Synthesis of Poly(ethylene glycol)–Dopamine Conjugates and Their Controlled Drug-Release Behaviors

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Received 12 May 2010; accepted 14 November 2010
DOI 10.1002/app.33768
Published online 14 March 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Dopamine (DA) was covalently linked via succinic anhydride spacers to poly(ethylene glycol)s (PEGs) with average molecular weights of 4000 (PEG4000), 6000 (PEG6000), and 10,000 (PEG10000). The chemical modification of the PEGs was conducted by a two-step protocol: (1) the preparation of PEG having carboxylic end groups and (2) the synthesis of PEG4000–DA, PEG6000–DA, and PEG10000–DA. The controlled drug-release studies were performed in pH 1.1, 7.4, and 9.0 buffer solutions, The results demonstrate that under the same conditions, the rate of hydrolysis for PEG10000–DA was the slowest among three prodrugs, and a greater amount of DA could be detected being released from the prodrug matrices in the presence of α-chymotrypsin in a buffer solution with pH 8.0. Also, these novel prodrugs could slowly release the active drug molecules and improve the pharmacokinetics of DA. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 1992–1998, 2011

Key words: biological applications of polymers; conjugated polymers; synthesis

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder associated primarily with a loss of dopamine (DA) neurons in the nigrostriatal system. Typical symptoms include tremors, rigidity, slowed body movements, unstable postures, and difficulty walking.[1] It results from the degeneration of DA-producing nerve cells in the brain, specifically in the substantia nigra and the locus coeruleus. DA is the neurotransmitter that stimulates the motor neurons, the nerve cells that control muscle movement. When DA production is depleted, as in PD, the patient is unable to control movement and coordination.[2] The conversion of DA, a water-soluble catecholamine that does not cross the blood–brain barrier (BBB), into the corresponding L-amino acid, levodopa (L-DOPA), enables DA delivery to the brain; this has been the mainstay of the treatment of PD for nearly 40 years.[3] The use of L-DOPA to deliver DA to the brain is a BBB drug-delivery strategy that uses large a neutral amino acid transporter type 1 (LAT1), one of the carrier-mediated transport systems within the BBB. Upon crossing the BBB through LAT1, L-DOPA is converted back to DA within the brain by the aromatic amino acid decarboxylase. Unfortunately, conversion of L-DOPA to DA also occurs in peripheral tissues as well, especially in the gut. Therefore, only a small fraction of L-DOPA is able to reach the brain, the primary site of drug action. Thus, the unwanted side effects associated with treatment with L-DOPA during the beginning of therapy are mainly caused by its premature conversion to DA in peripheral tissues.[4] The main disadvantages of L-DOPA are its low water solubility, sensitivity to chemical and enzymatic oxidation, and peripheral decarboxylation.[5] The plasma half-life of L-DOPA also only averages 1.3 h.[6] With regard to the previously discussed limitation of L-DOPA, Dalpiaz and coworkers[7,8] prepared a prodrug (glu-dopamine) by coupling DA to D-glucose via a succinic spacer. They demonstrated that DA conjugation to glucose allowed it to induce therapeutic effects against PD after intravenous administration.

Polymer drug-delivery systems can often compensate some shortcomings of small molecular drugs, such as side effects, limited water solubility, poor biocompatibility, biostability, and immunogenicity.[9] Recently, more research has shown that poly(ethylene glycol) (PEG) is an ideal carrier of polymer prodrugs.[10,11] PEG is known to be a nontoxic, nonimmunogenic, biocompatible, and water-soluble polymer used in biotechnology, biomaterials, and pharmaceutics. PEG can be chemically activated and may be attached to a compound at single or multiple sites. The covalent attachment of PEG to a protein

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Contract grant sponsor: National Nature Science Foundation of China; contract grant number: 20374040.
Contract grant sponsor: Nature Science Foundation of Shannxi; contract grant number: 09JK791.

has been used to improve the circulating half-life, decrease the immunogenicity, and reduce the proteolytic degradation. This approach of the covalent attachment of PEG to a protein or other active agent is commonly referred to as *PEGylation*.[12,13]

As it is well known, a large molecule can barely cross the BBB. However, it is fortunate that PEGylated liposome nanoparticles were proven to be successful in brain drug delivery.[14,15] The Fishburn group[16] also discovered that when PEG polymers were attached to small-molecule drugs, a drug's delivery profile could be optimized by the modification of its ability to cross biological membranes, such as the membranes associated with the gastrointestinal barrier, the BBB, and the placental barrier. The conjugation of PEG to a met-enkephalin analogue, [*D*-Pen(2,5)]-enkephalin, induced significant changes in a number of factors, including elimination half-life, volume of distribution, protein binding, hydrophilicity, receptor binding, P-glycoprotein (P-gp) affinity, metabolism, and membrane transport; this resulted in an improved analgesic effect.[17] PEG-treated poly(alkyl cyanoacrylate) nanoparticles were shown to cross the BBB and accumulate at high densities in the brain in experimental allergic encephalomyelitis.[18] Also, a retroconvection-enhanced delivery method has been developed to improve the entry of intravenously administered therapeutics within solid brain tumors by increasing the blood-to-brain transfer of macromolecules.[19] This technology should be helpful to macromolecules crossing the BBB in the brain delivery of polymer prodrugs. On a side note, Kortekaas et al.[20] showed that PD patients have reduced P-gp function in the BBB. The uptake of the drug verapamil was significantly elevated in the midbrain of PD patients relative to controls. This implies that reduced P-gp function of PD patients in the BBB is beneficial to the transfer of the drug to the brain. In fact, Pardridge[21] transported PEGylated liposome nanoparticles across the BBB by a new transvascular gene therapy of PD.

On the basis of the previously discussed studies, we attempted to prepare a PEG–DA prodrug by the attachment of PEG to a small molecular drug DA with superiority for prolonging the pharmacological activity, enhancing the absorption, and providing a protection against DA loss. The prodrug PEG–DA could release DA by cleaving enzymatically in the brain tissue. The synthesis method for the PEG–DA prodrug has been rarely reported. Kalcic et al.[22,23] covalently bonded DA to a styrene/maleic anhydride copolymer and poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] to prepare two polymeric prodrugs (styrene-maleic anhydride copolymer (SMA)–DP and poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)] (PHEA–DP)) as potentially more stable forms of dopamine (DA). We used succinic anhydride as a linker to modify the two end hydroxyl group of PEGs with different molecular weights, and the modified PEGs with two end carboxyl group were linked to DA by an amide-type bond to obtain several PEG–DA conjugates. Their detailed molecular structures were characterized via NMR and Fourier transform infrared techniques. The conjugates showed markedly improved solubilities and chemical stabilities at acidic pH and also at physiological pH. Furthermore, study *in vitro* on drug release from these prodrugs in various media demonstrated that a significant controlled release effect for DA was achieved.

EXPERIMENTAL

Materials and methods

DA was obtained from Aldrich Chemical Co.(Beijing, China). Analytical-grade PEGs with average molecular weights of approximately 4000 (PEG4000), 6000 (PEG6000), and 10,000 (PEG10000) were made by Tian-Tai Fine Chemical, Ltd. (Beijing, China). α-Chymotrypsin was purchased from Kaiyau Medicine Co. (Beijing, China). A dialtic bag (cutoff molecular weight = 3500) was obtained from Huamei Biochemical Co. (Beijing, China). Succinic anhydride was a chemical grade from Guoyao Chemical Co. (Shan-Xi, China); triethylamine, 1,4-dioxane, methanol, ethyl ether, and other chemicals were analytical grade and were made in China. They were used as received without further purification.

^{1}H-NMR measurements were conducted on a Varian Inova 400 spectrometer Varian, Palo Alto, CA, USA at room temperature with $CDCl_3$ as a solvent. Infrared spectroscopy experiments were preformed on a Specode 75 model (Carl Zeiss, Jena, Germany) with KBr as the sample holder. Ultraviolet–visible spectra were recorded on a UV-1700 spectrophotometer (Beijing Beyond Technology Development Co., Ltd., Beijing, China). Differential scanning calorimetry (DSC; MDSC 2910, TA Instruments) was used to determine the thermal behaviors of DA and the prodrugs. The scan rate was set to 20°C/min within the temperature range 30–300°C.

Preparation of PEG–$(COOH)_2$

PEG4000–$(COOH)_2$, poly(Ethylene glycol) modified by two succinic anhydrides, structure in Scheme 1 PEG6000–$(COOH)_2$, and PEG10000–$(COOH)_2$ were synthesized according to a previously published procedure.[24,25]

Synthesis of the PEG4000–DA conjugate

In a 100-mL, three-necked, round-bottom flask equipped with a magnetic stirring bar and a reflux condenser, PEG4000–$(COOH)_2$ (3.0 g), DA (0.27 g), DCC N,N-dicyclohexylcarbodimide (0.25 g), and

CH_2Cl_2 (24 mL) were added under stirring and heated to reflux for 24 h. The solvent was evaporated *in vacuo*, the system was cooled to room temperature, and the product was obtained by its precipitation into 20 mL of ethyl ether and then filtered. The product was washed three times with ethyl ether, dicyclohexyl urea was removed, and finally, a white crystal was obtained and dried *in vacuo*.

The fact that no free drug of DA existed in the polymer prodrug was confirmed by thin-layer chromatography and DSC measurements. A standard curve was made by pure DA in a water solution. The content of DA in the conjugate was calculated by comparing the slopes of the standard curve of DA at a 280.0-nm wavelength on the basis of the release of DA in alkaline media after 1 h at 60°C. It was confirmed that the DA content in the conjugate was 6.8%.

^{1}H-NMR ($CDCl_3$, 400 MHz, δ, ppm): 6.7 (m, 2H, Ar—H), 6.4 (s, 1H, Ar—H), 2.8 (m, 2H, Ar—CH_2—CH_2), 3.5 (m, 2H, N—CH_2—CH_2), 2.6 (m, 2H, O=C—CH_2—CH_2), 2.6 (m, 2H, O=C—CH_2—CH_2), 3.6 (m, 180H, PEG).

The same procedure was used to prepare PEG6000–DA and PEG10000–DA. The DA contents in the PEG6000–DA and PEG10000–DA conjugates were 4.9 and 1.83%.

Drug-release tests via hydrolysis

The drug-release experiments via hydrolysis were carried out at pH 1.1 (0.002*M* glycine/0.002*M* KCl adjusted to pH 1.1 with HCl), pH 7.4 (0.005*M* Na_2HPO_4/0.001*M* KH_2PO_4 adjusted to pH 7.4 with NaOH), pH 8.0 [0.080*M* tris(hydroxymethyl)aminomethane (Tris)/0.100*M* $CaCl_2$ adjusted to pH 8.0 with HCl], and pH 10.0 (0.012*M* Na_2CO_3/0.008*M* $NaHCO_3$ adjusted to pH 10.0 with NaOH) at 37 ± 0.1°C.[26,27] PEG–DA was first put into a dialtic bag and sealed; it was then was immersed in a buffer solution of 25 mL. After a suitable time interval, 5.0 mL of the solution released was withdrawn, and another 5.0 mL of fresh buffer solution was added to maintain the system at a stable volume.[28] Standard curves were made by pure DA in various buffer solutions. The concentration of the DA released was analyzed and calculated by the comparison of the slopes of the standard curves at 280.0 nm. Each experiment was repeated three times.

Enzymatic hydrolysis experiment

The hydrolytic stability of PEG–DA to α-chymotrypsin was assessed in a 0.08*M* Tris buffer, 0.1*M* $CaCl_2$ buffer solution at pH 8.0.[29] A 10^{-5}*M* α-chymotrypsin solution (200 μL) and 0.001*M* HCl were added to 2 mL of a PEG–DA solution containing 7.1 mg of DA and was then put it into a dialtic bag, sealed, and incubated in 40 mL of a buffer solution at 37 ± 0.1°C. After a suitable time interval, 5.0 mL of the solution released was withdrawn, and 5.0 mL of fresh buffer solution was added to maintain the system with a stable volume. A standard curve was made by DA in pH 8.0 buffer solutions. The concentration of DA released was analyzed and calculated by the comparison of the slopes of the standard curves at 280.0 nm. Each experiment was repeated three times.

Determination of the DA content in the conjugates

The DA content was estimated by both high performance liquid chromatography and ultraviolet spectroscopy. The ultraviolet spectroscopy data were obtained by the comparison of the absorbances of DA and the conjugate at 280.0 nm. The high performance liquid chromatography data were obtained by the determination of the concentration of free DA after total hydrolysis of the polymer–drug conjugates. Both measurement results were in good accordance.

RESULTS AND DISCUSSION

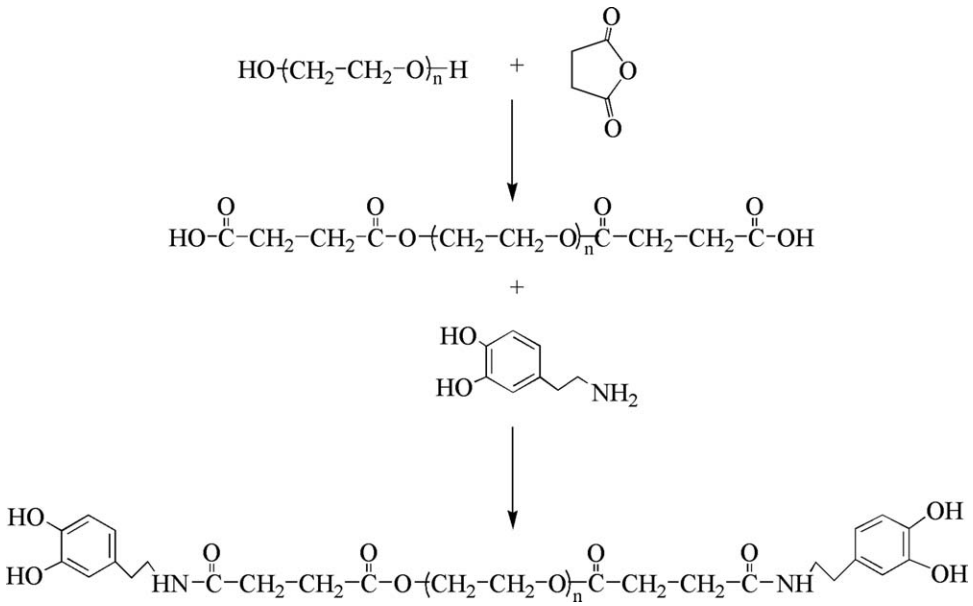
Synthesis of the PEG–DA conjugate

The DA could react chemically with PEG, but it needed to use a succinic anhydride linkage. To fulfill this goal, first, the reaction of DA with succinic anhydride to obtain an intermediate of PEG with active carboxylic terminal was necessary. Then, the PEG–DA conjugate was synthesized by reaction of the intermediate with DA. The detailed chemical route is shown in Scheme 1.

The amide bond in the PEG–DA conjugate could be hydrolyzed or enzymatically hydrolyzed via human body fluid. This is the fundamental mechanism for realizing the controlled drug release for DA with pharmacological activity. The detailed release process is shown in Scheme 2. With this mechanism, the PEG–DA conjugate was first hydrolyzed into *N*-3,4-dihydroxyphenylethyl succinamide [Scheme 2(a)] and PEG [Scheme 2(b)]; then, *N*-3,4-dihydroxyphenylethyl succinamide was further hydrolyzed into DA [Scheme 2(c)] and succinic acid [Scheme 2(d)]. In the experiment, we obtained the intermediate *N*-3,4-dihydroxyphenylethyl succinamide as a brown oil.

ANAL. Calcd for $C_{12}H_{15}NO_5$: C, 56.91%; H, 5.97%; N, 5.53%. Found: C, 57.18%; H, 5.91%; N, 5.59%. ^{1}H-NMR (CD_3OD, *d*): 2.28 (m, 2H, —$COCH_2CH_2$—CO—), 2.42 (m, 2H, 7-CH_2), 2.51 (m, 2H, —$COCH_2$ CH_2—CO—), 3.16 (m, 2H, 8-CH_2), 6.39 (d, 1H, 6-H), 6.52 (s, 1H,2-H), 6.55 (d, 1H, 5-H).

Thin-layer chromatography and DSC measurements were used to look for the existence of free DA in the polymer prodrug, and the results are shown in Figure 1. Pure DA's melting point was at 241–243°C;



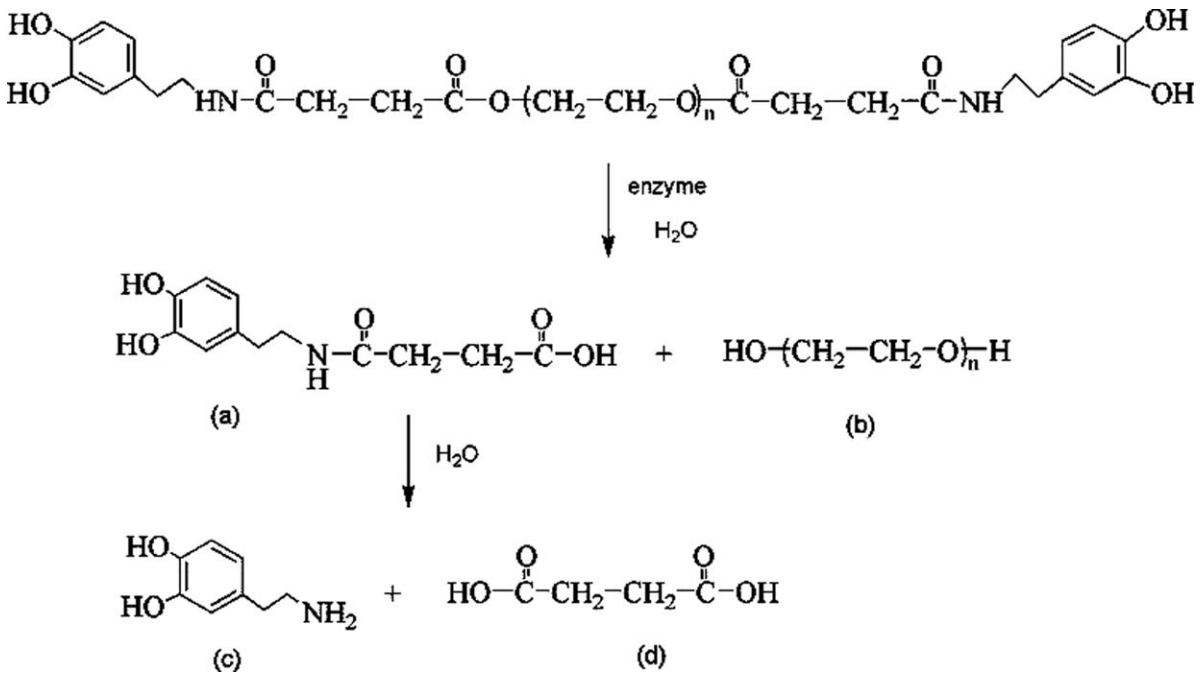
Scheme 1 Schematic of the synthesis route for PEG–DA.

however, no peak was visible near DA's melting point for the three polymer prodrugs. A carbonyl CO stretching with an intense strong band at 1630 cm^{-1} and an N—H bond peak at 3350 cm^{-1} was observed in the infrared spectrum for three PEG–DA based conjugates (Fig. 2). These results indicated that three polymer–drug conjugates had already formed.

Controlled release *in vitro*

To obtain some preliminary information about the potential use of PEG–DA as a drug-delivery system for a prolonged release, the hydrolysis process for three prodrugs *in vitro* was studied in a buffer solution at pH 1.1, 7.4, and 9.0 and at pH 8.0 in the presence and absence of α-chymotrypsin.

Figure 3 presents the hydrolysis rates of PEG4000–DA at three pH levels. As shown, PEG4000–DA was subjected to hydrolyzation to 80.2% at pH 1.1 and to 91.2% at pH 7.4. After 3 h, the hydrolyzation percentage could reach 70.0% at pH 9.0. Figure 4 depicts the hydrolytic behavior for PEG6000–DA. Clearly, at pH 7.4, the hydrolyzation percentage could reach 90.2%, and at pH 1.1 and 9.0, the



Scheme 2 Schematic of the hydrolysis or enzymatic hydrolysis route for PEG–DA.

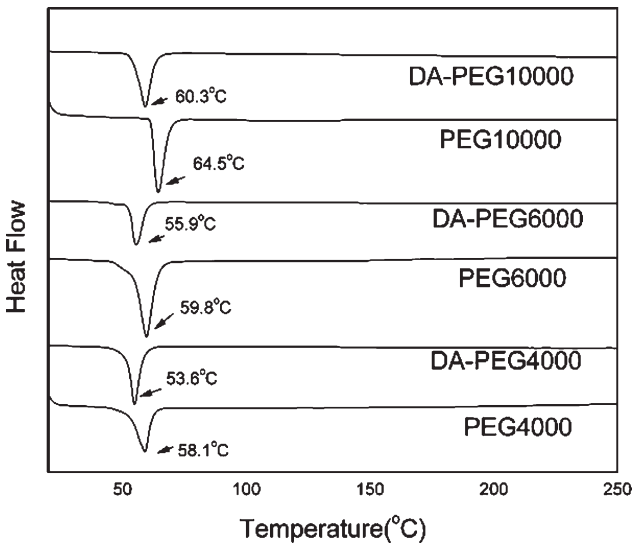


Figure 1 DSC thermograms of PEG and PEG–DA.

hydrolyzation percentage could reach 69.8 and 54.7%, respectively. Figure 5 depicts the same hydrolytic behavior for PEG10000–DA. The hydrolyzation percentages were 48.4% at pH 1.1 and 54.8 and 35.0% at pH 7.4 and 9.0, respectively.

The release rates of DA from the three prodrugs were all relatively faster within 3 h. The hydrolysis rate of DA from PEG4000–DA, PEG6000–DA, and PEG10000–DA were calculated to be 26.7, 23.2, and 16.1%/h at pH's of 1.1 and 7.4, respectively. The hydrolysis rates were calculated to be 23.2, 18.2, and 11.7% at pH 9.0. The hydrolysis rate of DA from PEG10000–DA was the slowest among the three prodrugs under the same conditions.

The release rates of DA from the three prodrugs were all the fastest at pH 7.4. The release rates were

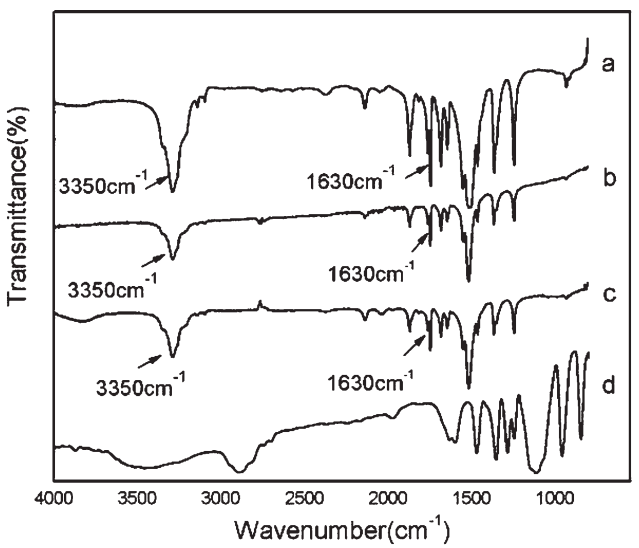


Figure 2 IR spectra of (a) PEG4000–DA, (b) PEG6000–DA, (c) PEG10000–DA, and (d) PEG10000.

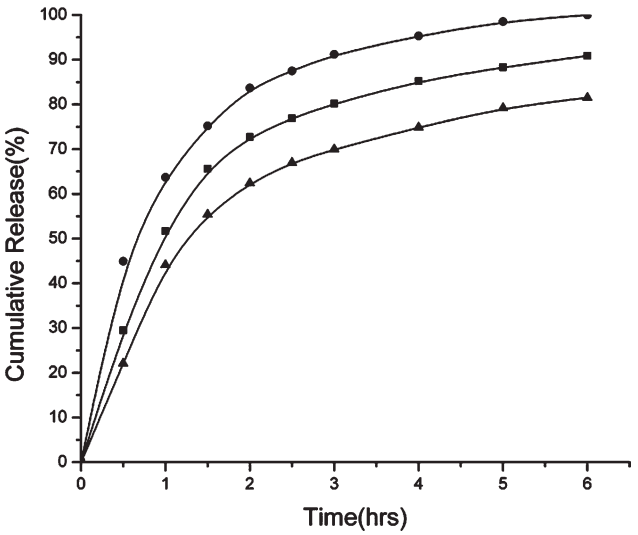


Figure 3 Release of DA in buffer solutions at 37°C from PEG4000–DA: (■) pH 1.1, (●) pH 7.4, and (▲) pH 9.0.

the slowest at pH 9.0. The phenomenon may have been related to the structure of the DA molecule.

α-Chymotrypsin, one of the digestive enzymes secreted by the pancreas, belongs to a family of enzymes that can cleave proteins into small peptides. It is also widely known for its ability to catalyze the hydrolysis of ester bonds and amide bonds.[30] To understand the effect of α-chymotrypsin, the release of DA from PEG–DA prodrugs in the presence and in the absence of α-chymotrypsin at pH 8.0 was studied to determine the release rates.[31]

As shown in Figure 6, 89.4% of DA was released from PEG4000–DA after 3 h in the presence of α-chymotrypsin, and 82.6% of DA was released in the absence of α-chymotrypsin. The hydrolysis rates of DA from PEG4000–DA were calculated to be 15.9

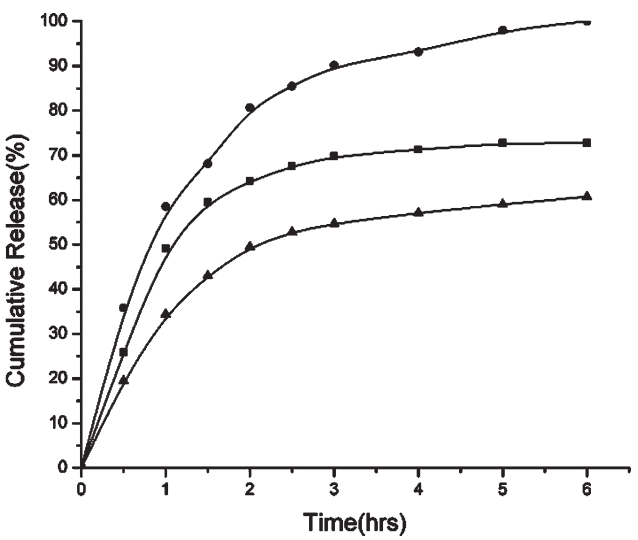


Figure 4 Release of DA in buffer solutions at 37°C from PEG6000–DA: (■) pH 1.1, (●) pH 7.4, and (▲) pH 9.0.

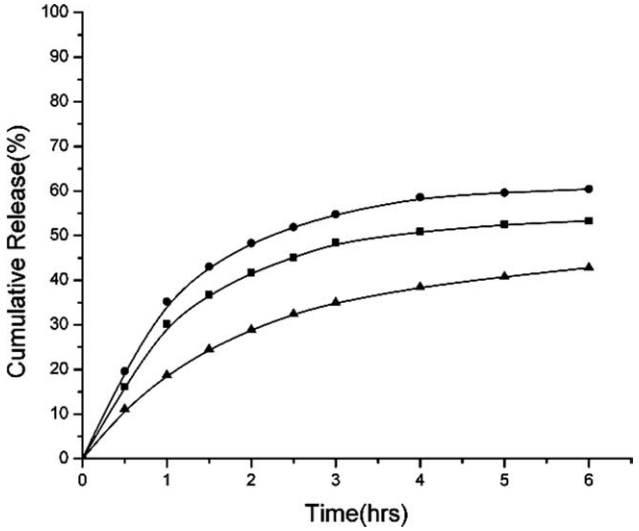


Figure 5 Release of DA in buffer solutions at 37°C from PEG10000–DA: (■) pH 1.1, (●) pH 7.4, and (▲) pH 9.0.

and 15.3%/h at pH 8.0 with and without α-chymotrypsin within 6 h.

As shown in Figure 7, 86.2% of DA was released from PEG6000–DA in the presence of α-chymotrypsin, and 78.2% was released in the absence of α-chymotrypsin after 3 h. The hydrolysis rates of DA from PEG6000–DA were calculated to be 15.1 and 14.0%/h at pH 8.0 with and without α-chymotrypsin within 6 h.

As shown in Figure 8, 42.5% of DA was released from PEG10000–DA in the presence of α-chymotrypsin, and 37.3% was released in the absence of α-chymotrypsin after 3 h. The hydrolysis rates of DA from PEG10000–DA were calculated to be 7.7 and 7.3%/h at pH 8.0 with or without α-chymotrypsin within 6 h.

On the basis of the experimental results, more DA was released in the presence of α-chymotrypsin than

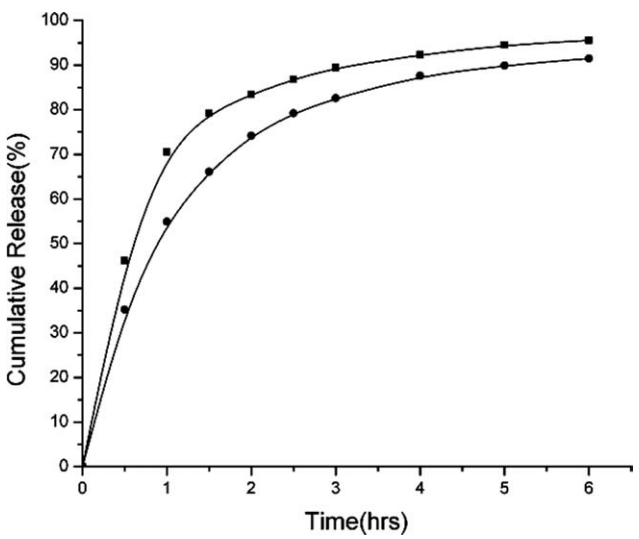


Figure 6 Release of DA from PEG4000–DA in a 0.08*M* Tris buffer at pH 8.0 in (■) the presence and (●) the absence of α-chymotrypsin.

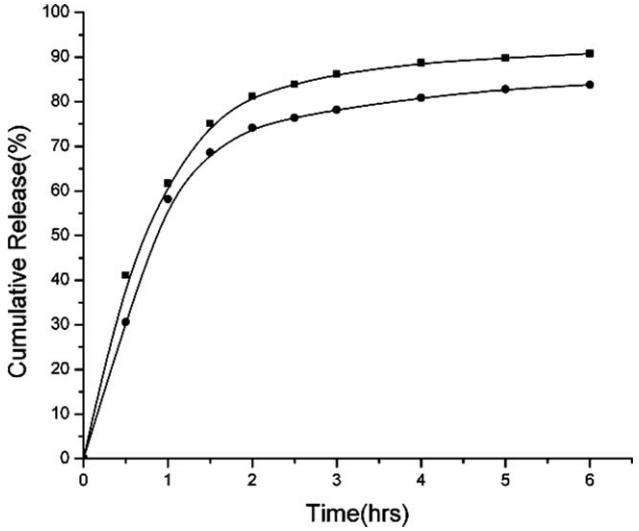


Figure 7 Release of DA from PEG6000–DA in a 0.08*M* Tris buffer at pH 8.0 in (■) the presence and (●) the absence of α-chymotrypsin.

in its absence according under the same testing conditions. However, there existed little difference between the release rates of DA from the three prodrugs under the conditions with or without α-chymotrypsin. Interestingly, it was clear that PEG10000–DA was the most stable prodrug in various buffer solutions and in the presence or absence of α-chymotrypsin. These results suggest that the amide bond between DA and PEG4000 may have been easily susceptible to its environmental conditions.

CONCLUSIONS

Three novel polymer prodrugs were prepared via the linking of DA to PEGs with different molecular

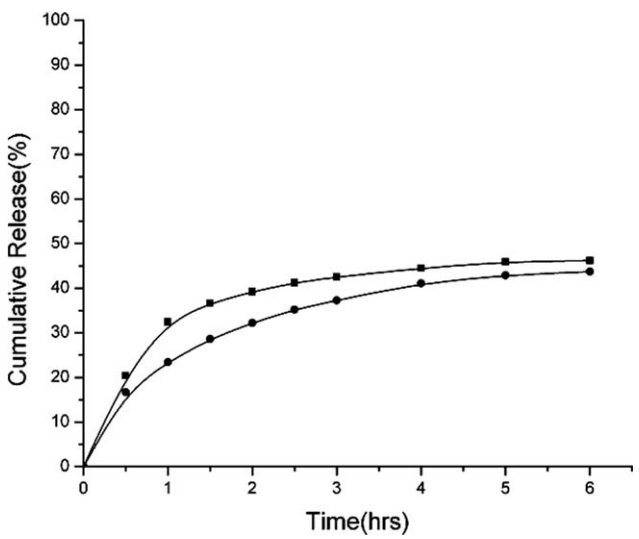


Figure 8 Release of DA from PEG10000–DA in a 0.08*M* Tris buffer at pH 8.0 in (■) the presence and (●) the absence of α-chymotrypsin.

weights with succinic anhydride as a spacer. PEG-based prodrugs showed an ability to increase the solubility of DA, whereas the DA molecules bonding via an amide bond could guarantee the drug release. The three prodrugs were relatively stable in different pH buffer solutions during the inspection of their release behaviors *in vitro*. The hydrolysis rate for PEG10000–DA was the slowest among the three polymer prodrugs under the same conditions. Thus, PEG4000–DA showed more susceptibility to pH values. With the same buffer solution and time period, more DA was released in the presence of α-chymotrypsin than in its absence. The three prodrugs slowly released the active drug molecules and improved the pharmacokinetics of DA.

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