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SHORT COMMUNICATION

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An improved interfacial coacervation technique to fabricate biodegradable nanocapsules of an aqueous peptide solution from polylactide and its block copolymers with poly(ethylene glycol)

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Abstract *ABA* block copolymers of polylactide and poly(ethylene glycol) as amphiphilic bioabsorbable polymers were synthesized by the ring-opening polymerization of DL-lactide onto poly(ethylene glycol) (PEG 2000 or PEG 6000) and their structures were characterized on the basis of proton NMR. Biodegradable nanocapsules of an aqueous insulin solution were prepared from the block copolymers and polylactide by an improved interfacial coacervation technique. The results showed that the diameters of the nanocapsules were mainly dependent on the ratio of the two chains in the block polymers. The size of the nanocapsules decreased with an increase in the amount of surfactant used. More insulin solution resulted in an enlargement of the nanocapsules in diameter. In an optimum condition, biodegradable nanocapsules could be achieved with a size around 250 nm with a narrow distribution. The encapsulation percentages of insulin were larger in the nanocapsules from the PEG 2000 copolymers than in those from the PEG 6000 analogs and changed with the ratios of the blocks in the block copolymers.

Key words Biodegradable nanocapsule · Insulin · Polylactide · Block copolymer of polylactide and poly(ethylene glycol) · Drug delivery system

Introduction

Polylactide (PLA) is one of the most prominent bioabsorbable polymers with nontoxicity and good biocompatibility. So PLA and its copolymers have been widely used not only in biomedical devices but also in drug delivery systems. PLA and poly(lactide–glycolide) (PLGA) could be processed into microspheres [1, 2] by an oil-in-water emulsification–evaporation process and into injectable nanoparticles [3–8] by a salting-out process or by interfacial deposition. However, they were only effective to entrap some hydrophobic medicines such as steroidal contraceptives and a few antibiotics. When a drug was hydrophilic, its encapsulation percentage was quite low because the drug was loaded generally by adsorption on the surface of PLA microspheres and nanoparticles. For example, Muller et al. [9] entrapped a hydrophilic substance with DL-PLA nanoparticles by adsorption to give an encapsulation percentage of only 6%.

Many peptide or protein medicines were applied in clinics recently. Most of them were hydrophilic and easily biodegradable in the human body. If they could be entrapped into biodegradable nanocapsules with diameters smaller than 500 nm, their life in vivo would be prolonged and the corresponding dose could be easily absorbed, control-released and/or targeted after injection or oral administration. The question is how to fabricate the nanocapsules to entrap a hydrophilic drug. Although PLA or PLGA microcapsules have been made to entrap a peptide, a solution using a double emulsion–evaporation technique, their diameters ranged from 37 to 125 μm [10, 11]. An interfacial coacervation technique for the fabrication of smaller microcapsules with a

diameter of 15 μm was reported in 1964 [12]. An improved procedure was developed recently in our laboratory to prepare biodegradable nanocapsules with diameters of about 250 nm. PLA and its amphiphilic block copolymers with poly(ethylene glycol) (PEG) as the biodegradable polymers were employed instead of collodion, which was hard to biodegrade in vivo and water-miscible acetone was used to replace ether as the organic solvent. The amphiphilic bioabsorbable polymers were synthesized by the ring-opening polymerization of DL-lactide onto PEG as described previously [13, 14] and adapted to fabricate the nanocapsules by the improved interfacial coacervation technique. Insulin was encapsulated as a model of a hydrophilic peptide drug. The effects of some process conditions on the nanocapsule diameter and the insulin encapsulation percentage were investigated.

Experimental

Materials

DL-Lactic acid was purchased from Guangfu Institute of Fine Chemicals in Tianjin. PEGs, PEG 2000 and PEG 6000, were purchased from Tianjin Tiantai Chemical Company and were dried at 60 °C in a vacuum oven before use. Insulin (27 IU/mg) was supplied by Tianjin Biochemical Reagent Factory. Dextrin T-70 and Tween 20 as the biochemical reagents and stannous octoate with a purity of 95% were obtained from Sigma Company. Glycerol trioleate was made in the Second Factory of Chemical Reagents of Shanghai. DL-Lactide, melting point 125–127 °C, was prepared from DL-lactic acid according to the procedure described previously [15] and crude products were recrystallized three times in ethyl acetate. Other reagents and solvents were of analytical reagent grade made in China.

Methods

Synthesis of PLA–PEG–PLA block copolymer

DL -Lactide was mixed with PEG in a polymerization tube at the different proportions listed in Table 1. After stannous octoate (0.01% molar ratio to lactide) as a catalyst had been added, the mixture was gradually heated to 100 °C in a vacuum to remove volatiles. Then, the reaction system was purged with nitrogen. The temperature was then raised to 180 °C and kept for 30 h. The products were extracted with a cold mixture of ethyl acetate and *n*-heptane (2:1 v/v) to remove the possible homopolymer of lactide and then with distilled water to deplete unreacted PEG. The copolymers as residues were purified by repeated deposition and dried in a vacuum oven at 60 °C for 3 days.

Preparation of biodegradable nanocapsules of aqueous insulin solution from PLA and PLA–PEG–PLA copolymers

One of the PLA–PEG–PLA copolymers (200 mg), PLA(100 mg) and glycerol trioleate (0.2 ml) were dissolved in acetone (10 ml) to give an organic phase. Insulin solution (1 g/l, 6 ml) in 0.01 mol/l hydrochloric acid was an inner aqueous phase. The outer aqueous phase consisted of Tween 20 (0.5%) as a nonionic surfactant and Dextrin T-70 (1.0%) as a steric stabilizer in water (20 ml). The inner aqueous phase was added to the organic phase at room temperature under magnetic agitation to afford an emulsion. After half an hour, the emulsion formed was added to the outer aqueous phase under stirring to obtain nanocapsules of insulin solution in a colloidal suspension. Finally, the suspension was concentrated at 20 °C under vacuum to evaporate acetone and a little water.

Characerization of the block copolymers and the nanocapsules

^{1}H NMR spectra of the PLA–PEG–PLA copolymers were recorded using a Varian 400M NMR spectrometer using chloroform-*d* as solvent and tetramethylsilane as the internal standard. The mean diameter and size distribution of the nanocapsules were estimated by dynamic laser scattering using an INNDVO 300/BI9000AT photon correlation spectroscope (Brookhaven Co., USA). The encapsulation percentage of insulin was determined according to

Table 1 Preparation and characterization of the polylactide (*PLA*)–poly(ethylene glycol) (*PEG*)–PLA copolymers

Copolymer formed[a]	Feeding lactide (g)	Feeding PEG (g)	Theoretical C_{PLA} (%)[b]	Calculated C_{PLA} (%) from ^{1}H NMR[c]	Calculated M_n from ^{1}H NMR[d]
$P_{2k\text{-}15}$	0.9	5.1	15	13.2	2304
$P_{2k\text{-}30}$	1.8	4.2	30	29.8	2849
$P_{2k\text{-}52}$	2.8	3.0	52	45.1	3643
$P_{2k\text{-}70}$	4.2	1.8	70	68.0	6250
$P_{2k\text{-}90}$	5.4	0.6	90	89.4	18868
$P_{6k\text{-}30}$	1.8	4.2	30	25.8	8086
$P_{6k\text{-}50}$	3.0	3.0	50	41.2	10204
$P_{6k\text{-}70}$	4.2	1.8	70	63.5	16438
$P_{6k\text{-}90}$	5.4	0.6	90	86.6	44776
PLA	6.0	0.0	100	100	32,000

[a] In the copolymer samples $P_{x\text{-}y}$, 2k or 6k means that the molecular weight of the PEG used is 2000 or 6000, and the number y is the weight percentage of the PLA portion in a copolymer

[b] C_{PLA} means the weight percentage of the PLA portion in a copolymer; the theoretical C_{PLA} is calculated from the amounts of the feeding lactide and PEG

[c] Experimentall C_{PLA} is calculated from the areas of the peaks at 5.12 ppm (C**H** in PLA) and 3.6 ppm (C**H**$_2$ in PEG) in ^{1}H NMR according to the formula $C_{PLA} = M_{PLA}/(M_{PEG} + M_{PLA})$, $M_{PLA} = 6.5455(S_{5.12}/S_{3.6})$ because of $S_{3.6}/[4(M_{PEG}/44)] = S_{5.12}/[M_{PLA}/72]$, where M_{PEG} and M_{PLA} are the mean molecular weights of the PEG and PLA blocks in the copolymers, respectively, $S_{5.12}$ and $S_{3.6}$ are the peak areas at 5.12 ppm (C**H** in PLA) and 3.6 ppm (C**H**$_2$ in PEG) in ^{1}H NMR, respectively, and the numbers 44 and 72 are the molecular weights of the units –CH_2CH_2O– and $OCH(CH_3)CO$–, respectively, in the block copolymers

[d] M_n is the mean molecular weight of a copolymer calculated following the formula $M_n = M_{PEC} + M_{PLA}$

the measurement of free insulin in the nanocapsule suspension after separation of insulin and nanocapsules by gel permeation chromatography (GPC) using Dextrin gel G-75 as a stationary phase and tris(hydroxymethyl)aminomethane/HCl buffer (pH 7.1) as an eluant.

Results and discussion

Synthesis and ^{1}H NMR characterization of the PLA–PEG–PLA copolymers

Nine block copolymers of DL-lactide with PEG were synthesized by ring-opening polymerization as described previously [13, 14]. The ratio of the PLA block to PEG was varied in the range 15–90%. Their structures including the terminal groups were characterized by the ^{1}H NMR spectra [14]. The results showed that a PLA–PEG–PLA block polymers of *ABA* type was mainly formed. A little PLA–PEG copolymer of *AB* type was detected to coexist in some samples with low PLA portions (especially less than 50%) because a weak but sharp signal of the terminal OH group in the PEG moiety appeared at 2.63 ppm in their ^{1}H NMR spectra. According to the area ratio of the peaks at 5.12 ppm (C**H** signal in PLA blocks) and 3.6 ppm (C**H**$_2$ signal in PEG block) respectively, in the ^{1}H NMR spectra, the PLA portion and the mean molecular weights of the block copolymers were calculated and are listed in the Table 1. It was found that the experimental ratios of the PLA block to the PEG portion calculated from the NMR signals were only slightly lower than the theoretical ones calculated from the feeding amount of lactide and PEG in the polymerization, suggesting that the monomer conversion should be quite high. The data in the Table 1 show that the number-average molecular weight (M_n) of the copolymers increased with the PLA weight portion in the PLA–PEG–PLA copolymers (C_{PLA}), (while the molecular weight of PEG was constant. Moreover, the molecular weights of the PLA–PEG–PLA copolymers from PEG 6000, in a range from 8086 to 44776, were correspondingly much higher than those of the block copolymers from PEG 2000. When the feeding weight of PEG 6000 was equal to that of PEG 2000, the number of terminal OH groups was 3 times less, so more lactide monomers should be propagated onto one PEG 6000 molecule to give higher M_n.

Fabrication of the biodegradable nanocapsules

Nanocapsules were fabricated from the synthesized block copolymers by two stages of dispersion in the improved interfacial coacervation technique. The formation mechanism of the nanocapsules proposed is shown in Fig. 1. When an aqueous insulin solution was dispersed in an acetone phase containing an amphiphilic copolymer, PLA and glycerol trioleate, the copolymers were transferred from the bulky acetone phase onto the interface of the aqueous microdroplets to form a layer because the PEG portion of the copolymer was hydrophilic. It is known that acetone is miscible with water, so water molecules in the microdroplets would diffuse across the layer into the acetone phase to some extent. However, insulin might be kept within the microdroplets because it had a large molecular weight and its four ammonium groups $-NH_3^+$ (including two terminal $-NH_3^+$ groups and the residual $-NH_3^+$ groups from lysine and arginine) would be bound by hydrogen bonds with oxygen atoms of PEG loops in PLA–PEG–PLA copolymers as in crown ether. If the emulsified system was again mixed in the bulky outer aqueous phase containing a nonionic surfactant and a polymeric stabilizer, acetone would be dissolved in water to force the further interfacial coacervation of PLA and glycerol trioleate in acetone solution onto the interface. As a result, a thick membrane could be formed out of the microdroplets. The surfactant and polymeric stabilizer should move to the outer surface and make their hydrophilic parts face the outer aqueous phase. The secondary stage of this procedure was similar to the interfacial polymer deposition described by Fessi et al. [16]. Finally, a nanocapsule system was obtained after acetone was removed by vacuum evaporation at room temperature.

Influence of the PLA–PEG–PLA copolymeric structures on the diameter of the nanocapsules

The diameters of the nanocapsules from different block copolymers were measured by a dynamic laser scattering technique and are listed in Table 2. The results show that the values of the polydispersity were generally not larger than 0.2, indicating that the size distribution of the nanocapsules was quite narrow. That was due to the

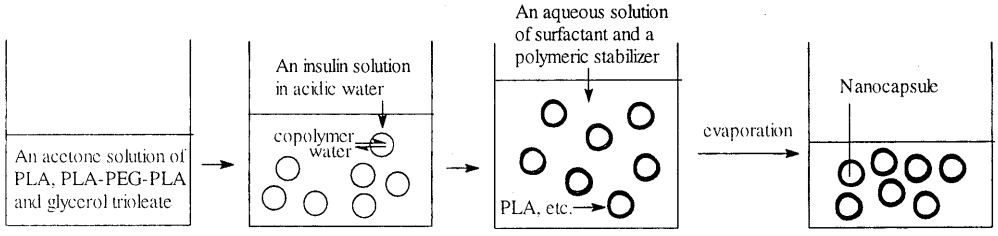

Fig. 1 Schematic diagram of the mechanism proposed for the formation of biodegradable nanocapsules by the improved interfacial coacervation technique

Table 2 Fabrication and diameter characterization of insulin nanocapsules of insulin solution from the block copolymers and PLA

Sample number	Copolymers used	Mean diameter of the nanocapsule (nm)	Polydispersity
1	$P_{2k\text{-}15}$	283	0.1676
2	$P_{2k\text{-}30}$	133	0.1797
3	$P_{2k\text{-}52}$	119	0.1450
4	$P_{2k\text{-}70}$	625	0.1472
5	$P_{2k\text{-}90}$	231	0.1857
6	$P_{6k\text{-}30}$	426	0.1746
7	$P_{6k\text{-}50}$	668	0.1601
8	$P_{6k\text{-}70}$	726	0.2383
9	$P_{6k\text{-}90}$	565	0.2189
10	PLA	726	0.2949

stabilizing function of the amphiphilic PLA–PEG–PLA copolymers on the microdroplets in the first stage of dispersion. However, the diameters changed a lot for the nanocapsules with different block polymers. It was known that the compositions of the inner aqueous phase, the acetone phase, and the outer aqueous phase would influence the size of the nanocapsules formed. By keeping other factors constant, the amphiphilic properties of the copolymers, dependent on the total molecular weight and the molecular weights of each block, should be the main factors. Generally speaking, the higher the molecular weight of the PLA portion or the total M_n, the larger the nanocapsules formed. The diameters of the nanocapsules from the PLA copolymers of PEG 6000 ranged from 426 to 726 nm, comparable with the large size (726 nm) of the nanocapsules from pure PLA; however, for the nanocapsules from the copolymers of PEG 2000, smaller diameters were observed because the molecular weights of the PLA blocks were lower. When the molecular weight of the PLA portion was less than that of PEG in the copolymers $P_{2k\text{-}15}$, $P_{2k\text{-}30}$ and $P_{2k\text{-}52}$, the smallest nanocapsules, with diameters of about 100 nm, were formed. It was also noted that nanocapsules with a mean diameter of 231 nm were formed from the copolymer $P_{2k\text{-}90}$ with a total molecular weight of 18,868 Da. This implies that the diameters of the nanocapsules were not only dependent on the molecular weights of the PLA portions or an entire copolymer but also on the stability of the nanocapsules early formed during the secondary stage of dispersion. If the nanocapsules formed in the first stage of dispersion were kept stable in the following procedure, their diameters should be small as expected. That was the case with the nanocapsules from the copolymer $P_{2k\text{-}90}$.

Influence of the other preparation conditions for the biodegradable polymers

The influence of other factors on the formation of the biodegradable nanocapsules can be seen in the Table 3. If twofold insulin solution was employed as in sample 11, smaller nanocapsules were formed. This implies that insulin in an acidic medium acts as a surfactant just like most proteins and polypeptides, and often existed on the interface of the water-in-oil emulsion. More glycerol trioleate in the acetone phase results in a decrease in the diameters of the nanocapsules as in samples 12 and 14 because glycerol trioleate makes the acetone solution less water-miscible and is helpful for the dispersion of the inner aqueous phase in the first stage. As a rule, more surfactants, including Tween 20 and Dextrin T-70, in the outer aqueous phase promote the dispersion in the second stage and prevent the nanocapsules formed from aggregation so as to afford much smaller sizes of the nanocapsules; that was the case for samples 13 and 14. In addition, the application of a large amount of the surfactant could make the size distribution of the nanocapsules prepared very narrow: the corresponding polydispersity measured by the laser scattering technique was less than 0.1.

Insulin encapsulation into the nanocapsules of the block copolymers

In order to prove that insulin was really encapsulated in the nanocapsules prepared, the encapsulation percent-

Table 3 Effects of the preparation conditions for the biodegradable polymers on the sizes of the nanocapsules. For all samples, the concentration of insulin in 0.01 M hydrochloric acid is 1 g/l; The ratio of the acetone phase to the outer water phase is 1:2. The amphiphilic copolymer is $P_{2k\text{-}90}$ with a concentration of 20 g/l in acetone and the concentration of PLA is 10 g/l

Sample number	Feeding amount of insulin solution (ml)	Feeding amount of glycerol trioleate (ml)	Concentration of components in outer water phase (%)		Mean diameter of nanocapsule (nm)	Polydispersity
			Dextrin T-70	Tween 20		
5	6	0.2	1	0.5	231	0.1857
11	12	0.2	1	0.5	138	0.1172
12	6	0.3	1	0.5	152	0.1372
13	6	0.2	1.5	0.75	88	0.0939
14	6	0.3	1.5	0.75	139	0.0705

age of insulin in some nanocapsules was also measured. Generally speaking, insulin solution could not be entirely trapped in the capsules as reported to date. If the amount of leaked insulin is determined, the encapsulation percentage can be calculated; however, the direct measurement of free insulin in the suspension was often interfered with by the nanocapsules. The nanocapsules prepared were too small in size to be separated with free insulin by common techniques such as filtration, centrifugation and dialysis. In this study, GPC was chosen to separate free insulin with the nanocapsules in the suspension. The encapsulation percentages were calculated through the measurement of free insulin and the data are listed in the Table 4. The result showed that all nanocapsules from the amphiphilic copolymers synthesized could encapsulate insulin with percentages from 28.82 to 70.58%, much more than that from pure PLA (18.85%), indicating that the amphiphilic block copolymers played an important role for the encapsulation of insulin. The properties of the copolymers influenced the encapsulation of insulin during the two stages of dispersion. The insulin encapsulation percentages (30.35–70.58%) of the PLA–PLA–PEG nanocapsules from PEG 2000 were generally higher than those (28.82–42.92%) of the copolymer nanocapsules from PEG 6000. When the molecular weights of the block copolymers from PEG 2000 were low, or when their PLA portions were small, these very hydrophilic copolymers could easily transfer from the acetone phase onto the interface of the inner aqueous microdroplets just after the inner aqueous phase was added to acetone. Therefore, not so much water and insulin in the inner aqueous phase diffused (dissolved) into the acetone phase before a capsule was formed. The nanocapsule from the block polymer $P_{2k\text{-}15}$ was an excellent example; the encapsulation percentage for insulin was up to 70.58%. If the molecular weight of a block copolymer or its hydrophobic PLA portion was high, its transfer rate from acetone to the interface was slow, so it could not prevent water and insulin in the aqueous microdroplet from entering the acetone phase. As a result, a low encapsulation percentage of insulin was finally obtained. That was the case with the block copolymers from PEG 6000.

Another important factor affecting the encapsulation percentage of insulin should be the stability of the nanocapsules formed in the first stage of dispersion while they were again dispersed in the outer aqueous phase. If the molecular weights of the three blocks in the PLA–PEG–PLA copolymers of *ABA* type such as $P_{2k\text{-}70}$ and $P_{6k\text{-}70}$ were almost same, the corresponding nanocapsules formed in the first stage of dispersion would be unstable when they were again dispersed in the bulky aqueous phase. The nanocapsules would be broken through an inversion of the hydrophilic portion in both copolymers from the inner aqueous phase to the outer aqueous phase, so insulin in the capsules would leak into the outer aqueous phase and the lowest encapsulation percentages (30.35 and 28.82%, respectively) were observed. If the PEG portion was much longer than the PLA blocks in the copolymers, such as $P_{2k\text{-}15}$, or if each of the PLA blocks was much longer than the PEG block in the copolymers, such as $P_{2k\text{-}90}$ and $P_{6k\text{-}90}$, the corresponding nanocapsules formed in the first stage would be kept stable while they were again dispersed in the bulky aqueous phase. So the high encapsulation percentages of insulin should result.

Table 4 Encapsulation percentage of insulin in the fabricated nanocapsules from the amphiphilic copolymers and PLA

Sample number	Copolymers used	Encapsulation percentage of insulin
1	$P_{2k\text{-}15}$	70.58
2	$P_{2k\text{-}30}$	37.85
3	$P_{2k\text{-}52}$	46.49
4	$P_{2k\text{-}70}$	30.35
5	$P_{2k\text{-}90}$	49.23
6	$P_{6k\text{-}30}$	32.35
7	$P_{6k\text{-}50}$	32.90
8	$P_{6k\text{-}70}$	28.82
9	$P_{6k\text{-}90}$	42.92
10	PLA	18.85

Conclusion

Two series of PLA–PEG–PLA block copolymers as t amphiphilic bioabsorbable materials were synthesized by the ring-opening polymerization of lactide onto PEG 2000 and PEG 6000, respectively. The molecular weights and PLA portions of the copolymers were controlled by a change of the feeding amounts of lactide and PEG. Nanocapsules of insulin solution were fabricated from the copolymers and PLA by a novel two stages of dispersion as an improved interfacial coacervation technique. The diameters and insulin encapsulation percentages of the nanocapsules were dependent on the total molecular weights and/or those of the blocks in the copolymers and other preparation conditions. Using the PLA–PEG–PLA copolymer $P_{2k\text{-}90}$ with a molecular weight of 18,868 Da and a PLA portion of 89.4% in weight, nanocapsules with diameters around 250 nm were successfully prepared to encapsulate insulin with a percentage of 49.23%. Such a novel technique in the preparation of bioabsorbable nanocapsules proved promising for the encapsulation and controlled release of hydrophilic peptide drugs.

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