Polymer Bulletin 59, 311–318 (2007)
DOI 10.1007/s00289-007-0774-9

Polymer Bulletin

Novel amino acid-based polymers for pharmaceutical applications

Viktória Torma[1], Tamás Gyenes[1], Zoltán Szakács[2], Béla Noszál[3], Árpád Némethy[1], Miklós Zrínyi[1] (✉)

[1]Department of Physical Chemistry and Material Sciences, Budapest University of Technology and Economics, H-1521 Budapest, Hungary

[2]Laboratory for Speciation, Drug and Trace Analysis, Institute of Chemistry, Eötvös Loránd University, Pázmány Péter sétány 1/a, H-1117 Budapest, Hungary

[3]Department of Pharmaceutical Chemistry, Semmelweis University, Research Group for Drugs of Abuse and Doping Agents, Hungarian Academy of Sciences, Hőgyes Endre u. 9, H-1092 Budapest, Hungary

E-mail: zrinyi@mail.bme.hu; Fax: 36 1 463 3565

Received: 12 February 2007 / Revised version: 20 March 2007 / Accepted: 16 April 2007
Published online: 27 April 2007 –

Summary

Entirely amino acid-based polymers were prepared by side-chain attachment to polysuccinimide derived from the thermal polycondensation of aspartic acid. Following deprotonation of various amino acid ester hydrochlorides by a secondary amine, the restored primary amino groups initiated the ring-opening of succinimide to form amide bonds. ^{1}H and ^{13}C NMR measurements revealed that the mole fraction of the introduced amino acid side chains could be controlled by the reaction time, while no hydrolysis of methyl ester groups was observed. The synthesized polymers contain exclusively amino acids, which makes them promising candidates as base materials of controlled drug delivery systems.

Introduction

In recent pharmaceutical applications, controlled delivery of drugs became a topic of primary importance [1- 4]. This includes the controlling of release kinetics, which can be accomplished, among others, by long circulating colloidal nanoparticles [3]. The (usually small) drug molecule can be attached either covalently (in this case, its specific cleavage is required on the site of action) or through secondary forces to the macromolecular carrier. Using molecular carrier system usually reduces toxic side effects and can enhance bioavailability or membrane penetration properties. Targeting of the drug to specific organs or tissues usually requires further conjugation to an appropriate ligand recognized by the cells of interest [5]. The carrier vehicles are preferably composed of biocompatible building blocks, so that only non-toxic small molecules are liberated upon their enzymatic degradation.

In the present study, polymers composed entirely of amino acids are synthesized as potential starting materials of biodegradable [6] drug carriers. Their precursor polymer

was polysuccinimide (PSI), synthesized by thermal polycondensation of aspartic acid [7,8]. This reaction requires no coupling reagents or protecting group, since the formation of succinimide rings is a thermodynamically favored process. The formed imide linkages can be opened by nucleophilic agents, such as the hydroxide ion, leading to poly(aspartate), which is technically important e.g. as scale inhibitor [9]. Nitrogen donor reactants, like ethanolamine create side chains on PSI through amide bonds [10-12], with a potential biomedical application as plasma expander [13]. Although amine moieties are ubiquitous in biomolecules, they are usually protonated (zwitterionic) at physiological, neutral or acidic conditions and the positive charge destroys their nucleophilicity.
In order to modify PSI by zwitterionic ammonium-containing biomolecules (e.g. proteinogenic amino acids or oligopeptides), their nucleophilicity should be restored. Our novel synthetic approach makes use of an auxiliary base to deprotonate and thus activate the ammonium group of amino acid methyl esters. The prepared polymers are characterized by multinuclear (^{1}H and ^{13}C) NMR measurements.

Materials and methods

Preparation of polysuccinimide based polymers

L-aspartic acid (puriss, 99.0%), phosphoric acid (a.r., 85%), methanol (p.a. 99.8%), citric acid monohydrate (a.r., 99.5%), ethyl acetate (a.r., 99%) were obtained from Reanal (Hungary). Mesitylene (Fluka, purum, 98%), sulfolane (Aldrich, 99 %), dimethyl sulfoxide (DMSO, Fluka, purum, 99%), dibutylamine (DBA, Riedel-de Haën, 99%) and phenylalanine methyl ester hydrochloride (HPheOMe · HCl, Fluka, puriss, 99%) from SIGMA-ALDRICH were used. Methyl esters of amino acids, like serine methyl ester hydrochloride (HSerOMe · HCl, 99%), glycine methyl ester hydrochloride (HGlyOMe, 99%) were purchased from Bachem (Germany). All reagents and solvents were used without further purification.

Preparation of Polysuccinimide (PSI)
PSI was synthesized by thermal polycondensation of aspartic acid in a solvent mixture of mesitylene and sulfolane, using phosphoric acid as catalyst [14]. The average molecular mass of PSI was determined by static light scattering to be 73000 and its purity was >98 mol%, as confirmed by integration of ^{1}H NMR peak areas.

Preparation of poly[(O^{1}-methyl-glycyl)-aspartate-co-succinimide] (PGA)
0.97 g of PSI (corresponding to 0.01 mol succinimide monomer unit) was dissolved in 10 ml of DMSO. 1.5 g (0.012 mol) of HGlyOMe · HCl and 1.55 g (0.012 mol) of DBA was dissolved in 10 ml DMSO and added to the PSI solution under vigorous stirring at room temperature. After elapsing a reaction time of 3 days, PGA was precipitated by adding 300 ml of citric acid buffer (pH=2.5). Integration of ^{1}H NMR peak areas revealed that 90% of succinimide units were functionalized by GlyOMe.

Preparation of poly[(O^{1}-methyl-phenylalanyl)-aspartate-co-succinimide)] (PFA)
0.97 g (0.01 mol succinimide monomer unit) PSI was dissolved in 10 ml of DMSO. In a separate beaker, 2.6 g (0.012 mol) of HPheOMe · HCl and 1.55 g (0.012 mol) of dibutylamine was dissolved in 10 ml DMSO. The latter solution was added to the first

one under vigorous stirring. After 3 days reaction time at room temperature, the functionalized polymer was precipitated by adding 300 ml of citric acid buffer (pH=2.5). From ^{1}H NMR integrals, a purity of 90 mol% (relative to the mole number of repeating units in the main chain) and a functionalization degree of 30 mol% were established.

Preparation of poly[(O^{1}-methyl-seryl-aspartate)-co-succinimide] (PSA)

0.97 g of PSI (corresponding to 0.01 mol succinimide unit) was dissolved in 10 ml of DMSO. 1.86 g (0.012 mol) of HSerOMe and 1.55 g (0.012 mol) of DBA was dissolved in 10 ml of DMSO and added to the PSI solution under vigorous stirring at room temperature. After a 7-days reaction, PSA was precipitated by addition of 60 ml ethyl acetate. ^{1}H NMR peak integrals of this crude product indicated that 67% of PSI units were opened and functionalized by SerOMe. Since PSA is a hydrophilic polymer, its small-molecule impurities (DBA, DMSO and excess of PheOMe) could be removed by dialyzing the reaction mixture against distilled water for 2 weeks, yielding a final purity of 95%. PSA can be isolated from this aqueous solution by removing water by vacuum, addition of methanol and finally precipitation by ethyl acetate.

NMR Spectroscopy of polymers

50 to 120 mg of native or amino acid-functionalized PSI samples were dissolved in 0.8 ml DMSO-d_6 and characterized on a two-channel Varian Inova 600 MHz spectrometer equipped with a waveform generator, a pulsed field gradient (PFG) unit and a dual inverse broad-band probehead. Quantitative ^{1}H NMR spectra were recorded with a relaxation delay of 16 s, acquisition time of 3 s and averaging at least 32 transients In order to assign the side-chain of functionalized PSI, standard Varian 2D pulse sequences were used: *z*-TOCSY (7 kHz DIPSI-2 spinlock for a mixing time of 80 ms, 256 t_1 increments of 4 transients each), NOESY (150 to 300 ms mixing time, 256 t_1 increments with 16 transients each), multiplicity-edited HSQC using adiabatic 180° ^{13}C-pulses (256 t_1 increments with 4 transients each) and multiple-bond HMQC (optimized for 8 Hz ^{1}H-^{13}C couplings, 32 transients and 400 to 512 t_1 increments). All 2D sequences were supplied by Varian with the VnmrJ software package (version 2.1B), which was used both for acquisition and spectral processing. The NMR spectra were calibrated to resonance lines of residual DMSO-d_5 in the solvent (2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C). Deconvolution of overlapping ^{1}H peaks was performed by using an evaluation copy of MestReC 4.8.6.0 (MestreLab Research).

Results and discussion

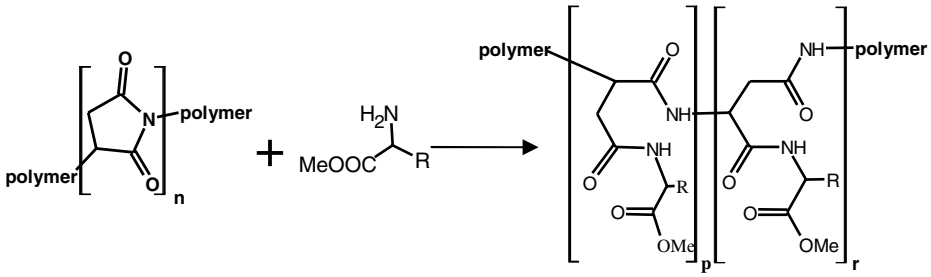
Preparation and characterization of polysuccinimide

PSI was synthesized by slight modification of the method described by Tomida and coworkers [8,14]. The amount of H_3PO_4 catalyst was gradually increased and the fractions of irregular structural elements and branched units in the PSI chains were determined by ^{1}H NMR spectroscopy. Details on these investigations will be published in due course. In the following functionalization processes, the PSI batch

prepared using 16% catalyst by mass (relative to the precursor aspartic acid) was applied.

Synthesis and Structural Characterization of Functionalized PSI

In the hitherto published synthetic approaches, PSI is modified by reactants bearing unprotonated amine group(s) [11,15-21]. For biomedical applications, however, functionalization by a biocompatible amine reactant is required. Natural α-amino acids are ideal candidates to introduce side chains of various polarity, acid-base properties and aromatic motives to PSI, provided that the nucleophilicity of their primary ammonium group is restored by deprotonation. The auxiliary base used for this purpose should ideally not initiate degradation of succinimide repeating units and, from the point of view of homogeneous synthesis, both the free base and its acidic salt product should exhibit adequate solubility in the DMSO solvent.
Most amino acids are insoluble in DMSO, therefore we used their commercially available methyl ester hydrochlorides. Several organic and inorganic bases were tested for effective deprotonation of their primary ammonium groups. Secondary amines with bulky organic groups, like dibutylamine (DBA) proved to be optimal. In principle, the nucleophilic character of secondary bases may also initiate the ring-opening of succinimide repeating units. However, if deprotonation is conducted under a control of ammonium:DBA molar ratio and it also precedes the introduction of PSI to the reaction mixture, the risk of direct DBA-succinimide reaction can be minimized. Since the succinimide carbonyls provide more electrophilic sites for the DBA nitrogen atom (Scheme 1), hydrolysis of methyl ester groups is also expected to be a minor side-reaction.



Scheme 1. Modification of polysuccinimide by a dibutylamine-deprotonated amino acid methyl ester. *n*, *p* and *r* are polymerization degrees, where $n = p + r$.

Characterization of PSI-based polymers by NMR

In the ^{1}H NMR spectra of modified polymers, the signals of residual succinimide units could be detected (see Figure 1 and Table 1 for the assigned chemical shifts), pointing to incomplete functionalization.
In course of NMR assignment of the synthetically introduced structural elements, ^{1}H-^{13}C HSQC spectra were recorded first to identify CH and CH_2 groups. TOCSY spectra were then used to build isolated NH-CH-CH_2 fragments, which may belong either to the main-chain α- or β-aspartate or to the side-chain amino acid ester. The

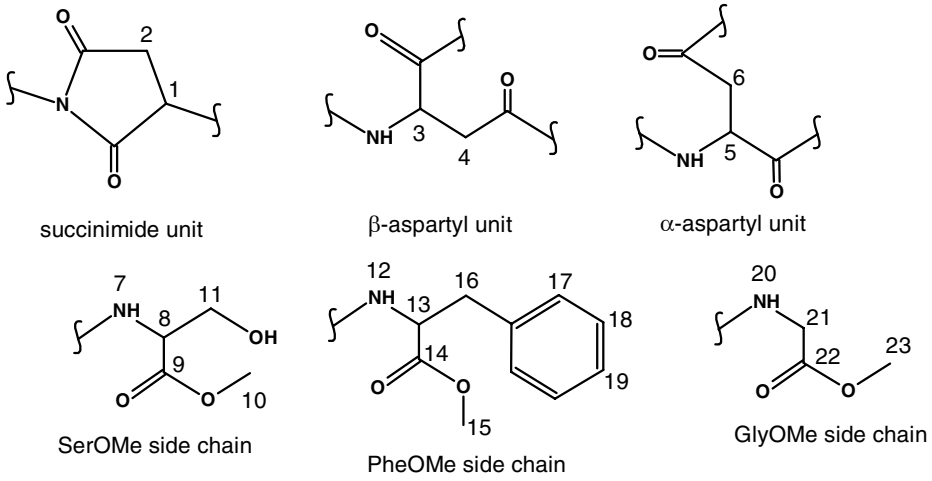


Figure 1. Numbering of each nucleus for assignments of the attached side chains (below) and most main-chain repeating units (above) of functionalized PSI polymers

Table 1. ^{1}H and ^{13}C NMR chemical shifts of assigned structural fragments of functionalized PSI in DMSO-d_6 at room temperature (see Fig. 1 for numbering of nuclei)

assignt	^{13}C chem. shift (ppm)	^{1}H chem. shift (ppm)	assignt	^{13}C chem. shift (ppm)	^{1}H chem. shift (ppm)
1	47.2	5.22-5.40	13	53.7	4.44
2	33.9	2.70, 3.16	14	171.8	
3	49	5.03-5.07	15	51.7	3.58
4	34	2.44, 3.05-3.10	16	36.7	2.90, 2.97
5		5.11-5.19	17		7.19
6		2.34-2.44, 3.15	18		7.26
7		8.31	19		7.19
8	54.6	4.31	20		8.39
9	171.0		21	41.7 / 40.4[a]	3.75 / 3.84[a]
10	51.7	3.61	22	170.4	
11	61.1	3.60, 3.67	23	51.7	3.62
12		8.49			

[a] Two structurally inequivalent GlyOMe sides chain have been identified in the functionalized PSI

unambiguous assignment of these structural units requires the elucidation of their connectivities: either by identifying spatially close proton-proton pairs in NOESY spectra or, more preferably, on the basis of three-bond ^{1}H-^{13}C coupling in the connecting ^{1}H-C-NH-^{13}CO fragment of neighboring units (HMBC or multiple-bond HMQC spectra). The latter strategy was proven to be effective for side-chain assignment (see Figure 1 and Table 1), confirming also that no methyl ester hydrolysis occured during functionalization. However, no long-range ^{13}C-^{1}H correlation peaks could be observed between α-protons of the aspartate monomer units and adjacent carbonyl groups, even by averaging 256 transients or more. Presumably, the polymer

chain is more rigid in the functionalized polymers as compared to PSI or its hydrolysis product poly(aspartate) [22,23], thus rendering the ^{13}C relaxation properties of carbonyls even more unfavorable. The single exception was the GlyOMe-functionalized PSI, where the steric bulk plays presumably the least role among the studied systems.
Consequently, no information emerged from our NMR study to the molar ratio of α- and β-aspartyl repeating units or their sequencing (ratios of αα, αβ and ββ diads). For the less rigid poly(aspartate), sequencing data have already been published [22].

Possibilities of further functionalization

Unreacted succinimide units in the functionalized polymers represent reactive sites, hence open the way to further synthetic modifications and applications. Analysis of the methyne region 5.30-5.00 ppm provides a convenient means of quantifying the residual succinimide level, as demonstrated previously for poly(aspartate) [22,24]. In accordance with the cited publications, signals in the 5.40-5.22 ppm region exhibit no amide connectivity in TOCSY spectra, thus these are assigned to the unopened succinimide units. The α- and β-aspartyl units give rise to several overlapping peaks ranging from 5.20 to 5.00 ppm, which are shown to be coupled with amide groups near 8.5 ppm in DMSO-d_6 solvent. Thus, spectral deconvolution of these regions (see Fig. 2) and taking the ratio of the total integral of the 5.40-5.22 ppm region to that of

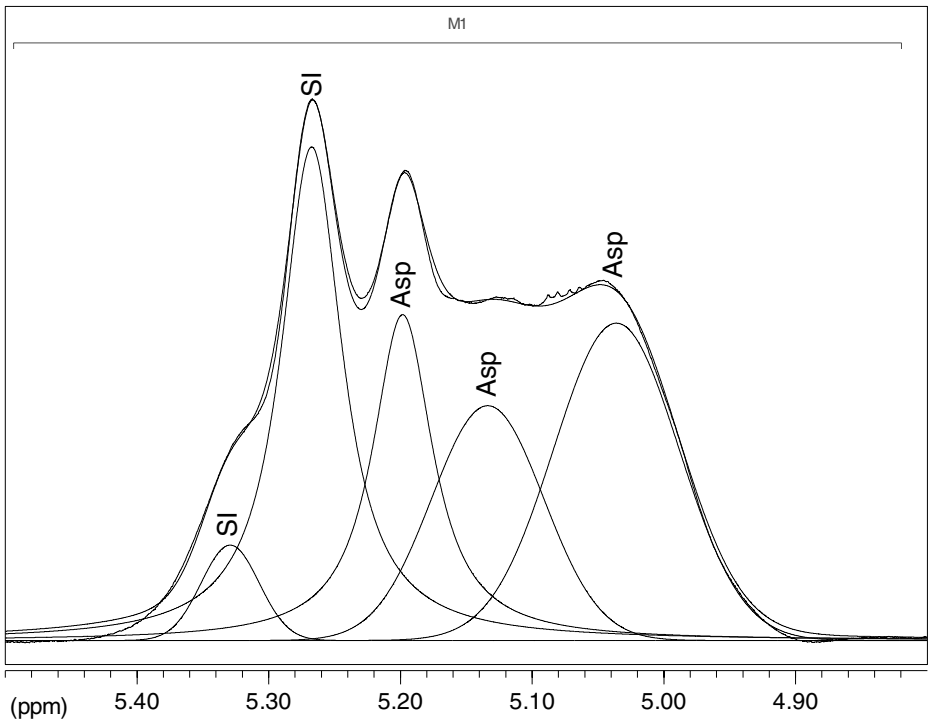


Figure 2. Deconvolution of the α-CH region of the main chains of poly[(O^1-methyl-seryl-aspartate)-*co*-succinimide] in DMSO-d_6. Peak labels: SI = unreacted succinimide, Asp = aspartyl repeating unit in the main chain

the 5.20-5.00 ppm interval yields the proportion of opened succinimide units. The distinction of succinimide and aspartate α-CH peaks by deconvolution can even be performed in D_2O (or, by using the dpfgse solvent suppression, in H_2O), thus enabling a similar quantitation for hydrophilic polymers like PSA in that solvent. Since the opening of succinimide rings does not necessarily mean functionalization, relative "mole number" of side chain amino acid esters can be assessed either by integrating the peak area of their α-CH groups (4.5-3.8 ppm for various side chains) or that of their OCH_3 resonance. Both approaches yield comparable degrees of functionalization (see Materials and Methods) within the estimated error of NMR analysis (2-3%). The impact of reaction time was also investigated in case of PSA: a 3-days-reaction time allowed the attachment of 35 mol% SerOMe side chains, while 7 days increased this conversion to 67%.

Since methyl ester moieties of the amino acid side chains offer additional points to synthetic modification, special care was taken to preserve O^1-methyl ester groups during preparation. Since no attached amino acid side chains with carboxylic groups were observed in ^1H and ^{13}C NMR spectra, their proportion is estimated to be less than 1%. These methyl ester groups can later be removed under conditions of mild alkali hydrolysis (pH ca. 9), where no cleavage of amide bonds is expected.

Conclusion

By covalent attachment of amino acid esters to polysuccinimide, novel polymers containing solely amino acids were prepared and characterized by multinuclear NMR spectroscopy. The ration of grafted amino acid to succinimide was easily controlled by reaction time and the ester groups are shown to be preserved during synthesis. The new polymers can find applications as base materials of drug-carrier systems, while the synthetic strategy is supposed to be generally applicable for preparation further PSI-based biocompatible and biodegradable polymers.

Acknowledgements. This research was supported by the EU – Hungarian Government joint fund (GVOP – 3.2.2 – 2004 – 07 – 0006/3.0).

References

1. Gyenes T, Zrínyi M (2001) Acta Pharmaceutica Hungarica 4:405
2. Roseman TJ, Mansdorf SZ (1983) Controlled Release Delivery Systems. Dekker In., New York and Basel
3. Sahoo SK, Labhasetwar V (2003) Drug Discovery Today 8:1112
4. Petrak K (2005) Drug Discovery Today 10:1667
5. Hudecz F, Remenyi J, Szabo R, Koczan Gy, Mezo G, Kovacs P, Gaal D (2003) J Mol Recognition 16:288
6. Ouchi T, Shiratani M, Jinno M, Hirao M, Ohya Y (1993) Macromol Chem Rapid Commun 14:825
7. Kovács J, Könyves I, Pusztai A (1953) Experientia 12:459
8. Tomida M, Yabe M, Arakawa Y, Kunioka M (1997) Polymer 38:2791
9. Ross RJ, Low KC, Shannon JE (1997) Mater Performance 36:53
10. Antoni G, Arezzini C, Cocola F, Gazzei G, Neri P (1979) Il Farmaco Ed. Pr. 34:146
11. Giammona G, Carlisi B, Cavallaro G, Pitarresi G, Spampinato S (1994) J. Controlled Release, 29:63
12. Nakato T, Kusuno A, Kakuchu T (2000) J. Polym. Sci. Part A. 38:117

13. Neri P, Antoni G, Benvenuti F, Colola F, Gazzei G (1973) J. Med. Chem. 16:893
14. Tomida M, Nakato T, Matsunami S, Kakuchi T (1997) Polymer 38:4733
15. Giammona G, Pitarresi G, Tomarchio V, Spadaro G (1994) Colloid Polym Sci. 12:1637
16. Giammona G, Pitarresi G, Tomarchio V, Cavallaro G, Mineo M, (1996) J. Controlled Release 41:195
17. Tachibana Y, Kurisawa M, Uyama H, Kakuchi T, Kobayashi S (2003) Chem. Commun., 8:106
18. Tachibana Y, Kurisawa M., Uyama H., Kakuchi T, Kobayashi S (2003) Chem. Lett 32: 374
19. Park HD, Kim JH, Kim SH, Kim YH (1999) Polymer (Korea) 23:247
20. Kim JH, Lee JH, Yoon SW (2002) Ind Eng Chem 8:138
21. Neuse EW, Perlwitz A, Schmitt S (1990) Angew. Macromol. Chem. 181:153
22. Wolk SK, Swift G, Paik YH, Yocom KM, Smith RL, Simon ES (1994) Macromolecules 27:7613
23. Matsubara K, Nakato T, Tomida M (1998) Macromolecules 31:1466
24. Matsubara K, Nakato T, Tomida M (1997) Macromolecules 30:2305