IOP Conf. Series: Materials Science and Engineering **381** (2018) 012069 doi:10.1088/1757-899X/381/1/012069

Preparation and Degradation of Lysozyme-loaded Microspheres Based on Polyurethane

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Abstract. A series of block polyurethanes based on poly (3-hydroxybutyrate) (PHB-diol, Mn=6180) and poly (ethylene glycol, Mn=1500) segments were synthesized by a facil way of solution polymerization. The chemical structure was systematically characterized by ^{1}H, nuclear magnetic resonance spectrum (NMR) and Fourier transorm infrared spectroscopy. The polyurethanes microspheres were loaded with lysozyme by double emulsion solvent evaporation and the influence of environment on degradation process was investigated. It was stated that an accelerated degradation process was carried out at pH=9 and 37°C. The cumulative release of drug-loaded microspheres was achieved about 85% in phosphate buffer solution when the degradation time reaches 20 weeks. Moreover, lower degradation of the microspheres was observed after 12 weeks in the release medium. Results showed that the microspheres exhibit different degradable performance in different media from surface erosion to diffusion bulk collapsing.

1. Introduction

Polyhydroxybutyrate is a type of biodegradable biomaterial with good hot workability [1, 2]. However, PHB chains are hydrophobic and have high crystallinity. Modifications are mainly concentrated in biosynthetic modification [3, 4], macro monomer reaction modification [5, 6] and radiation method. PCL was introduced into the main chain of PHB via one-step solution polymerization [7]. The shape memory material with hydrophobicity and mechanical properties was prepared by introducing polylactic acid (PLA) into PHB matrix [8]. The copolymer network with excellent thermal stability was obtained which used polyethylene glycol diacrylate and diacrylate polyhydroxybutyrate valerate as monomers [9]. The polyurethane based on polyhydroxybutyrate and different molecular weight polyethylene glycols were prepared by using toluene-2, 4-diisocyanate (TDI) as a coupling agent [10, 11].

It is found that drug-loaded microsphere of PHB has good release properties [12, 13]. However, the high hydrophobicity of PHB limited further development in the field of sustained release[14]. Polyurethane possesses appropriate mechanical properties and excellent process ability by adjusting the chemical composition, which has been widely studied in drug controlled release. Micro phase separation structure also provides storage sites and release channels for drugs. Microspheres containing protein or peptide as controlled release devices have been widely used for the treatment of human diseases and animal health. Various techniques are available to encapsulate proteins into biodegradable microspheres such as W/O/W double emulsion, organic phase separation, supercritical fluid, and spray drying techniques. Among them, the W/O/W double emulsion technique is a well-used process.

2. Experimental

2.1. Materials

Polyhydroxybutyrate (PHB, Mn-7.0 ×105 ±10% g/mol) was purchased from Zhejiang Tianan Tech Co. Ltd. (Zhejiang, China). All PHB samples were purified by dissolving in chloroform followed by filtration and precipitation in ether before use. 1,6-hexamethylene diisocyanate (HDI) (99%, Sigma, USA) were used without further purification. Chloroform, p-Toluene sulfonic acid (PTSA), ethylene glycol were all from Kemiou Reagent Development Center (Tianjin, China) and used as received.

2.2. Preparation of dihydroxy-terminated PHB oligomers(PHB-diol)

Dihydroxyl terminated PHB oligomers (PHB-diol) were prepared by Tran's esterification between the purified PHB materials and ethylene glycol using p-toluene sulfonic acid as catalyst. Typically, purified PHB (10 g) was dissolved in 100 ml of chloroform and refluxed in nitrogen. Subsequently p-toluene sulfonic acid (4.8 g) and ethylene glycol (20 g) were added. The reaction temperature was controlled at 60°C. The reaction was carried out under reflux for 6~10 h depending on required molecular weight of PHB-diol. The resultant solution was washed with distilled water for 3 times, concentrated and dried under reduced pressure.

2.3. PHB-PEG urethane preparation

A series of polyurethanes (PUHE) were synthesized by different molar ratios of polyethylene glycol (Mn=1500 g/mol) and PHB-diols with different molecular weight. Polyethylene glycol was dried in a 25 mL two-necked tube at 40°C under vacuum overnight. Then, 20 mL of anhydrous 1, 2-dichloroethane was added to the flask, and any trace of water in the system was removed through azeotropic distillation with only 5 mL of 1,2-dichloroethane being left in the flask. When the flask was cooled down to 75°C, 0.20 g of HDI (1.2×10-3mol) and two drops of tin octoate (8×10-3 g) were added sequentially. The reaction mixture was stirred at 80°C under nitrogen atmosphere for 48 h. The resultant copolymer was precipitated in diethyl ether and further purified in a mixture of methanol and diethyl ether.

2.4. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy (FTIR) was measured by a Bio-Rad FTS135 (Bio-Rad, USA) spectrophotometer. PHB-co-PEG block polyurethane (PUHE) was dissolved in chloroform, coated on a KBr pellet and dried before test.

2.5. ^{1}H, NMR spectrum

The chemical structure and monomer composition were determined by 1H, NMR using a ADVANCE III 400MHz NMR spectrometer. The NMR spectrum was obtained at room temperature in CDCl3 (20 mg/mL) with tetramethylsilane (TMS) as an internal standard.

2.6. Preparation of lysozyme-PHB/PEG microspheres

The lysozyme -PHB/PEG microspheres were prepared by double emulsification solvent evaporation method. Lysozyme solution (W1) of 3 mL 3% w/v was injected into 15 mL 3% w/v PHB-PEG chloroform solution (O) under stirring (23,000 rpm) for 2–3 min to form the primary emulsion (W1/O). Then the primary emulsion was dripped into a 150 mL 1% w/v PVA solution (W2) at a rate of 5000 rpm for 4–5 min to form the secondary emulsion (W1/O/W2). High speed emulsification was achieved by homogenizer. Both lysozyme solution (W1) and the primary emulsion (W1/O) were added by injection through 0.2 mm injector in 15 s. The finial W1/O/W2 emulsion was left to evaporate under magnetic stirring (500 rpm) for 4–5 h[15]. The microspheres were separated by centrifugation at 5000 rpm for 5 min and washed three times with 100 mL of deionized water to remove PVA residues. Finally, the microspheres were lyophilized.

2.7. In vitro lysozyme release

Microspheres (50 mg) were placed in triplicate into Eppendorf tubes and incubated in 10 mL release medium (PBS buffer, pH = 7.4, 0.1 mol/L) under agitation (100 rpm) at 37 ± 0.5°C. At desirable time intervals, the microspheres suspension was centrifuged at 5000 rpm for 20 min. The supernatant (10mL) was withdrawn and replaced with 10 mL fresh release medium. The amount of lysozyme released was determined by measuring the lysozyme content in the supernatant. The lysozyme concentrations in the supernatants were determined by UV spectrophotometry at 281 nm. The corresponding adsorption amounts were calculated, and the adsorption kinetics curve was plotted, through which the equilibrium adsorption time was obtained.

3. Results and discussions

3.1. Characterization of infrared absorption spectra

To investigate the compositions of as-prepared PHB-diol and PUHE polyurethanes, FTIR is employed as shown in Figure 1. It is confirmed by the band at 3435cm^{-1} corresponding to the hydrogen-bonded hydroxyl groups with alkoxy, and this band appears in the spectrum of PHB-diol while it appears very weak in that of original PHB. As is shown in Figure1(c), the band near 3435 cm^{-1} attributed to bonded –NH– groups is overlapped with hydrogen-bonded hydroxyl groups with PEG, and absorption peak appears become more distinct. The band near 1723cm^{-1} corresponds to C=O stretching modes. The amide absorption at 1546 cm^{-1} is the characteristic peak of urethane, which demonstrates the copolymerization between PHB and PEG.

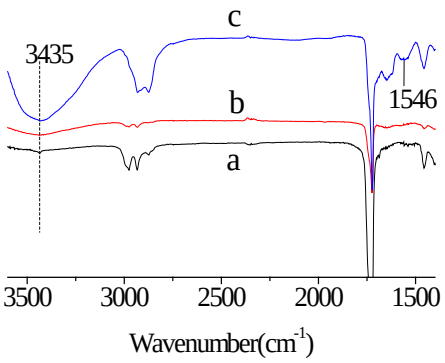

Figure 1. Infrared absorption spectra of (a)PHB,(b)PHB-diol and (c)PHB-PEG

3.2. Characterization of proton nuclear magnetic resonance spectra (^{1}HNMR)

The ^{1}H NMR ($CDCl_3$) spectra of polyurethanes(PUHE) are shown in Figure2: 5.21–5.29 (due to protons b), 4.93 (due to protons a and a′), 4.20(due to protons f and f′), 1.26 (due to protons c), 2.44-2.63 (due to protons d), 3.17 (due to protons g and g′), 3.66(due to protons e,e′,f and f′), 1.48(due to protons h and h′), 1.32(due to protons i and i′).

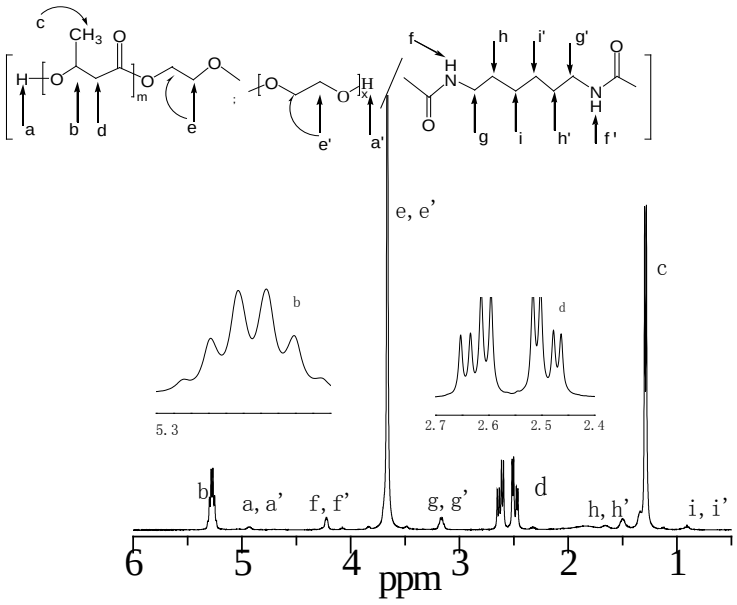

Figure 2. [1]HNMR spectra of polyurethane PHB-PEG

3.3. Effect of pH on release performance of drug-loaded microspheres

As shown in the Figure3, it was discussed that the effect of pH on the release properties of lysozyme, where pH was 7.4, 5 and 9.The release rate of the microspheres is the fastest in the alkaline medium, followed by the neutral medium, and the release rate of the acidic medium is the smallest. The reason may be that in the neutral buffer solution, water can penetrate into the gaps of the polymer chains through the pore on the surface of the microspheres, and initiates the random rupture of the polymer chains. At the same time, the carboxyl groups located on the surface of the microspheres can be neutralized by OH^- in the aqueous medium. Therefore, as the degradation progresses, more and more pores are formed to provide an efficient mass transfer channel for accumulated degradation products.

However, the presence of a large amount of H^+ replaces the weakly acidic carboxyl group and thus the hydrolysis of the ester bond was accelerated. And the acidic gradient formed by acidic degradation products is inhibited, and the autocatalytic effect no longer plays a major role in the degradation process of microspheres, so the release rate of drug microspheres in the acidic environment is minimal.

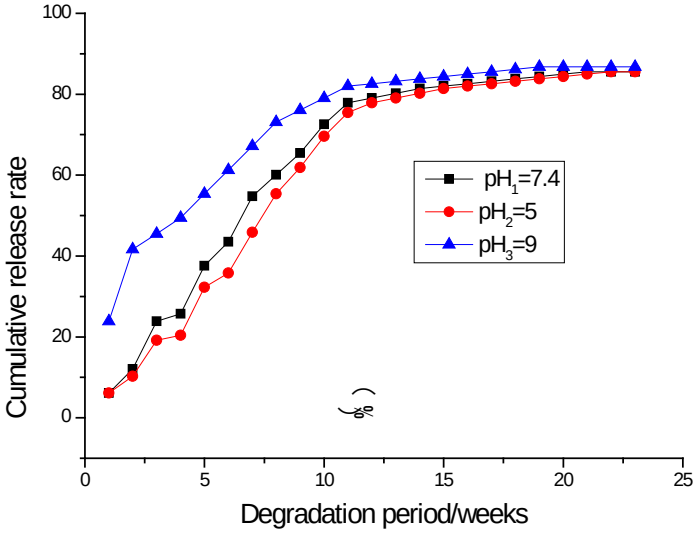

Figure 3. In vitro release kinetics of lysozyme -loaded microspheres

3.4. SEM analysis of microspheres after hydrolysis

The morphology of microspheres degraded in PBS were observed by SEM. It was shown that degradation occurred gradually from the surface to the inside. The surface morphology change accompanying with structural deterioration of the microspheres and an increase in porosity and cracks were observed throughout the course of degradation lasting over 2 weeks(Figure 4(a)), 5weeks(Figure 4(b))and 10 weeks (Figure 4(c)). The microspheres with higher PHB content in the copolymer (Figure 4(d), PHB%=75%) showed greater hydrolytic stability to degradation probably due to the preventing the entry of the hydroxyl and water molecules [16]. When the microspheres based on the copolymer incorporating the higher amount of PEG, (Figure 4(e), PHB%=50%; Figure 4(f), PHB%=25%), it presented more extensive cracks and network structures in progressive 2 weeks, suggesting extensive degradation (Figure 4 (e, f)).

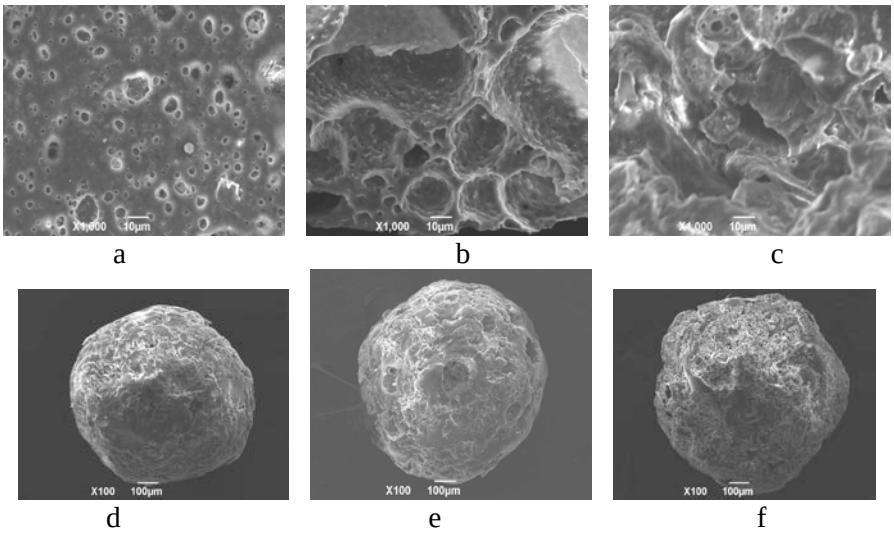

Figure 4. The morphology of microspheres after hydrolysis

4. Conclusions

A series of poly (PHB/PEG urethane)s having different compositions were synthesized. The microspheres based on polyurethane were loaded with lysozyme by double emulsion solvent evaporation and the influence of environment on degradation process was investigated. The cumulative release of drug-loaded microspheres was achieved about 85% in phosphate buffer solution at pH 7.4 at 37.5°C for a period of 20 weeks. SEM studies revealed that the the microspheres exhibit different degradation patterns in different media from surface erosion to diffusion bulk collapsing. And the microspheres with a larger amount of the hydrophilic PEG component led to a faster hydrolytic degradation.

5. Acknowledgements

The authors are grateful to 2017 Shaanxi Provincial Key Research and Development Plan (2017GY-180) and Innovation Training Program for Undergraduates of Xi'an Shi You University for financial support.

6. References

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