Available online at www.sciencedirect.com

ScienceDirect

Mendeleev Commun., 2024, **34**, 365–368

Mendeleev Communications

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

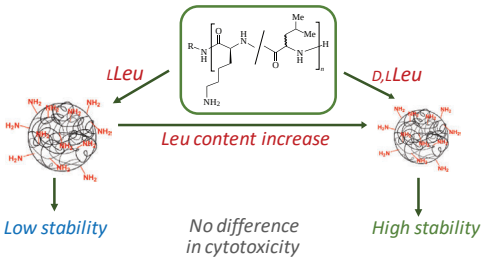
Evgenia G. Korzhikova-Vlakh,*[a,b] Natalia N. Zashikhina,[a] Evgeniia G. Stulova,[b] Apollinariia Yu. Dzhuzha[a] and Viktor A. Korzhikov-Vlakh[b]

[a] *Institute of Macromolecular Compounds, Russian Academy of Sciences, 199004 St. Petersburg, Russian Federation. E-mail: vlakh@hq.macro.ru*

[b] *Institute of Chemistry, St. Petersburg State University, 198584 St. Petersburg, Russian Federation*

DOI: 10.1016/j.mencom.2024.04.017

Copolymers of L-lysine and L/D,L-leucine differing in amino acid composition were synthesized and characterized for their self-assembly into nanoparticles. The effect of polypeptide composition on critical aggregation concentration, hydrodynamic diameter of nanoparticles, their stability and cytotoxicity was explored and discussed.



Keywords: copolymers, lysine, leucine, amphiphilic polypeptides, self-assembly, critical aggregation concentration, nanoparticles.

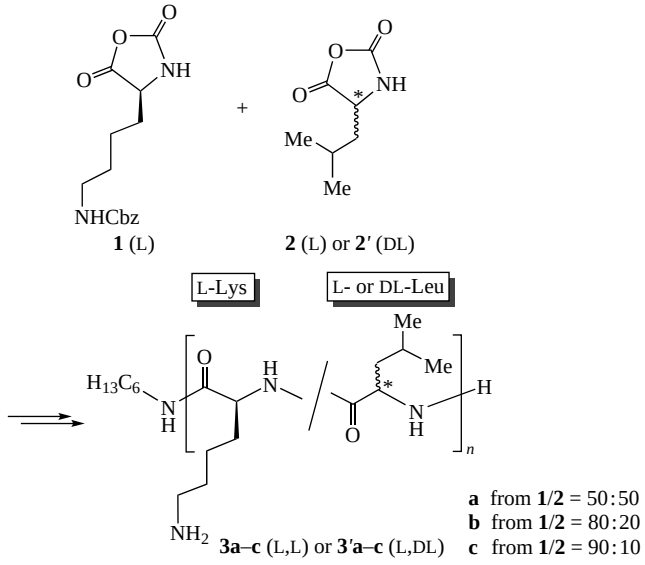
Today, amphiphilic copolymers capable of self-assembly are of great interest as polymeric systems for the delivery of small drugs, peptides and nucleic acids.[1,2] Amphiphilic block-, graft- and random copolymers in aqueous media can form a variety of morphologies such as polymeric spherical and worm-like micelles, polymersomes and vesicles as well as nanogels.[3–5] Among the existing synthetic copolymers, amphiphilic poly(amino acids) or polypeptides with random sequence have a number of advantages. These include biodegradability down to non-toxic natural metabolites as amino acids, biocompatibility, diversity of natural and non-natural amino acids with multiple side-chain functionality, as well as the ability to form secondary structures (α-helixes and β-sheets).[6,7]

Recently, the formation of nanoparticles from amphiphilic block-copolymers based on polyserine-*b*-polyphenylalanine,[8] polylysine-*b*-polyphenylalanine,[9] or polyserine-*b*-poly(glutamic acid)[10] and random copolymers based on poly(lysine-*co*-phenylalanine),[11] poly(lysine-*co*-2-aminoisobutyric acid),[12] or poly(glutamic acid-*co*-phenylalanine)[13] have been reported. The most convenient and controlled route to synthesize such polypeptides is ring opening polymerization (ROP) of *N*-carboxyanhydrides (NCAs) of α-amino acids with the use of primary amines as initiators.[7,14–16] Amphiphilic block-copolymers would undergo self-assembling into polymeric micelles or polymersomes[7] while amphiphilic random copolymers form physically stabilized nanogels.[10]

Polypeptide nanoparticles are widely considered as delivery systems for substances with different physicochemical properties: small drugs such as cytostatic and anti-inflammatory drugs,[17–20] peptides,[13] and nucleic acids.[21–23] The lysine-containing polypeptides proved to be the efficient systems for delivery of various genes, *e.g.* pDNA, mRNA, siRNA.[21–25] Recently, we have reported the efficient intracellular delivery of mRNA using nanoparticles self-assembled from amphiphilic copolymer of lysine and isoleucine.[24]

The aim of this study was the synthesis and characterization of the amphiphilic copolymers of L-lysine (LLys) and L or D,L-leucine (LLeu or D,LLeu) containing different ratios of the amino acids. Special attention was also paid to the influence of the chirality of hydrophobic amino acid (LLeu or D,LLeu) on copolymer self-assembly and properties of the nanoparticles.

Synthesis of copolymers of LLys and L- or D,LLeu was performed by ROP of ε-benzyloxycarbonyl-L-lysine NCA **1** with either L- or D,L-leucine NCAs **2** or **2′** (Scheme 1) in 1,4-dioxane (4 wt%) at 25 °C for 48 h with the use of *n*-hexylamine as initiator (for synthetic details and product



Scheme 1

characterization, see Online Supplementary Materials). The initial **1**/**2** ratios were set as 50 : 50, 80 : 20 and 90 : 10 (mol%). The monomers/*n*-$C_6H_{11}NH_2$ ratios were equal to 1 : 100. The yields of copolymers were in the range of 62–86%. At this step, copolymers containing Cbz protective group at lysine units were hydrophobic and could be analyzed for their molecular weights and dispersity by size-exclusion chromatography (SEC) in DMF containing 0.1 M LiBr at 60 °C. According to SEC analysis, the number average molecular weight (M_n) and dispersity ($Đ$) of copolymers of LLys^{Cbz} and LLeu obtained at monomer ratios of 50 : 50, 80 : 20 and 90 : 10 were: M_n in the range of 14500–24700; $Đ$ in the range of 1.20–1.31. For copolymers of LLys^{Cbz} and D,LLeu, they were: M_n in the range of 21900–37800; $Đ$ in the range of 1.21–1.36 (see Online Supplementary Materials, Table S1). In both cases, the molecular weight of copolymers increased with increasing Lys content in the polymerization mixture due to the differences in molecular weights of the monomers since M(Lys^{Cbz}) ≈ 2 M(Leu). The dispersity of the copolymer samples was almost independent of the chirality of hydrophobic amino acid, while the molecular weight of copolymers was higher when racemic Leu NCA **2′** was used.

The composition of final copolymers **3a–c**/**3′a–c** was determined by the quantitative HPLC-MS analysis for amino acids obtained after acidic removal of the Cbz protective groups (Figure 1). Regardless of whether LLeu or D,LLeu was used for the synthesis, all copolymers **3**/**3′** were enriched with this hydrophobic amino acid. However, a more pronounced effect was observed when enantiopure LLeu was used at monomer content of 50 and 20 mol%.

After deprotection, copolymers **3a–c**/**3′a–c** become amphiphilic and capable of self-assembling in aqueous media. In this study, nanoparticles were obtained *via* gradient phase inversion (dialysis) method during purification of deprotected copolymers, lyophilization and storage at 4 °C. Before use, a weighed sample of polymer nanoparticles was redispersed under short ultrasound treatment (10–20 s) in deionized water or buffer solution depending on the goal.

First, the effect of Leu content and chirality on the hydrodynamic diameter (D_H) and polydispersity index (PDI) of the self-assembled nanoparticles was evaluated. Despite using LLeu or D,LLeu, the hydrodynamic diameter was found to decrease with increasing Leu content [Figure 2(*a*)]. An increase in hydrophobicity leads to a lower solubility of the copolymer in water, and consequently to stronger hydrophobic interactions and the formation of more compact structures. In addition, partial replacement of LLeu with DLeu in copolymer resulted in a dramatic decrease in the hydrodynamic diameter of the self-assembled polypeptide structures. It is known that the self-organization of (poly)peptides and the properties of the formed structures depend not only on their amino acid composition, but also on the chirality of the amino acids included in the (poly)-

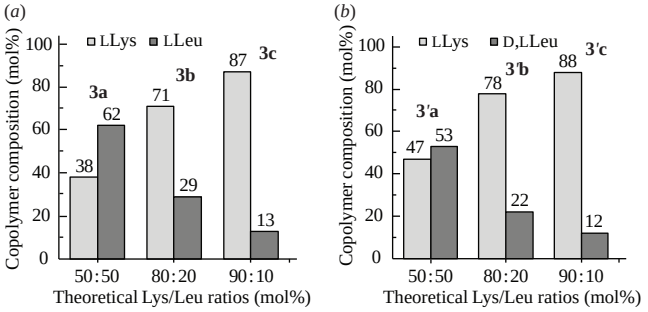


Figure 1 Composition of random copolymers (*a*) **3a–c** of L-lysine with L-leucine and (*b*) **3′a–c** of L-lysine with D,L-leucine synthesized at different ratios of monomers **1** and **2**/**2′**.

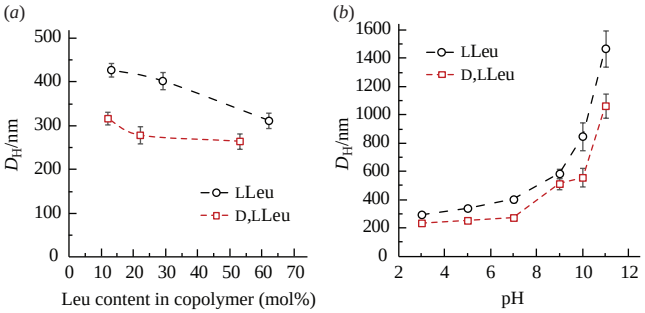


Figure 2 Dependence of hydrodynamic diameter of nanoparticles based on copolymers **3a–c**/**3′a–c** of L-lysine and L/D,L-leucine on (*a*) content of hydrophobic amino acid (0.01 M phosphate buffer, pH 7.4) and (*b*) pH value (water, pH is adjusted with 0.01 M HCl/NaOH solution, intermediate copolymer compositions).

peptides.[26,27] For example, the study of the L- and D-stereoisomers of amyloid peptides showed that the formation of amyloid fibrils was L-stereospecific. Moreover, mixing of enantiomeric peptides altered the morphology and mechanical properties of the self-assembled peptide systems.[26,27] Used in this study LLeu belongs to the amino acids responsible for the formation of the β-folded secondary structure of proteins and polypeptides.[28] Thus, the differences in the hydrodynamic diameters of nanoparticles formed from self-assembled amphiphilic copolymers of close composition based on LLys and LLeu or D,LLeu may be related to the different conformation of polypeptides.

PDI values for nanoparticles formed from LLeu-derived **3a–c** and those derived from D,LLeu **3′a–c** were in the range of 0.27–0.36 and 0.26–0.28, respectively. According to the literature, nanoparticles with PDI values up to 0.3 are considered acceptable as drug delivery systems.[29–31] As expected, the values of zeta-potential of the surface of nanoparticles were positive and lied in the range of 48–62 mV. For both series of polypeptides, zeta-potential values increased with increasing lysine content in the copolymer (Table S2).

The study of hydrodynamic diameter of nanoparticles depending on pH of the medium showed some influence of the pH on the characteristics of nanoparticles [see Figure 2(*b*)]. In the case of LLeu-based nanoparticles **3a–c**, the change in pH from 3 to 9 was accompanied by an increase in the hydrodynamic diameter of the nanoparticles from 200 to 600 nm. When the pH reached values of above 10, a pronounced aggregation of nanoparticles was detected. At the same time, nanoparticles **3′a–c** based on racemic Leu did not practically change their size until pH 7 and started pronounced aggregation at pH ⩾8. The results obtained are in agreement with the known properties of Lys (pK_a of ε-amino group of Lys is 10.5) supported by a decrease in surface zeta-potential values (see Online Supplementary Materials, Figure S1).

In addition, the critical aggregation concentration (CAC) values for self-assembly of amphiphilic copolymers of different composition were determined by conductometry method (Figure S2). Figure 3 summarizes the obtained results. For the copolymers under study, the CAC values were in the range of 11.7–22.9 μg ml^{-1} that corresponded to 0.6–1.5 μM. For the series of copolymers containing LLeu **3** and D,LLeu **3′**, molar CAC values demonstrated the same tendency, namely, they decreased with increasing content of hydrophobic leucine in the copolymer. This is in agreement with the reported data for self-assembly of other amphiphilic copolymers.[32,33]

The stability of nanoparticles based on the synthesized copolymers was investigated in two model media: (1) 0.01 M phosphate buffer solution, pH 7.4, and (2) 0.01 M phosphate buffer solution, pH 7.4, with the addition of papain, an enzyme with broad substrate specificity and active at the pH range of

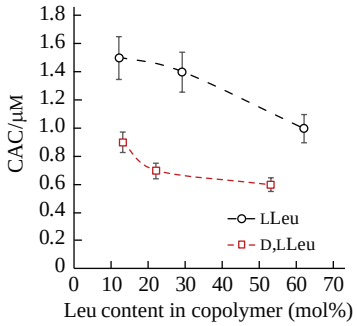


Figure 3 Trends of changes in molar CAC values depending on the hydrophobic leucine in copolymer of L-lysine with L/D,L-leucine (**3**/**3′**).

5.0–7.5 (see Online Supplementary Materials, section S6). Stability was evaluated by incubating the nanoparticle dispersion at 37 °C for 30 days and measuring the hydrodynamic diameter of the particles at specified time intervals (Figure 4). In buffer medium, a gradual aggregation of nanoparticles based on polymers **3a–c** was observed after 10 days of incubation. Addition of the enzyme to the buffer medium promoted a sharp aggregation of **3a–c** particles due to active degradation of hydrophilic fragments leading to the exposure of the hydrophobic fragment to the aqueous solution and, as a consequence, aggregation of nanoparticles. Complete degradation of **3a–c** particles was observed after 20 days. At the same time, semi-epimeric **3′a–c** nanoparticles were stable in buffer solution and much more stable in enzyme-containing medium compared to their fully L-analog. The results obtained are in agreement with the literature data. For example, the replacement of L-amino acids with their D-stereoisomers in the KKVVVFKVFKVKFKKK† peptide affected the secondary structure, stability, and antimicrobial activity of the peptide. In particular, amino acid substitutions for D-stereoisomers in the middle of the amino acid sequence disrupted the alpha-helix structure resulting in complete loss of peptide activity. At the same time, the stability of the peptide in serum significantly increased due to the substitution of L-amino acids into D-stereoisomers.[34]

Finally, the cytotoxicity of the obtained nanoparticles was investigated in normal cells, namely, human embryonic kidney cells (HEK293) and human lung epithelial cells (BEAS-2B) (see Online Supplementary Materials, section S7). In all cases, the incubation time of nanoparticles with cells was 24 h. From both series, copolymers with maximum leucine **3a** and **3′a** content were tested. The calculated values of half-maximal inhibition concentration IC_{50} for the tested samples are summarized in

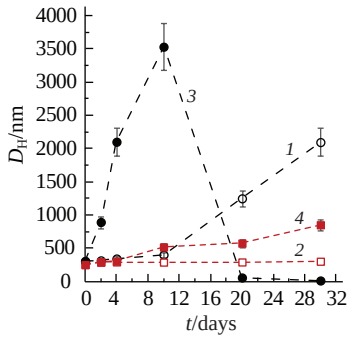


Figure 4 Stability of nanoparticles based on copolymer samples of L-lysine and L/D,L-leucine (**3a**/**3′a**) on time of incubation (37 °C) in 0.01 M sodium phosphate buffer solution (pH 7.4): (*1*) **3a**, (*2*) **3′a**, (*3*) **3a** + papain and (*4*) **3′a** + papain.

† Standard one-letter abbreviations used to represent peptide sequences: K – lysine, V – valine, F – phenylalanine.

Table 1 Cytotoxicity of neat nanoparticles based on different copolymers **3a**/**3′a** of L-lysine and L/D,L-leucine and those covered with heparin (24 h) in HEK293 and BEAS-2B cell lines.[a]

Sample	D_H/nm	Zeta-potential/mV	IC_{50}/μg ml^{-1}	
			HEK293	BEAS-2B
3a	312 ± 38	48 ± 2	53 ± 7	33 ± 3
3′a	265 ± 22	55 ± 1	61 ± 3	38 ± 2
3a/heparin	570 ± 58	–22 ± 1	>1000	>1000
3′a/heparin	456 ± 51	–26 ± 1	>1000	>1000

[a] Characteristics were measured by DLS and ELS (H_2O, 25 °C).

Table 1. For polypeptides containing LLeu or D,LLeu, the IC_{50} values for the corresponding cells were comparable and indicated cytotoxicity of the tested cationic nanoparticles. In general, the values obtained are in agreement with the known properties of positively charged polypeptide nanoparticles enriched with Lys and/or Arg. The IC_{50} values determined using normal and cancer cells were found to be dependent on the size and composition of the cationic polypeptide nanoparticles and lay in the range of 15–80 μg ml^{-1} (see refs. 7, 35, 36). Despite this fact, the cationic nature of the polypeptides is necessary to provide efficient binding of nucleic acids or other negatively charged drugs. Moreover, cationic amphiphilic (poly)peptides are known to possess antimicrobial properties.[7,34] At the same time, if necessary, the positive charge of the nanoparticle surface can be shielded by coating them with negatively charged polymers, *e.g.*, carrageenan, heparin.[11] Indeed, covering of polypeptide **3a**/**3′a** nanoparticles with heparin allowed one to recharge the particle surface and avoid the cytotoxicity of nanoparticles.

In conclusion, increasing leucine content and partial replacement of LLeu by DLeu to their racemic mixture in the copolymers with LLys leads to self-assembly into more compact nanoparticles in aqueous media. Moreover, nanoparticles formed from copolymers of LLys with DLLeu demonstrated higher stability both in buffer and enzyme-containing media without apparent changes in biological properties *in vitro*.

This study was supported by Russian Science Foundation (grant no. 21-73-20104). HPLC-MS analysis was carried out in Chemical Analysis and Materials Research Centre of the Research Park of Saint-Petersburg State University.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2024.04.017.

References

1 F. Perin, A. Motta and D. Maniglio, *Mater. Sci. Eng., C*, 2021, **123**, 111952.
2 A. M. Bodratti and P. Alexandridis, *Expert Opin. Drug Delivery*, 2018, **15**, 1085.
3 K. Kita-Tokarczyk, J. Grumelard, T. Haefele and W. Meier, *Polymer*, 2005, **46**, 3540.
4 I. Jarak, M. Pereira-Silva, A. C. Santos, F. Veiga, H. Cabral and A. Figueiras, *Appl. Mater. Today*, 2021, **25**, 101217.
5 Y. Sun, X. Lyu, Z. Li and Y. Huang, *Polymer*, 2017, **112**, 325.
6 Z. Li, Y. Zheng, J. Yan, Y. Yan, C. Peng, Z. Wang, H. Liu, Y. Liu, Y. Zhou and M. Ding, *ChemBioChem*, 2023, **24**, e202300132.
7 M. Stepanova, A. Nikiforov, T. Tennikova and E. Korzhikova-Vlakh, *Pharmaceutics*, 2023, **15**, 2641.
8 Z. Zhao, Y. Wang, J. Han, K. Wang, D. Yang, Y. Yang, Q. Du, Y. Song and X. Yin, *Int. J. Nanomed.*, 2014, **9**, 5849.
9 X. Zhou, X. Su and C. Zhou, *Eur. Polym. J.*, 2018, **100**, 132.
10 N. N. Sudareva, I. I. Tarasenko, D. N. Suslov, O. M. Suvorova, K. A. Kolbe, G. Y. Yukina, M. L. Tyndyk, Yu. G. Zmitrichenko and E. G. Korzhikova-Vlakh, *Mendeleev Commun.*, 2024, **34**, 18.

11 N. Zashikhina, S. Gladnev, V. Sharoyko, V. Korzhikov-Vlakh, E. Korzhikova-Vlakh and T. Tennikova, *Int. J. Mol. Sci.*, 2023, **24**, 3702.
12 I. Tarasenko, N. Zashikhina, I. Guryanov, M. Volokitina, B. Biondi, S. Fiorucci, F. Formaggio, T. Tennikova and E. Korzhikova-Vlakh, *RSC Adv.*, 2018, **8**, 34603.
13 N. N. Sudareva, O. M. Suvorova, I. I. Tarasenko, N. N. Saprykina, N. V. Smirnova, S. G. Petunov, A. S. Radilov, A. S. Timin, E. G. Korzhikova-Vlakh and A. D. Vilesov, *Mendeleev Commun.*, 2020, **30**, 25.
14 Z. Song, Z. Han, S. Lv, C. Chen, L. Chen, L. Yin and J. Cheng, *Chem. Soc. Rev.*, 2017, **46**, 6570.
15 G. J. M. Habraken, M. Peeters, C. H. J. T. Dietz, C. E. Koning and A. Heise, *Polym. Chem.*, 2010, **1**, 514.
16 I. V. Averianov, M. A. Stepanova, I. V. Gofman, A. Lavrentieva, V. A. Korzhikov-Vlakh and E. G. Korzhikova-Vlakh, *Mendeleev Commun.*, 2022, **32**, 810.
17 N. Zashikhina, E. Gandalipov, A. Dzhuzha, V. Korzhikov-Vlakh and E. Korzhikova-Vlakh, *J. Microencapsulation*, 2023, **40**, 1.
18 S. Lv, Z. Tang, M. Li, J. Lin, W. Song, H. Liu, Y. Huang, Y. Zhang and X. Chen, *Biomaterials*, 2014, **35**, 6118.
19 S. S. Desale, S. M. Raja, J. O. Kim, B. Mohapatra, K. S. Soni, H. Luan, S. H. Williams, T. A. Bielecki, D. Feng, M. Storck, V. Band, S. M. Cohen, H. Band and T. K. Bronich, *J. Controlled Release*, 2015, **208**, 59.
20 X. Li, J. Liu, H. Chen, Y. Chen, Y. Wang, C. Y. Zhang and X.-H. Xing, *Green Chem. Eng.*, 2023, **4**, 173.
21 O. Korovkina, D. Polyakov, V. Korzhikov-Vlakh and E. Korzhikova-Vlakh, *Molecules*, 2022, **27**, 8495.
22 M. Thompson and C. Scholz, *Nanomaterials*, 2021, **11**, 1119.
23 C. He, X. Zhuang, Z. Tang, H. Tian and X. Chen, *Adv. Healthcare Mater.*, 2012, **1**, 48.
24 I. Pilipenko, O. Korovkina, N. Gubina, V. Ekimova, A. Ishutinova, E. Korzhikova-Vlakh, T. Tennikova and V. Korzhikov-Vlakh, *Int. J. Mol. Sci.*, 2022, **23**, 5363.
25 G. X. Zhao, H. Tanaka, C. W. Kim, K. Li, D. Funamoto, T. Nobori, Y. Nakamura, T. Niidome, A. Kishimura, T. Mori and Y. Katayama, *J. Biomater. Sci., Polym. Ed.*, 2014, **25**, 519.
26 H. Wadai, K. Yamaguchi, S. Takahashi, T. Kanno, T. Kawai, H. Naiki and Y. Goto, *Biochemistry*, 2005, **44**, 157.
27 D. Gupta, R. Sasmal, A. Singh, J. P. Joseph, C. Miglani, S. S. Agasti and A. Pal, *Nanoscale*, 2020, **12**, 18692.
28 K. Fujiwara, H. Toda and M. Ikeguchi, *BMC Struct. Biol.*, 2012, **12**, 18.
29 S. Zhang and C. Wang, *Nano-Struct. Nano-Objects*, 2023, **35**, 100994.
30 M. Danaei, M. Dehghankhold, S. Ataei, F. Hasanzadeh Davarani, R. Javanmard, A. Dokhani, S. Khorasani and M. Mozafari, *Pharmaceutics*, 2018, **10**, 57.
31 E. J. Cho, H. Holback, K. C. Liu, S. A. Abouelmagd, J. Park and Y. Yeo, *Mol. Pharm.*, 2013, **10**, 2093.
32 Z. Jiang, I. Blakey and A. K. Whittaker, *Polym. Chem.*, 2017, **8**, 4114.
33 H. Tan, J. Mao, W. Zhang, B. Yang, X. Yang, Y. Zhang, C. Lin, J. Feng and H. Zhang, *Polymers*, 2020, **12**, 955.
34 S. Y. Hong, J. E. Oh and K.-H. Lee, *Biochem. Pharmacol.*, 1999, **58**, 1775.
35 L. Wen, A. Gao, Y. Cao, F. Svec, T. Tan and Y. Lv, *Macromol. Rapid Commun.*, 2016, **37**, 551.
36 J. Qu, S. Peng, R. Wang, S.-T. Yang, Q.-H. Zhou and J. Lin, *Colloids Surf., B*, 2019, **181**, 315.

Received: 12th January 2024; Com. 24/7367