Water Soluble Poly(ethylene glycol) Prodrug of Silybin: Design, Synthesis, and Characterization

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ABSTRACT: Silybin, the main component of silymarin, is an antihepatotoxic agent. But it presents numerous challenges associated with its poor aqueous solubility which has been realized as the major problem in its dosage form development and clinical application. The objective of our study was to solubilize silybin by designing and synthesizing its aqueous soluble prodrug using high aqueous soluble polymeric carrier—poly(ethylene glycol) (PEG). A novel soluble silybin prodrug was synthesized with a linear PEG and succinic ester linkage, and was extensively characterized using proton NMR, FTIR, and TOF-MS. Furthermore, the prodrug was evaluated for its drug loading capability which was 6.65% and the solubility was 800 mg/mL. The results indicate significantly higher solubility of the prodrug in comparison with silybin. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 3230–3235, 2008

Key words: drug delivery systems; conjugated polymers; hydrophilic polymers; synthesis; water-soluble polymers

INTRODUCTION

Silymarin, a polyphenolic isolated from the milk thistle plant, *Silybum marianum*, is an antihepatotoxic agent, which has been used to treat a range of liver and gallbladder disorders, including cirrhosis, hepatitis, and jaundice, and to protect the liver against poisoning from chemical and environmental toxins. Derivatives of milk thistle have been used as herbal remedies for more than 2 centuries. It was revealed that silymarin consists of a large number of flavonolignans, including silybin, isosilybin, silydianin, and silychristin, in which silybin is the main component.[1,2]

Drugs are mainly hydrophobic organic compounds. Usually, the low solubility of biologically active compound could influence their bioavailability, and is often a limiting factor for their applicability. The extremely poor aqueous solubility has been realized as the major problem in the development of oral solid dosage form of silybin and remains as a challenge of its clinical application.[3] Therefore, the solubility enhancement of silybin is an important task in pharmaceutical technology, which could lead to a better bioavailability.

For years, a broad variety of methodologies to increase aqueous solubility of silybin has been developed, among them, its conjugate with a soluble polymer as a prodrug strategy is one of the most promising ones. A prodrug is a biologically inactive derivative of a parent drug molecule, and in order to release the active drug, it usually requires hydrolyze or enzymatic transformation within the body. Usually, a prodrug has improved delivery properties over the parent molecule. A conjugation of a drug with a polymer forms so-called ''polymeric prodrug.'' Polymeric prodrugs have a great many advantages over their low-molecular-weight precursors.[4] Because of these advantages, for decades, the delivery of biomolecules using polymeric materials has attracted considerable attention and polymeric prodrugs have lead into a new era of polymeric drug delivery systems (PDDS).[5]

Poly(ethylene glycol) (PEG) is a highly investigated water-soluble polymer without any side effect and is widely used as a covalent modifier of biological macromolecules as well as a carrier for low-molecular-weight drugs. PEG is amphiphilic and dissolve in organic solvents as well as in water, its high aqueous solubility makes it a versatile candidate for the prodrug conjugation. It is also nontoxic, biocompatible and can be eliminated by a combination of renal and hepatic pathways thus making it ideal to employ in pharmaceutical applications, the FDA has

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approved PEG for human intravenous (i.v.), oral, and dermal applications.[6]

PEG conjugates are classical prodrugs with high solubility, either monomethoxy PEG (mPEG) or dihydroxyl PEG is used for prodrug modification, and they can be functionalized and conjugated with drug and other biological components. Conjugation was carried out with two terminal available hydroxyl groups exist at the end of the polymer chain (or just one in the case of the most used monomethoxy PEG), which can be functionalized and conjugated to the biocomponents.

The aim of our study was to synthesize a new kind of water-soluble silybin prodrug, in which PEG was used as solubilizing moiety, in order to enhance the solubility of the drug, improve its delivery and render it a better bioavailability, which offers the silybin prodrugs the great potential of further clinical application.

In this study, mPEG (MW 2000) has been used to synthesize silybin-PEG prodrug, and the produrg has been characterized by FTIR, ^{1}H-NMR, and MS. The method of measuring the loading capability of prodrug was set up, and the solubility of it was also measured.

EXPERIMENTAL

Materials

Silybin was procured from Panjin Green Biological Development Co., China, and its purity was claimed to be 95%. mPEG 2000 was obtained from Fluka, U.S. Succinic anhydride, dicyclohexyl carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), which were all chemical grade, were purchased from Sinopharm Chemical Reagent Co., China. *N*-hydroxysuccinimide (NHS) was received from Yangzhou Baosheng Biochemical Co., China. Sephadex G-25 glucan medium was procured form Pharmacia and SP-700 macroporous resin was obtained form Mitsubishi Chemical Co., Japan, which were used for purifying the product. Other solvents or reagents were all analytical grade or higher, which must be anhydrated before use.

Melting points were determined in open capillaries and were uncorrected, using a RY-1 melting point detector which was purchased from Tianjin Tianfen Analysis Instrument Factory. UV absorbance was obtained using a UV–Vis spectrophotometer which was purchased from Shanghai Huxi Analysis Instrument Factory Co.

Synthesis of PEG-silybin prodrug

Synthesis of PEG-acid (1)

Anhydrous mPEG (MW2000) (40 g, 20 mmol) which was dissolved in anhydrous chloroform (150 mL) was reacted with succinic anhydride (2.5 g, 25 mmol) in the presence of catalytic amount of pyridine (2 mL). After refluxing for 48 h, the reaction mixture was evaporated to dryness at reduced pressure, and the residue was suspended in 50 mL of saturated sodium hydrogen carbonate aqueous solution. After filtering at reduced pressure, the solution was extracted with ethyl acetate (2 × 30 mL), followed by cooling to 0°C, then the combined organic solutions were acidified with hydrochloric acid (2 mol/L) to pH 2 and extracted with chloroform (3 × 60 mL). The combined organic solutions were dried over sodium sulfate overnight and concentrated to a small volume at reduced pressure. The resulting slurry was added to absolute diethyl ether with string and was put at 0°C for forming. The white precipitate achieved was filtered and dried at reduced pressure, then 30 g of product **1** (yield 75 %, m.p. 50–52°C) was obtained.

Synthesis of PEG-NHS active ester (2)

Compound **1** (20 g, 5 mmol) in 50 mL of anhydrous chloroform was cooled to 0°C, followed by adding dicyclohexyl carbodiimide (0.73 g, 7 mmol). After stirring at 0°C for 20 min, *N*-hydroxysuccinimide (0.8 g, 7 mmol) in 100 mL of anhydrous DMF which was cooled to 0°C previously was added, and the mixture was reacted at 0°C for 2 h. After reacting at room temperature for 24 h, the reaction mixture was evaporated to dryness at reduced pressure, and the residue was suspended in 50 mL of anhydrous chloroform. The solutions was filtered and concentrated to a small volume at reduced pressure, then 150 mL of anhydrous absolute diethyl ether was added into the resulting slurry, and the mixture was put at 0°C for forming overnight. The white precipitate achieved was filtered and dried at reduced pressure, then 16.5 g of product **2** (82.5%, m.p. 49–52°C) was obtained.

Synthesis and purification of PEG-silybin prodrug (3)

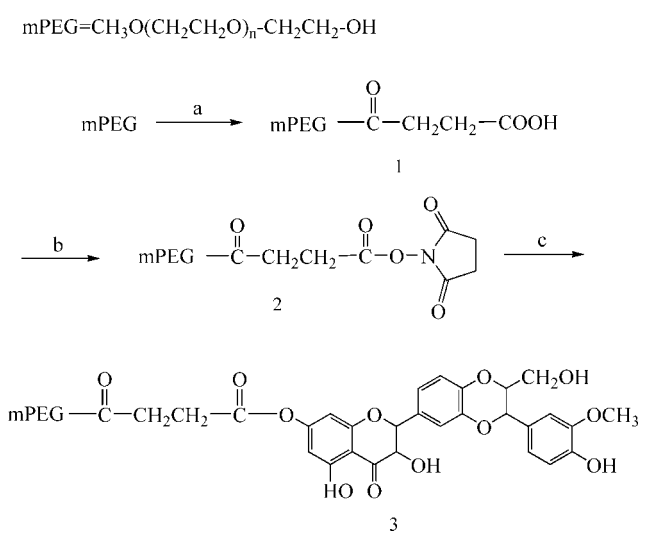
Dried silybin (1.1 g, 2.4 mmol) and 4-dimethylaminopyridine (0.3 g, 2.4 mmol) was dissolved in anhydrous DMF (10 mL), followed by adding dropwise compound **2** in 15 mL of anhydrous DMF in 30 min, then the mixture was stirred at 50°C for 24 h. The reaction mixture was evaporated to dryness at reduced pressure, and the residue was suspended in 30 mL of water. The solutions was filtered and extracted with chloroform (3 × 10 mL), and then the combined organic solutions were washed by hydrochloric acid (1 mol/L) and water and were dried over sodium sulfate overnight. The solution was evaporated to dryness at reduced pressure, and the

residue was recrystallized in isopropyl alcohol. The crude product **3** obtained which was pale yellow precipitate.

The product was separated from the unreacted silybin and other low molecular weight undesired substances by SP-700 macroporous resin column and sephadex G-25 glucan medium column. Elution with distilled water removed the undesired substances in macroporous resin column, while at 70% acetone the desired compound was recovered from it. Then the solution collected was concentrated to a small volume at reduced pressure, and the pure product was separated from Sephadex G-25 glucan medium column, whose rate of flow (R_f) was 0 in thin layer chromatography (TLC) when the developing agent was *n*-butanol-acetic acid-water (7 : 1 : 2). The product was freeze-dried to be obtained, which was pale yellow precipitate afforded 2.5 g (62%, m.p. 56–58.5°C).

Characterization

The Fourier-transform infrared (FTIR) spectrum of PEG-silybin prodrug was recorded using a Shimadzu FT-IR-8400S fourier-transform infrared spectrometer at room temperature using KBr pallet. The ^{1}H-NMR spectra were determined on a Bruker (AVACE) AV-500 spectrometer using $CDCl_3$ as solvent. Chemical shifts (δ) were given in ppm using tetramethylsilane as an internal reference. Mass spectra were performed on Agilent 1100LC/ TOF MS spectrometer.



Scheme 1 Route of synthesis of PEG-silybin prodrug. Reagents and conditions: (a) succinic anhydride, pyridine, anhydrous $CHCl_3$, rt, 48 h; (b) *N*-hydroxyl succinic anhydride, dicyclohexyl carbodiimide, anhydrous DMF, anhydrous $CHCl_3$, 24 h; (c) silybin, 4-imethylaminopyridine, anhydrous DMF, 50°C, 24 h.

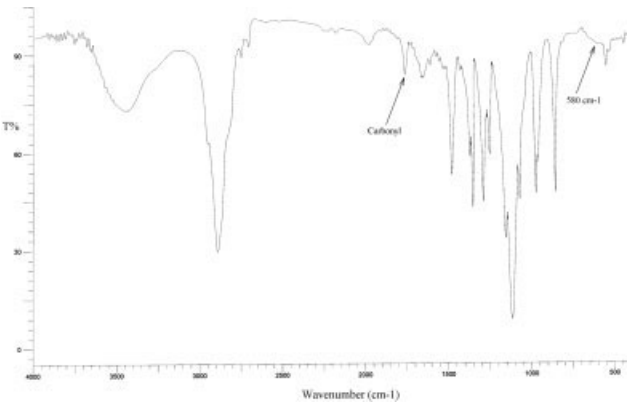


Figure 1 IR spectra of mPEG-silybin prodrug.

Analysis of the drug loading capability

The UV absorbance of native silybin in methanol was determined at 286 nm for eight different concentrations ranging from 0.108 μg/mL to 0.444 μg/mL. From the standard plot of absorbance versus concentration, PEG conjugated silybin derivatives were dissolved in methanol at an approximate concentration of 82 μg/mL and the UV absorbance of these compounds at 286 nm was determined. Using this value and employing the standard plot obtained from the above, the concentration of silybin in the sample was determined. Dividing this value by the sample concentration provided the percentage of silybin in the sample, and then the value of drug loading capability (the percentage of native drug in the prodrug) of this prodrug could be obtained.

Measurement of the solubility of the prodrug

PEG-substituted silybin was prepared by the methods described, the solubility of which was estimated by adding water in small portions until dissolution occurred. At the temperature of 25°C, an exact amount of pure compound **3** (100 mg) was weighed into a volumetric flask (2 mL), 25 μL of distilled water was added per time using continuously adjustable pipette which was followed by sonicated for 30 s. When dissolution occurred and a flowable liquid was obtained, the total volume of water was used for calculations. On the basis of the value of water added, the solubility of the PEGylated prodrug was calculated.

RESULTS AND DISCUSSION

Design, synthesis, and characterization of PEG-silybin prodrug

The bioconjugate, PEG-silybin prodrug was successfully synthesized using a three-step-procedure as shown in Scheme 1.

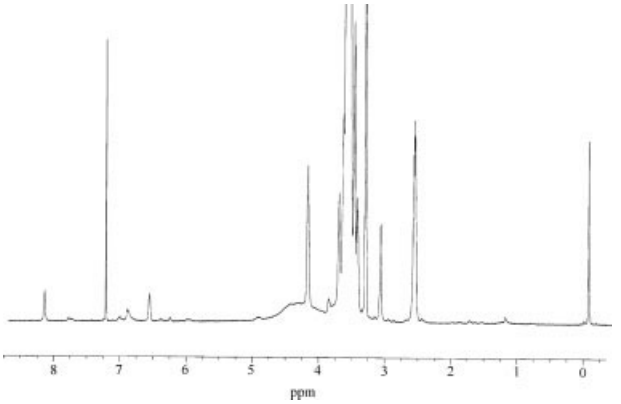


Figure 2 ^{1}H-NMR spectra of mPEG-silybin prodrug.

Native PEG has two sites available for the conjugation (mPEG has only one), and then active ingredient could be conjugated, which is conjugated to the distal end of the polymeric carrier. Under most conditions, this procedure requires certain modifications of the polymer. PEG, containing hydroxyl groups can be acetylated with anhydrides to form an ester terminating to free carboxylate groups.[7]

In this study, mPEG was reacted with succinic anhydride in the presence of catalytic amount of pyridine to introduce the functional carboxy groups, which make it possible to attach the hydroxyl of silybin to the backbone of PEG. Then the PEG acid was treated with *N*-hydroxyl succinic anhydride (NHS) using DCC as coupling agent and converted to the active ester, this methodology called ''active ester approach'' was introduced in order to accelerate the reaction and make the next reaction to take place easily and selectively on the phenolic hydroxyl group of the active ingredient, according to the published procedures.[8] Finally, silybin was introduced to backbone of polymer by a standard coupling procedure with the aids of DMAP. The product was purified using SP-700 macroporous resin column and Sephadex G-25 glucan medium column.

Figure 1 shows the FTIR spectra of PEG-silybin prodrug. From the prodrug spectrum, it was found that distinctive absorption bands appear at 1733 cm^{-1} (carbonyl group). Compared with that of mPEG, the changes in 580 cm^{-1} was found in the spectra of the product. The ^{1}H-NMR spectrum of the prodrug was given in Figure 2. The ^{1}H-NMR assignments of PEG-silybin was as follows: ^{1}H-NMR ($CDCl_3$)δ = 2.62 (—CO—CH_2—CH_2—CO—); δ = 3.20 (—OCH_3); δ = 3.3–3.8 (—O—CH_2—CH_2—O—); δ = 4.0–5.0 (—CH_2—O—, —CH—O—); δ = 6.0–7.0 (Ar—H); δ = 8.2 (Ar—OH) and the peak of PEG was discovered at 3.5. In Figure 3, the mass spectra of product, the peak in 1007.5782 was $[M+H+Na]^{2+}$, and the peak in 1015 was $[M+H+K]^{2+}$.

Drug loading capability

A prodrug is a reversible derivative of a parent drug molecule, which is biologically inactive before being activated by the specific conditions in the targeted site, so actually, only the parent drug has curative effect. Drug loading capability is an important parameter for the design of prodrug, which is also a central datum for its clinical application.

In our prodrug, the polymer backbone, PEG, and the spacer between PEG and silybin had no ultraviolet absorption, so all the absorption was contributed by silybin, whose intensity was positive correlation with the concentration of silybin. According to the method which had been set up (described in Section Analysis of the drug loading capability), the stand-

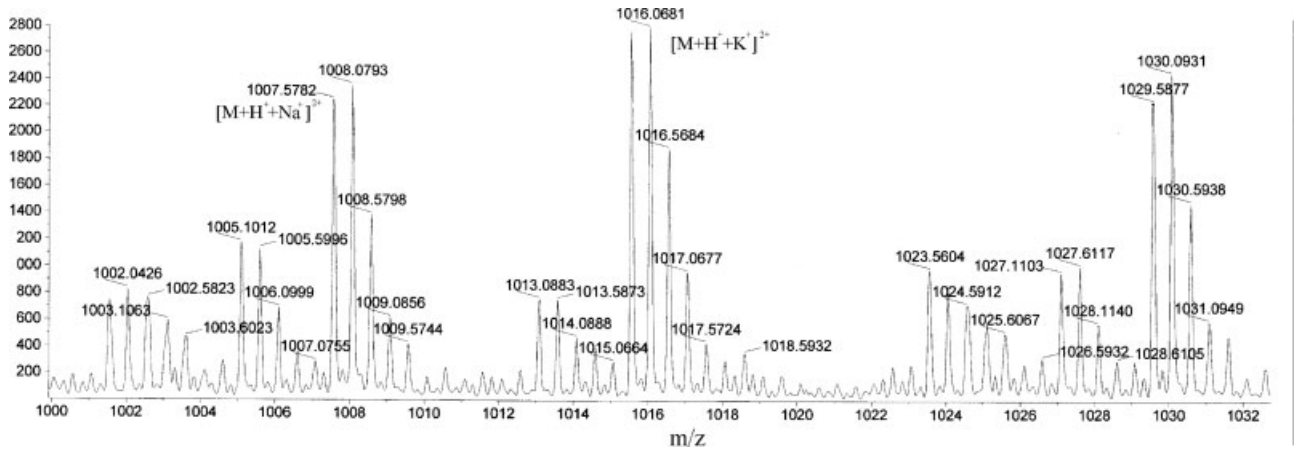


Figure 3 MASS spectra of mPEG-silybin prodrug.

ard plot was obtained (Fig. 4) and the drug loading capability of this prodrug was 6.56%.

If all of the binding sites available on polymeric carriers were conjugated with silybin, the drug loading capability would be 18.66% (the calculated value in Table I), which suggested only 35.6% of terminals of mPEG were conjugated with silybin successfully, maybe because of the steric hindrance of the polymer influencing the process of reaction.

Native PEG has only two binding sites available for the conjugation, therefore, only two drug molecules or other active ingredient could be conjugated. This limits the loading capacity of this kind of polymeric carriers, which is one of the major disadvantages of linear PEG polymer. In the next phase of our research, it will be an effective method to introduce branched spacer[9,10] to the polymeric carrier system to increase the amount of binding sites in order to enhance drug loading capability of prodrugs.

Solubility of the prodrug

An ideal drug should have enough aqueous solubility, but in fact, the solubility of biologically active compounds is often a limiting factor for their applicability. The extremely poor aqueous solubility might influence the transport of drugs in vivo and lead to a low bioavailability, which is realized as the major problem in the development of drugs and remains as a challenge of their clinical application. Therefore, the solubility enhancement is an important task in pharmaceutical technology.

High aqueous solubility makes PEG polymer a versatile candidate for the prodrug conjugation, and enhancing the solubility of drug is one of the major tasks to design PEG prodrugs.

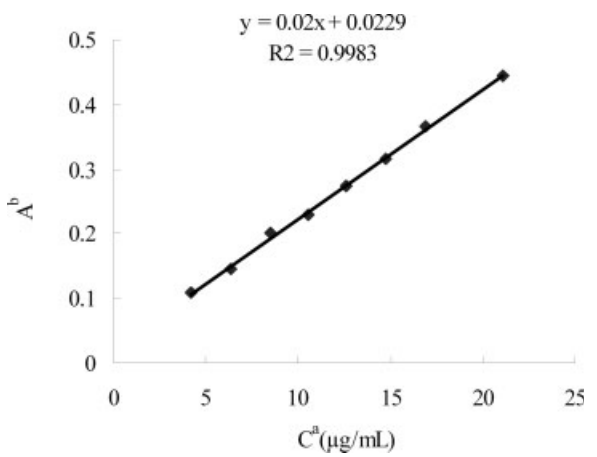


Figure 4 Standard plot of UV ABS versus silybin content. [a]Concentration of silybin in methanol, [b]UV absorbance of native silybin in methanol determined at 286 nm.

TABLE I
Drug Loading Capability of PEG-Silybin Prodrug

Sample	C^a (μg/mL)	A^b	Calculated[c] (%)	Observed[d] (%)
mPEG-Silybin	82	0.132	18.66	6.65

[a] Concentration of mPEG-silybin prodrug in methanol.
[b] UV absorbance of native silybin in methanol determined at 286 nm.
[c] Drug loading capability of the prodrug if all of the binding sites on polymeric carriers were conjugated with silybin.
[d] Drug loading capability obtained of mPEG (2000)- silybin prodrug in this study.

For high-molecular-weight polymer such as PEG, it is easy and authentic to observe its dissolving process, so it is efficient to measure the solubility of prodrug by direct observation as the literature has reported.[11]

As the methods described in Section measurement of the Solubility of the prodrug, the total volume of water used to make dissolution of 100 mg of prodrug occur and to obtain a flowable liquid was 125 μL. On the basis of the value of water added, the solubility of this PEGylated prodrug was calculated to be 800 mg/mL, and the equivalent solubility of silybin was 52.5 mg/mL which was enhanced in a large amount comparing with the value of silybin (0.0401 mg/mL). In PEG-based prodrug system, the solubility was mainly dominated by the solubility of PEG polymer, so in our PEG-silybin prodrug, the solubility of prodrug was close to the level of PEG polymer.

CONCLUSIONS

The PEG-silybin prodrug with succinic ester linkage was synthesized and characterized successfully, and the results of its drug loading capability and solubility were obtained. In this study, solubilization of silybin from the PEG-based prodrug was achieved. The solubility of PEG-silybin prodrug was 800 mg/mL, which was enhanced remarkably, by application of the high aqueous solubility carrier PEG. It would facilitate the development of dosage from design of silybin and its clinical application.

Further studies may be warranted to assess the PEG-based prodrugs pharmacokinetics and to design PEG prodrugs with higher drug loading capability by introducing branched spacers into the prodrugs.

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