

Biodegradable polysarcosine with inserted alanine residues: Synthesis and enzymolysis

Peng Zhou¹, Tianlun Shen^{1,2}, Wanli Chen³, Jihong Sun², Jun Ling^{1,*}

¹ MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China.

² Department of Radiology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310016, China.

³ Center of Analysis & Measurement, Zhejiang University of Technology, Hangzhou 310014, China.

* Corresponding author: lingjun@zju.edu.cn

Abstract: Polysarcosine (PSar), a water-soluble polypeptoid, is gifted with biodegradability *via* random ring-opening copolymerization of sarcosine- and alanine-*N*-thiocarboxyanhydrides catalyzed by acetic acid in controlled manners. Kinetic investigation reveals the copolymerization behavior of the two monomers. The random copolymers, named PAS, with high molecular weights between 22.0 and 43.6 kg/mol and tunable Ala molar fractions varying from 6% to 43% are able to be degraded by porcine pancreatic elastase within 50 days in mild conditions (pH=8.0 at 37 °C). Both the biodegradation rate and water solubility of PAS depend on the content of Ala residues. PAS with Ala fractions below 43% are soluble in water while the one with 43% Ala self-assembles in water into nanoparticles. Moreover, PAS are non-cytotoxic at the concentration of 5 mg/mL. The biodegradability and biocompatibility endow the Ala-containing PSar with potential to replace PEG as protective shield in drug-delivery.

Key words: Poly(alanine-*r*-sarcosine), biodegradability, biocompatibility, alanine-NTA, sarcosine-NTA.

Precision pharmacotherapy *in vivo* is becoming a popular and promising research field where drug-delivery method plays a vital role in sending drugs to nidus.¹⁻² Since some pharmaceuticals or medicinal proteins are hydrophobic, it is necessary to modify the hydrophobes with hydrophiles in order to increase their water solubility and hydrodynamic diameters, thereby impeding their self-aggregation or interaction with cells in blood.³⁻⁴ In addition, the protective outerwear is able to prevent drugs from antibodies that will result in unsuccessful therapy to the most extent.⁴

Poly(ethylene glycol)s (PEG) are artificial and non-ionic hydrophilic polymers, which are widely used in biomedicine by covalent or non-covalent conjugation to stabilize drugs during transport.⁵⁻¹¹ The first two complexes of proteins with PEG were PEGylated albumin and liver catalase which exhibited non-immunogenicity and extension of circulation time in blood while maintaining bioactivity of the proteins.¹²⁻¹³ Later, microspheres such as poly(lactide acid-*r*-glycolic acid) and liposomes were modified with PEG to prolong their circulating life *in vivo*.¹⁴⁻¹⁷ PEG served as a shield to keep ionic substances such as DNA and poly(L-lysine) away from blood or organs to minimize toxicity.¹⁸⁻¹⁹ While PEGs with high molecular weight (MW) up to 50 kg/mol are favored for loading small molecules to improve enhanced permeability and retention effect as well as stealth effect, the excretion of high MW PEG should be taken into account.¹¹ Non-biodegradability is another characteristic of high MW PEG since PEG can do harm to body due to accumulation.^{7, 11, 20} Besides, PEGylated drugs have been revealed to induce immunogenicity in recent years, which will have serious impact on patients.²¹⁻²³ It calls for a substitution that works as well as PEG but also covers the shortages of PEG. However, not all the non-ionic hydrophilic polymers are qualified to replace PEG.

Polysarcosine (PSar), *i.e.* poly(*N*-methyl glycine), is an excellent candidate.^{11, 24-26} PSar is made of sarcosine, the endogenous amino acid in muscle tissues, and is mimetic to polyalanine but is highly water soluble.²⁷⁻²⁸ The methyl substituted amide group becomes the key factor that PSar displays limited affinity to protein, therefore prohibiting thrombus.²⁹⁻³¹ The absence of H-bond donor also endows PSar with non-immunogenicity because H-bond donor seems to be essential for reacting with

antibody.³²⁻³⁴ Many researches have been reported in recent years that the biocompatible, non-cytotoxic and stealth-like PSar does well in biomedicine.^{25-26, 35-40} With the same problem as PEG has, PSar is non-biodegradable and there has been no successful enzymolysis of PSar so far, indicating the excretion of PSar is non-negligible.⁴¹ Another methylated glycine, L-alanine (Ala), is one of the native amino acids as an isomer of sarcosine. Polyalanine (PAla) is digestible by enzyme.⁴²⁻⁴³ However, PAla is not soluble in water and most organic solvents because of its secondary structures of α -helix and β -sheet folding, which limits its biomedical application. In contrast, PSar is soluble in water, alcohol, *N,N*-dimethylformamide, chloroform, acetonitrile, *etc.* We propose that PSar containing randomly distributed Ala residues inside the backbone, *i.e.* random copolymer of poly(Ala-*r*-Sar) will evolve biodegradability to serve as a perfect drug coat. On the other hand, with the incorporation of sarcosine residue in the PAla backbone, *i.e.* poly(Ala-*r*-Sar) with high fraction of Ala residues, the solubility of PAla will be certainly improved. The only report of copolymer of sarcosine and alanine is back to 1978 when Kricheldorf investigated the copolypeptide by the polymerization of Ala- and Sar-*N*-carboxyanhydrides without the information of copolymerization behavior and properties of the copolymers.⁴⁴

Nowadays, ring-opening polymerization (ROP) of α -amino acid *N*-thiocarboxyanhydrides (NTA) becomes a fashionable way to synthesize tailored polypeptides or polypeptoids with high MW. Although Kricheldorf *et al.* found NTA and *N*-substituted NTA (NNTA) were not promising to synthesize high MW polypeptides and polypeptoids because of physical death and stabilization of thiocarbonate group by solvation, respectively,⁴⁵ there has been many efforts paving the way for well-controlled ROP of NTA monomers.^{37, 46-58} Acetic acid (HAc) catalyzes polymerizations of NTA⁵⁴ and NNTA⁵⁵ to overturn common sense to the most extent because researchers used to think that HAc would inactivate the amino groups at the polymer chain end and impede propagation. Nevertheless, to the best of our knowledge, the copolymerization of NTAs and NNTAs has not been reported yet. The random copolymerization of Ala-NTA and Sar-NTA is well worth investigating not only to

understand the copolymerization behaviors but also to fabricate biodegradable PSar.

To investigate the syntheses of random copolymers of poly(Ala-*r*-Sar), abbreviated as PAS, we start with the homo-polymerization of Ala-NTA. The polymerization of Ala-NTA initiated by benzylamine (BA) in MeCN solution (Sample 1 in Table 1) encounters physical death of active chain end⁴⁵ and yields white precipitation within half an hour. The white solid with high dispersity (\mathcal{D}) above 2 is characterized by ¹H NMR (Figure S2A). The copolymerization of Ala-NTA and Sar-NTA still yields precipitation but becomes more controllable than homo-polymerization of Ala-NTA. Both the MeCN-insoluble and MeCN-soluble parts (Sample 2A and 2B in Table 1) contain both Ala and Sar residues according to ¹H NMR analyses (Figure S2). These phenomena evidence that Ala-NTA is able to copolymerize with Sar-NTA in a more controllable way than homo-polymerization of Ala-NTA. The following content improves the controllability of copolymerization.

Table 1. Polymerization of Ala-NTA and Sar-NTA ^a

Sample	Ala: Sar: BA: HAc ^b	Solvent	Temp. (°C)	Time (h)	Yield (%)	Number ^c		Ala (%) ^d	M_n ^e (kg/mol)	\mathcal{D} ^e
						Ala	Sar			
1	15:0:1:0	MeCN	25	42	>99	10	0	-	1.4	2.05
2A ^f	15:17:1:0	MeCN	25	42	>99	15	13	54	3.2	1.26
2B ^f	15:17:1:0	MeCN	25	42	>99	9	11	45	2.7	1.36
3	13:32:1:90	MeCN	25	43	87.6	12	27	31	5.3	1.13
4	50:48:1:200	MeCN	25	137	97.6	48	49	49	11.8	1.09
5	10:90:1:200	MeCN	35	42	85.7	8	70	10	11.9	1.10
6	100:100:1:400	DMAc	60	66	87.2	71	94	43	22.0	1.08
7	53:447:1:1000	DMAc	60	48	82.1	26	391	6	43.6	1.25
8	0:500:1:1000	DMAc	60	48	84.8	0	460	-	50.9	1.19

^a Homo- or copolymerization of Ala-NTA and Sar-NTA. The initial total NTA concentration is 0.6 mol/L. ^b The molar ratios of initial feeds. ^c The number of amino acid residues in polymers, calculated by ¹H NMR. ^d Molar fraction of Ala residues in copolymers. ^e Determined by SEC. ^f Products in precipitation (2A) and solution (2B) are characterized separately.

The copolymerizations of Ala-NTA with Sar-NTA are living and controlled promoted by HAc. No precipitation is observed during the polymerization in the presence of HAc (Figure 1A and Sample 3 in Table 1). Sample 3 contains both Ala and Sar residues (Figure 1B) whose proton signals locate at 1.02~1.39 ppm (methyl groups of Ala residues), 2.64~3.12 ppm (methyl groups Sar residues) and 3.63~4.90 ppm (α -H). The number of Ala and Sar residues in copolymer is close to the initial ratio of [NTA]:[BA], and the SEC curve (Figure 1D) exhibits a symmetrically unimodal peak and narrow dispersity ($D = 1.13$). The identical diffusion coefficient ($-10.6 \text{ m}^2/\text{s}$) of the protons in both Sar and Ala residues is detected in DOSY NMR spectrum (Figure 1C), further confirming the involvement of two residues in the same backbone. The MALDI-ToF MS of Sample 3 shows a monomodal distribution, and every population is attributed to poly(methyl glycine) carrying BA chain-end and a cation (Figure 1E and 1F). With the initial [NTA]:[BA] ratio of 100 (Sample 4 and 5 in Table 1), excellent controllability of polymerization, quantitative yield and no precipitation are observed, producing PAS copolymer with high fraction of Ala residues (49%) and low D of 1.09 (Sample 4 in Table 1).

^{13}C NMR, HSQC and HMBC are employed to examine the random architecture of Sample 4. Firstly, ^{13}C NMR and HSQC help to determine alkyl carbons of polymer chain (Figure 1G and S3). The carbon signals of methyl groups of Ala (C_a) and Sar (C_d) residues are located in 18.0 ppm and 35.2 ppm, respectively, while the signals in 44.6 ppm (C_b) and between 47.3~51.9 ppm (C_c) are attributed to α -carbons. Secondly, the correlation signals of H_e-C_1 , H_d-C_4 , H_d-C_3 and H_e-C_2 in HMBC spectrum demonstrate the existence of Ala-Ala, Sar-Ala, Sar-Sar and Ala-Sar diads (Figure S4A, B, C and D, respectively). These carbonyl groups of four diads in ^{13}C NMR spectrum (Figure 1G) are distinguished according to the carbonyl signals of four model molecules of *N*-ethyl acetamide, *N,N*-dimethyl acetamide, *N,N*-dimethyl propionamide and *N*-ethyl propionamide which locate at 168.8, 169.6, 172.6 and 172.5 ppm, respectively (Figure S5).

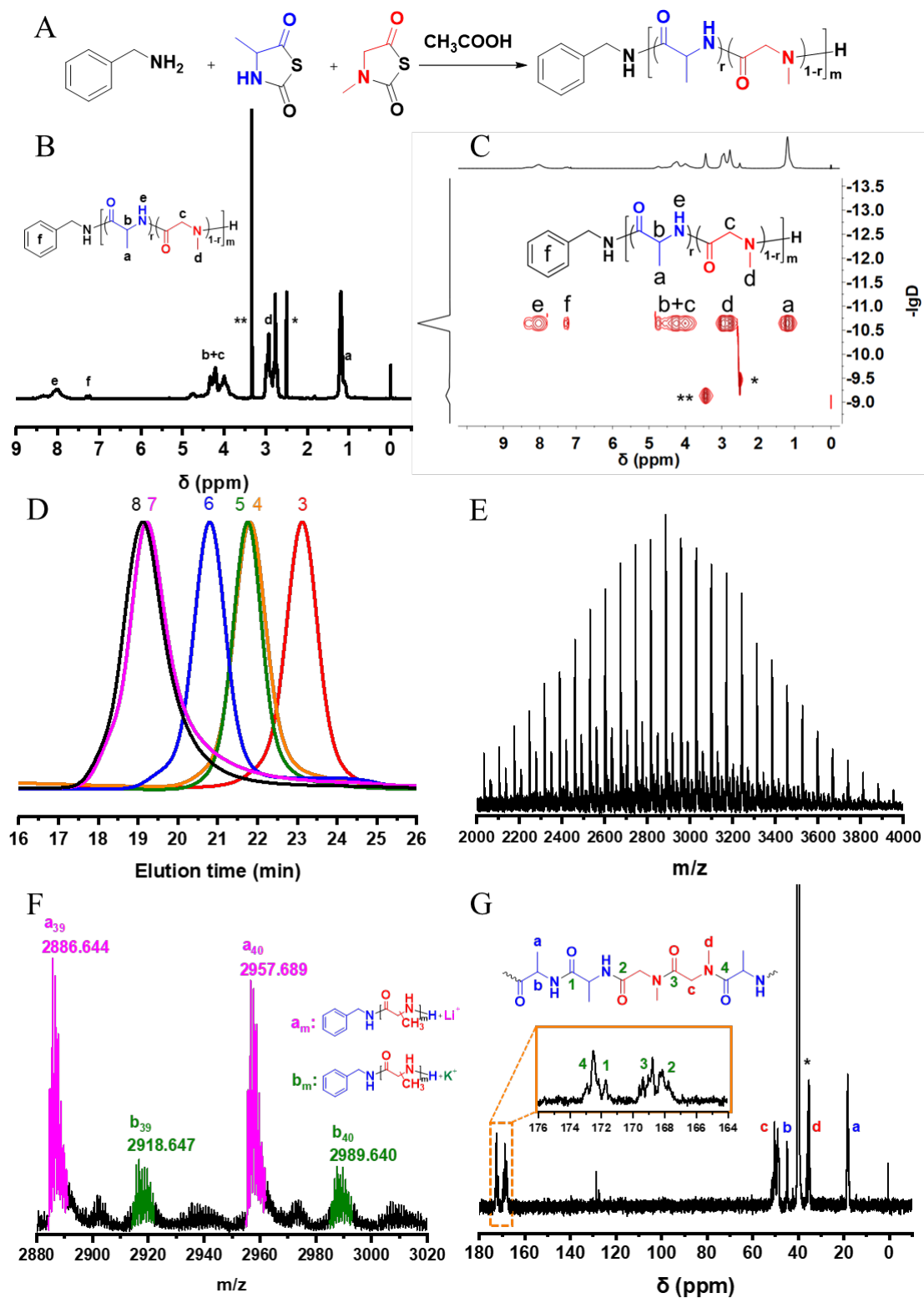


Figure 1. (A) Synthesis of PAS by the random copolymerization of Ala-NTA with Sar-NTA. (B) ^1H NMR and (C) DOSY spectra of PAS where * and ** represent DMSO and water, respectively. (D) SEC traces of copolymers and homo-PSar with the same serial numbers in Table 1. (E) MALDI-ToF MS of Sample 4 in Table 1 with (F) a zoom-in

view and corresponding structures. (G) ^{13}C NMR spectrum with a zoom-in view of PAS, *: DMSO.

The copolymerization kinetics are investigated and the time-dependent ^1H NMR spectra are shown in Figure S6. Both Ala-NTA and Sar-NTA exhibit pseudo-first-order kinetics (Figure S7). The linear $\ln([M]_0/[M]_t)$ versus time plots reveal the livingness of polymerization and determine the comparable apparent polymerization rate constants (k) of Sar-NTA ($3.66 \times 10^{-3} \text{ min}^{-1}$) and Ala-NTA ($2.02 \times 10^{-3} \text{ min}^{-1}$). The molar fractions of the two amino acid residues in PAS keep close to 50% during copolymerization (Figure S8), indicating a random distribution of Ala and Sar residues along the backbone.

We synthesize PAS with high MW (22.0~50.9 kg/mol) and narrow D (within 1.25) containing Ala residues of 0%, 6% and 43% (Sample 8, 7 and 6 in Table 1 and Figure 1D, respectively) and explore their degradation behaviors in the presence of enzyme. The mixtures of 7 mg PAS with 70 mg porcine pancreatic elastase (PPE) in 2 mL phosphate buffered saline are kept under 37 °C for 0, 25 and 50 days. Not surprisingly, PSar homopolymer (Sample 8 in Table 1) cannot be digested by PPE after 50 days (Figure 2A). On the contrary, the SEC peak of the PAS containing 6% Ala (Sample 7 in Table 1) shifts to lower MW side, and the molecular weight at peak (M_p) decreases by 8% after enzymolysis over 50 days (Figure 2B). It is more obvious that the PAS with 43% Ala (Sample 6 in Table 1) is completely digested within 50 days since there is no detectable PAS in SEC (Figure 2C). The introduction of Ala residues in PAS backbone enables its biodegradability by PPE in mild condition, and the degradation rate increases with the content of Ala residues.

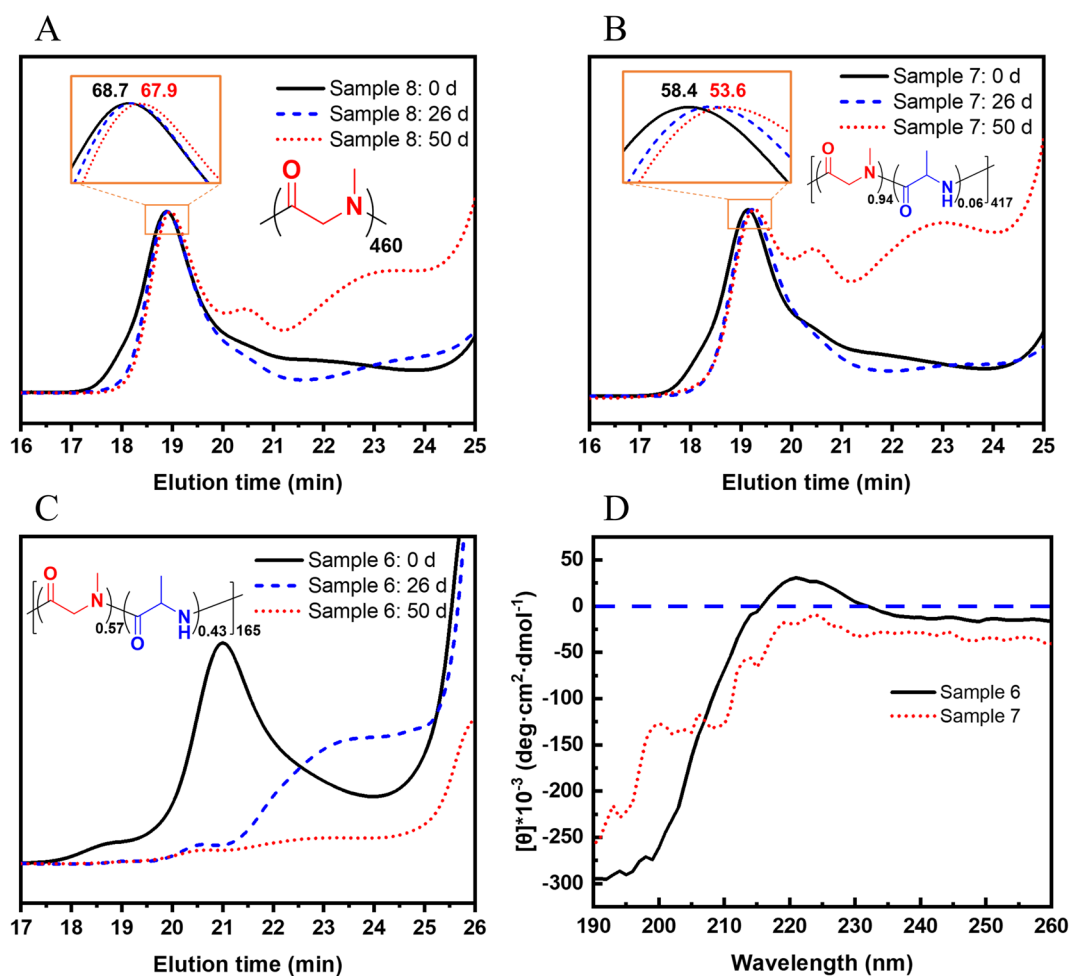


Figure 2. (A, B and C) SEC traces of PAS that hydrolyzed by PPE for 0 day (black solid line), 26 days (blue dash line) and 50 days (red dot line). The zoom-in views show M_p of PAS before and after enzymolysis for 50 days. The elution time of PPE is between 20~24 min, which overlaps that of degraded PAS. (D) CD spectra of PAS.

As PAla is easy to form β -sheet conformation, it is appealing to characterize the secondary structure in PAS. Circular dichroism (CD) spectra (Figure 2D) illustrate a random coil for PAS with 6% Ala (Sample 7) and an extended 3_1 -helical structure for PAS with 43% Ala (Sample 6).⁵⁹ The random architecture of PAS is responsible for the fact that neither α -helix nor β -sheet folding is formed. The hydrophobicity and secondary structure of Sample 6 are driving forces for PAS to self-assemble into nanoparticles in water, and the average hydrodynamic diameter is *c.a.* 230 nm by dynamic lighting scattering (DLS) (Figure 3A). Besides cooperating with other hydrophobes, PAS itself is qualified as a drug-carrier.

For further application of PAS in living bodies, the biocompatibility of PAS is assessed using cell viability test with CCK-8 as indicator. Two copolymers (Sample 6 and 7 in Table 1) at various concentrations are incubated with NIH 3T3 cells at 37 °C. As presented in Figure 3B, the tested cells remain relative cell viability over 100% after incubating for 24 h with concentration of PAS as high as 5 mg/mL. Thus, the *in vitro* cell viability elucidates PAS to be non-cytotoxic.

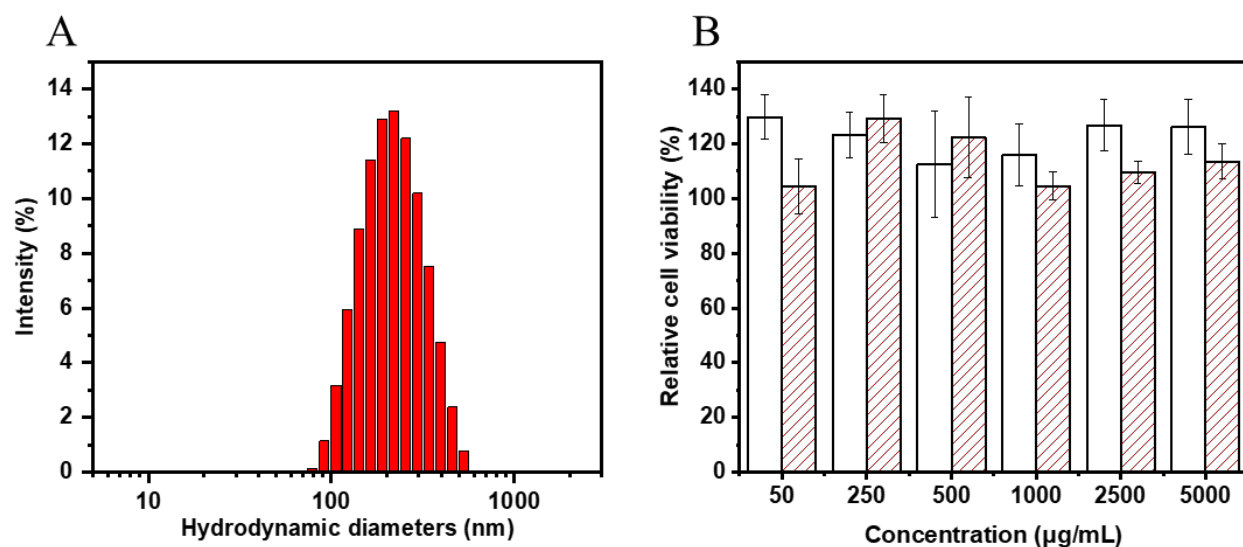


Figure 3. (A) DLS result of PAS nanoparticles from Sample 6 in Table 1. (B) Relative cell viability of PAS (Sample 6 (blank) and 7 (slash) in Table 1) for NIH 3T3 cells after incubation at 37 °C for 24 h with concentration of PAS between 50 and 5000 µg/mL.

In conclusion, we present a synthetic approach of PAS as a biodegradable PSar with randomly inserted Ala residues *via* ring-opening copolymerization of Ala-NTA with Sar-NTA in the presence of HAc. The well-controlled copolymerization produces random copolymers with designable DPs and narrow dispersity ($D < 1.25$). Both Ala-NTA and Sar-NTA show pseudo-first-order characteristics according to kinetics study, and the consumption of the former is slightly slower. PAS with high MW (22.0~43.6 kg/mol) and tunable contents of Ala residues varying from 6% to 43% can be digested by PPE at different rates of enzymolysis. The biodegradability and non-toxicity make PAS a promising biomaterial as an alternative of PEG in drug-delivery applications.

Supporting Information

Experimental section, characterization of NTA monomers and polymers, kinetics and enzymolysis studies.

Conflicts of interest

The authors declare no financial conflicts.

Acknowledgement

Financial support by the National Natural Science Foundation of China (21871232) and the Key Research and Development Program of Zhejiang Province (2019C03014).

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