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Effect of L- or D,L-leucine content on self-assembly and properties of its amphiphilic copolymers with L-lysine

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S1. Materials

Triphosgene (98%), ε-*N*-benzyloxycarbonyl-L-lysine (ε-Cbz-Lys-OH) (≥99.0%), α-pinene (99.0%), *n*-hexylamine (99.0%), trifluoromethanesulfonic acid and trifluoroacetic acid (≥98.0%) were purchased from Sigma-Aldrich (Germany). L-Leucine and D-leucine were supplied by Bide Pharmatech Ltd (China). The solvents dioxane, tetrahydrofuran, petroleum ether, ethyl acetate, diethyl ether, dimethylformamide and acetonitrile, and salts used to prepare buffer solutions were purchased from Vekton (Russia). Buffer solutions were prepared by dissolving salts in deionized water and additionally filtered through Millipore Merck membrane filters (Germany) with a pore size of 0.22 μm.

Cell experiments were performed using 96-well plates (Biovitrum, Russia). For cell experiments, basal media DMEM (Biovitrum, Russia), fetal bovine serum (EBS, Biochrom GmbH, Germany), penicillin (10000 U/mL) and streptomycin (10 mg/mL) solutions (Biochrom GmbH, Germany) were used. Human embryonic kidney cells (HEK 293) and human lung epithelial cells (BEAS-2B) were used for cytotoxicity studies.

S2. Monomer synthesis

The synthesis was carried out in a three-neck flask equipped with a $CaCl_2$ tube, a thermometer to monitor the reaction temperature, and an inert gas (argon) supply tube.

LLys^{Cbz} *NCA* **1**

Before the reaction, a suspension of 5.0 g (17.8 mmol) of ε-*N*-benzyloxycarbonyl-L-lysine in 200 mL of freshly distilled dioxane was prepared in a flask with 5.6 mL of α-pinene. Into the reaction medium heated to 50 °C was added 1.7 g (5.9 mol, 1/3 equivalent relative to the amount of acid) of triphosgene and left under vigorous stirring in an inert gas stream for 1 h. Thereafter, 1/20 eq. of triphosgene was added to the reaction medium 3 times every 30 min. The heating was turned off one hour after the addition of the last portion of triphosgene. After the reaction mixture

cooled to room temperature, the unreacted amino acid was filtered off on a Schott filter. The filtrate was evaporated on a rotary evaporator, after which it was poured into 150 mL of petroleum ether and left overnight in the refrigerator. The purification was carried out by 3-fold recrystallization in ethyl acetate/petroleum ether system and dried in a vacuum desiccator. The purity of the compound was confirmed by ^{1}H NMR spectroscopy and comparison of spectra with literature data. The yield of Lys^{Cbz} NCA was 5.2 g (96%).

LLeu NCA **2**

For synthesis 3.0 g (22.8 mmol) and 2.2 g (7.6 mmol) of triphosgene were taken, dioxane was used as a solvent. The general methodology was the same as described in the previous paragraph. The yield of LLeu NCA was 2.7 g (76%).

DLeu NCA

For synthesis 1.5 g (11.4 mmol) and 1.1 g (3.8 mmol) of triphosgene were taken, dioxane was used as a solvent. The general methodology was the same as described for Lys^{Cbz} NCA. The yield of DLeu NCA 1.3 g (72%).

S3. Polymer 3 or 3' Synthesis and Deprotection

Copolymerization of NCAs **1** with **2/2'** was carried out in dry solvent, for which a 4% solution of monomers in dioxane was prepared. After preparation of the NCA solution, a solution of the initiator *n*-hexylamine in the ratio [M]/[I]=100 was added to the polymerization vessel. The polymerization mixture was thermostated at 25 °C for 48 h. After two days, the obtained product was precipitated with 3 to 5 times excess of diethyl ether. The precipitate was separated by centrifugation at 7000 rpm for 10 min. After decantation of the solution, the precipitate was washed with excess diethyl ether. The obtained copolymers were air dried at room temperature. The yields of copolymers varied in the range of 62-86%.

To deprotect the Cbz-group from the ε-amino group of lysine, 1 mL of ice-cold trifluoroacetic acid was added to 50 mg of copolymer and left to stir for 60 min. Thereafter, 50 µL of trifluoromethanesulfonic acid was added to the resulting suspension. The reaction was carried out under stirring for 5 h, after which 5 mL of DMF was added to the reaction mixture and the resulting solution was transferred to a dialysis bag (MWCO 1000). Dialysis was carried out against water for 48 h. The precipitate was separated by centrifugation and freeze-dried.

S4. Characterization of Copolymers

The molecular weight characteristics and dispersibility of polymer samples **3/3'** were analyzed by size-exclusion chromatography (SEC) using a Prominence Shimadzu LC-20 system

with a refractometer detector RID 10-A (Kyoto, Japan) and containing a tandem of two columns Agilent PLgel MIXED-D (7.5 × 300 mm, 5 μm) (USA). A 0.1 M solution of LiBr in DMF was used as eluent. The mobile phase flow rate was 1.0 mL/min, and the analysis temperature was 60 °C. Molecular weights and dispersity of the obtained copolymers were calculated using polymethylmethacrylate standards with molecular masses in the range from 17,000 to 250,000 and dispersity below 1.14. The calculations were performed using GPC LC Solutions software (Shimadzu, Kyoto, Japan).

In order to quantify the amino acid composition of copolymers **3/3'**, hydrolysis of the copolymers to free amino acids was performed. For hydrolysis, 100 mL of 6H HCl with 0.0001% phenol content was prepared. The copolymer sample of 4 mg was put into the ampoule for hydrolysis and hydrolysis solution in the amount of 1 mL/0.5mg of copolymer was added. Then the ampoule was sealed and incubated at 110 °C for 3 days. After hydrolysis, the solution was evaporated to dryness, washed with water and evaporated again until neutral pH. The precipitate was then dissolved in 0.5 mL of water and the resulting mixture was analyzed by ion-exchange HPLC with mass spectrometric detection. Calibration curves was pre-built for standard solutions of lysine and leucine with concentrations in the range of 0.05-0.5 μg/mL. The analysis was performed using a LC-20 Prominence Shimadzu high-performance liquid chromatograph; mobile phase – 6 mM H_3PO_4, mobile phase flow rate – 1.0 mL/min, analysis time – 15 min.

Table S1. Molecular weight characteristics of copolymers of L-lysine pre-**3** and L/D,L-leucine pre-**3'** obtained at different monomer ratios.

Copolymer	$[Lys^{Cbz}\ NCA]_0$/ $[Leu\ NCA]_0$	M_w	M_n	$Đ$
P(LLys-*co*-LLeu) pre-**3a**	50/50	17400	14500	1.20
P(LLys-*co*-LLeu) pre-**3b**	80/20	26600	20800	1.28
P(LLys-*co*-LLeu) pre-**3c**	90/10	32300	24700	1.31
P(LLys-*co*-D,LLeu) pre-**3'a**	50/50	26500	21900	1.21
P(LLys-*co*-D,LLeu) pre-**3'b**	80/20	48700	36100	1.35
P(LLys-*co*-D,LLeu) pre-**3'c**	90/10	51400	37800	1.36

S5. Formation of Nanoparticles and Their Characterization

Simultaneous purification of copolymers from low molecular weight impurities and formation of polymer nanoparticles were carried out by phase inversion (dialysis) from an organic solvent (DMF) into a water. The samples were freeze dried and stored at 4 °C.

The size and ζ-potential of the obtained samples were measured by dynamic light scattering method on a Malvern Zetasizer NanoZS instrument. To redisperse the nanoparticles, 1 mL of water or aqueous buffer solution with the required pH was added to 1 mg of copolymer and subjected to brief exposure to ultrasound (30 s). All measurements were performed at least 3 times.

To cover nanoparticles with heparin, 1 mg of heparin was used per 1 mg of nanoparticles. Fixation of heparin on the particle surface was confirmed by changing the surface zeta-potential from positive to negative in water.

Table S2. Characteristics of nanoparticles based on copolymers of L-lysine **3** and L/D,L-leucine **3'** (0.01 M sodium phosphate buffer, pH 7.4, 25 °C).

Copolymer	Copolymer composition (mol%)		D_H (nm)	PDI	ζ-potential (mV)
	Lys	Leu			
P(LLys-*co*-LLeu) **3a**	38	62	312 ± 8	0.36 ± 0.07	48.3 ± 2.1
P(LLys-*co*-LLeu) **3b**	71	29	403 ± 9	0.30 ± 0.07	60.8 ±1.6
P(LLys-*co*-LLeu) **3c**	87	13	428 ± 5	0.27 ± 0.06	61.9 ± 0.9
P(LLys-*co*-D,LLeu) **3'a**	47	53	265 ± 2	0.28 ± 0.05	55.3 ± 1.1
P(LLys-*co*-D,LLeu) **3'b**	78	22	279 ± 4	0.26 ± 0.08	57.9 ± 0.8
P(LLys-*co*-D,LLeu) **3'c**	88	12	317 ± 5	0.26 ± 0.11	61.5 ± 0.5

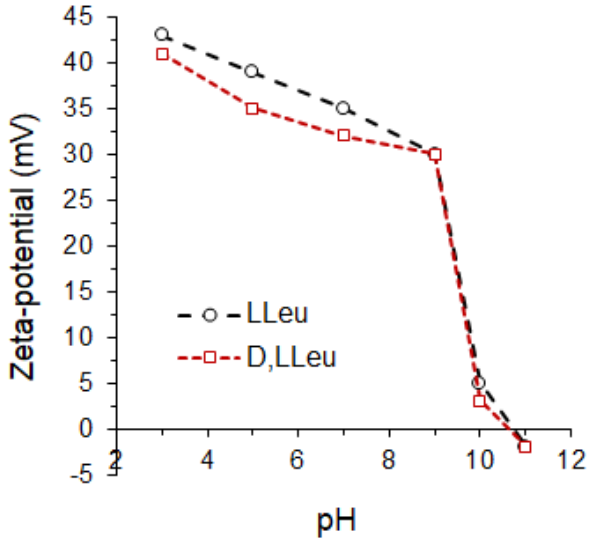
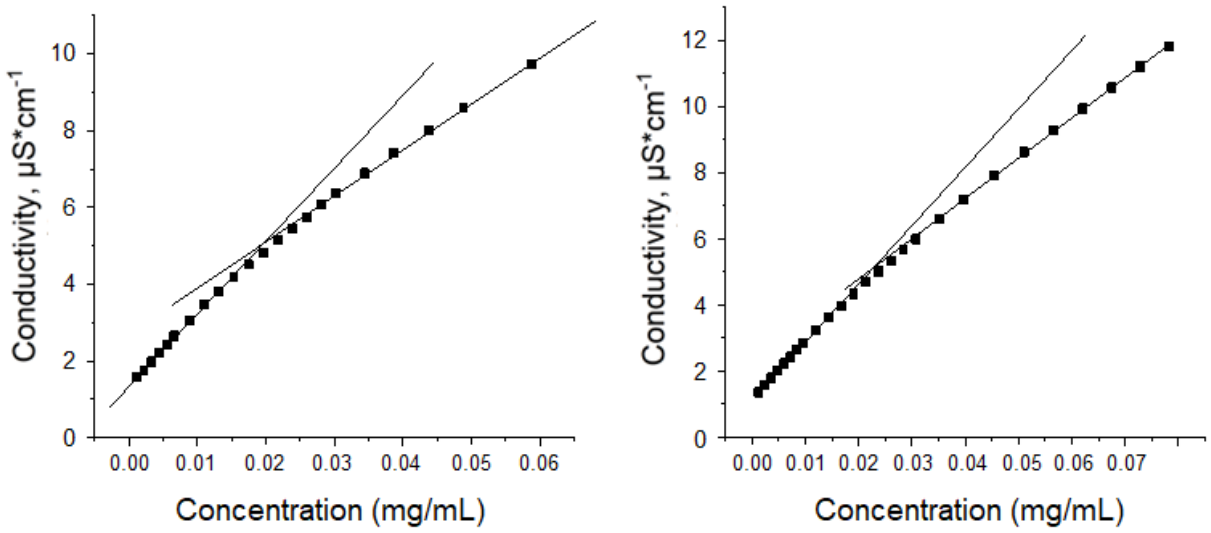
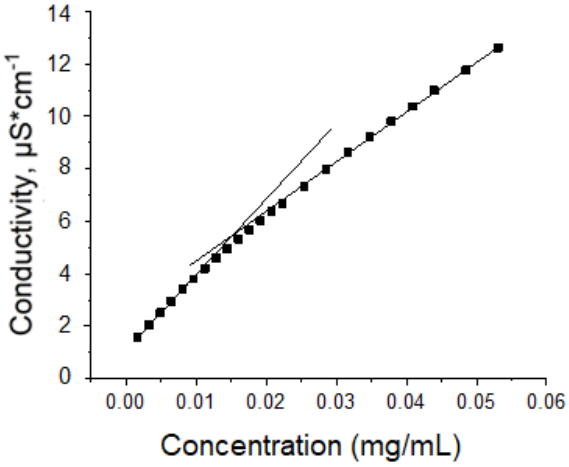


Figure S1. Dependence of zeta-potential of nanoparticles based on copolymers of L-lysine and L/D,L-leucine on pH (water with pH adjusted with 0.01M NaOH/HCl solution, 25 °C).



(*a*)

(*b*)



(*c*)

Figure S2. Dependences of conductivity on copolymer concentration (determination of CAC by conductometry method): (*a*) P(LLys-*co*-LLeu) **3a** (38/62 mol%), (*b*) P(LLys-*co*-D,LLeu) **3'a** (47/53 mol%), (*c*) P(LLys-*co*-D,LLeu) **3'b** (78/22 mol%).

S6. Stability of Nanoparticles *in vitro*

The stability of nanoparticles of **3/3'** was studied in 2 model media: 1) 0.01 M phosphate buffer solution, pH 7.4 at 37 °C and 2) 0.01 M phosphate buffer solution, pH 7. 4 containing papain. In the case of phosphate buffer solution containing papain, 0.5 mL of a solution containing the enzyme with a concentration of 0.1 mg/mL was added to 0.5 mL of particle suspension (2 mg/mL) in buffer solution. The process was monitored by dynamic light scattering method within 30 days.

S7. Biological Evaluation

The cytotoxicity was investigated using the CellTiter-Blue (CTB) reagent assay. 96-well plates were used for the experiment. In each well, 8000 cells were seeded in 100 μL of culture medium and cultured for 24 h. The culture medium was then aspirated and 200 μL of culture medium containing nanoparticles of different concentrations (4-1000 ng/mL) was added (n = 3). The cells were incubated for 24 h, after which the medium was removed and 100 μL of STB reagent solution in basal medium (1:10) was added to each well. Cells were incubated in a CO_2 incubator for 2 h at 37°C and solution fluorescence measurements using Fluoroscan Ascent reader (Thermo Fisher Scientific Inc., USA) were performed. The data were normalized as a percentage relative to the control, i.e. fluorescence intensity of solutions of wells containing cells incubated without test substances. Values of the concentration at which half of the cells survive (IC_{50}) were calculated from the obtained sigmoidal curve using OriginPro 8 program.