixed solvent H$_2$O/THF 100/15 (v/v).

(E) DLS analysis on size distributions and TEM images of self-assembled PEG-poly(α-L-Lys)$_{15}$-b-poly(β^3-HPhG)$_{15}$ (c = 1 mg/mL) in H$_2$O solvent.

polypeptides was very close to the theoretical value, showing a linear increase after adding each two blocks and with a narrow dispersity (Đ = 1.18–1.32) (Figure 5C). In another demonstration on the sequence-tunable polymerization for polypeptide synthesis, we iteratively added Boc-α-L-Lys NCA or α-BLG NCA into the reaction of PEG-NH$_2$-initiated β^3-LCHG NTA polymerization in CHCl$_3$/H$_2$O (95/5, v/v), resulting in poly-α/β-peptides with insertion of α-amino acid block into predefined position along the polypeptide chain owing to the dramatically faster reaction rate of NCA in CHCl$_3$/H$_2$O (95/5, v/v) (Figure 5D), which was supported by the observed characteristic ^{1}H NMR signals for both Ha, Hb in poly(α-BLG) block and H$_1$, H$_2$, H$_4$ in poly(Boc-α-L-Lys) block within obtained poly-α/β-peptides (Figure 5E).

Sequence-control of block copolymers is very important for the nanostructure of the polymer materials and have diverse applications, such as drug encapsulation and delivery, which is highly relied on the reproducible synthesis of copolymers (Feng et al., 2013; Ge et al., 2020; Krumm et al., 2012; Miao et al., 2017; Nederberg et al., 2011; Wang et al., 2006; Xu and Pu, 2021; Zhang et al., 2019). Above sequence-tunable copolymerization on a mixture of Boc-α-L-Lys NCA and Bn-β^3-LCHG NTA in CHCl$_3$/H$_2$O (95/5, v/v) was found to have excellent reproducibility, generating a PEG-terminated triblock copolymer of PEG-poly(Boc-α-L-Lys)-b-poly(Bn-β^3-LCHG) with overlapped GPC traces, almost identical M_n and dispersity (M_n = 6.7–6.8KDa, Đ = 1.13–1.14) among repeats from six batches of reactions (Figures 6A and 6B and Table S1). In addition, the

obtained PEG-functionalized block-like poly-α/β-peptide with different amino acid component underwent self-assembly spontaneously to form different nanostructures (Figure 6C). Specifically, DLS and TEM characterization indicated that self-assembly of PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ in THF/H$_2$O (15/100, v/v) gave micelles with particle size of 244 nm and PDI value of 0.25 (Figure 6D); however, the assembly of PEG-poly(α-L-Lys)$_{15}$-b-poly(Bn-β^3-HPhG)$_{15}$ gave a fibrous structure with particle size of 328 nm and PDI value of 0.22 (Figures 6E, and S32). This proof-of-concept demonstration showed the power of this controlled-polymerization strategy to synthesize poly-α/β-peptides with predefined sequence that defines the self-assembly, resulting nanostructure and applications.

Conclusions

We demonstrate a promising strategy for sequence-tunable synthesis of polypeptides utilizing the copolymerization on α-NCA and β-NTA. The polymerization is tunable by the reaction solvent to give random-like copolymers of poly-α/β-peptides in THF and block-like copolymers of poly-α/β-peptides in a mixed solvent of CHCl$_3$/H$_2$O (95/5, v/v). This polymerization strategy represents a breakthrough in facile synthesis of poly-α/β-peptides with tunable sequence and resistance to enzymatic degradation, implying great potential in protein mimicking study to explore folding, self-assembly, and biological functions.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103124.

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AUTHOR CONTRIBUTIONS

R.L. directed the whole project. M.Z. and R.L. conceived the idea, proposed the strategy, designed the experiments, evaluated the data, and wrote the manuscript together. M.Z. performed the majority of the experiments. J.Z. conducted polymerization kinetics study and participated in sequence-controlled copolymerization. L.L. performed DLS and TEM characterization on assembled poly-α/β-peptides. X.X. and S.D. conducted the antimicrobial assays. Y.W., J.X., Z.C. and Z.J. contributed to the synthesis of monomers and MALDI-TOF-MS characterization. All authors proofread the manuscript.

DECLARATION OF INTERESTS

R.L. and M.Z. are co-inventors on a patent application covering reported α/β-peptide polymer synthesis and application. All remaining authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Staphylococcus epidermidis	ATCC	ATCC49134
Pseudomonas aeruginosa (intermediate resistance to carbenicillin and piperacillin)	ATCC	ATCC9027
Escherichia coli (E. coli R01, R07)	Shanghai Ruijin Rehabilitation Hospital	N/A
Acinetobacter baumannii (A. b R02, R03)	Shanghai Ruijin Rehabilitation Hospital	N/A
Pseudomonas aeruginosa (P. a R05, R12)	Shanghai Ruijin Rehabilitation Hospital	N/A
Klebsiella pneumonia (K. p R02, R11)	Shanghai Ruijin Rehabilitation Hospital	N/A
Chemicals, peptides, and recombinant proteins		
Nε-Boc-L-lysine	Adamas	01048879; CAS: 2418-95-3
L-glutamic acid γ-benzyl ester	Adamas	28910C; CAS: 1676-73-9
β-alanine	Adamas	13484A; CAS: 107-95-9
3-amino-3-phenylpropionic acid	Adamas	01096013; CAS: 614-19-7
1-benzyl L-aspartate	Adamas	01110161; CAS: 7362-93-8
1-methyl L-aspartate	Adamas	01035418; CAS: 17812-32-7
cis-2-Amino-1-cyclohexanecarboxylic acid	Bidepharm	BD121558-1g; CAS: 5691-20-3
4-tert-Butylbenzylamine	TCI	B1587-5ml; CAS: 39895-55-1
mPEG-NH2	Ruixibio	Cat#R-1001-2k
Software and algorithms		
ChemDraw Professional 15.1	PerkinElmer	https://www.perkinelmer.com/category/chemdraw
Others		
SepaBean machine equipped with Sepaflash columns	Santai Technologies Inc. in China	http://www.santaitech.com/cn/
Bruker AVANCE III 400	Bruker	https://www.bruker.com/en/products-and-solutions/mr/nmr.html
Bruker Ascend 600	Bruker	https://www.bruker.com/en/products-and-solutions/mr/nmr.html
Waters XEVO G2 TOF mass spectrometer	Waters	https://www.waters.com/nextgen/us/en.html
AB SCIEX TOF/TOFTM 5800	SCIEX	https://sciex.com/
Waters GPC instrument equipped with a Waters 1515 isocratic HPLC pump, a Brookhaven BI-MwA multi-angle light scattering detector and a Waters 2414 refractive index detector	Waters	https://www.waters.com/nextgen/us/en.html
SHIMADZU LC 20AR HPLC System	Shimadzu Scientific Instruments	https://www.ssi.shimadzu.com/
Model ZEN3600	Malvern Instruments Ltd., UK	https://www.malvernpanalytical.com/en
JEOL JEM-1400 TEM	JEOL Ltd.	http://www.jeol.com.cn/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Runhui Liu (rliu@ecust.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyse data sets/code. All data are described in the main text and methods.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacteria used in this study include *Staphylococcus aureus* USA300 (methicillin-resistant), *Staphylococcus epidermidis* ATCC49134, *Bacillus subtilis* BR-151, *Escherichia coli* JM109, *Pseudomonas aeruginosa* ATCC9027 (intermediate resistance to carbenicillin and piperacillin), and 9 clinically isolated pathogens from Shanghai Ruijin Rehabilitation Hospital including *Escherichia coli* (*E. coli* R01, R07), *Acinetobacter baumannii* (*A. b* R02, R03), *Pseudomonas aeruginosa* (*P. a* R05, R12), and *Klebsiella pneumonia* (*K. p* R02, R11). All 14 strains of Gram positive and Gram negative bacteria were cultured individually in Luria–Bertani (LB) medium for 9 h at 37°C under shaking at 200 rpm.

METHOD DETAILS

Synthesis of α-NCAs
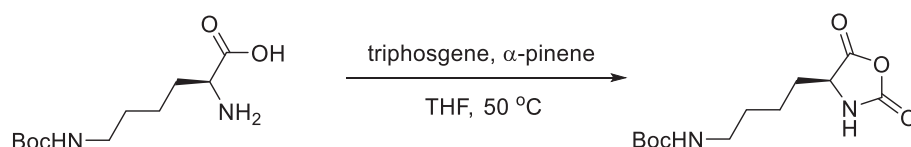
N^{ε}-*tert-butyloxycarbonyl-α-L-lysine N-Carboxy Anhydride (Boc-α-L-Lys NCA).* Boc-α-L-Lys NCA was synthesized by following the previously reported method with slight modification (Zhang et al., 2018) N^{ε}-Boc-α-L-Lys (4.9 g, 20.0 mmol), α-pinene (7.4 g, 54.0 mmol) and triphosgene (2.7 g, 9.0 mmol) were added into 200 mL anhydrous THF. The reaction suspension was stirred and heated to 50°C under a nitrogen environment for 2 h. The reaction mixture was concentrated under vacuum and the obtained residue was dissolved in ethyl acetate (EtOAc, 100 mL) followed by washing with ice water (3 × 50 mL) and cold brine (1 × 50 mL) successively. The collected organic phase was dried over anhydrous MgSO$_4$ and concentrated under reduced pressure to give a crude product, that was purified from recrystallization using anhydrous EtOAc/n-hexane three times to give the pure Boc-α-L-Lys NCA as a needle-like colorless crystal (2.5 g, 45.9% yield). ^{1}H NMR (400 MHz, CDCl$_3$, Figure S1): δ 6.99 (s, 1H), 4.66 (br, 1H), 4.33 (dd, J = 6.4, 4,8 Hz, 1H), 3.13 (s, 2H), 1.96-2.07 (m, 1H), 1.79-1.90 (m, 1H), 1.47-1.61 (m, 4H), 1.45 (s, 9H), ^{13}C NMR (100 MHz, CDCl$_3$, Figure S2): δ 170.15, 156.85, 152.46, 80.09, 57.72, 31.01, 29.47, 28.66, 21.44. ESI-MS: m/z calculated for C$_{12}$H$_{19}$N$_2$O$_5$[M-H]$^-$:271.1; Found 271.1.
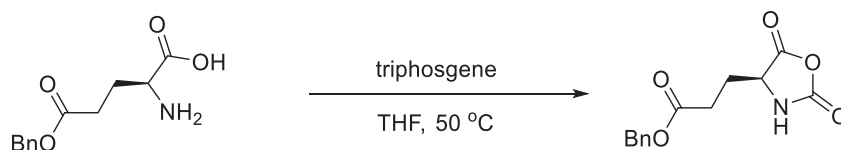

γ-Benzyl-L-glutamate N-carboxyanhydride (α-BLG NCA). α-BLG NCA was synthesized by following the precedent literature with slight modification. (Wu et al., 2018) L-glutamic acid γ-benzyl ester (4.46 g, 20 mmol) and triphosgene (2.7 g, 9.0 mmol) were dissolved in 200 mL anhydrous THF. The reaction was stirred and heated to 50 °C under a nitrogen environment for 2 h. The reaction mixture was concentrated and dissolved in 100 mL EtOAc following by washing with ice water (1 × 100 mL) and cold brine (1 × 100 mL)

successively. The obtained organic layer was then dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give a crude product that was purified from recrystallization in anhydrous EtOAc/ n-hexane three times to give the pure α-BLG-NCA as a needle-like colorless crystal (4.0 g, 76.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$, Figure S3): δ 7.41–7.30 (m, 5H), 6.67 (s, 1H), 5.14 (s, 2H), 4.38 (t, *J* = 6.0 Hz, 1H), 2.59 (t, *J* = 6.8 Hz, 2H), 2.32–2.23 (m, 1H), 2.16-2.04 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, Figure S4): δ 172.62, 169.60, 152.13, 135.43, 128.94, 128.82, 128.59, 67.34, 57.15, 30.03, 27.12. HRESI-MS: m/z calculated for $C_{13}H_{13}NNaO_5$ [M+Na]$^+$: 286.0691; Found 286.0692.

Synthesis of β-NTAs
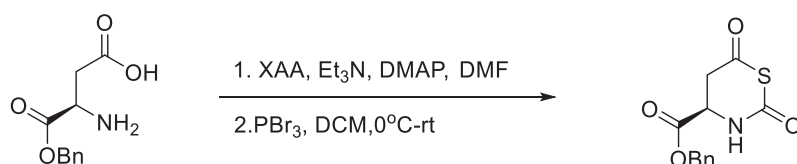
### *β[3]-Benzyl-L-carboxyhomoglycine N*-thiocarboxyanhydride (Bn-β[3]-LCHG NTA).

Bn-β[3]-LCHG NTA was synthesized by following a precedent procedure with modifications (Zhou et al., 2020). To a reaction flask containing 100 mL anhydrous DMF was added 1-benzyl L-aspartate (4.47 g, 20.0 mmol), S-ethoxythio-carbonyl mercapto-acetic acid (XAA, 3.6 g, 20.0 mmol), Et_3N (10.1 g, 100.0 mmol) and DMAP (1.8 g, 2.0 mmol). The reaction mixture was stirred vigorously at room temperature under a nitrogen environment for 48 h. The reaction mixture was concentrated and dissolved in 100 mL EtOAc followed by washing with deionized water (3 × 50 mL) and saturated brine (1 × 50 mL) successively. The obtained organic layer was then dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give a crude intermediate that was purified through a silica gel column chromatography to give the pure intermediate (5.4 g, 17.3 mmol). The intermediate was then dissolved in anhydrous DCM (200 mL) and treated with PBr_3 (4.7 g, 17.3 mmol) at 0°C under nitrogen. The reaction mixture was allowed to stir at rt for 6 h, and then was washed sequentially with ice water (3 × 100 mL) and saturated brine (1 × 100 mL). The obtained organic phase was dried over $MgSO_4$, filtered and concentrated to afford the crude product that was purified by recrystallization from EtOAc/n-hexane to give the pure Bn-β[3]-LCHG NTA as a white fluffy solid (3.8 g, 71.6% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$, Figure S5): δ 7.30–7.45 (m, 5H), 6.83 (s, 1H, NH), 5.27 (s, 2H), 4.40–4.47 (dt, *J* = 2.8, 10.4 Hz, 1H), 3.23 (dd, *J* = 2.8, 16.4 Hz, 1H), 2.96 (dd, *J* = 10.4, 16.4 Hz, 1H). EI-MS: m/z calculated for $C_{12}H_{11}NO_4S$ [M]$^+$: 265.0; Found 265.0.
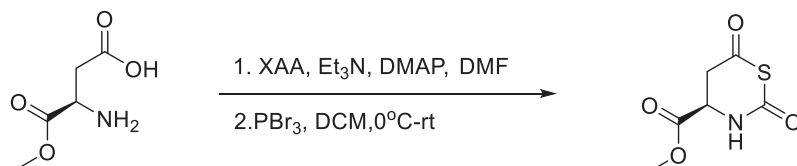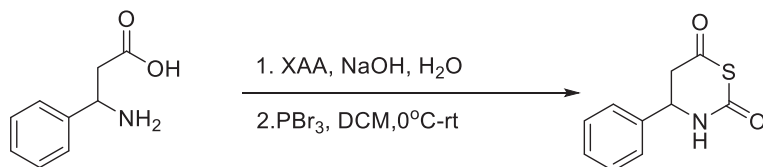

β[3]-Methyl-L-carboxyhomoglycine N-thiocarboxyanhydride (Me-β[3]-LCHG NTA).

Me-β[3]-LCHG NTA was prepared as a white crystal in 67.5% yield over two steps from 1-methyl L-aspartate, using a similar method described above for Bn-β[3]-LCHG NTA synthesis. ^{1}H NMR (400 MHz, $CDCl_3$, Figure S6): δ 7.20 (s, 1H), 4.40–4.47 (m, 1H), 3.86 (s, 3H), 3.20 (dd, *J* = 3.2, 16.4 Hz, 1H), 3.01 (dd, *J* = 9.6, 16.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, Figure S7): δ 193.84, 168.84, 164.13, 54.01, 51.23, 42.78. HREI-MS (Figure S8): m/z calculated for $C_6H_7NO_4S$ [M]$^+$: 189.0096; Found 189.0094.
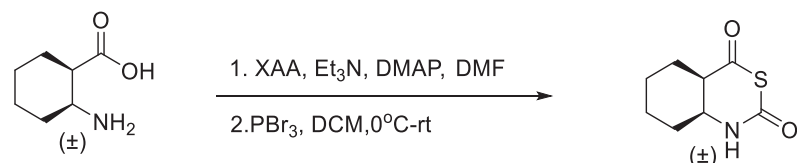
Cis-2-aminocyclohexanecarboxylic acid N-thiocarboxyanhydride ($\beta^{2,3}$-CH($\pm$) NTA). $\beta^{2,3}$-CH($\pm$) NTA was prepared as a white crystal in 53.8% yield over two steps from cis-2-aminocyclohexanecarboxylic acid using the method described above for Bn-β^3-LCHG NTA synthesis. ^{1}H NMR (400 MHz, CDCl$_3$, Figure S9): δ 7.39 (s, 1H), 3.72–3.92 (m, 1H), 2.7–2.9 (m, 1H), 1.98–2.12 (m, 1H), 1.84–1.94 (m, 1H), 1.60–1.79 (m, 4H), 1.38–1.56 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$, Figure S10): δ 199.13, 166.25, 50.54, 49.68, 29.60, 24.43, 23.38, 21.20. HRESI-MS (Figure S11): m/z calculated for C$_8$H$_{10}$NO$_2$S [M-H]$^-$: 184.0432; Found 184.0433.
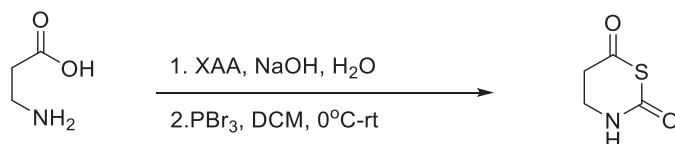
β-Alanine N-thiocarboxyanhydride (β-HG NTA). β-HG NTA was synthesized by following a precedent procedure described above for Bn-β^3-LCHG NTA synthesis with modifications. The mixture of β-alanine (1.8 g, 20.0 mmol) and S-ethoxythiocarbonyl mercapto-acetic acid (XAA, 3.6 g, 20.0 mmol), NaOH (4.0 g, 100.0 mmol) were dissolved in 100 mL deionized water. The reaction mixture was stirred vigorously at rt for 72h followed by acidification with 2 N HCl to pH = 3 and then extraction with ethyl acetate (3 × 100 mL). The combined organic phase was washed with saturated brine (1 × 100 mL) then dried over anhydrous MgSO$_4$ and concentrated under reduced pressure to give the crude intermediate, which was purified through a silica gel column chromatography to give the pure intermediate (2.7 g, 15.2 mmol). The pure intermediate was dissolved in anhydrous DCM (200 mL) and then was treated with PBr$_3$ (4.1 g, 15.2 mmol) at 0°C under nitrogen. The reaction mixture was allowed to stir at rt for 6 h and then washed sequentially with ice water (3 × 100 mL) and saturated brine (1 × 100 mL). The organic phase was dried over MgSO$_4$, filtered and concentrated to afford the crude product that was further purified by recrystallization from EtOAc/n-hexane to give pure β-HG NTA as a white fluffy solid (1.2 g, 45.7% yield over two steps). ^{1}H NMR (400 MHz, CDCl$_3$, Figure S12): δ 7.49 (brs, 1H), 3.56–3.61 (m, 2H), 2.80–2.86 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$, Figure S13): δ 196.05, 166.47, 40.22, 37.77. HREI-MS (Figure S14): m/z calculated for C$_4$H$_5$NO$_2$S [M]$^+$: 131.0041; Found 131.0027.


β^3-D/L-homophenylglycine N-thiocarboxyanhydride (β^3-HPhG NTA). β^3-HPhG NTA was prepared as a white crystal in 82.8% yield over two steps from 3-amino-3-phenylpropionic acid using the method described above for β-HG NTA synthesis. ^{1}H NMR (400 MHz, CDCl$_3$, Figure S15): δ 7.28–7.40 (m, 3H), 7.18–7.24 (m, 2H), 5.97 (brs, 1H), 4.46–4.54 (m, 1H, NHC<u>H</u>), 3.32 (dd, J = 4.0, 14.0 Hz, 1H), 2.91 (dd, J = 9.6, 14.0 Hz, 1H). EI-MS: m/z calculated for C$_{10}$H$_9$NO$_2$S [M]$^+$: 207.0; Found 207.0.

Copolymerization on α-NCA and β-NTAs in THF

To a reaction vial equipped with a magnetic stir bar was added the α-NCA and β-NTA at a predefined molar ratio in an appropriate solvent, followed by addition of a primary amine initiator. The polymerization reaction was stirred at room temperature for 2–4 days until completion as monitored by TLC and HPLC (>99% conversion). The reaction mixture was poured into 45 mL cold n-hexane to precipitate out the crude polypeptide as a light yellow solid, which was collected after centrifugation and dried under air flow. The crude product was purified via the dissolution-precipitation process thrice (2.0 mL THF/45 mL n-hexane) and then drying under vacuum. Copolymerization on α-NCA and β-NTA in THF was conducted by following the

general procedure described above. To prepare poly-α/β-peptides with variable structures, α-NCA, β-NTA and the primary amine initiator were individually prepared as stock solutions at a concentration of 0.2 M in anhydrous THF under nitrogen and then mixed as needed for different copolymerization. To prepare poly-α/β-peptides with variable chain length (Table 1, entry 8–11), α-NCA, β-NTA and the primary amine initiator were individually prepared as stock solutions at a concentration of 0.78 M in anhydrous THF under nitrogen and then mixed as needed for different copolymerization. The variable ratio of α:β amino acid subunits within the polypeptide chains was controlled by the initial ratio of Boc-α-L-Lys NCA and Bn-β^3-LCHG NTA in the reaction mixture.
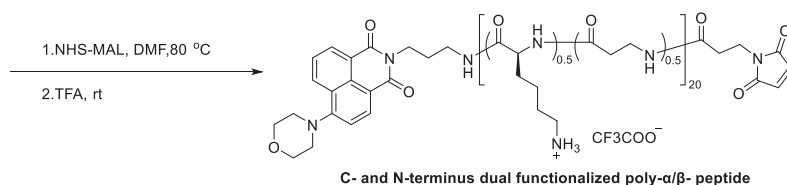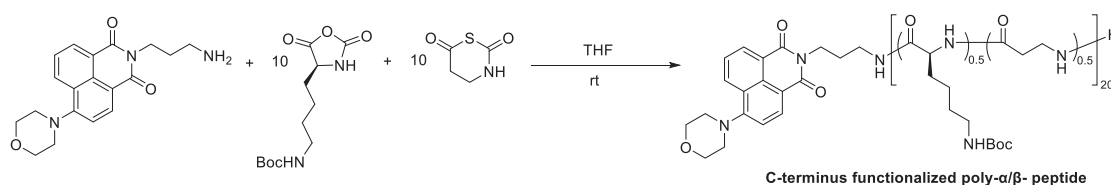
Synthesis of α/β-peptide triblock copolymer in THF

1st block of α/β peptide copolymer

α/β peptide diblock copolymer

α/β peptide triblock copolymer
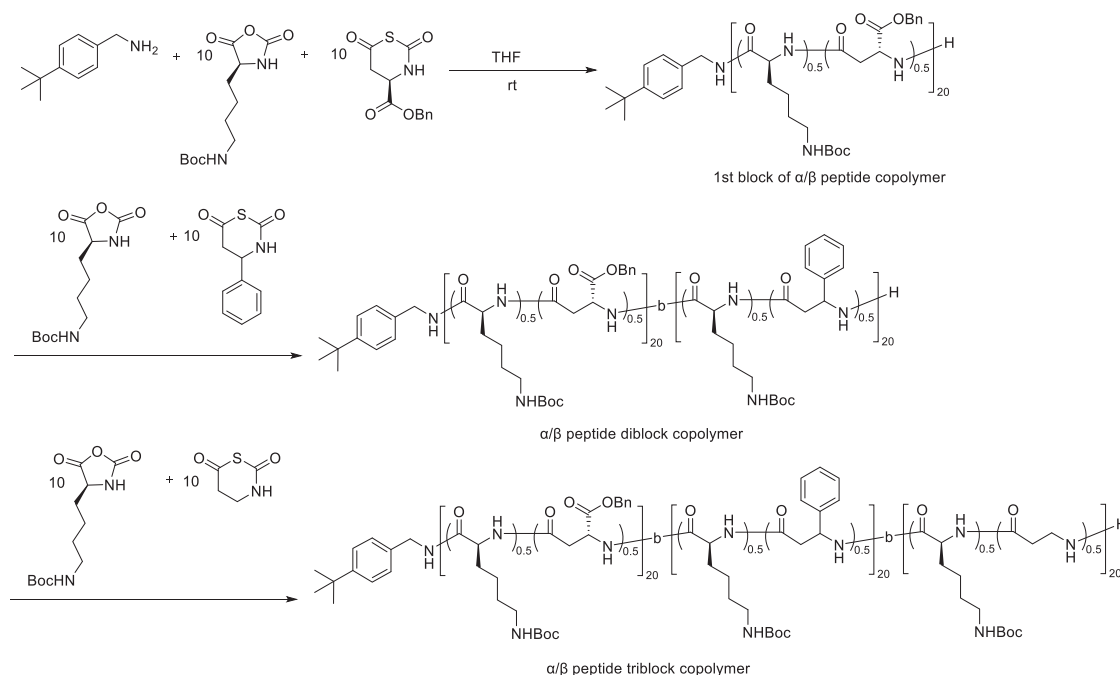
For the synthesis of α/β-peptide triblock copolymer, the α-NCAs, β-NTAs and tBuBnNH₂ were respectively dissolved in anhydrous THF to a final concentration of 0.2 M. The first block of poly-α/β-peptide was prepared from the mixture of 2 mL Boc-α-Lys NCA, 2 mL Bn-β^3-LCHG NTA and 200 μL tBuBnNH₂ to give the poly(Boc-α-L-Lys$_{0.5}$/Bn-β^3-LCHG$_{0.5}$)$_{20}$ (M_n = 4.7 kg/mol, Đ = 1.13, characterized by GPC) in 87.5% yield after three cycles of dissolution-precipitation process (2.0 mL THF/45.0 mL n-hexane). The purified poly(Boc-α-L-Lys$_{0.5}$/Bn-β^3-LCHG$_{0.5}$)$_{20}$ was dissolved in 100 μL anhydrous THF to a final concentration of 0.2 M as the initiator, followed by adding 1 mL of Boc-α-Lys NCA (54.4 mg, 0.2 mmol) and 1 mL of β^3-HPhG NTA (41.4 mg, 0.2 mmol) into the reaction flask. The polymerization reaction was continued for 72h to give the α/β-peptide diblock copolymer poly(Boc-α-L-Lys$_{0.5}$/Bn-β^3-LCHG$_{0.5}$)$_{20}$-b-poly(Boc-α-L-Lys$_{0.5}$/β^3-HPhG$_{0.5}$)$_{20}$ (M_n = 8.1 kg/mol, Đ = 1.22, characterized by GPC) in 88.7% yield after three cycles of dissolution-precipitation process (2.0 mL THF/45.0 mL n-hexane). The purified diblock copolymer was dissolved in 100 μL anhydrous THF to a final concentration of 0.2 M as the initiator, followed by adding 1 mL of Boc-α-Lys NCA (54.4 mg, 0.2 mmol) and 1 mL of β-HG NTA (26.2 mg, 0.2 mmol) into the reaction flask. The polymerization reaction was continued for 72h to give the α/β-peptide triblock copolymer poly(Boc-α-L-Lys$_{0.5}$/Bn-β^3-LCHG$_{0.5}$)$_{20}$-b-poly(Boc-α-L-Lys$_{0.5}$/β^3-HPhG$_{0.5}$)$_{20}$-b-poly(Boc-α-L-Lys$_{0.5}$/β-HG$_{0.5}$)$_{20}$ (M_n = 11.1 kg/mol, Đ = 1.14, characterized by GPC) in 82.3% yield after three cycles of dissolution-precipitation process (2.0 mL THF/45.0 mL n-hexane).

The synthesis of C- and N-terminal dual functionalization of poly-α/β-peptide in THF
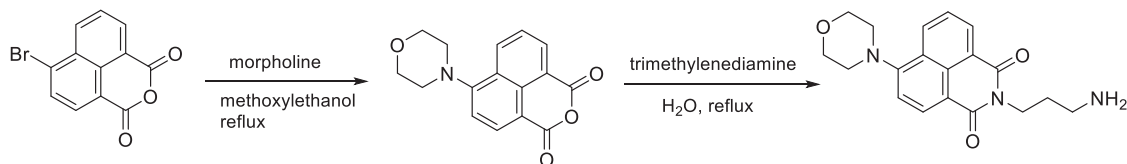
2-(3-aminopropyl)-6-morpholino-1H-benzo[de]isoquinoline-1,3(2H)-dione (Dye-NH₂). Dye-NH$_2$ was used as the initiator for the copolymerization of α-NCAs and β-NTAs. Dye-NH$_2$ was synthesized by following previously reported procedure to give the pure product as a yellow solid in 25.3% yield over two steps (Zhou et al., 2020). Structure characterization of Dye-NH$_2$ was consistent to the report in literature. ^{1}H NMR (400 MHz, CDCl$_3$, Figure S16): δ 8.58 (dd, J = 0.8, 7.2 Hz, 1H), 8.51 (d, J = 8.0 Hz, 1H), 8.42 (dd, J = 0.8, 8.4Hz, 1H), 7.70 (dd, J = 8.4, 7.2 Hz, 1H), 7.23 (d, J = 8.0 Hz, 1H,), 4.27 (t, J = 6.8 Hz, 2H), 4.02 (t, J = 4.4 Hz, 4H), 3.27 (t, J = 4.4 Hz, 4H), 2.76 (t, J = 6.6 Hz, 2H), 1.90 (p, J = 6.8 Hz, 2H), 1.79 (s, 2H).


C-terminus functionalized poly-α/β- peptide

C- and N-terminus dual functionalized poly-α/β- peptide

The C-terminal functionalization of poly-α/β-peptide was demonstrated by the copolymerization of Boc-α-L-Lys NCA and β-HG NTA. First, Dye-NH$_2$, Boc-α-L-Lys NCA and β-HG NTA was dissolved in anhydrous THF individually to a final concentration of 0.2 M. Then 200μL Dye-NH$_2$ was added into the mixture of 2 mL Boc-α-L-Lys NCA and 2 mL β-HG NTA, and the reaction mixture was stirred at rt for 72 h in a dark environment. After the reaction was complete, the reaction solution was poured into cold n-hexane (45.0 mL) to precipitate out the crude product that was collected by centrifugation and dried under air flow. The purified C-terminal functionalized poly-α/β-peptide Dye-poly(Boc-α-L-Lys$_{0.5}$/β-HG$_{0.5}$)$_{20}$ was obtained in 92.7% yield after three cycle of dissolution-precipitation in a mixed solvent (2.0 mL THF/45.0 mL n-hexane) and then was characterized by GPC (Figure S17).

The purified Dye-poly(Boc-α-L-Lys$_{0.5}$/β-HG$_{0.5}$)$_{20}$ (31.0 mg, 10.0 mmol) and 3-Maleimidopropionic acid N-hydroxysuccinimide ester (NHS-MAL, 13mg, 50.0 mmol) were dissolved in 500 μL anhydrous DMF and the reaction mixture was stirred for 18 h at 80°C under nitrogen. When reaction was complete, the reaction mixture was cooled to rt and concentrated under reduced pressure to give the crude product. The C-and N-terminal dual functionalized poly-α/β-peptide Dye-poly(Boc-α-L-Lys$_{0.5}$/β-HG$_{0.5}$)$_2$-MAL was purified vis three cycles of the dissolution-precipitation process using a mixed solvent (2.0 mL THF/45.0 mL n-hexane). The collected solid was washed with 2 mL MeCN once then was dried under vacuum to give a yellow solid (20.5mg, 62.5% yield).

Dye-poly(Boc-α-L-Lys$_{0.5}$/β-HG$_{0.5}$)$_2$-MAL (20.5 mg, 6.3 mmol) was continuously treated with 2 mL of trifluoro-acetic acid (TFA) at rt for 2 h under gentle shaking to remove the side-chain *N*-Boc protecting group. After TFA was removed under air flow, the crude product was purified via three cycles of the dissolution-precipitation process in a mixed solvent (0.5 mL MeOH/45.0 mL Et$_2$O) to give the deprotected poly-α/β-peptide Dye-poly(α-L-Lys$_{0.5}$/β-HG$_{0.5}$)$_2$-MAL as yellow solid in the form of TFA salt (20.0 mg, 88.3% yield). ^{1}H NMR (600 MHz, D$_2$O, Figure S18) confirmed that the morpholino-naphthalimide fluorophore group and the maleimide group were successfully conjugated to the C-terminal and N-terminal of poly-α/β-peptide, respectively.

Kinetics study on copolymerization of α-NCA and β-NTA in THF
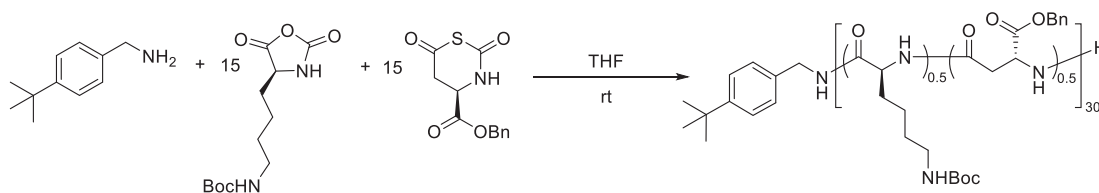

The kinetics study on copolymerization of α-NCAs and β-NTAs in THF was performed as described here. Boc-α-Lys NCA, Bn-β^3-LCHG NTA and *t*BuBnNH$_2$ were dissolved in anhydrous THF individually to a final concentration of 0.2 M. To the mixture of 1.0 mL Boc-α-Lys NCA, 1.0 mL Bn-β^3-LCHG NTA and 67 μL *t*BuBnNH$_2$ was added triphenylmethane (TPM, 4.0 mg) as the internal standard. The reaction mixture was stirred at rt in a nitrogen purged glovebox. Real-time monitoring of remaining Boc-α-Lys NCA and Bn-β^3-LCHG NTA was performed by measuring the relative ratio of the absorbance peak area at 200 nm between the Boc-α-Lys NCA/TPM and Bn-β^3-LCHG NTA/TPM using HPLC to calculate the remaining percentage. A mixed solvent of MeCN and H$_2$O was used as the mobile phase, with MeCN increasing incrementally from 50% to 90% within 9 min, holding at 90% for 8 min, and decreasing from 90% to 50% within 3 min at a flow rate of 1.0 mL/min using a Luna Omega polar C18 column. The remaining percentage of NCA and NTA versus time was calculated from below equation:

$$\text{Remaining percentage (\%)} = \frac{[M]_t}{[M]_0} \times 100\%$$

whereas $[M]_t$ is the concentration of the monomer at a given time, $[M]_0$ is the initial concentration of the monomer.

The synthesis of HDP-mimicking amphiphilic poly-α/β-peptide libraries in THF
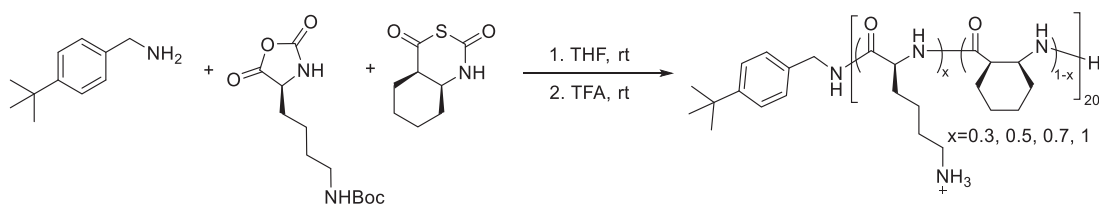
The synthesis and characterization of HDP-mimicking amphiphilic poly(α-L-Lys$_x$/β^{2,3}-CH$_{1-x}$)$_{20}$ libraries were performed as described here. In a nitrogen purged glovebox, *t*BuBnNH$_2$, Boc-α-L-Lys NCA and β^{2,3}-CH NTA were individually dissolved in anhydrous THF to a final concentration of 0.2 M as the stock solution. Then 100μL *t*BuBnNH$_2$ was added into 2.0 mL mixture of Boc-α-L-Lys NCA stock solution and β^{2,3}-CH NTA stock solution in a volume ratio of x/1-x, and the reaction mixture was stirred at rt for 72 h under nitrogen. When the reaction was complete, the obtained polymers were purified by repeated dissolution-precipitation process (2.0 mL THF/45.0 mL n-hexane) three times to give poly(Boc-α-L-Lys$_x$/β^{2,3}-CH$_{1-x}$)$_{20}$ in 85.7–93.5% yield. The purified poly(Boc-α-L-Lys$_x$/β^{2,3}-CH$_{1-x}$)$_{20}$ was treated with 2 mL TFA at rt for 2 h under gentle shaking to remove the side-chain *N*-Boc protecting group, followed by removing

TFA under air flow to give the crude product. The crude product was purified by the dissolution-precipitation process (0.5 mL MeOH/45.0 mL Et$_2$O) three times. The purified poly(α-L-Lys$_x$/$\beta^{2,3}$-CH$_{1-x}$)$_{20}$ was dissolved in milli-Q and lyophilized to give the final product in the form of TFA salts. The polymers were and characterized by ^{1}H NMR (Figures S22–S25) and used for further antibacterial activity test.

### Copolymerization on α-NCA and β-NTA in CHCl$_3$/H$_2$O (95/5, v/v)

Copolymerization on α-NCA and β-NTA in CHCl$_3$/H$_2$O (95/5, v/v) was operated at ambient condition (Song et al., 2019). To the reaction mixture of α-NCA and β-NTA (with variable α:β ratio) at total final concentration of 0.1 M in CHCl$_3$/H$_2$O (95/5, v/v) was quickly added a solution of the initiator in CHCl$_3$/H$_2$O (95/5, v/v) under vigorous stir. The reaction was stirred at room temperature in an open flask until the polymerization reaction was completed as monitored by TLC and HPLC (>99% conversation). The reaction solution was concentrated under vacuum and the obtained residue was purified by the dissolution-precipitation process (2.0 mL THF/45 mL n-hexane) thrice to give the product as a white solid.

The controlled copolymerization on Boc-α-L-lys NCA and Bn-β^3-LCHG NTA in CHCl$_3$/H$_2$O (95/5, v/v)
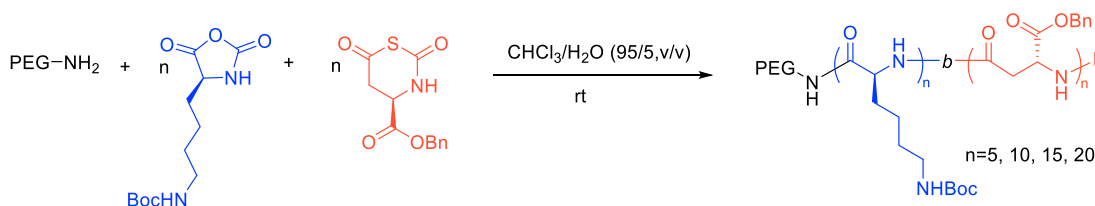
The copolymerization of Boc-α-L-lys NCA and Bn-β^3-LCHG NTA in CHCl$_3$/H$_2$O (95/5, v/v) was operated as follows: At ambient condition, Boc-α-L-Lys NCA, Bn-β^3-LCHG NTA and PEG-NH$_2$ were respectively dispersed in a mixture of 0.2 M CHCl$_3$/H$_2$O (95/5, v/v). 150 μL, 75 μL, 50 μL and 37.5 μL of PEG-NH$_2$ in CHCl$_3$/H$_2$O (95/5, v/v) were respectively added into the reaction mixture of 750 μL of Boc-α-L-lys NCA and 750 μL of Bn-β^3-LCHG NTA in CHCl$_3$/H$_2$O (95/5, v/v) under vigorous stir. When the polymerization reaction was complete monitored by TLC and HPLC (conversation>99%), the reaction solution was poured into cold n-hexane (45.0 mL) to precipitate out the crude product. The crude product was purified via the dissolution-precipitation process thrice (2.0 mL THF/45 mL n-hexane) and then characterized by GPC using DMF as the mobile phase at flow rate of 1 mL/min (Figures S30 and S31).

Kinetics study on copolymerization of α-NCA and β-NTA in CHCl$_3$/H$_2$O (95/5, v/v)
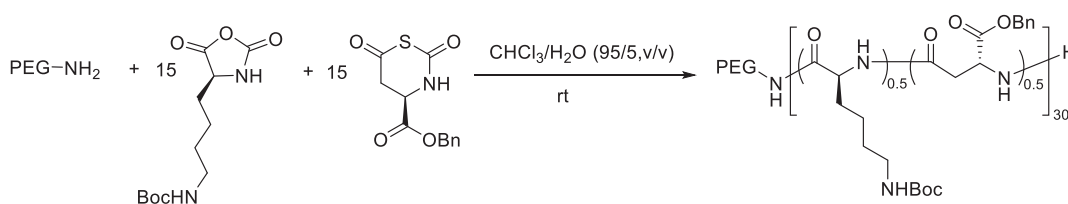

The kinetics study on the copolymerization of α-NCAs and β-NTAs in CHCl$_3$/H$_2$O (95/5, v/v) was described here. At ambient condition, Boc-α-L-lys NCA (54.4 mg, 0.2 mmol) and Bn-β^3-LCHG NTA (53.0 mg, 0.2 mmol) were weighed out and dispersed in a mixed solvent CHCl$_3$/H$_2$O (2000μL/106μL, v/v) under vigorous stir. Then 67μL of 0.2M PEG-NH$_2$ (M_n = 2000) solution in CHCl$_3$/H$_2$O (95/5, v/v) and 4 mg TPM was quickly added into the reaction mixture, and the reaction was stirred at rt in an open flask at an environment of 60% relative humidity. The real-time monitoring of remaining Boc-α-Lys NCA and Bn-β^3-LCHG NTA was performed by measuring the relative ratio of the absorbance peak area at 200 nm between the Boc-α-Lys NCA/TPM and Bn-β^3-LCHG NTA/TPM using HPLC to detect the remaining monomers percentage in copolymerization using CHCl$_3$/H$_2$O (95/5, v/v) as the solvent. A mixed solvent of MeCN and H$_2$O was used as the mobile phase,

with MeCN increasing incrementally from 50% to 90% within 9 min, holding at 90% for 8 min, and decreasing from 90% to 50% within 3 min at a flow rate of 1.0 mL/min using a Luna Omega polar C18 column. The reactivity ratio of Boc-α-Lys NCA and Bn-β^3-LCHG NTA within the copolymerization reaction, using a mixed solvent of CHCl$_3$/H$_2$O (95/5, v/v), was shown in Figure S20.

*The synthesis of sequenced-controlled decablock poly-α/β-peptide via α-NCAs and β-NTAs copolymerization in CHCl$_3$/H$_2$O (95/5, v/v).* At ambient condition with 60% relative humidity, Boc-α-L-Lys NCA, Bn-β^3-LCHG NTA, and Me-β^3-LCHG NTA as monomers and PEG-NH$_2$ as initiator were respectively dispersed in a mixture of 0.2 M CHCl$_3$/H$_2$O (95/5, v/v). The sequence of poly-α/β-peptide was controlled by copolymerization with step-wise addition of 15:5 α-NCA:β-NTA in the form of each diblock poly-α-peptide-b-poly-β-peptide. First, 750 μL Boc-α-L-Lys NCA, 250 μL Bn-β^3-LCHG NTA and 50 μL PEG-NH$_2$ were mixtured and vigorously stirred in CHCl$_3$/H$_2$O (95/5, v/v) at rt in an open flask, when the polymerization reaction was complete monitored by TLC and HPLC (conversation>99%), the reaction solution was directly characterized by GPC without purification, then the mixture of 750 μL Boc-α-L-Lys NCA and 250 μL Me-β^3-LCHG NTA was added into the polymer reaction to prepare the second diblock. The polymerization would not stop until to give the decablock poly-α/β-peptide and the resulting sequenced-controlled decablock poly-α/β-peptide was obtained 248.6 mg in 90.2% yield.

Synthesis of sequence-controlled poly-α/β-peptide in CHCl$_3$/H$_2$O (95/5, v/v) by incorporating α-peptide sequence into β-peptide backbone. In an open flask at ambient condition, 4.0 mL of 0.2 M Bn-β^3-LCHG NTA in CHCl$_3$/H$_2$O (95/5, v/v) and 40 μL of 0.2 M PEG-NH$_2$ in CHCl$_3$/H$_2$O (95/5, v/v) were mixed and vigorously stirred at room temperature. A solution of 200 μL 0.2 M Boc-α-L-Lys NCA in CHCl$_3$/H$_2$O (95/5, v/v) and 200 μL 0.2 M α-BLG NCA in CHCl$_3$/H$_2$O (95/5, v/v) was alternatively added into above reaction every 4 hours. After adding α-NCA into the reaction each time, the reaction was monitored by HPLC until the consumption of α-NCA to have a new block of α-peptide sequence. The reaction continued with the presence of Bn-β^3-LCHG NTA in the reaction flask for another two hours to have a new block of the β-peptide sequence along the polypeptide chain. Then α-NCA was added to the reaction flask again as described above to obtain another new block of α-peptide sequence. Repeated addition of α-NCA into the polymerization reaction flask was conducted until total 10 blocks of α-peptide sequence were inserted into the backbone of the β-peptide to obtain the sequence-controlled poly-α/β-peptide. The obtained polymer was purified by the dissolution-precipitation process (2.0 mL THF/45 mL n-hexane) thrice to give the final product of 238.6 mg (88.5% yield) as white solid. The poly-α/β-peptide was characterized by ^{1}H NMR using poly-(Boc-α-L-Lys)$_5$ and poly-(α-BLG)$_5$ for comparison.

The synthesis of self-assembling poly-α/β-peptides in CHCl$_3$/H$_2$O (95/5,v/v)
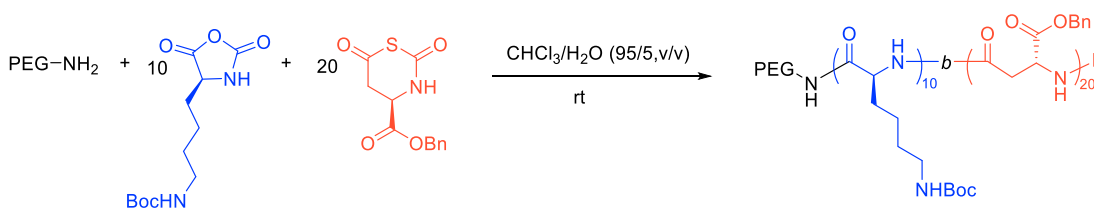
At ambient condition, Boc-α-L-lys NCA (27.2 mg, 0.1 mmol) and Bn-β^3-LCHG NTA (53.0 mg, 0.2 mmol) was respectively weighed out and dispersed in a mixture of CHCl$_3$/H$_2$O (1500μL/81μL, v/v,) with vigorous stir, followed by addition of 50 μL 0.2 M PEG-NH$_2$ (M_n = 2000) solution in CHCl$_3$/H$_2$O (95/5, v/v) quickly into the reaction. The reaction mixture was stirred at rt in an open flask at an environment of 60% relative humidity. When the polymerization reaction was complete, the reaction mixture was poured into 45.0 mL cold n-hexane to precipitate the crude product that was purified from the dissolution-precipitation process (2.0 mL THF/45.0 mL n-hexane) three times to give the final product PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ in 75.2–95.5% yield within our repeats of this reaction in six batches.
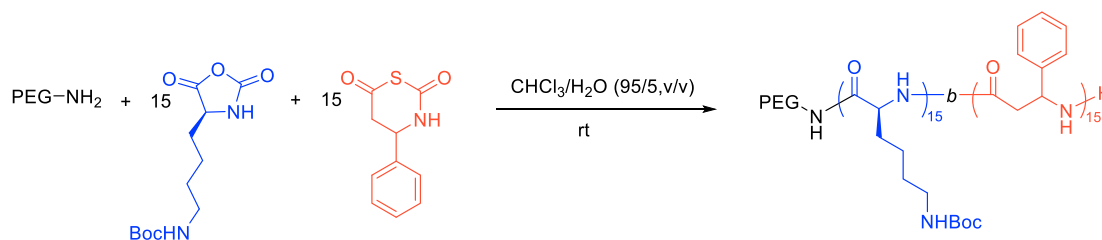
PEG-poly(Boc-α-L-Lys)$_{15}$-b-poly(β^3-HPhG)$_{15}$ was synthesized by following above procedure in preparing PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$

Minimum inhibitory concentration (MIC) assay

14 strains of Gram positive and Gram negative bacteria were cultured individually in Luria–Bertani (LB) medium for 9 h at 37°C under shaking at 200 rpm. After centrifugation at 4000 rpm for 5 min, the bacteria were collected and re-suspended in Mueller–Hinton (MH) medium to 2×10^5 CFU/mL as the working suspension. Poly(α-L-Lys)$_{20}$, poly(α-L-Lys$_{0.7}$/β^{2,3}-CH$_{0.3}$)$_{20}$, poly(α-L-Lys$_{0.5}$/β^{2,3}-CH$_{0.5}$)$_{20}$ and poly(α-L-Lys$_{0.3}$/β^{2,3}-CH$_{0.7}$)$_{20}$ were diluted to concentrations ranging from 1.56 μg/mL to 200 μg/mL by a two-fold gradient dilution in a 96-well plate. After mixing equal volumes of bacterial cell suspension (50 μL) and polypeptide solution (50 μL) in each well, the 96-well plates were incubated at 37°C for 9 h. Then the OD value in each well on the 96-well plate was collected on a SpectraMax® M2 plate reader. MH medium only and bacteria in MH medium was used as the blank and the positive control, respectively. The percentage of bacterial cell survival was calculated from the equation (% *cell growth* $= A_{600}^{polymer} - A_{600}^{blank} / A_{600}^{control} - A_{600}^{blank} \times$ 100), and plotted against polymer concentration to give the dose-response curves of antibacterial activities for polypeptides. Measurements were performed in duplicates, and the experiments were repeated at least twice.

Proteolytic resistance test on HDP-mimicking amphiphilic poly-α/β-peptides

Proteolytic resistance of poly(α-L-Lys)$_{20}$ and poly(α-L-Lys$_{0.5}$/β^{2,3}-CH$_{0.5}$)$_{20}$ was evaluated with hydrolysis kinetics of polypeptide using ^{1}H NMR analysis, by following previously reported literature with modifications (Chin et al., 2018). Specifically, 5 mg of poly(α-L-Lys)$_{20}$ and 5 mg of poly(α-L-Lys$_{0.5}$/β^{2,3}-CH$_{0.5}$)$_{20}$ were individually dissolved in 0.5 mL PBS buffer (pH 7.4) using D$_2$O as solvent. Trypsin was dissolved in 0.5 mL PBS buffer (pH 7.4) using D$_2$O as solvent to a final concentration of 10 mg/mL. 0.5 mL of above polypeptide solution and 12.5 μL of above trypsin solution were mixed and the mixture solution was detected by ^{1}H NMR at various time intervals. The integration of the CH peak on lysine backbone in proton NMR for each data collecting time is plotted against the time for data collecting after the polypeptide is incubated with the enzyme. The value of half-life in the presence of trypsin (Figures S27–S28) showed that the incorporation of β-amino acid residue, β^{2,3}-CH, increased half-life of poly(L-Lys)$_{20}$ against trypsin from 34 min to 60 min. The value of half-life of poly(α-L-Lys)$_{20}$ and poly(α-L-Lys$_{0.5}$/β^{2,3}-CH$_{0.5}$)$_{20}$ against proteinase K23 was detected from the mixture of 5 mg of polypeptide and 0.5 mg proteinase K23 using a similar method described above for trypsin treatment. The result (Figures S29 and S30) showed that the incorporation of β-amino acid residue, β^{2,3}-CH, increased the half-life of poly(L-Lys)$_{20}$ against proteinase K23 from 340 min to 1080 min.

Characterization of self-assembled poly-α/β-peptides

PEG-poly(Boc-α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ and PEG-poly(Boc-α-L-Lys)$_{15}$-b-poly(β^3-HPhG)$_{15}$ were respectively treated with 2 mL of TFA at rt for 2 h under gentle shaking to remove the side-chain N-Boc protecting group. The deprotected polypeptides were purified from the dissolution-precipitation process (2.0 mL THF/45.0 mL n-hexane) for three times. The obtained PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ was dissolved in deionized water (1.0 mg/mL) and then was diluted with 15% (v/v) THF to a final concentration of 0.87 mg/mL. The mixture was stirred for 1h and then was allowed to stand still for 18 hours at room temperature to allow the assembly of polypeptides. The stock solution of self-assembled PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$ was filtered through a 2 μm polytetrafluoroethylene (PTFE) filter before DLS and TEM characterization. The obtained PEG-poly(α-L-Lys)$_{15}$-b-poly(β^3-HPhG)$_{15}$ was dissolved in

deionized water to the final concentration of 1.0 mg/mL, and was subjected to self-assembly by the same procedure described above for PEG-poly(α-L-Lys)$_{10}$-b-poly(Bn-β^3-LCHG)$_{20}$.

QUANTIFICATION AND STATISTICAL ANALYSIS

The reactivity ratio of Boc-α-Lys NCA and Bn-β^3-LCHG NTA in THF and CHCl$_3$/H$_2$O (95/5, v/v) was calculated respectively using the Lynd method by following a precedent procedure (Yasir et al., 2020) (Figures S19 and S20). Lynd equation for the evaluation of reactivity ratio is given below:

$$X = 1 - f_{1,o}\left[1 - \left(1 - \frac{[M]_1}{[M]_{1,0}}\right)\right] - (1 - f_{1,o})\left[1 - \left(1 - \frac{[M]_1}{[M]_{1,0}}\right)\right]^{r_2}$$

$$X = 1 - f_{1,o}\left[1 - \left(1 - \frac{[M]_x}{[M]_{x,0}}\right)\right]^{r_1} - (1 - f_{1,o})\left[1 - \left(1 - \frac{[M]_x}{[M]_{x,0}}\right)\right]$$

whereas, r_1 and r_2 means the reactivity ratio of Boc-α-Lys NCA and Bn-β^3-LCHG NTA respectively.

$[M]_1$ and $[M]_x$ are the concentrations of NCA and NTA at a given time, respectively.

$[M]_{1,0}$ and $[M]_{x,0}$ is the initial concentration of NCA and NTA, respectively.

$f_{1,0}$ is the initial mole fraction of NCA.

X is the total conversion.