Supporting Information

Self-assembled polypeptide micelles for fungal keratitis treatment

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1. Materials

L-methionine, L-leucine, N_{ε}-carbobenzyloxy-L-lysine, n-butylamine, dimethylacetamide (DMAc), 33% HBr in acetic acid solution, and trifluoroacetic acid were obtained from Aladdin (Shanghai, China). Phenyl chloroformate was purchased from J&K Scientific (Shanghai, China). Ethyl acetate and petroleum ether obtained from Tianjin ZhiYuan Reagent Co., Ltd (Tianjin, Chian). Diethyl ether was purchased from Luoyang Chemical Reagent Factory (Luoyang, China). Cell counting Kit-8 (CCK-8) was purchased from Dojindo (Japan). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Solarbio (Beijing, China).

2. Animals and cells

Health C57BL/6 mice (6–8 weeks) were purchased from Liaoning Changsheng Biotechnology Co., Ltd., Gaoxin District, Benxi, Liaoning, China. All animal care and experimental procedures were reviewed and approved by the Ethics Committee for Experimental Animal Care of Henan Eye Institute (Approval No. HNEECA-2025-0001). All experiments were conducted in accordance with the guidelines established by the National Institutes of Health. In addition, all procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The human corneal epithelial cell line HCE-2 was obtained from American Type Culture Collection (Manassas, VA, USA). *Fusarium solani* (*F. solani*) used in this study were supplied by the Henan Eye Institute.

3. Syntheses and characterization of polypeptides

The three activated amino acid monomers, N_{α}-phenyloxycarbonyl-N_{ε}-carbobenzyloxy-L-lysine (NPCbzLys) N_{α}-phenyloxycarbonyl-L-leucine (NPLeu), and N_{α}-phenyloxycarbonyl-L-methionine (NPMet), were synthesized as reported by Endo et al [1]. They will not be further described.

3.1 Synthesis of Bu-$CbzLys_{35}$

NPCbzLys was dissolved in anhydrous DMAc. Then, n-butylamine was added into the above solution under the protection of nitrogen. The reaction was allowed to proceed for 2 d at 60 °C. Subsequently, the reaction mixture was precipitated into a large amount of diethyl ether, filtered, washed with diethyl ether, and dried under vacuum for 24 h to get the final product as a white solid.

3.2 Synthesis of Bu-$CbzLys_{35}$-b-Leu_{12} and Bu-$CbzLys_{35}$-b-Met_{13}

Bu-$CbzLys_{35}$ was dissolved in anhydrous DMAc, and then NPLeu or NPMet, was added into the above solution under the protection of nitrogen. The reaction was allowed to proceed for 2 d at 60 °C. Subsequently, the reaction mixture was precipitated into a large amount of diethyl ether, filtered, washed with diethyl ether, and dried under vacuum for 24 h to get the final product as a white or yellowish solid.

3.3 Synthesis of Bu-$(CbzLys_{0.75}$-co-$Leu_{0.25})_{32}$ and Bu-$(CbzLys_{0.75}$-co-$Met_{0.25})_{60}$

NPCbzLys and NPLeu (or NPMet) were dissolved in anhydrous DMAc. After all monomers were completely dissolved, n-butylamine was added into the above solution. The reaction was allowed to proceed for 48 h at 60 ^{o}C. Subsequently, the reaction mixture was precipitated into a large amount of diethyl ether, filtered, washed with diethyl ether, and dried under vacuum for 24 h to get the product as a white or yellowish solid.

3.4 Deprotection of the above four protected polypeptides

First, each protected polypeptide was dissolved into TFA, then 33% HBr/CF3COOH was added into the above solution under the protection of nitrogen. Second, the reaction was allowed to proceed for 3 h under ice bath. Last, the reaction mixture was precipitated into a large amount of diethyl ether,

washed with diethyl ether, and dried under vacuum for 24 h to get the product as a yellowish or white solid.

Details of all these syntheses can be found in Table S1–S2.

3.5 characterization of polypeptides

The structure of the synthesized polypeptides was characterized by ^{1}HNMR spectra on a Bruker AVANCE III 400 MHz spectrometer (Massachusetts, USA) using DMSO-d_6 as NMR solvents.

4. Preparation and characterization of polypeptide nano-micelles

4.1 Preparation of polypeptide nano-micelles

10 mg peptide was dissolved in 1 mL of DMSO and was added dropwise to 9 mL of pure water under ultrasonication. The above dispersion was ultrasonicated for 5 min to obtain the micelle dispersion. Finally, the dispersion was purified by dialysis against distilled water over night.

4.2 Characterization of polypeptide nano-micelles

The morphology of the nano-micelles was investigated using a standard TEM instrument (JEM-1400Flash, Tokyo, Japan). The size distribution and zeta potential of the nano-micelles was examined by a Dynamic Light Scattering (DLS) instrument (Zetasizer Nano ZS90, Worcestershire, UK).

4.3 Determination of the critical micelle concentration (CMC)

The CMCs of K-b-L and K-b-M micelles were determined by a pyrene fluorescence probe method according to a reported procedure [2]. Briefly, pyrene was used as a fluorescence probe and the normalized fluorescence intensity ratio, I_{372nm}/I_{383nm}, was plotted against polymer concentration in DI water. The CMC values were estimated from the turning points of the resulting curves. As shown in Figure S11 and Figure S12, the CMCs of K-b-L and K-b-M micelles were approximately 30 μg/mL and 100 μg/mL, respectively.

4.4 Stability of K-b-L micelles in different media

The colloidal stability of K-b-L micelles was evaluated by DLS after mixing the micelle dispersion (1 mg/mL) with different media at a volume ratio of 1:1. For PBS stability evaluation, K-b-L micelles were mixed with 1× PBS and the hydrodynamic size was monitored at 0, 1, 3, 12, and 24 h. For serum-containing medium evaluation, K-b-L micelles were mixed with serum-containing F12 medium and the hydrodynamic size was monitored at 0, 1, and 24 h.

5. In vitro antifungal activities against Fusarium solani

The antifungal activities of each polypeptide against Fusarium solani (F. solani) cells were studied by MTT assay. the fungal cells were seeded with RPMI 1640 medium onto a 96-well plate at 10^4 per well, then each polypeptide was added to the plate and obtained final concentrations of 0-500 μg/mL. After 48 h of incubation at 28 °C, MTT assay was used and the OD_{490} values was recorded by the Cytation5 Microplate Reader (Biotek Winooski, USA). The antimicrobial activities were calculated according to the OD_{490} values. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of each polypeptide at which no visible microbial growth is observed. The fungal survival rate was calculated according to following formula.

$$\textit{Fungal survival rate (\%)} = \frac{A_2 - A_0}{A_1 - A_0} \times 100$$

Where A_2 denotes the absorbance of the test group, A_1 denotes the absorbance of the control group (fungi wells without drug treatment), and A_0 denotes the absorbance of the blank control group (MTT wells without fungi).

6. In vitro cytotoxicity in human corneal epithelial cell line HCE-2

The cytotoxicity of each polypeptide against HCE-2 was studied using a CCK-8 assay. First, HCE-2 cells were seeded onto 96-well plates at a density of 8 × 10^3 cells per well, and cultured in 5% CO_2 at 37 °C for 24 h. Then, the medium was removed and a fresh medium (100 μL) containing different concentrations of each polypeptide (0, 12.5, 25, 50, 100 μg/mL) was added. After incubation for 24 h, the medium was removed and a fresh medium (100 μL) containing 10% CCK-8 solution was added to each well for an additional 4 h at 37 °C. Finally, the cell viabilities were calculated according to the OD values at 450 nm measured by an Enzyme Labeler (PerkinElmer EnVision, England). The cell viabilities were calculated according to following formula.

$$\textit{HCE-2 viabilities (\%)} = \frac{A_2}{A_1} \times 100$$

Where A_2 denotes the absorbance of the test group, A_1 denotes the absorbance of the control group (cell wells without drug treatment).

7. In vivo evaluation of antifungal activity in C57BL/6 mice

We established a mice model of *F. solani*-induced fungal keratitis as follows [3]. Fifteen healthy C57BL/6 mice with normal ocular surfaces and no signs of cataract, opacity, or leukoplakia were used in this study. Mice were first anesthetized, their whiskers were trimmed, and the periocular area was disinfected with iodophor. Topical anesthesia was then achieved using proparacaine hydrochloride eye drops, with care taken to fully expose the cornea. Under a surgical microscope (Topcon OMS-90, Japan), a 2 mm diameter area on the right cornea was marked using a sterile trephine, while the left eye served as an untreated control. The corneal epithelium within the marked region was carefully removed using a sterile blade, followed by superficial stromal injury created via a cross-scratch technique. A fungal spore suspension (10^4 CFU/mL, 5 μL) was subsequently applied to the corneal surface to induce fungal keratitis. Twelve hours post-inoculation, the mice were randomly assigned into three groups (n = 5 per group): negative control (normal saline), K-b-L nano-micelle treatment (20 μg/mL), and positive control (voriconazole, 25 μg/mL). Each group received 5 μL of the corresponding treatment three times daily for 7 consecutive days. The left eyes were not treated and excluded from statistical analysis.

Twelve hours after model establishment was defined as day 0. Slit-lamp imaging was performed to evaluate the initial lesion status and confirm consistency among groups, followed by clinical scoring based on previously reported criteria. After treatment, corneal changes were monitored on days 1, 3, 5, and 7 using slit-lamp examination. Images were recorded and independently scored by experienced clinicians. On day 7 after treatment, corneal tissues were collected from each group, along with tissues from an untreated normal mouse as a control. The samples were fixed in FAS fixative, paraffin-embedded, and sectioned at a thickness of 5 μm. Hematoxylin and eosin (H&E) staining was performed to evaluate corneal tissue morphology and assess the degree of tissue recovery among the different groups.

8. In vivo evaluation of systemic toxicity in C57BL/6 mice

Healthy mice were treated with K-b-L nanomicelles (5 μL, 20 μg/mL) for 7 consecutive days. After treatment, the mice were euthanized by overdose anesthesia, and major organs (heart, liver, spleen, lung, and kidney) were collected. The harvested tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. H&E staining was performed to evaluate potential histopathological changes and assess the systemic toxicity of the nano-micelles.

9. Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using a two-tailed Student's t-test or one-way analysis of variance (ANOVA) with GraphPad Prism 9.5. Differences were considered statistically significant at $P < 0.05$, with significance levels indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

Table S1. Summarized syntheses of protected polypeptides.

Synthesis	Initiator	Monomer	Time	Product	Quantity	Yield
1	Butylamine (11 mg, 73.14, 0.152 mmol)	NCbzLys (3.656 g, 400, 9.14 mmol)	2 d	Bu-$CbzLys_{35}$	2.5 g	100%
2	Bu-$CbzLys_{35}$ (0.6 g, 9200, 0.065 mmol)	NPLeu (0.23 g, 251, 0.92 mmol)	2 d	Bu-$CbzLys_{35}$-Leu_{12}	0.61 g	85%
3	Bu-$CbzLys_{35}$ (0.61 g, 9200, 0.066 mmol)	NPMet (0.24 g, 269, 0.89 mmol)	2 d	Bu-$CbzLys_{35}$-Met_{13}	0.64 g	88%
4	Butylamine (2.56 mg, 73.14, 0.035 mmol)	NCbzLys (0.85 g, 400, 2.13 mmol) and NPLeu (0.35 g, 251, 1.39 mmol)	2 d	Bu-$(CbzLys_{0.75}$-co-$Leu_{0.25})_{60}$	0.58 g	81%
5	Butylamine (24.6 mg, 73.14, 0.336 mmol)	NCbzLys (3.07 g, 400, 7.68 mmol) and NPMet (0.89 g, 269, 3.31 mmol)	2 d	Bu-$(CbzLys_{0.72}$-co-$Met_{0.28})_{32}$	2.45 g	98%

Table S2 Summarized de-protection reaction of polypeptide.

Synthesis	Protected peptide	Solvent	Acid	Product	Quantity
1	Bu-CbzLys$_{35}$-Leu$_{12}$ (304 mg)	5 mL TFA	0.7 mL 33% HBr/ CH_3COOH	Bu-Lys$_{35}$-Leu$_{12}$	260 mg
2	Bu-CbzLys$_{35}$- Met$_{13}$ (300 mg)	2 mL TFA	1 mL 33% HBr/ CH_3COOH	Bu-Lys$_{35}$-Met$_{13}$	321 mg
3	Bu-(CbzLys$_{0.75}$-co-Leu$_{0.25}$)$_{60}$ (298 mg)	5 mL TFA	0.7 mL 33% HBr/ CH_3COOH	Bu-(Lys$_{0.75}$-co-Leu$_{0.25}$)$_{60}$	288 mg
4	Bu-(CbzLys$_{0.72}$-co-Met$_{0.28}$)$_{32}$ (839 mg)	8 mL TFA	1.5 mL 33% HBr/ CH_3COOH	Bu-(Lys$_{0.72}$-co-Met$_{0.28}$)$_{32}$	900 mg

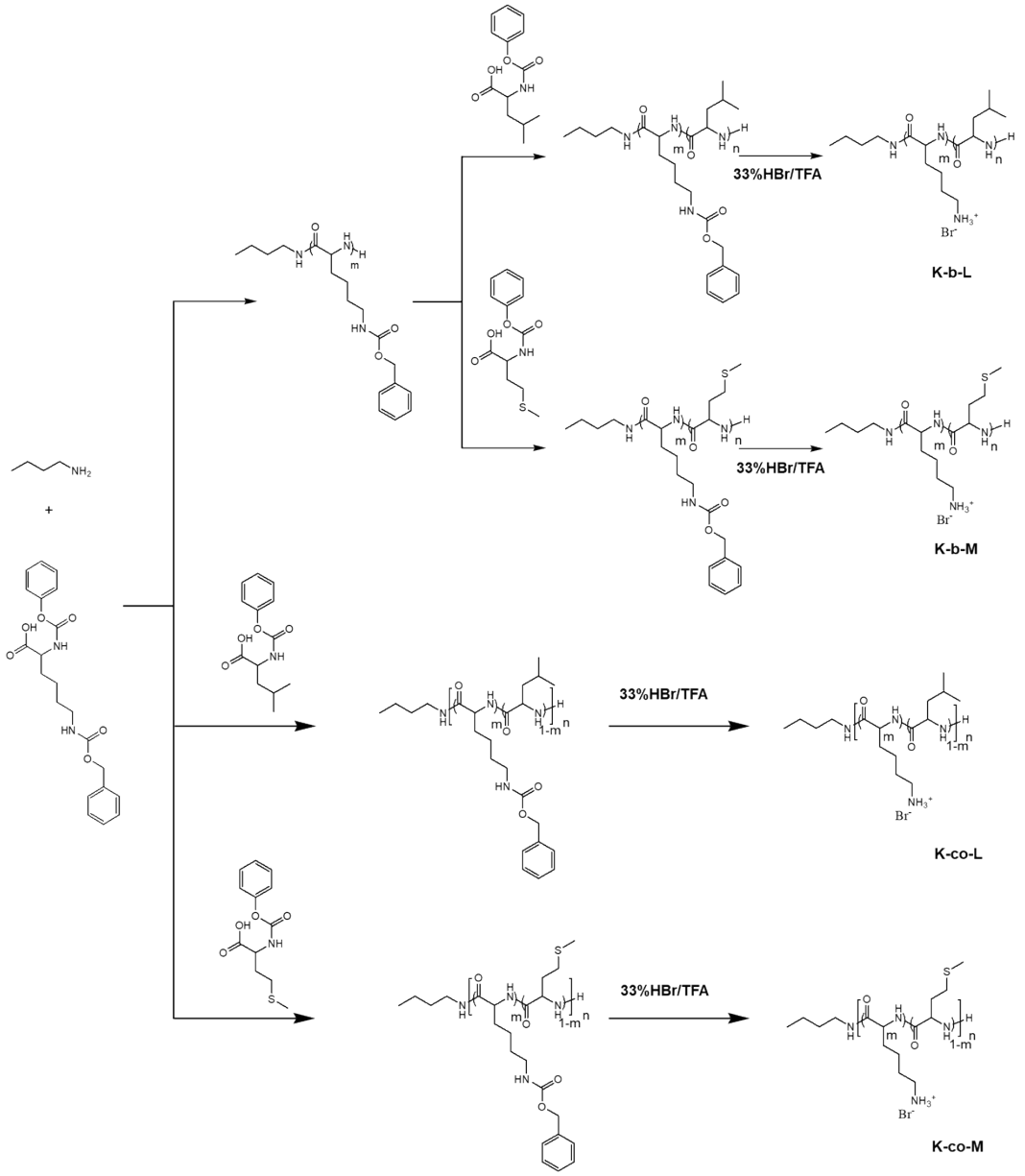

Figure S1. Synthetic route of four antifungal peptides.

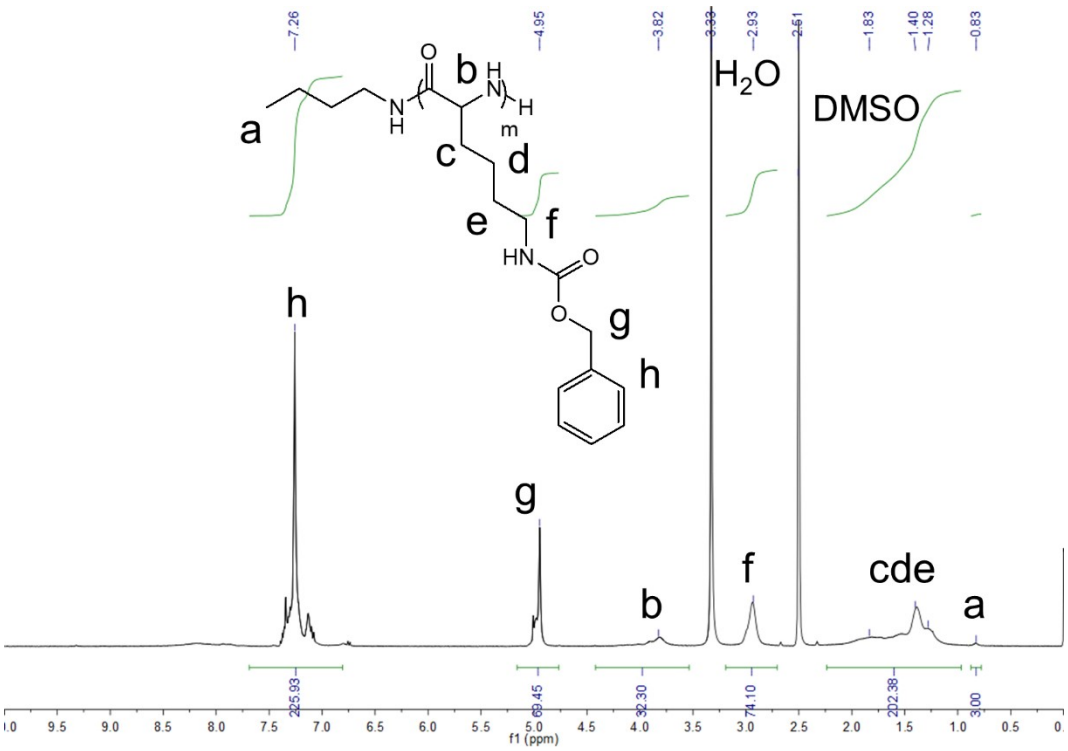

Figure S2. ^{1}H NMR spectrum of Bu-CbzLys$_{35}$ (solvent: DMSO-d_6).

According to the integrals of the signals a and b, the average number of CbzLys units is approximately 35.

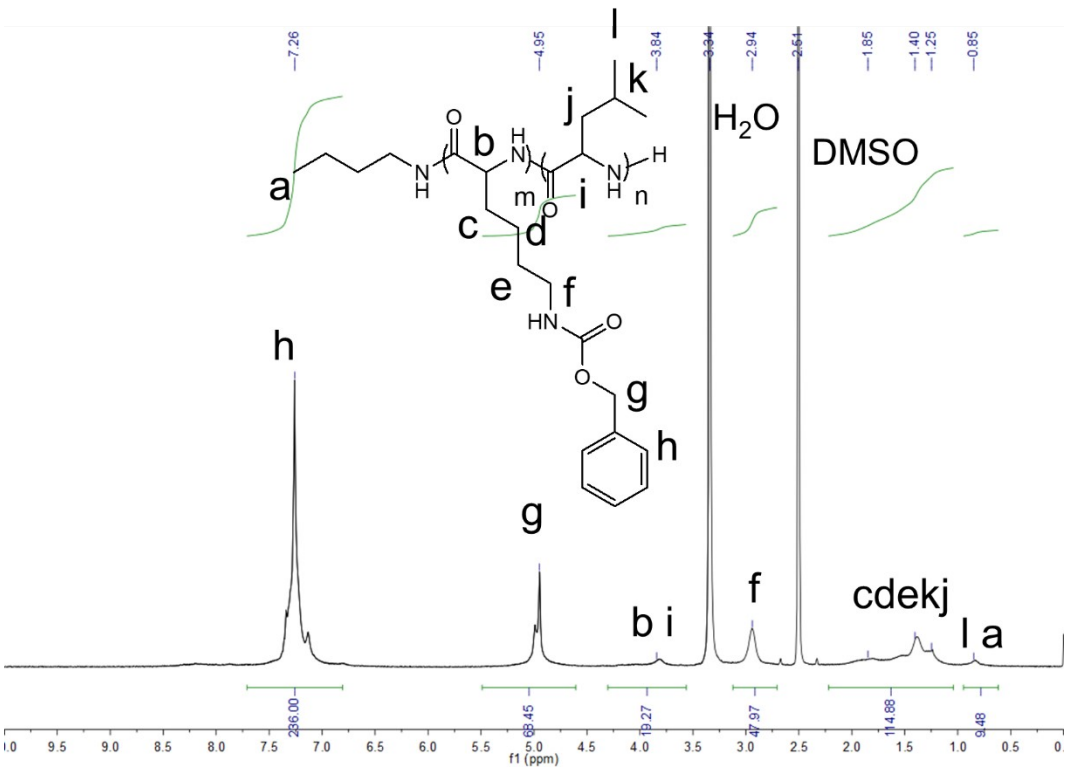

Figure S3. ^{1}H NMR spectrum of Bu-CbzLys$_{35}$-Leu$_{12}$ (solvent: DMSO-d$_6$).

The average chain length is hard to calculate from ^{1}H NMR due to that the product isn't well dissolved in the solvent.

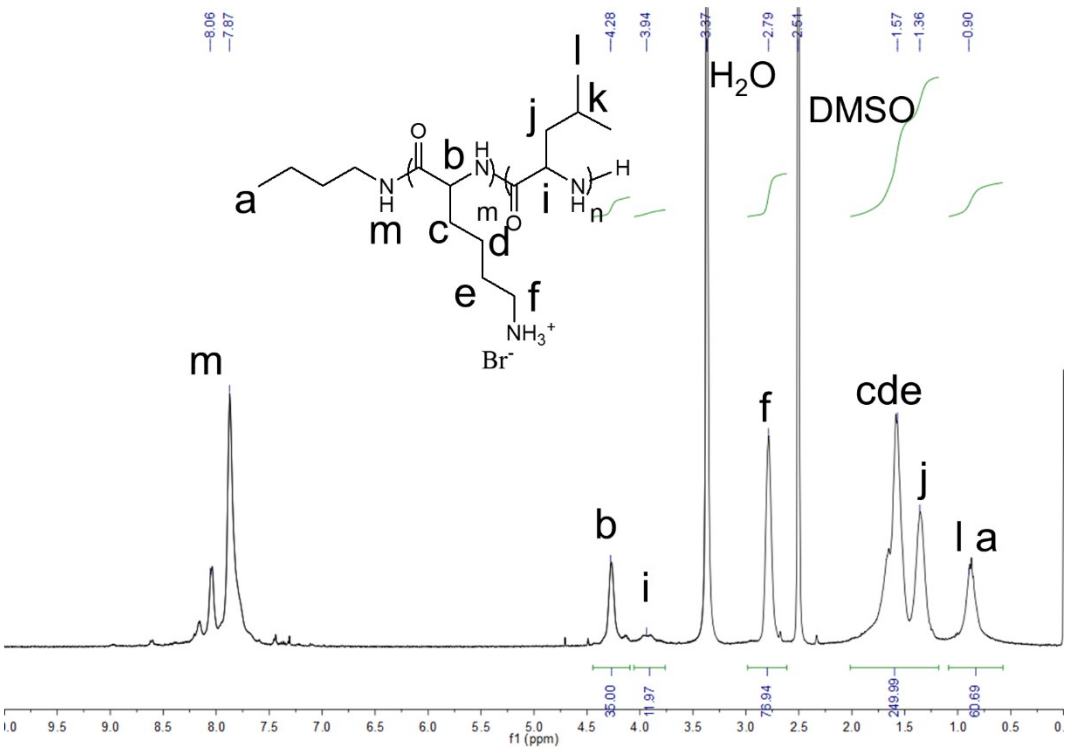

Figure S4. ^{1}H NMR spectrum of Bu-Lys$_{35}$-Leu$_{12}$ (solvent: DMSO-d$_6$).

The disappearance of the benzyl proton peak indicates the successful removal of benzyl group. The average number of Leu units is approximately 12, as calculated from the integrals of the signals b and i.

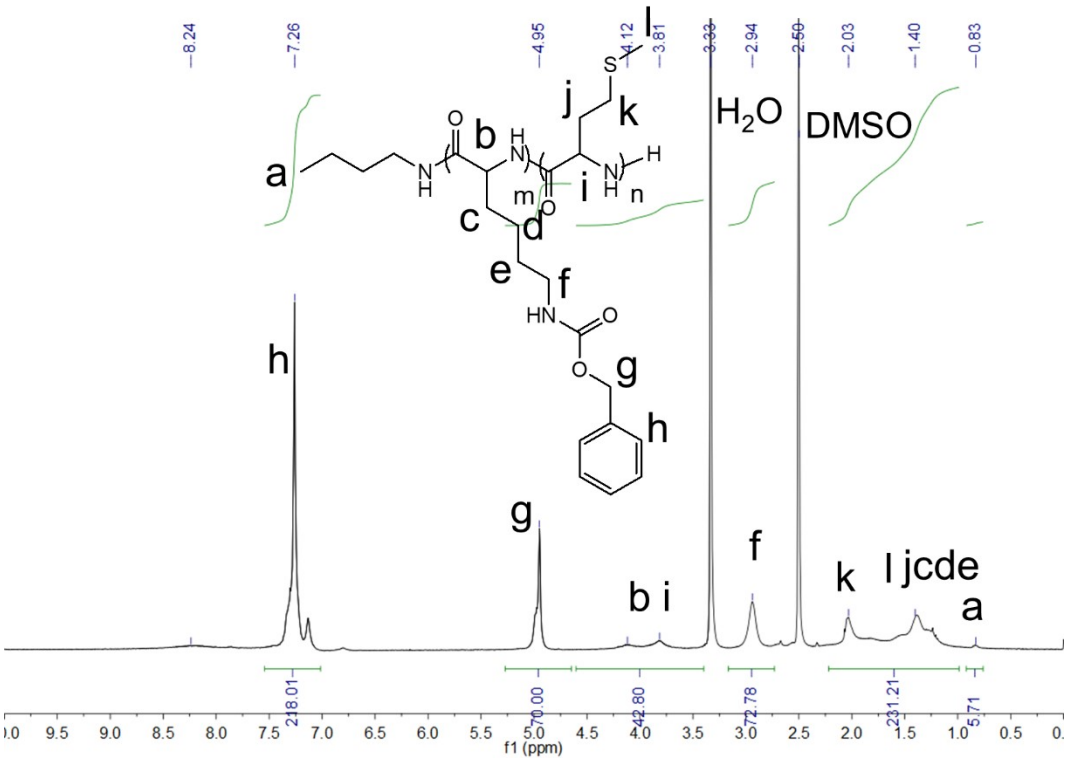

Figure S5. ^{1}H NMR spectrum of Bu-CbzLys$_{35}$-Met$_{13}$ (solvent: DMSO-d$_6$).

The average chain length is hard to calculate from ^{1}H NMR due to that the product isn't well dissolved in the solvent.

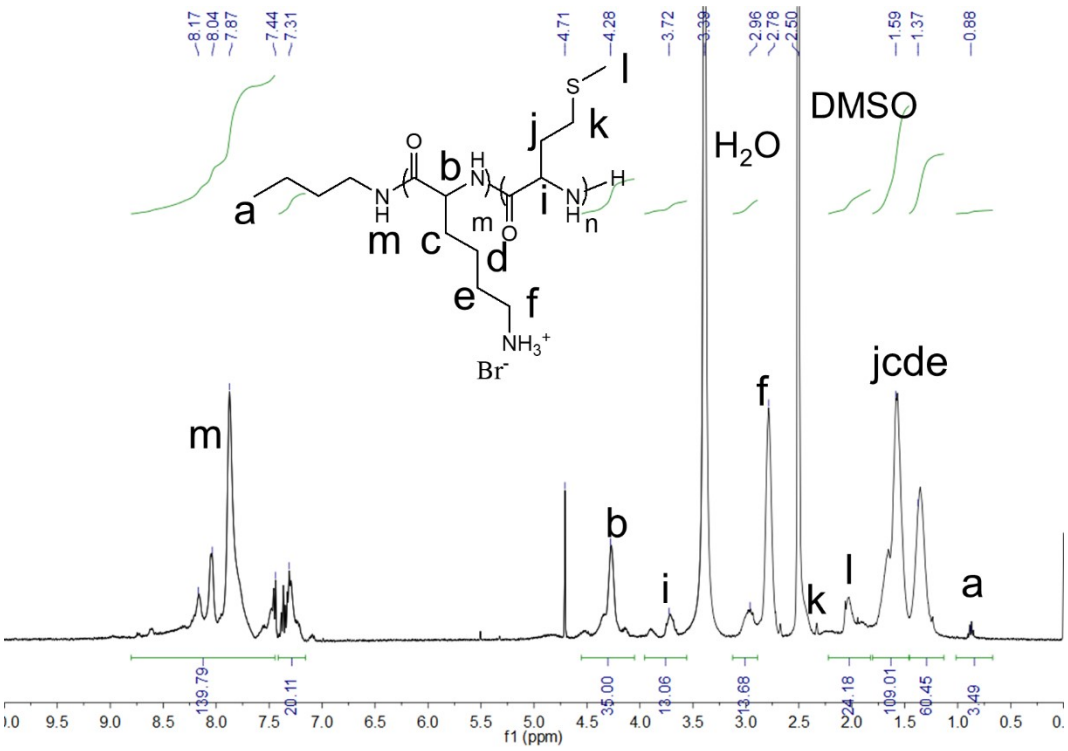

Figure S6. ^{1}H NMR spectrum of Bu-Lys$_{35}$-Met$_{13}$ (solvent: DMSO-d$_6$).

The disappearance of the benzyl proton peak indicates the successful removal of benzyl group. The average number of Met units is approximately 13, as calculated from the integrals of the signals b and i.

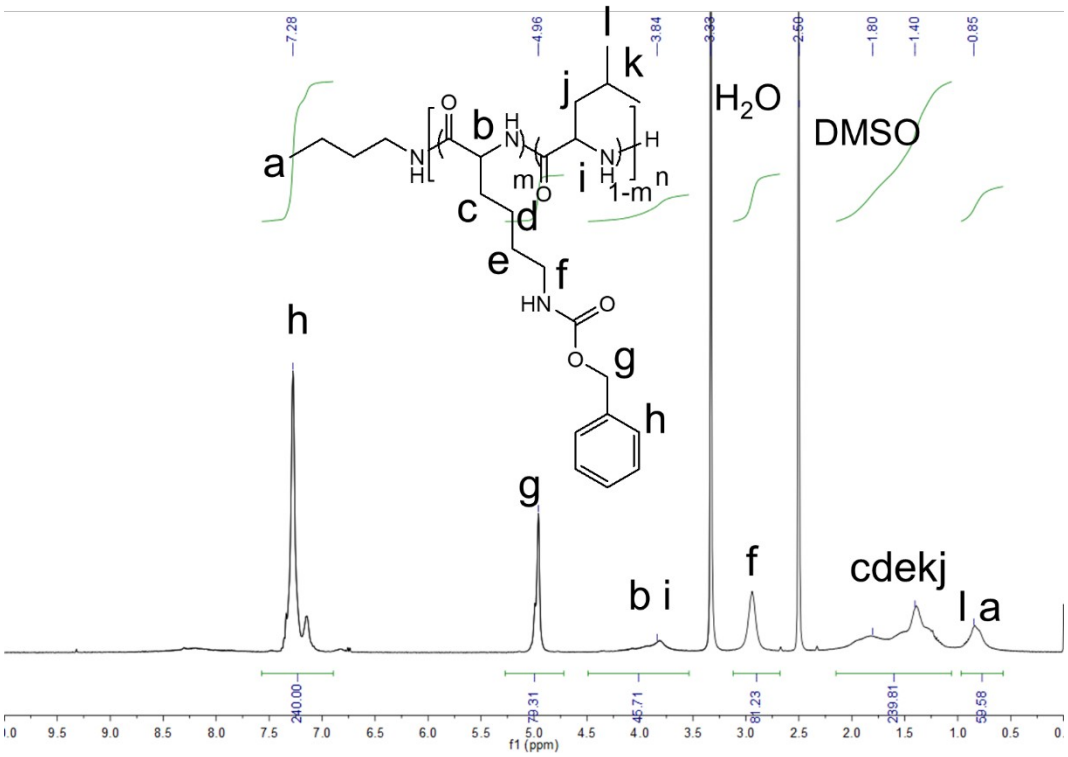

Figure S7. ^{1}H NMR spectrum of Bu-(CbzLys$_{0.75}$-co-Leu$_{0.25}$)$_{60}$ (solvent: DMSO-d$_6$).

The average chain length is hard to calculate from ^{1}H NMR due to the overlap of peaks. Therefore, the average chain length is estimated from the yield of the reactions summarized in Table S1.

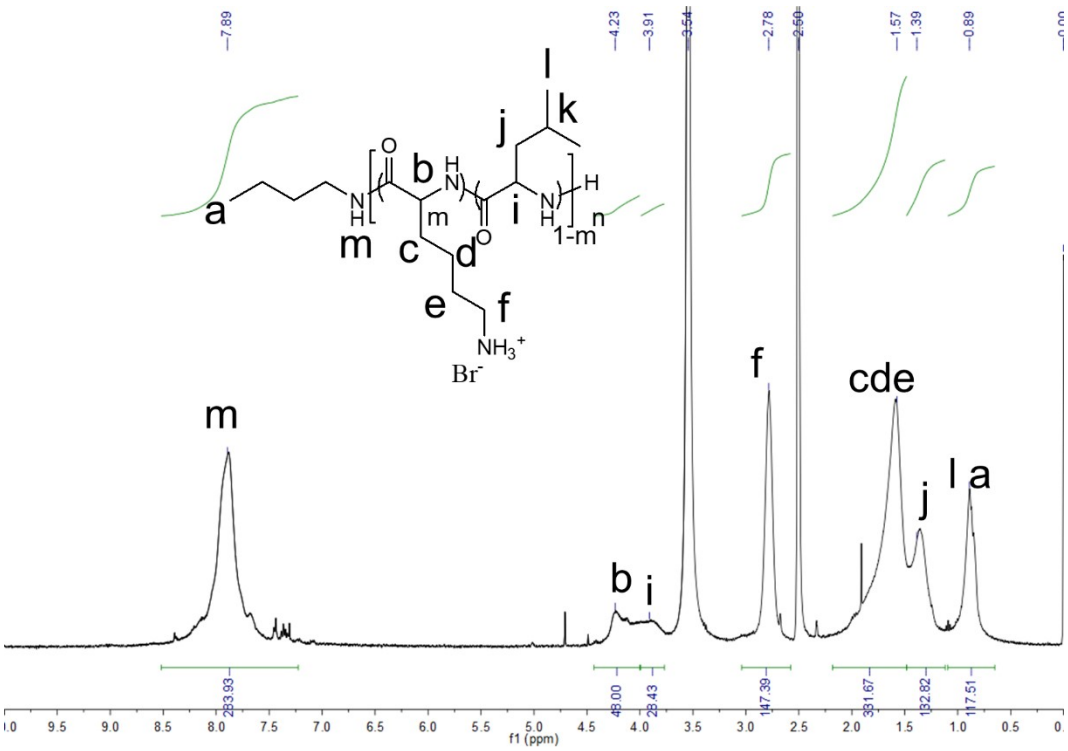

Figure S8. ^{1}H NMR spectrum of Bu-(Lys$_{0.75}$-co-Leu$_{0.25}$)$_{60}$ (solvent: DMSO-d$_6$).

The disappearance of the benzyl proton peak indicates the successful removal of benzyl group.

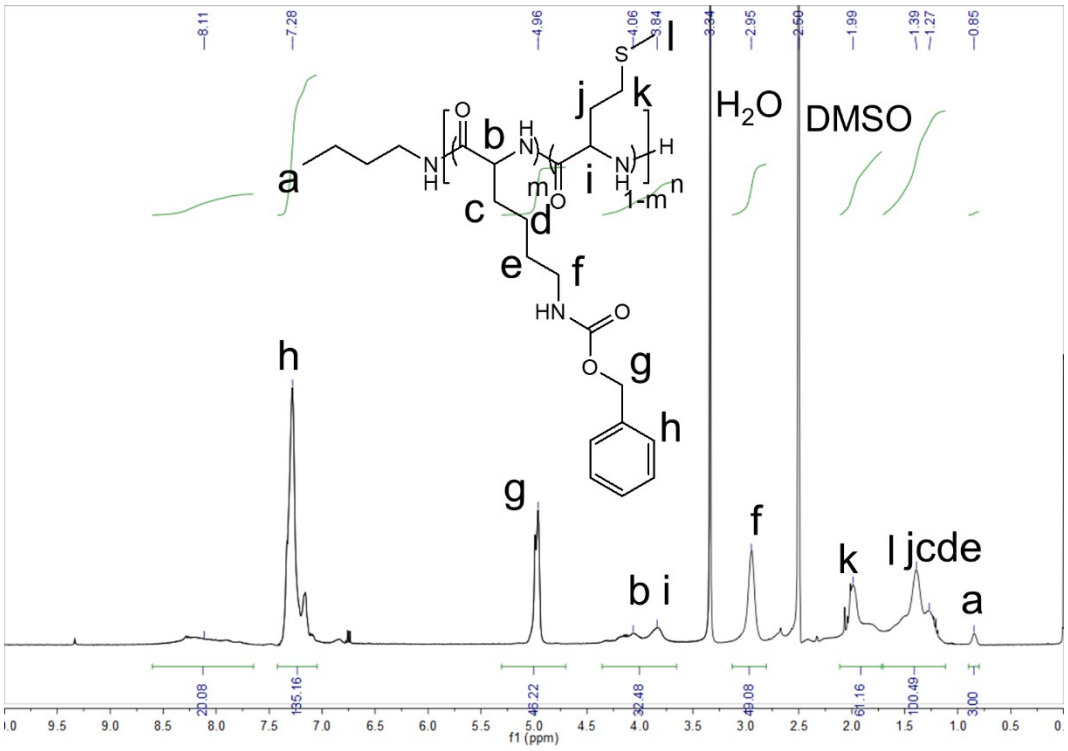

Figure S9. ^{1}H NMR spectrum of Bu-(CbzLys$_{0.72}$-co-Met$_{0.28}$)$_{32}$ (solvent: DMSO-d$_6$).

According to the integrals of the signals a and b, each polymer chain contains 32 amino acids in average. According to the integrals of the signals a and g, the average number of Lys units was calculated as approximately 23. When we minus 32 (the number of average chain length) by 23, the average number of Lys units in one polymer chain, we calculated that each polymer chain contains 9 Met in average. Again, there are statistical variations of these values.

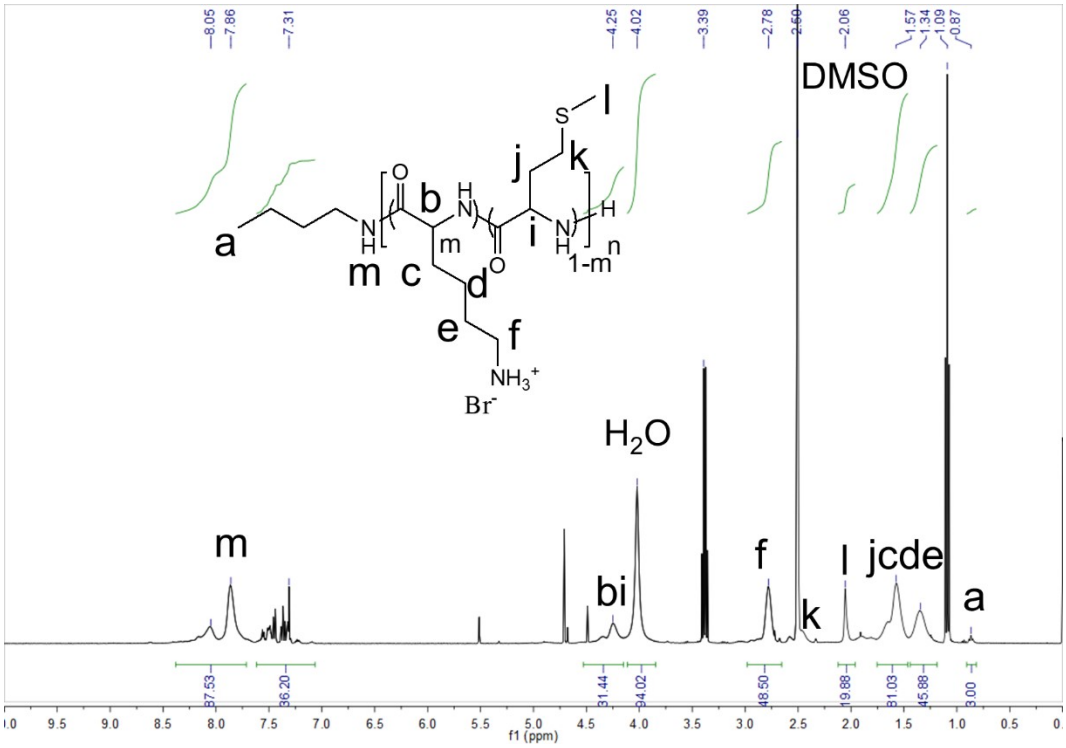

Figure S10. ^{1}H NMR spectrum of Bu-($Lys_{0.72}$-co-$Met_{0.28}$)$_{32}$ (solvent: DMSO-d_6).

The complete removal of Cbz groups was confirmed by the disappear of –***C*H$_2$**- signal (at around 5.0 ppm) in Cbz groups.

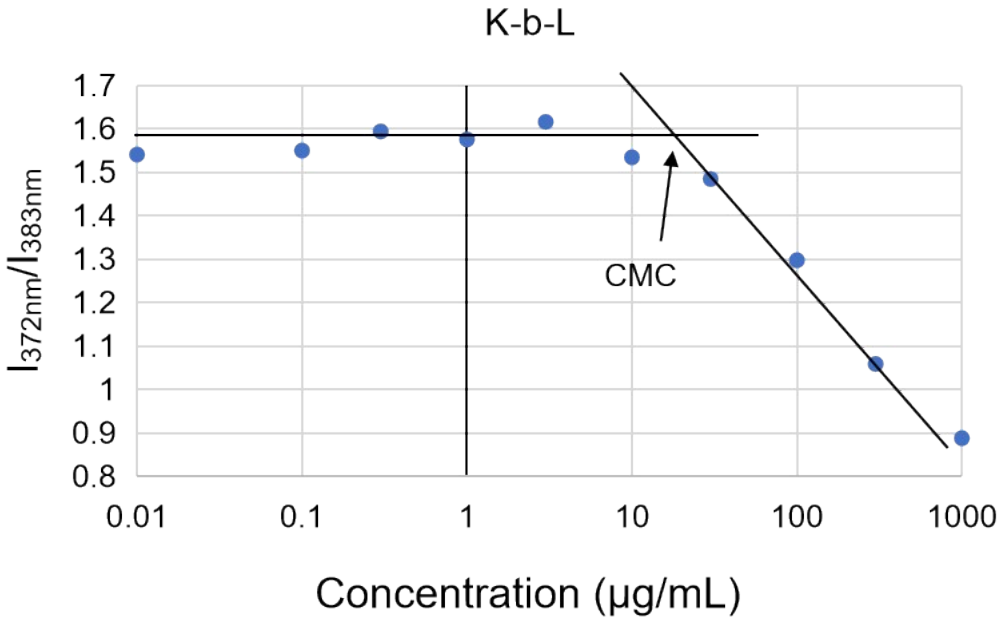

Figure S11. Normalized intensity ratio (I_{372nm}/I_{383nm}) of pyrene fluorescence measured as a function of K-b-L concentration in DI water.

The CMC was estimated as around 30 μg/mL.

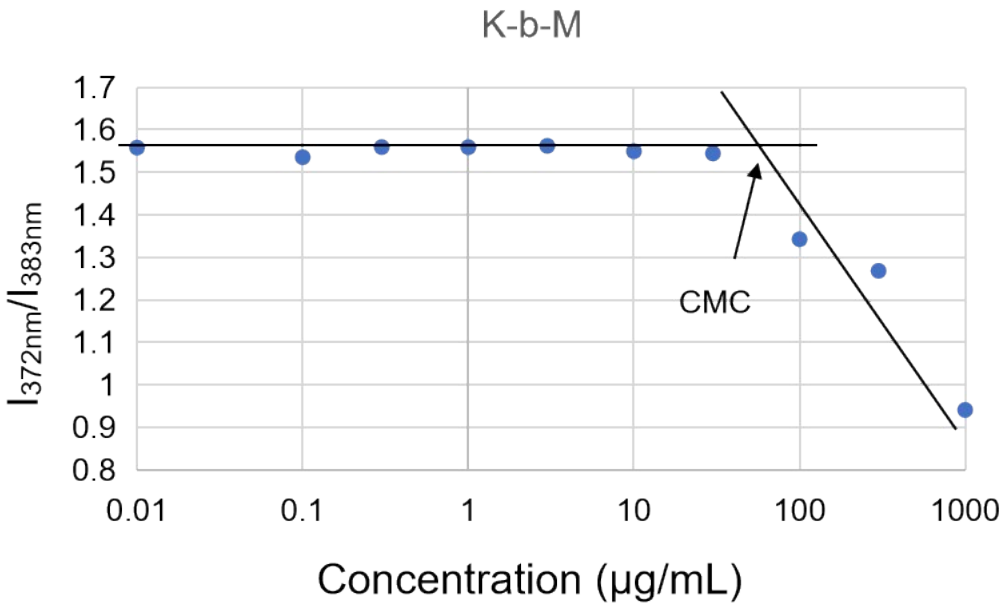

Figure S12. Normalized intensity ratio (I_{372nm}/I_{383nm}) of pyrene fluorescence measured as a function of K-b-M concentration in DI water.

The CMC was estimated as around 100 μg/mL.

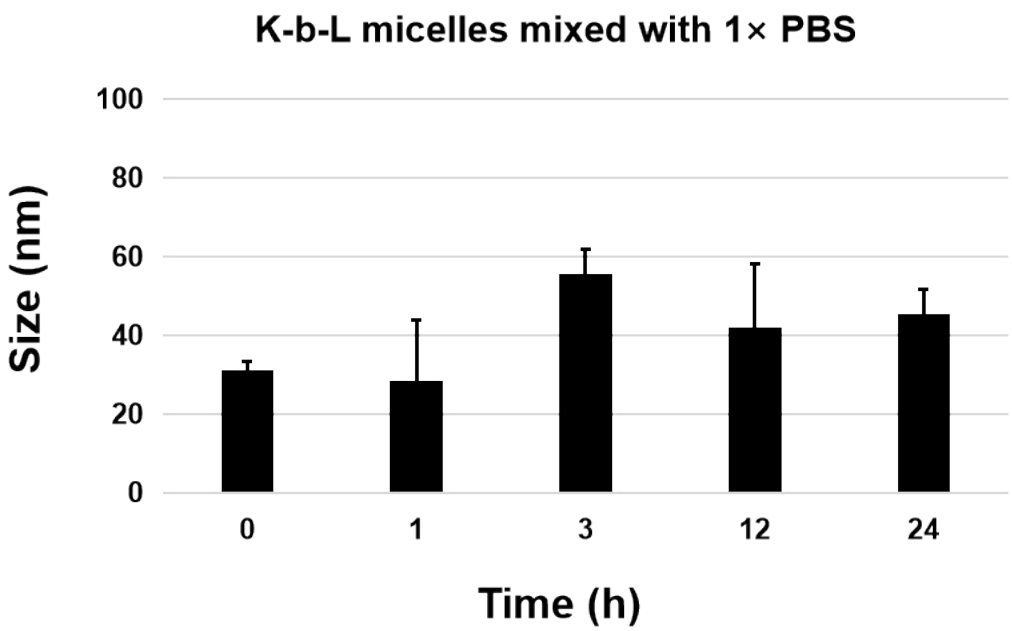

Figure S13. Time-dependent changes in the hydrodynamic size of K-b-L micelles after mixing with 1× PBS at a volume ratio of 1:1, as measured by DLS.

As shown in Figure S13, the particle size remained relatively stable in PBS over 24 h.

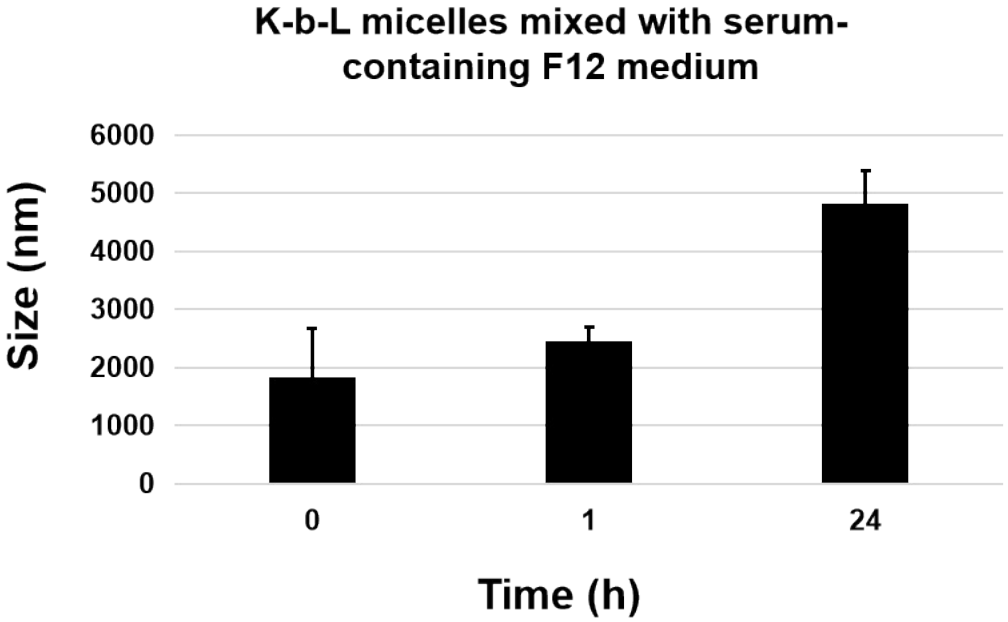

Figure S14. Time-dependent changes in the hydrodynamic size of K-b-L micelles after mixing with serum-containing F12 medium at a volume ratio of 1:1, as measured by DLS. After mixing with serum-containing F12 medium, visible white precipitates appeared immediately, indicating loss of colloidal stability. Consistently, DLS detected apparent particle sizes above 1800 nm, which further increased over time, confirming pronounced aggregation in the presence of serum components.

Reference

[1] Kamei Y, Nagai A, Sudo A, et al. Convenient synthesis of poly (γ-benzyl-L-glutamate) from activated urethane derivatives of γ-benzyl-L-glutamate[J]. Journal of polymer science part A: polymer chemistry, 2008, 46(8): 2649-2657.

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[3] Zhao M, Geng X, Zeng Q, et al. Cu-Single Atoms/Clusters-Nanoenzymes Trigger and Integrate Tandem Effect to Synchronously Boost Antifungal and Anti-Inflammation for Fungal Keratitis[J]. Advanced Healthcare Materials, 2026, 15(8): e04085.