Silk-Inspired Polyurethane Containing GlyAlaGlyAla Tetrapeptide. I. Synthesis and Primary Structure

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ABSTRACT: Biomedical polyurethane (PU) and silk fibroin have similar molecular architecture in their primary and aggregate structure, both of which have imido bonds and microphase separation, and they have been employed as scaffold materials for biomedical applications. As the featured peptide sequence of silkworm silk protein, GlyAlaGlyAla (GAGA) tetrapeptide was synthesized by using traditional liquid-phase peptide synthesis method with Boc-protected alanine and glycine as starting materials, and was transformed to its derivative with two end-amido groups. The derivative was incorporated into the backbone of the classical synthetic copolyurethane as a chain extender to form a silk-inspired polyurethane. Elemental analysis showed the nitrogen content in the silk-inspired PU was higher than that in the butanediol-extended PU but lower than the theoretical value, indicating the chain extender containing GAGA was partially incorporated into the PU chain. The results from [1]H-nuclear magnetic resonance, Fourier transform infrared spectra, Raman scattering spectra and pyrolysis-gas chromatography/mass spectrometry qualitatively identified the primary structure of the silk-inspired polyurethane. This concept and strategy may allow the fabrication of a new class of thermoplastic polyurethane elastomer to mimic the structure and properties of silk fibers if in fiber form, which combines traditional synthetic polymer chains with peptide sequences. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 235–242, 2010

Key words: polyurethanes; peptides; block copolymers; microstructure; silk

INTRODUCTION

Polyurethanes (PUs) are usually synthesized in the reaction of di- or polyfunctional hydroxy compounds with di- or polyfunctional isocyanates, which contain soft segments and hard segments.[1] Naturally occurring silk fibroins are characterized by β-sheet crystalline domains [e.g., (GlyAlaGlyAla-GlySer)$_n$ for *Bombyx mori* silk fibroin, and (Ala)$_n$ for *Nephila clavipes* dragline silk fibroin] that are dispersed in a continuous amorphous domain composed of amino acids having bulky side groups.[2] Both polyurethanes and silk fibroins have similar molecular architecture in their primary structure (imido links) and aggregate structure (microphase separation).[3]

To evaluate the relationship between structure and properties of such kind of fibrous proteins as well as the artificial production of high performance silk-like fibers, a variety of peptides and polymers based on silk fibroin model have been prepared by chemical method or genetic bioengineering.[4–16] Various templates, including all-peptide, peptide-nonpeptide, and all-nonpeptide models, for the design of synthetic silk-protein–like materials with hierarchical microstructure were explored. Yamamoto and coworkers[4,5] obtained a silk-like poly(amino acid) fiber created by self-assembly of poly(α, L-lysine) hydrobromide and poly(α, L-glutamic acid) sodium salt at their aqueous solutions. Sogah and coworkers[6–8] reported on an approach in which a recombinantly designed copolymer combining poly(alanine) with poly(ethylene glycol) was produced. Shao et al. attained a GlyAlaGlyAla (GAGA)-contained reactive production (diamine species)
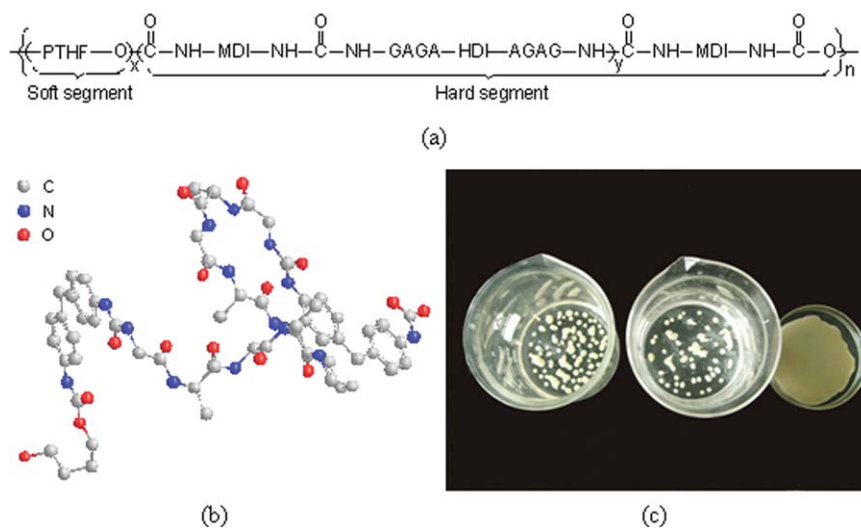
Figure 1 Chemical structure and macroshape of the silk-inspired PU containing GlyAlaGlyAla tetrapeptide: (a) Chemical structure; (b) Simulated molecular structure; (c) Macroshape of coagulation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

synthesized from 1,6-hexamethylene diisocyanate (HDI) and amino-protected amino acids. This diamine, HDI, and a polyisoprene oligomer having two end-hydroxy groups were employed to produce silk-protein–like multiblock copolymers.[9,10] The starting materials are similar to those for typical polyurethane synthesis. Hammond and coworkers produced a series of silk-like PUs containing poly(ethylene oxide) (PEO) soft segments and 1,6-hexamethylene diisocyanate-1,4-butanediol (HDI-BDO) hard segments (no any peptide links), in which PEO soft segments were semicrystalline and reinforced the PU matrix.[11,12]

Despite the large amount of research that is performed on silk-protein–like materials, relatively few reports have described the fiber-forming polymers, with good solubility, for mimicking the structure and properties of silk fibers. This article reports on a stepwise polymerization reaction of classical prepolymer (4,4′-methylene diphenyl diisocyanate-*co*-polytetrahydrofuran, MDI-PTHF-MDI) with a special diamine chain-extender containing GAGA tetrapeptide links (Fig. 1), attempting to obtain a copolymer combining the structure and properties of synthetic polyurethane with those of natural silk fibroin. The primary structure of the synthesized polyurethane is determined by means of elemental analysis (EA), [1]H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy, transmission Fourier transform infrared (FT-IR) spectroscopy and Raman spectroscopy.

EXPERIMENTAL

Materials and reagents

Chloroform (Sinopharm Chemical Reagent Co., Ltd., China, abbreviated as SCRC, 99.0%) and *N*-methyl-

morpholine (NMM; SCRC, 98.0%) were distilled before use. *N*,*N*′-dimethylformamide (DMF; SCRC, 99.5%) and dimethyl sulfoxide (DMSO; SCRC, 99.0%) were used after dehydration with 4 Å molecular sieves (SCRC, Φ 3–5 mm) for 2 days. *N-tert*-butoxycarboxyl-L-alanine (Boc-Ala-OH; Yangzhou Baosheng Biochemical Co., Ltd., 98.0%), *N-tert*-butoxycarboxyl-glycine (Boc-Gly-OH; Yangzhou Baosheng Biochemical Co., Ltd., China, 98.0%), *N*-hydroxysuccinimide (HOSu; SCRC, 97.0%), 1,6-hexamethylene diisocyanate (HDI; Alfa Aesar, 98.0%), 4,4′-diphenylmethane diisocyanate (MDI; Alfa Aesar, 98.0%), *N*,*N*′-dicyclohexyl carbodiimide (DCC; SCRC, 95.0%), and other reagents were used without further purification.

Synthesis of silk-inspired PU

The compound, HCl—H—GlyAlaGlyAla—NH(CH₂)₆ NH—AlaGlyAlaGly—H—HCl, was prepared and purified as described in the earlier report,[10] with double dose and some modifications. Briefly, it was synthesized stepwise in DMF, using traditional liquid-phase peptide synthesis method, employing Boc-Gly-OH, Boc-Ala-OH, and HDI as primary starting materials, using DCC and HOSu as the complex condensation reagents while using NMM as the alkali reagent. The Boc-protected intermediate products were purified by using silica gel (200–300 mesh) column chromatography eluted with a 1:1:1 (v/v/v) mixture of chloroform, methanol, and acetic acid. The protective group (Boc) was removed with hydrochloride-saturated acetic acid solution, and the product was purified by Soxhlet extraction with chloroform. The total yield was about 30%.
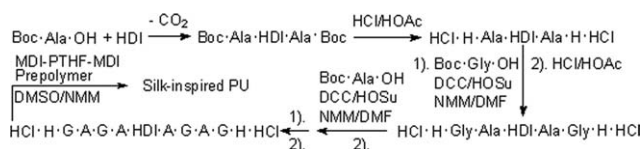
Figure 2 Total synthesis route for silk-inspired PU containing GlyAlaGlyAla tetrapeptide.

Subsequently, the classical two-step solution polymerization was employed to synthesize the polyurethane.[17] The diisocyanate was 4,4′-diphenylmethane diisocyanate (MDI, Bayer), the macrodiol was polytetrahydrofuran (PTHF, $M_w \approx 2000$, Aladdin Reagent, China), and the chain extender was the above-synthesized H—GlyAlaGlyAla—NH(CH$_2$)$_6$NH—AlaGlyAlaGly—H (denoted as AG4, transformed from HCl—H—GAGA—NH(CH$_2$)$_6$NH—AGAG—H—HCl by using NMM as the alkali reagent). The molar ratio of MDI/PTHF/chain extender AG4 was used as 2/1/1. The reaction medium was DMSO. In the first step, a prepolymer (MDI-PTHF-MDI) that contained free isocyanate at both ends of the chain was formed. In the second step, the prepolymer reacted with the chain extender to give the final PU completely. Figures 1 and 2 show the model structure of final PU product and the total synthesis route, respectively. The products were made into granules by being dripped into a mixture of methanol–water (3/1, v/v) and then coagulated.[17] The granules were further purified by extraction in toluene, dried, and stored in granular or flake forms. For comparison, the PU with a classical 1, 4-butanediol (BDO; Aladdin Reagent, China) chain extender was also synthesized in the same condition, which was denoted as BDO-extended PU. AG4-extended PU was a poly-(urethane urea), while BDO-extended PU was a poly-(urethane ether).

Elemental analysis

The two coagulated and purified PUs and other two control specimens (silk fiber from China local and PU Pellethane® 2363-80AE model from Dow Chemical) were quantitatively analyzed for the chemical elements carbon (C), hydrogen (H), and nitrogen (N). The leftover ratio was calculated as oxygen (O) content approximatively. According to JY/T 017-1996 (General rules for elemental analyzer, see Ref. 18 for detailed rationale), the analysis was performed with an automatic elemental analyzer Vario EL III (Elementar Analysensysteme Comp., Hanau, Germany).

^{1}H-Nuclear magnetic resonance spectroscopy

^{1}H-Nuclear magnetic resonance (^{1}H-NMR) experiments were carried out on a Bruker Avance 400 (400 MHz) spectrometer at 25°C. Dimethyl-*d*6 sulfox-ide (DMSO-*d*6) and tetramethylsilane (TMS) were used as solvent and internal standard substance, respectively. For comparison, the two uncoagulated and purified PUs and the chain extender AG4 were measured.

Fourier transform infrared spectroscopy

Fourier transform infrared (FT-IR) spectra were recorded with a Bruker Tensor 27 spectrometer from 4000 to 400 cm^{-1}, at 2 cm^{-1} resolution using 32 scans. The specimens of the two PUs and AG4 before coagulation were coated on potassium bromide (KBr) disks for testing at 25°C.

Raman spectroscopy

Raman scattering spectra of the two PUs and AG4 before coagulation were collected at 25°C on a Raman microscope DXR model, equipped with the Omnic software from Thermo Fisher Scientific. The excitation laser wavelength was 532 nm using a laser power level of about 6.0 mW.

Pyrolysis-gas chromatography/mass spectrometry

Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) was also used as a reliable and reproducible technique for providing abundant information on the primary structure of polymers. The pyrolysis of the samples of the synthetic silk-inspired PU and BDO-extended PU was carried out using a single-shot pyrolyzer PY-2020iS (Frontier Laboratories Ltd., Japan) interfaced to a gas chromatograph/mass spectrometer (Shimadzu GC/MS-QP2010). The experimental conditions were as follows: sample mass, about 0.05 mg; pyrolysis temperature, 750°C; pyrolysis time, 10 s; carrier gas, helium; total flow, 54 mL/min; split ratio, 50:1; capillary column, DB-5, 30 m × 0.25 mm × 0.25 μm (Length × internal diameter × thickness), held at 50°C for 2 min then temperature-programmed at 10°C/min up to 280°C and then holding for 15 min; injection temperature, 280°C.

Ions were generated by electron-impact (EI) ionization (electron energy 70 eV) in the ionization chamber of the mass spectrometer. The mass spectrometer was scanned from m/z 41 to m/z 400, with a cycle time of 0.3 s. EI mass spectra were acquired in the scan mode, and peak area data were used to obtain values of peak area percentage. The temperature of the interface and the ionsource were 280 and 200°C, respectively. Compounds were identified by using the NIST 05 and NIST 05s Library of Mass Spectra.

TABLE I
The Results of Elemental Analysis for Silk-Inspired PU and Controls

Specimen	C (%)	H (%)	N (%)	O (%)
Pellethane® 2363-80AE, PU from Dow Chemical	66.61	9.02	3.56	20.81
Silk fiber, from China local	43.93	6.64	16.79	32.64
BDO-extended PU	66.46	10.04	2.05	21.45
Silk-inspired PU	66.18	10.38	2.40	21.04

RESULTS AND DISCUSSION

Elemental analysis

Table I summarizes the results of elemental analysis. For analyzing reliability, elemental measurements were performed twice for each specimen. For each test, two specimens were made, and an average value was calculated. The second measurement differed from the first measurement by less than ±1% for each element, indicating high reproducibility of elemental measurements. As shown in Table I, the carbon content in the commercial Pellethane PU sample is much higher than that in silk fiber, while the nitrogen content in this commercial PU is much lower than that in silk fiber. The carbon content in the silk-inspired PU is slightly lower than that in the BDO-extended PU, and the nitrogen content in the silk-inspired PU is slightly higher than that in the BDO-extended PU. Therefore, the composition of silk-inspired PU is still a classical PU, as incorporation of amino acid alone does not achieve this.

Theoretically, the degree of polymerization of the PTHF ($M_w \approx 2000$) is around 23, so the subscript "x" in Figure 1 is 23, as well as the subscript "m" in Figures 3 and 4. The subscript "y" considers the pos-

sibility of the growing of the hard segments due to the reaction of a part of the chain extender with the excess of isocyanate.[19] In this work, the molar ratio of MDI/PTHF/chain extender AG4 (or BDO) was used as 2/1/1, so the chain extender and the isocyanate groups at the end of the prepolymer chains were completely reacted, i.e., $y = 1$. Therefore, the theoretical values of N content (%) in BDO-extended PU and silk-inspired PU are 2.14 and 6.22%, respectively. The N content in Pellethane (3.56%) is higher than that in the synthesized BDO-extended PU (2.05%), though their components are very similar. This may be attributed to the subscript "y" for Pellethane is higher than one. The N content in the silk-inspired PU (2.40%) is higher than that in the BDO-extended PU (2.05%), but far below the theoretical value (6.22%). This indicates the chain extender AG4 has been partially incorporated into the PU chain but not very adequately.

Even though the difference in compositions of silk-inspired PU and silk fiber is very significant, this compositional analysis still provides some important information for the biomimetic synthesis of silk-inspired biofiber, as the elements contained and even primary structure are not necessarily the
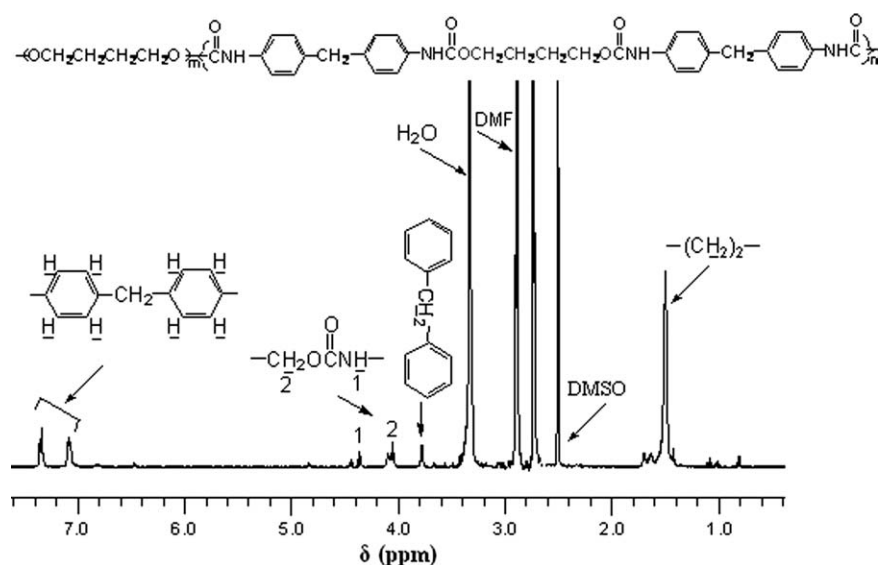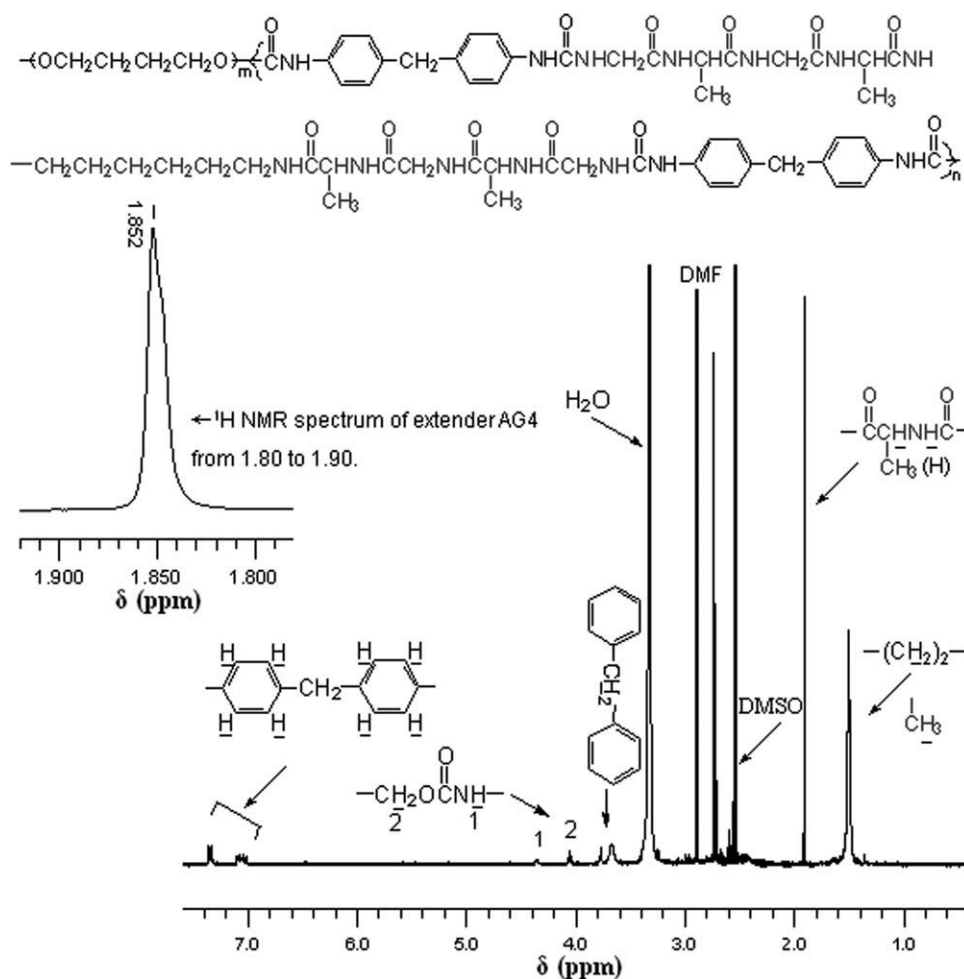

Figure 3 ¹H-NMR spectrum for the comparable BDO-extended PU.

**Figure 4** ^{1}H-NMR spectrum for the silk-inspired PU containing GAGA tetrapeptide links.

decisive factor for material properties. The secondary structure may play a more important role.[20,21]

^{1}H-NMR spectra

The model structure and ^{1}H-NMR spectra of as-synthesized BDO-extended PU and silk-inspired PU are included in Figures 3 and 4, respectively. The model structure shows the hard segments obtained by reacting MDI with the chain extender (BDO and AG4), and the soft segments constituted by the PTHF chains.[19] As shown in Figures 3 and 4. the different signals in the ^{1}H-NMR spectrum correspond to protons in different chemical environments. Similar chemical shifts of ^{1}H-NMR spectra of the two PUs were observed.

- $\delta = 1.50$ ppm in Figure 3: One multiplet (m) corresponding to the methylene protons $-(CH_2)_2-$ in the hard and the soft segments.
- $\delta = 1.50$ ppm in Figure 4: One multiplet (m) corresponding to the methylene protons $-(CH_2)_2-$ in the soft segments.

- $\delta = 2.51$ ppm in Figure 3 and $\delta = 2.52$ ppm in Figure 4: One singlet (s) due to the protons in the solvent DMSO.[22] DMF and DMSO were employed as reaction media in the course of AG4 and PU synthesis, respectively. It was difficult to remove them by evaporation and purification process due to their high boiling points, so the samples were less than analytically pure.
- $\delta = 2.74$, 2.90 ppm in Figure 3 and $\delta = 2.73$, 2.89 ppm in Figure 4: Two singlets (s) due to the protons ($2 \times -CH_3$) in the remnant solvent DMF.[22] To make the spectra clear, the chemical shift at about 8.0 of the other proton ($-CHO$) in the DMF was not included herein.
- $\delta = 3.35$ ppm in Figure 3 and $\delta = 3.33$ ppm in Figure 4: Two singlets (s) corresponding to the protons in trace-impurity water.
- $\delta = 3.81$ ppm in Figure 3 and $\delta = 3.75$ ppm in Figure 4: One multiplet (m) due to the protons bonded to the carbon in the α-position of phenyl (Ph) groups (Ph—CH_2—Ph). It corresponds to the isocyanate MDI.
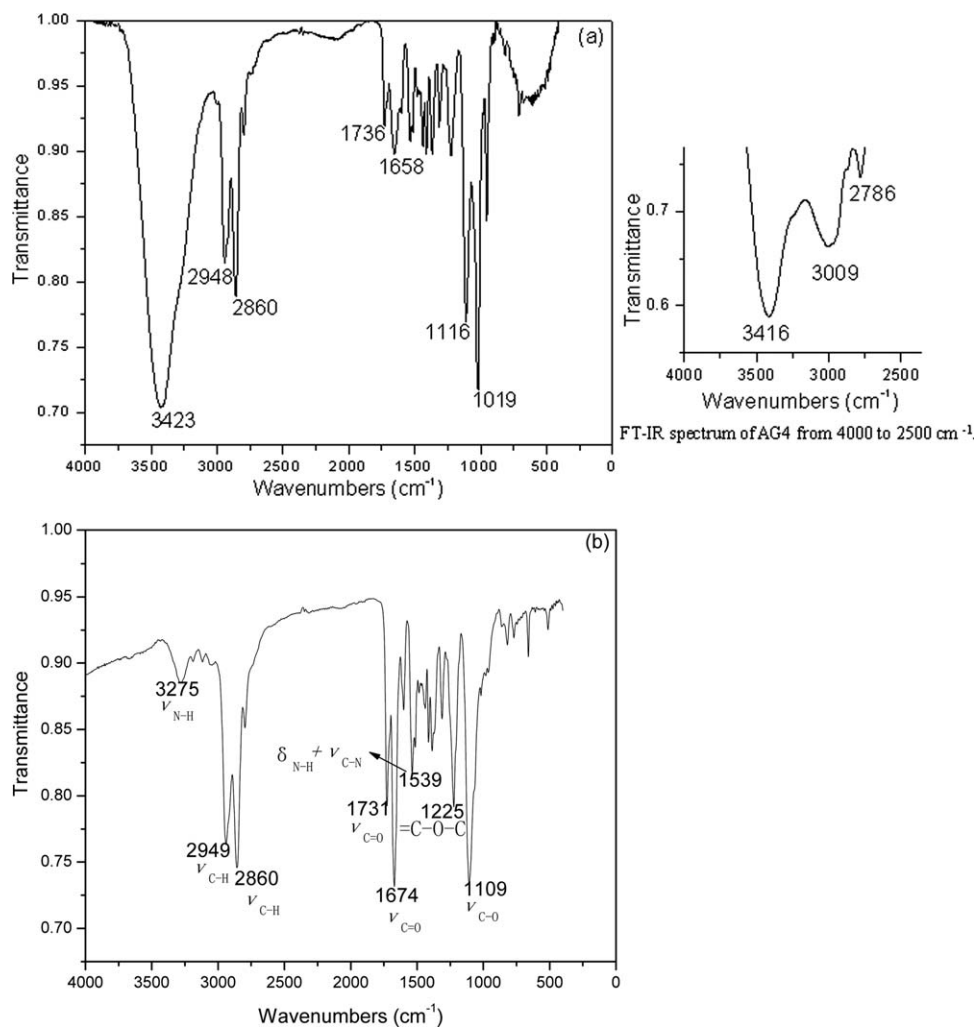
Figure 5 FT-IR spectra for: (a) the synthesized silk-inspired PU containing GAGA tetrapeptide links; (b) the comparable BDO-extended PU.

- $\delta = 4.08$ ppm in Figure 3 and $\delta = 4.07$ ppm in Figure 4: One triplet (*t*) corresponding to protons in α-position of the alkoxy group near a urethane group ($-CH_2OCONH-$).
- Low intensity broad signals at $\delta = 4.37$ ppm in Figure 3 and $\delta = 4.35$ ppm in Figure 4: corresponding to protons bonded to the nitrogen of the urethane group ($-CH_2OCONH-$).
- Two multiplets (*m*) at $\delta = 7.08, 7.35$ ppm in Figure 3 and $\delta = 7.06, 7.34$ ppm in Figure 4: corresponding to aromatic protons Ph (4H) in the isocyanate MDI.

The main difference of the ^{1}H-NMR spectra for the two PUs was also observed. As shown in Figure 4, a high intensity signal at $\delta = 1.91$ ppm appeared corresponding to the protons in the chain extender AG4. To confirm this, the ^{1}H-NMR spectra for AG4 was also recorded as shown at the left hand of Figure 4. A high intensity signal at $\delta = 1.85$ ppm was observed, while there was no signal at this

place in Figure 3. Compared with the related results of Ref. 19, there was no signals of our product PU at $\delta = 2.25$ ppm and $\delta = 4.10$ ppm, as there was no carbonyl α-protons ($-CH_2COO-$) and no proton in α-position of ester group ($-CH_2OCO-$) in the soft and the hard segment. Even though the signals of the synthesized PUs were of low intensity, the results clearly confirmed that the two products had typical PU structure and the AG4 was incorporated into the main chain of the silk-inspired PU.

FT-IR spectra

FT-IR has been used extensively both to identify and quantify chemical functionality and structure in polymers. This article examines the use of transmission FT-IR as a means of reliably identifying chemical changes in PU caused by varying chain extender. Generally, the strong hydrogen-bonded structure such as $-NH\cdots OC$ (O$-$) NH$-$, between the urethane linkages for a multiblock PU is easily
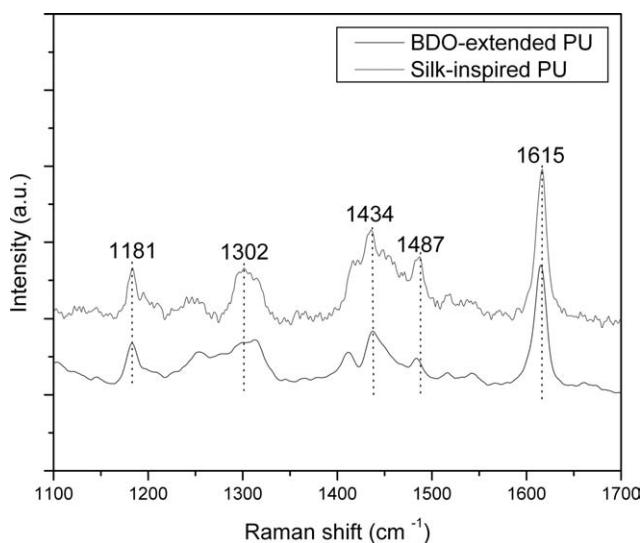
Figure 6 Partial Raman spectra for the synthesized silk-inspired PU containing GAGA tetrapeptide links (top red line) and the comparable BDO-extended PU (bottom black line).

formed.[23] There is a consensus in the infrared spectroscopic literature concerning the assignment of polyurethane infrared absorbance peaks. This includes the hydrogen-bonded N—H stretching (v_{N-H}) near 3280 cm^{-1}, the carbonyl stretching ($v_{C=O}$) near 1730 and 1700 cm^{-1}, the C—C benzene stretching near 1414 and 1516 cm^{-1}, the N—H bending + C—N stretching ($\delta_{N-H} + v_{C-N}$) near 1535 cm^{-1}, the C—N stretching near 1222 cm^{-1}, and the ether absorbance near 1110 cm^{-1}.[24,25] As shown in Figure 5(b), infrared absorbance peaks of the comparable BDO-extended PU are in accordance with the above peak assignments, besides C—H stretching (v_{C-H}) at 2949 and 2860 cm^{-1}. Absorbance peaks shown in Figure 5(a) are similar to those of Figure 5(b). The main difference of the FT-IR spectra of the PUs is at 3423 cm^{-1}. This peak does not appear on FT-IR spectra of BDO-extended PU. The strong absorbance peak near 3423 cm^{-1} can be attributed to nonbonded

N—H stretching.[24] As shown in Figure 5(a), this peak appears on FT-IR spectra of silk-inspired PU and AG4, as they both have many free N—H bonds on the main chains.

Raman spectra

Figure 6 shows Raman spectra for the synthesized silk-inspired PU containing GAGA tetrapeptide links and the comparable BDO-extended PU. There is no significant difference between them. It includes the C=C stretching (v_{Ar}) near 1615 cm^{-1}, the symmetric N=C=O stretching at 1434 and 1487 cm^{-1}, the C—H bending (δ_{C-H}) of urethane amide III near 1302 cm^{-1}, and the urethane amide near 1181 cm^{-1}. The data are consistent with the tentative Raman band assignments for the comparable BDO-extended polyurethane.[26] No peak was found on the Raman spectrum for the chain extender AG4. This phenomenon is difficult to explain presently however, it can be preliminarily concluded that the two products had typical PU structure.

Py-GC/MS

Py-GC/MS is a good way to investigate the thermal cracking of polymers. Since thermal degradation is done inside the mass spectrometer's ionization chamber, the high vacuum quickly removes the generated species from the reaction medium. Table II summarizes the seven main pyrolyzates of the synthesized silk-inspired PU containing GAGA tetrapeptide links and the comparable BDO-extended PU. Figure 7 shows the structure of the pyrolyzates. Similar pyrolyzates of the two PUs were observed. The pyrolyzates were mainly cyclic or linear ethers, esters or amides. This is consistent with related studies.[27] However, there are more butylene glycol monobutyl ether, 4,4'-methylenedi aniline, and 2-Propyltetrahydrofuran in the pyrolyzates of the comparable BDO-extended PU. There are more

TABLE II
Main Pyrolyzates of the Silk-Inspired Pu Containing Gaga Tetrapeptide Links and the Comparable BDO-Extended PU

|		Peak area (%)	
No.	Compound	Silk-inspired PU	BDO-extended PU
1	Butylene glycol monobutyl ether	17.64	37.66
2	N-tetrahydrofurfuryl hexanamide	17.35	17.55
3	N-tetrahydrofurfuryl pentanamide	16.68	0.64
4	Pentanedioic acid, 2-methyl-bis(1-methylpropyl) ester	12.97	12.37
5	Tetrahydro-2-furanylmethyl hexanoate	7.61	1.34
6	4,4'-methylenedi aniline	–	9.84
7	2-Propyltetrahydrofuran	–	6.74
8	Others	27.75	13.86
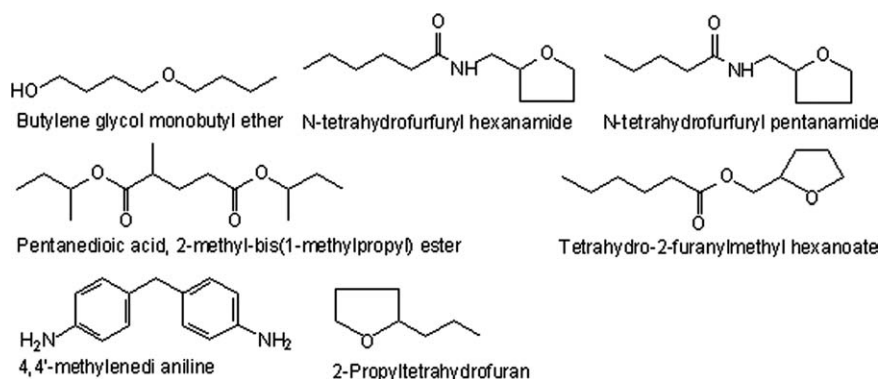
Figure 7 The chemical structure of the pyrolyzates.

N-tetrahydrofurfuryl pentanamide and tetrahydro-2-furanylmethyl hexanoate in the pyrolyzates of the silk-inspired PU. This can be attributed to more content of ethers and phenyls in the chain of BDO-extended PU and more content of amides in the chain of the silk-inspired PU.

CONCLUSIONS

In summary, a silk-inspired polyurethane containing GAGA tetrapeptide was synthesized by using the liquid-phase peptide synthesis and two-step solution polymerization with Boc-protected amino acid and the traditional starting materials for typical polyurethane synthesis. Elemental analysis showed the nitrogen content in the silk-inspired PU was higher than that in the BDO-extended PU but lower than the theoretical value, indicating the chain extender containing GAGA was partially incorporated into the PU chain. The primary structure of the silk-inspired PU was qualitatively identified by ^{1}H-NMR spectra, FT-IR spectra, Raman spectra, and Py-GC/MS. The concept and strategy of the silk-inspired polyurethane introduced may provide some information for the development of novel silk-like fibers, as polyurethane fibers have been manufactured commercially for many years. Further study on aggregate structure, solution property, and fiber spinning of the silk-inspired polyurethane is under way.

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