Polymer Chemistry

Cite this: *Polym. Chem.*, 2012, **3**, 498

www.rsc.org/polymers

PAPER

Pepsin-inspired polyurethanes containing a tyrosine–fumaric acid–tyrosine segment

Haoxiang Lu,[a] Wei Wang,[a] Zhen Zheng,[a] Peiyu Sun,[a] Xinling Wang*[a] and Feng-Chih Chang[b]

Received 12th October 2011, Accepted 2nd December 2011
DOI: 10.1039/c2py00481j

Two kinds of novel peptide mimetic pepsin-inspired degradable polyurethanes were synthesized and characterized. A synthetic pseudo tripeptide acted as chain extender and provided active sites for pepsin biodegradation. *In vitro* degradation behavior was investigated in simulated gastric fluid containing pepsin, and assessed by ^{1}H NMR, mass loss and SEM. The results indicated that the pseudo peptide bonds in polyurethanes were effectively digested by the action of pepsin. Furthermore, the rate and degree of pepsin-inspired polyurethanes in simulated gastric fluid containing pepsin could be controlled by alteration of the soft segments and activity of pepsin. The influence of the polymers on human umbilical vein endothelial cells was evaluated by WST-1 assay. The results indicated that the polymers sustained much higher cell viability than the controls.

Introduction

Recent years have witnessed a huge increase in requirements for biodegradable synthetic polymers for drug-delivery systems.[1–6] These polymers usually contained linear aliphatic polyesters or polycarbonates in the main chain or at the side chain to realize the degradation by the action of oxidation and hydrolysis.[7–9] However, cleavage of ester and carbonate bonds has uncertainty, mainly regarding the predictability of the degradation site, and in the uncontrollability of degradation in designated parts of the body. This may limit the applications of the biodegradable synthetic polymers in site-specific drug delivery systems.

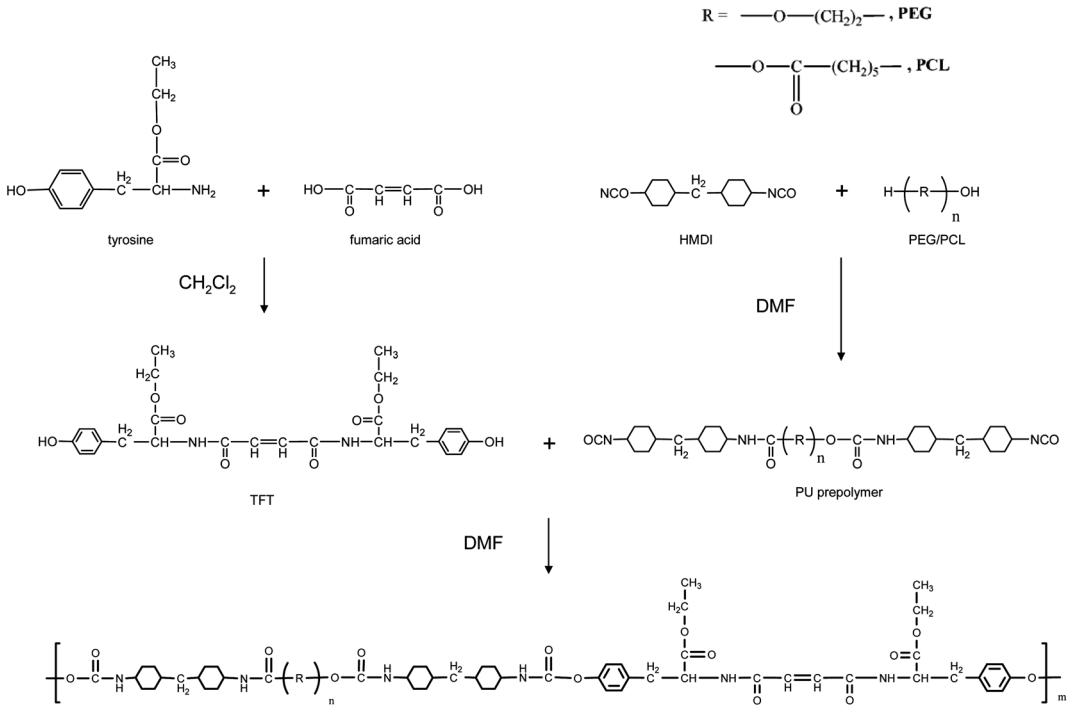
A site-specific drug delivery system refers to a novel drug delivery system which releases drug under the stimulus of the abnormal pH value, redox environment, enzyme and other specific chemicals of the diseased tissues to increase drug effect, and avoids drug delivery in normal parts of the body to decrease the damage to healthy tissues. For patients with gastric cancer, the oral stomach-specific drug delivery system, releasing drug under the stimulus of acid environment or pepsin, can alleviate their suffering from system chemotherapy and greatly facilitate the treatment. However, traditional acid sensitive drug carriers encounter difficulties in balancing the sustained-release time and site-specific delivery.

Here, we proposed to develop a novel pepsin-inspired drug carrier to break though the bottle-neck. It is known that pepsin is the principal proteolytic enzyme existing only in the gastric juices. Pepsin, unlike other peptidases, hydrolyzes only peptide bonds. Proteins may be digested by pepsin into peptides of varying length, oligopeptide and even free amino acids.[10] The cleavage is specific to peptide linkages formed by the aromatic amino acids like phenylalanine, tyrosine and tryptophan. The attractive properties, precisely controllable degradation and quick-response under mild conditions, induced peptide and polyamino acid to act as pepsin degradable biopolymers.[11,12] But the deficiencies, like over short lifetime *in vivo*, high price, difficult synthesis and process, may limit their applications.[13,14]

In order to overcome the limitation, we proposed to design a novel pseudo oligopeptide as the surrogates of peptide bonds supplier and introduced it into a selected polymer. As one of the premier synthetic polymers, polyurethanes have been employed as biomaterials because of their advantages such as low toxicity, biocompatibility, easily variable micro- and macro-structure, and easily controlled physicochemical properties.[15–18] In this work, we first synthesized a pseudo tripeptide formed by L-tyrosine and fumaric acid, a kind of natural bioactive unsaturated dicarboxylic acids acting as intermediates in the tricarboxylic acid cycle.[19] Then, it was incorporated into the main chain of polyurethanes to develop two kinds of peptide mimetic polymers (Scheme 1).

To make sure the peptide mimics have controllable pepsin-inspired degradable behavior, and good physical properties and cell viability, *in vitro* degradation tests were performed by the action of pepsin with a range of activity in a simulated gastric fluid environment. The degradation behavior was monitored by ^{1}H NMR spectroscopy, mass loss and SEM. The influence of the polymers on the viability of human umbilical vein endothelial cells (HUVECs) was studied by WST-1 assay. The peptide mimetic polyurethanes may possess a certain similarity to

[a]*School of Chemistry and Chemical Technology, Shanghai Jiaotong University, State Key Laboratory of Metal Matrix Composites (Shanghai Jiao Tong University), 800 Dongchuan Road, Shanghai, 200240, China. E-mail: xlwang@sjtu.edu.cn; zzheng@sjtu.edu.cn; Fax: +86-21-5474 1297; Tel: +86-21-5474 5817*
[b]*Department of Applied Chemistry, National Chiao-Tung University, Hsin-Chu, Taiwan, China*



Scheme 1 Synthesis of polyurethanes based on TFT.

pepsin-inspired polymers in oral stomach-specific drug delivery systems. In spite of them, the tyrosine-fumaric acid-tyrosine pseudo tripeptide (TFT) can act as a building block for design of pepsin-inspired polymers.

Experimental section

Materials

Fumaric acid, dibutyltin dilaurate (DBTDL) and methyledichloride (CH_2Cl_2) from Sino Chemical Reagent Co. Ltd; 1,6-hexamethylene diisocyanate (HMDI) from Bayer Co. Ltd; L-tyrosine ethyl ester hydrochloride from Aladdin Reagent Co. Ltd and 1-ethyl-3-(3-dimethyllaminopropyl) cabodiimide hydrochloride (EDC·HCl) from Shanghai Medpep Co. Ltd. were used without further purification. Triethylamine (TEA) and N,N-dimethylformamide (DMF) obtained from Sino Chemical Reagent Co. Ltd were dried before use. Polyethylene glycol (PEG, M_n: 600, 1000, 2000) obtained from Sigma Aldrich and polycaprolactone diol (PCL M_n: 1000, 2000) from Duosen Chemistry Co. Ltd were dehydrated for 3 h at 100 °C.

Measurements

Material characterization. NMR spectroscopy was performed on an Avance-400 spectrometer (Bruker, Switzerland) with deuterated DMSO as the solvent at 25 °C. Gel permeation chromatography (GPC) analyses were obtained on a Perkin Elmer Series 200, using DMF as the eluent. Molecular weights were estimated against polyethylene glycol standards.

The thermal behavior of the polyurethanes was examined by Differential Scanning Calorimeter (DSC), using a TA Instruments Q2000. DSC date was obtained at heating/cooling rates of 10 °C min^{-1} under a nitrogen flow.

Scanning electron microscopy (SEM) micrographs were recorded on a Hitachi S-2150 field-emission scanning electron microscope. The samples were sprayed with gold particles on the surface and mounted on an aluminum stub using electric adhesive tape.

Pepsin-inspired degradation. Polyurethanes were recoated as films with a depth of approximately 0.2 mm. The films were cut into 0.8 cm × 0.8 cm pieces. Simulated gastric fluid (pH = 1.7) was prepared from a sodium chloride solution (0.2% w/w) by proper dilution with hydrochloric acid. Pepsin solutions with different activities (5U, 10U, 20U and 40U) were used to investigate the effect of protease activity on the degradation of the polymers.

Polyurethane pieces were put into vials full of the pepsin solution separately and degraded at 37 °C while the control samples were immersed in simulated gastric fluid without pepsin. The pH value of the pepsin solution was monitored every 12 h and adjusted to 1.7 with hydrochloric acid. Samples were taken out at 1, 2, 4, 8, 16, 32 day intervals. Then, the films were washed with distilled water (3 × 10 mL) and dried in vacuum oven at 40 °C prior to any characterization.

Cell viability test. WST-1 (Beyondtime Bio-Tech, China) assay was employed to measure cell viability.[20] Polyurethane membranes were placed into a 24-well flat polystyrene culture plate (Corning). 500 μl Dulbecco's Modified Eagle's Medium (DMEM) of a human umbilical vein endothelial cell suspension (about thirty thousand cells per well) were transferred into each well and cultured for 48 h. 500 μl of fresh DMEM was used to substitute the original one before adding 1/10 (v/v) of WST-1 reagent. The cells were incubated for another 1 h, then 150 μl of the suspension in each well was transferred to a 96-well flat

plate (Corning). The absorbance of the suspension was measured at 450 nm using a microplate reader. 3 specimens were tested for each sample. The cells seeded on culture plate at the same time intervals were employed as controls (100% viability).

Synthesis

Pseudo trigopeptide (TFT). TFT was synthesized though a carbodiimide-mediated process between the amino group of 2 mol of L-tyrosine and the carboxyl group of 1 mol of fumaric acid. L-tyrosine ethyl ester hydrochloride, CH_2Cl_2 and TEA were first charged in a flask and stirred under nitrogen atmosphere for 0.5 h. Then, fumaric acid and EDC·HCl were added to the reactor and stirred in an ice-water bath under nitrogen atmosphere for 8 h. The CH_2Cl_2 solvent containing the indissoluble triethylamine hydrochloride was filtered off. The filtrate was washed with dilute hydrochloric acid and solid particles with light yellow colour precipitated between the aqueous phase and organic phase. The admixtures were filtered off again. The precipitate was washed with deionized water sufficiently and dried under vacuum. The yield was 81.2%. The ^{1}H NMR analysis were shown in Fig. 1. ^{1}HNMR (DMSO, ppm): 1.09 (6H, trip, CH_3, i); 2.87 (4H, quint, CH_2, h); 4.01 (4H, quint, CH_2, g); 4.41 (2H, quint, CH, f); 6.64 (4H, trip, benzene ring, e); 6.82 (2H, sing, CH, d); 6.98 (4H, trip, benzene ring, c); 8.80 (2H, doub, NH, b); 9.22 (2H, sing, OH, a).

Polyurethanes PU(PEG-HMDI-TFT). Segmented polyurethanes from PEG, HMDI and TFT were synthesized by conventional two step methods following the procedure found in literature.[21] Briefly, PEG and DMF were added to a three-necked flask in an oil bath at 70 °C under nitrogen. HMDI was then charged to the reactor at an NCO : OH ratio of the prepolymer equal to 2.05 : 1. Dibutyltin dilaurate (DBTDL) was added to the flask as a catalyst and the mixture was stirred for 2 h. Then, TFT dissolved in DMF was slowly added to the prepolymer solution and the reaction was allowed to proceed for 8 h. The product was precipitated in distilled water and dried at 60 °C under vacuum. Take PEG1000-HMDI-TFT for example, the IR and ^{1}H NMR spectra of polyurethanes were characterized. IR: ν (cm^{-1}) 3299 (N–H), 3069 (C–H benz), 2934, 2859 (C–H aliph), 1738 (C=O urethane), 1631 (C=O amide), 1549 (N–H urethane), 1093 (C–O ether). ^{1}H NMR (DMSO, 400 MHz): δ (ppm) 8.80 (2H NH in amide group), 7.11 (4H NH in urethane group), 6.99 and 6.63 (8H CH in benz), 6.83 (2H CH=CH in fumaric acid), 4.39 (2H, CH in tyrosine), 3.50 (88H, CH_2 in PEG1000).

Polyurethanes PU (PCL-HMDI-TFT). Segmented polyurethanes from PCL, HMDI and TFT were synthesized following a similar method to PU(PEG-HMDI-TFT). In the first step, PCL and HMDI were reacted at a 1 : 2 molar ratio in DMF. Dibutyltin dilaurate (DBTDL) was added to the flask as a catalyst. The reaction was carried out at 70 °C under nitrogen

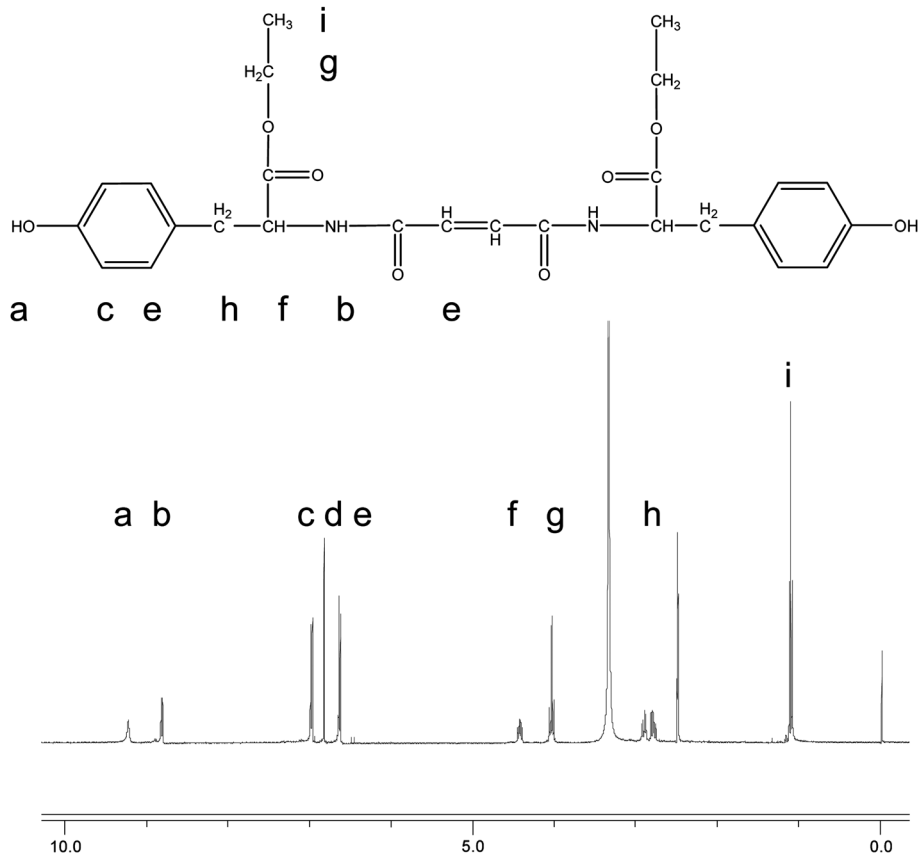


Fig. 1 ^{1}H NMR spectrum of TFT.

for 2 h. TFT was dissolved in DMF and slowly added to the prepolymer solution. The reaction was allowed to proceed for 6 h. The product was precipitated in anhydrous ethyl ether and dried at 60 °C under vacuum. Take PCL1000-HMDI-TFT for example, the IR and ^{1}H NMR spectra of polyurethanes were obtained. IR: ν (cm^{-1}) 3298 (N–H), 3075 (C–H benz), 2968 and 2925 (C–H aliph), 1738 (C=O urethane), 1713 (C=O ester), 1517 (N–H urethane). ^{1}H NMR (DMSO, 400 MHz): δ (ppm) 8.83 (2H NH in amide group), 6.90 (4H NH in urethane group), 6.97 and 6.64 (8H CH in benz), 6.83 (2H CH=CH in fumaric acid), 4.42 (2H, CH in tyrosine), 3.98, 2.23 and 1.51 (80H, CH_2 in PCL1000).

Results and discussion

The molecular weight and tensile properties of polyurethanes are shown in Table 1. The data indicate that the mechanical strength of PEG based polyurethanes was much lower than that of PCL based polyurethanes.

The thermal behavior of polyurethanes was assessed using DSC (Fig. 2). It could be observed that the glass transition temperature (T_g) was ranging from −4.9 °C to −49 °C for PEGPU and from −25 °C to −50 °C for PCLPU. The T_g of both PEG- and PCL-polyurethanes decreased with the increase of soft segment molecular weight. The PEG- polyurethanes exhibited higher T_g than the PCL-polyurethanes with the same soft segment molecular weight.

Only polyurethane containing PEG2000 showed crystallinity with T_m 28.5 °C. It may be attributed to the high soft segment molecular weight, which improved regularly chain packing. The polyurethanes containing PCL showed no crystallinity because of a higher degree of phase mixing between the soft segment and hard segment.

In vitro pepsin-inspired degradation behavior of the polyurethanes was examined to monitor the intensity of the hydrogen atoms in the pseudo peptide bond (δ = 8.8 ppm) and double bond of fumaric acid (δ = 6.8 ppm) by ^{1}H NMR spectroscopy. Fig. 3 and 4 showed the ^{1}H NMR spectra of PEG1000PU before and after 2, 4 and 8 days under the activity of pepsin 10U and 40U respectively. The peak of the hydrogen atoms in methylene group between the two hexamethylene groups of HMDI (δ = 0.8 ppm) was selected as the internal standard peak for the peak area analysis. The decreased intensity of the hydrogen atoms for both PU samples corresponded to digestion of pseudo peptide bonds. For PU samples digested by 10U pepsin, peak area of aim hydrogen atoms decreased about 15% after 2 days, about 35% after 4 days and around 60% after 8 days incubation. For PU samples digested by 40U pepsin, peak area of aim hydrogen atoms decreased about 30% after 2 days, about 60% after 4 days

Table 1 The molecular weights and tensile properties of polyurethanes

Polyurethanes	Diol	M_n	M_w	M_w/M_n
PEG600-HMDI-TFT	PEG600	28 450	63 088	2.21
PEG1000-HMDI-TFT	PEG1000	33 577	69 171	2.06
PEG2000-HMDI-TFT	PEG2000	27 660	58 622	2.11
PCL1000-HMDI-TFT	PCL1000	17 076	34 359	2.01
PCL2000-HMDI-TFT	PCL2000	29 785	62 395	2.09

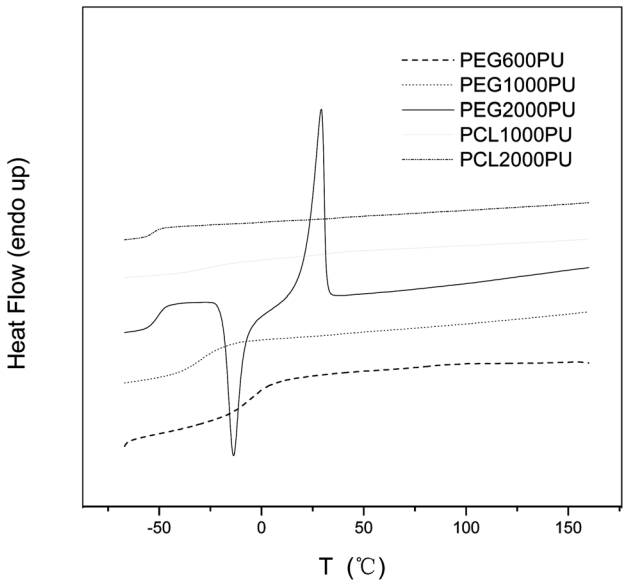


Fig. 2 DSC curves of polyurethanes.

and around 75% after 8 days incubation. With the increase of incubation time, peak area of aim hydrogen atoms kept decrease. It proved that the pseudo peptide bonds in polyurethanes were effectively cleaved by pepsin. Moreover, the decrease in intensity of the hydrogen atoms in benzene ring of tyrosine (δ = 6.9 and 6.6 ppm) and urethane group formed by phenolic hydroxyl group (δ = 9.2 ppm) could also be observed. The area of the peaks decreased about 5% after 2 days, about 20% after 4 days and around 35% after 8 days incubation under 10U pepsin. When the activity rose to 40U, the area of the peaks decreased about 15% after 2 days, about 35% after 4 days and around 50% after 8 days incubation. It indicated that after digestion of pseudo peptide bonds, the long chain PU broke into pieces. The fragments were much more susceptible to pepsin and the strong acid environment leading to the cleavage of parts of the urethane groups formed by phenolic hydroxyl groups. As a result, some

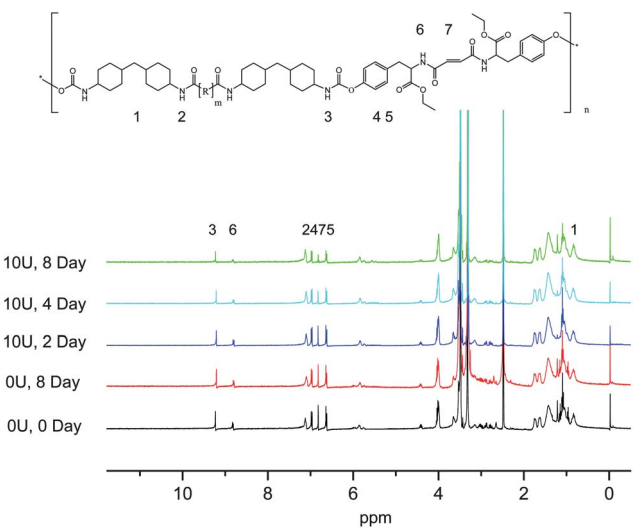


Fig. 3 ^{1}H NMR spectra of PEG1000PU before and after 2, 4 and 8 days of degradation mediated by 10U pepsin.

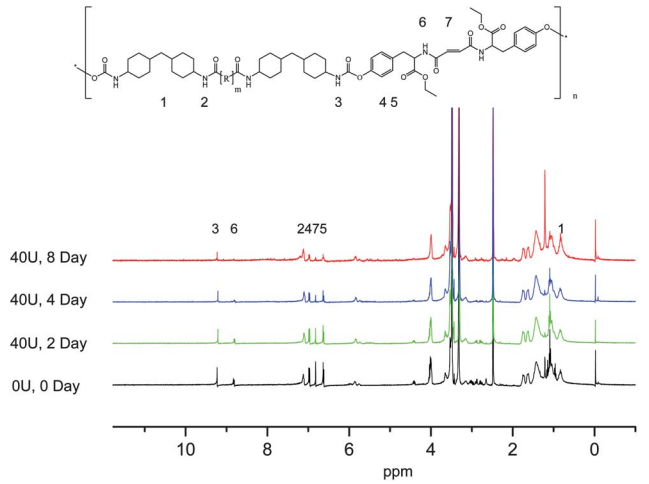
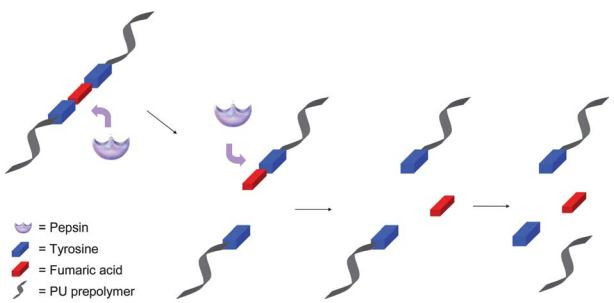


Fig. 4 ^{1}H NMR spectra of PEG1000PU before and after 2, 4 and 8 days of degradation mediated by 40U pepsin.

tyrosine was released into the incubation media and accelerated the collapse of PU (Scheme 2). For comparison, the control sample incubated in simulated gastric fluid solution (pH = 1.7) without pepsin was also characterized. The intensity of the hydrogen atoms in the pseudo peptide bond (δ = 8.8 ppm), double bond of fumaric acid (δ = 6.8 ppm) and benzene ring of tyrosine (δ = 6.9 and 6.6 ppm) showed almost no decrease after 8 days incubation revealing that acid seldom broke pseudo peptide bonds in the short term. The methylene of the soft segments and HMDI remained almost constant before and after degradation. The results of ^{1}H NMR spectroscopy proved that the degradation of the main chain mainly resulted from the collapse of pseudo peptide structures.

The changes in the weight of the PU samples at different time intervals was monitored and compared with the control samples. Fig. 5 and 6 showed the mass loss of samples exposed to simulated gastric fluid containing pepsin. The mass loss of both PEG1000 and PCL1000 based polyurethanes preceded a rapid increase during the first 4 days, followed a resembling linear increase at a lower rate during the second period up to 32 days. After 32 days incubation, PEG1000 based polyurethanes lost about 25% of their weight while PCL1000 based polyurethanes lost 15% because of the greater hydrophilicity of PEG1000. The hydrophobicity of PCL1000 based PU may lead to difficult access to the pepsin and then reduced the rate of degradation. As a contrast, the mass loss of control samples at the same time



Scheme 2 Schematic illustration of polyurethanes based on TFT degradation mediated by pepsin.

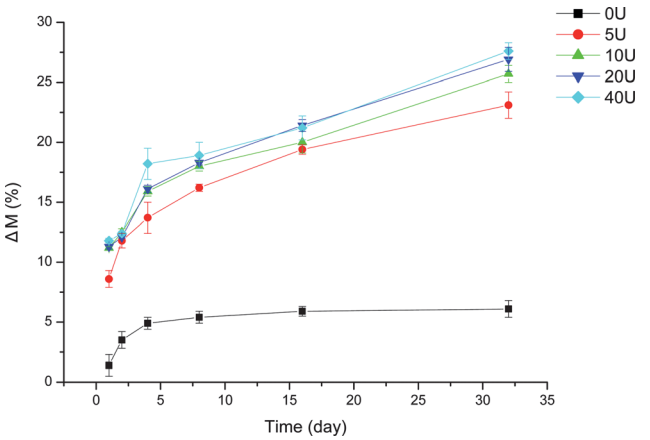


Fig. 5 Mass loss curves of PEG1000PU.

interval was about 6% and 3% for PEG1000 and PCL1000 based polyurethanes respectively, indicated that the degradation was accelerated greatly by pepsin. Still, the degradation rate of samples increased with the increase of activity of pepsin.

The SEM analysis of samples before and after degradation provided valuable proof for the digestion (Fig. 7). Numerous holes were observed on the surface of the PEG1000PU incubated for 4 and 8 days (Fig. 7b and 7c), compared to the smooth surface of original sample (Fig. 7a), indicating that the film underwent attack in the bulk. The original polyurethanes based on PCL1000 remained generally smooth, leaving only wrinkles on the surface (Fig. 7d). A mass of holes and grooves were observed on the surface of the film incubated for 4 days (Fig. 7e) and the holes and grooves became deeper after 8 days degradation (Fig. 7f), thus suggesting the degree of degradation persisted.

PCL based polyurethanes were selected and a WST-1 assay was carried out to quantitatively compare the biocompatibility of the novel polyurethanes by valuing the cell viability. The human umbilical vein endothelial cells were employed to detect the cytotoxicity of sample films and their effects on cells proliferation, and the cells seeded on a culture plate were counted as the control (Fig. 8). After culturing for 48 h, cells had undergone proliferation and a significant increase in cell viability was observed compared to the control sample with the cell viability of 209.1% for PCL1000PU and 188.9% for PCL2000PU. It was

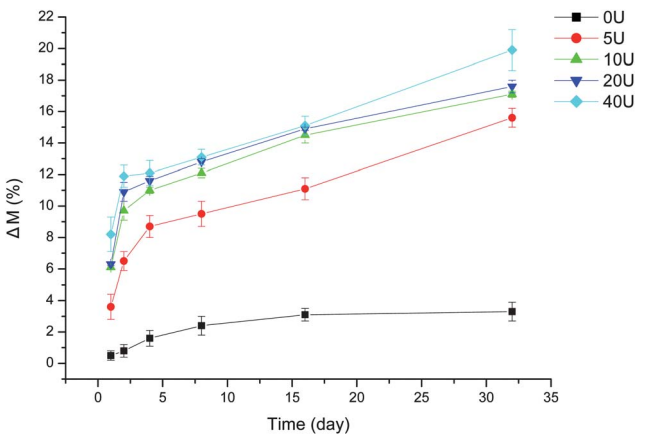


Fig. 6 Mass loss curves of PCL1000PU.

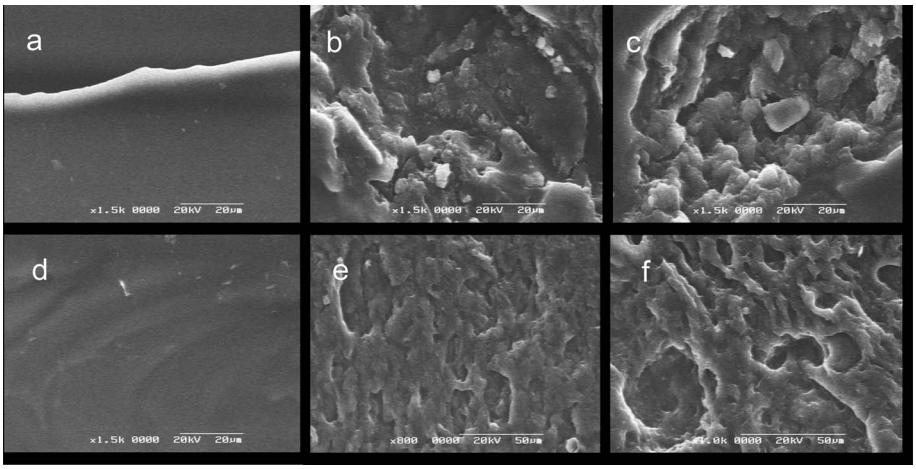


Fig. 7 SEM micrographs of the PEG1000PU and PCL1000PU after 0, 4 and 8 days of pepsin degradation. PEG1000PU before degradation (a), PEG1000PU after 4 days degradation (b), PEG1000PU after 8 days degradation (c), PCL1000PU before degradation (d), PCL1000PU after 4 days degradation (e), PCL1000PU after 8 days degradation (f).

shown that the amount of endothelial cells seeded on polyurethanes for 48 h was remarkably increased. It suggested that the sample has little negative influence on cellular proliferation. The distinct of viability between PCL1000PU and PCL2000PU might be due to different hydrophilicity and surface morphology.

Conclusion

Two series of novel peptide mimetic pepsin-inspired degradable polyurethanes have been successfully synthesized. The linear polymers were incubated with pepsin in simulated gastric fluid and the degradation behavior was monitored by ^{1}H NMR, mass loss and SEM. The results indicated that the pseudo peptide bonds in polyurethanes were effectively digested by the action of pepsin, and after that, parts of urethane groups formed by phenolic hydroxyl groups of tyrosine were cleaved to increase the collapse of polyurethanes. Furthermore, the rate and degree of pepsin degradation could be controlled by alteration of the soft segments and activity of pepsin. The result of cell viability test indicated the PCL1000PU based on TFT did not show a negative effect on endothelial cells and could act as potential biomaterials.

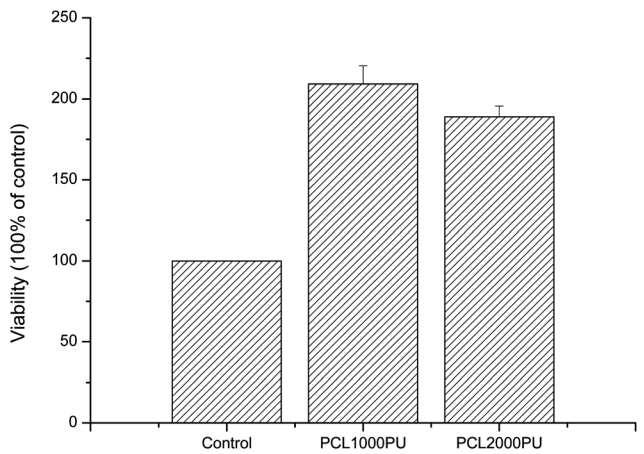


Fig. 8 Viability of human umbilical vein endothelial cells cultured on PCL based PU measured by the WST-1 assay after seeding for 48 h. HUVECs seeded on culture plates at the same time interval were used as a control.

Acknowledgements

Financial support from the National Science Foundation of China (20974061) and the Shanghai Leading Academic Discipline Project (No. B202) is gratefully acknowledged.

Reference

1 D. Hofmann, M. E. Castano, K. Kratz and A. Lendlein, *Adv. Mater.*, 2009, **21**, 1.
2 M. A. Dewit and E. R. Gillies, *J. Am. Chem. Soc.*, 2009, **131**, 18327.
3 G. Mihov, G. Draaisma, A. Dias and B. Turnell, *et al.*, *J. Controlled Release*, 2010, **184**, 46.
4 K. E. Broaders, S. J. Pastine, S. Grandhe and J. M. J. Frechet, *Chem. Commun.*, 2011, **47**, 665.
5 J. Liu, Z. Z. Jiang and W. M. Saltzman, *et al.*, *Biomaterials*, 2011, **32**, 6646.
6 L. J. Zhou, L. Q. Yu and Q. Fu, *et al.*, *Macromolecules*, 2011, **44**, 857.
7 Y. Hong, J. J. Guan and W. R. Wagner, *et al.*, *Biomaterials*, 2010, **31**, 4249.
8 H. Cheng, P. S. Hill and D. G. Anderson, *et al.*, *Adv. Mater.*, 2011, **23**, 95.
9 S. H. Kim, J. P. K. Tan and J. L. Hedrick, *Biomaterials*, 2011, **32**, 5505.
10 A. Muhammad and A. J. Jasim, *J. Chem. Soc. D*, 1969, 859.
11 S. K. Areida, D. P. Reinhardt and M. P. Marinkovich, *et al.*, *J. Biol. Chem.*, 2001, **276**, 1594.
12 S. Basu, L. P. Cunningham and P. J. Campagnola, *et al.*, *Biomacromolecules*, 2005, **6**, 1465.
13 L. Bracci, C. Falciani and B. Lelli, *et al.*, *J. Biol. Chem.*, 2003, **278**, 46590.
14 S. Thust and B. Koksch, *J. Org. Chem.*, 2003, **68**, 2290.
15 Y. Hong, J. J. Guan and W. R. Wagner, *et al.*, *Biomaterials*, 2010, **31**, 4249.
16 E. M. Rosenbauer, M. Wagner, A. Musyanovych and K. Landfester, *Macromolecules*, 2010, **43**, 5083.
17 P. Y. Sun, L. Y. Tian, Z. Zheng and X. L. Wang, *Acta Polym. Sin.*, 2009, **8**, 803.
18 H. T. Liu, W, L Xu, S. P. Zhao, J, J Huang, H, J Yang, Y, L Wang and C, X Ouyang, *J. Appl. Polym. Sci.*, 2010, **117**, 235.
19 M. Akane, K. Masafumi and H. Arai, *et al.*, *J. Bacteriol.*, 2008, **190**, 7170.
20 J. M. Worle-Knirsch, K. Pulskamp and H. F. Krug, *Nano Lett.*, 2006, **6**, 1261.
21 F. X. Li, Z. F. Liu and X. P. Liu, *et al.*, *Macromolecules*, 2005, **38**, 69.