Amino Acid-PEGylated Resveratrol and Its Influence on Solubility and the Controlled Release Behavior

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A set of polyethylene glycol (PEG)–resveratrol (RES) conjugates with amino acid as spacers was designed and synthesized to improve certain defects of resveratrol, such as poor solubility, its short half-life and the difficulty of obtaining controlled release. Amino acids, which are released along with RES, are necessary for human health and likely have a facilitating effect on the absorption of the drug. The prepared PEG conjugates were characterized by FT-IR, 1H-NMR, X-ray diffraction (XRD) and differential scanning calorimetry (DSC). Evaluation of free RES, loading capability, solubility and in vitro release of conjugates was also conducted. The results show that solubility in water of all the conjugates is over 900 mg·mL−1 and controlled release of RES was achieved. Therefore, the developed PEG conjugate is a favorable system for modifying the solubility and bioavailability of RES.

Key words conjugate; polyethylene glycol (PEG); amino acid; resveratrol (RES)

Resveratrol (RES, 1, structure shown in Fig. 1) [chemical name: (E)-3,4',5-trihydroxy stilbene, or 5-(4-hydroxy-styryl) benzene-1,3-diol] belongs to the stilbene family and was first isolated from the roots of white hellebore in 1924 and later found in many plants, such as grapes, peanuts and Polygonum cuspidatum. RES has received much attention in recent years because of its various health functions and its potential for use in medicinal applications. Many studies have demonstrated that RES has significant pharmacological activity and potential to be a chemopreventive and therapeutic agent for cancers, Alzheimer’s disease, cardiovascular disease, hyperlipidemia, ischemia disease and other diseases. Recently, RES has seen widespread use as a food ingredient and nutraceutical supplement in bread, cake and beverage products. RES is an important ingredient in wine because of its anti-cardiovascular activity. However, RES exhibits poor solubility in water; therefore, alcohol is sometimes used as a solvent to obtain the intended efficacy. This addition of alcohol, while advantageous in some respects, severely limits the use of RES because alcohol cannot be added to a wide variety of drugs. In addition, RES’s short biological half-life, instability in the presence of certain enzymes and oxidants and tendency to attach with metals in foods can result in limited bioavailability in vivo. Microspheres improved the stability of RES but the problem of bioavailability still exists. Cyclodextrin and nanoemulsion systems had also been reported to increase the bioavailability of RES but the stability issues still have because only intermolecular forces are utilized in these forms. Clearly, a better delivery system is required to compensate for the shortcomings of RES by enhancing its bioavailability and therapeutic effects while simultaneously reducing its associated variability and/or adverse effects.

Polyethylene glycol (PEG), as an oral vehicle material being accepted by the Food and Drug Administration (FDA), is a highly water-soluble, biocompatible, non-teratogenic, non-immunogenic, antigenic and non-toxic polymer that is easily eliminated from the human body and used in drug delivery system in many ways. Since polymer conjugates were proposed by Ringsdorf in 1975, the conjugate-mode approach has been expected to yield more efficient delivery systems for active agents. Several studies have reported on PEG-modified drugs, some of which describe the use of paclitaxel, camptothecin and doxorubicin in clinics. PEG conjugates enhance water solubility of drugs due to the highly hydrophilic character of PEG and the altered distribution and biological behavior of the drug in aqueous solutions. Additionally, PEG conjugates decrease the elimination or breakdown of the active agent and facilitate the sustained or controlled release of drugs. For these reasons, the PEG matrix has great potential in pharmaceutical industry applications to solubilize, stabilize and control the release of drugs. Because the end hydroxyl group of PEGs should be activated, succinyl or carboxymethyl groups, which were proved nontoxic to body, were selected as spacers to modify the two end hydroxyl group of PEGs and provide end carboxyl group to link with other drugs. In the current report, we designed a new set of PEG–RES conjugates (2 and 3, as shown in Fig. 1) with succinyl/carboxymethyl and various amino acids as linking arms in an effort to improve the solubility and bioavailability of RES for applications in pharmaceutics. The prepared PEG–RES conjugates are expected to release the corresponding beneficial amino acids accompanying RES in the body. Amino acids are biocompatible and essential to health; in particular, they can facilitate the absorption of active agents with no undesired side effects. In this work, RES modified by PENG2000 through succinyl/carboxymethyl and amino acids was developed, and the characterization and preliminary in vitro release of a new set of water-soluble PEG-amino acyl-RES conjugates were investigated. Characterizations were
conducted using FT-IR, differential scanning calorimetry (DSC) and $^1$H-NMR. The in vitro release test was conducted using the dialysis method. The synthesis, use and in vitro release evaluation of PEG conjugate as a prodrug system for RES has not yet been reported in the literature.

MATERIALS AND METHODS

General Experimental Procedures  RES (99.5% purity, HPLC) was synthesized in our laboratory according to a previously published method. The RES reference standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. The dialytic bag (cut-off molecular weight of 3500) was purchased from Solarbio Science & Technology Co., Ltd., Beijing, China. Analytic grade PEG2000 was purchased from Yongda Chemical Ltd., Tianjin, China. Chemical grade succinic anhydride was purchased from Bodi Chemical Co., Ltd., Tianjin, China. Amino acids, such as glycine, alanine, leucine, proline and reagents including dicyclohexylcarbodiimide (DCC), $N$-$N$-$4$-dimethylaminopyridine (DMAP) were all of industrial grade and purchased from Gongjia Chemical Co., Shanghai, China. Pyridine, isopropyl alcohol, anhydrous ether, chloroform, ethyl acetate, dichloromethane, dimethyl formamide (DMF) and other chemicals were of analytical grade and purchased from Shijiazhuang Modern Reagent Co., Ltd., Shijiazhuang, China. Acetonitrile used in HPLC analysis was chromatographically pure and purchased...
from Oceanpak Alexative Chemical Co., Ltd., Gothenburg, Sweden. Lipase with an activity of 100000 U/g was purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Water was twice distilled. Chemical reagents were used directly without further purification.

**Characterization.** FT-IR Spectra FT-IR spectra were recorded with a FT-IR spectrophotometer (FTS135, Bio-Rad, U.S.A.) using KBr as the sample holder, and samples were scanned from 400 to 4000 cm⁻¹.

**1H-NMR Spectra** 1H-NMR measurements were conducted on an AVANCE III-500 (WB) (Bruker, America) at room temperature using trimethylsilyl (TMS) as an internal standard and CDCl₃ as the solvent.

**Differential Scanning Calorimetry (DSC)** DSC analysis was performed using a DSC-TGA (SDT-2960, TA Co., U.S.A.). Approximately 4 mg of sample was placed into a platinum DSC sample cell, and the scan rate was set to 10°C·min⁻¹ within the temperature range 30–300°C under a nitrogen flow of 20 mL·min⁻¹.

**X-Ray Diffraction (XRD)** X-Ray diffraction patterns of RES, PEG2000 and the prepared conjugates were observed using a diffractometer (D8 Advance, Bruker Co., Germany) with a copper anode. The samples were scanned continuously from 5° to 60° (2θ) at a scan rate of 4°·min⁻¹, with a step size of 0.02°. The background was measured with a trace of Cu Kα radiation.

**Ultraviolet Spectrophotometry Analysis** The contents of RES in PEG composites and the water solubility of conjugates were determined using an ultraviolet spectrophotometer (UV 2501PC, Shimadzu, Kyoto, Japan). The maximum absorption was detected at a wavelength of 306 nm.

**HPLC Analysis** The amount of RES in the in vitro release test was determined by high-performance liquid chromatography (HPLC, LC-20AT VP, Shimadzu). The mobile phase was a mixture of acetonitrile and distilled water (40:60, v/v). The HPLC column was a Phenomenexluna-C18 (250×4.6 mm, 5 µm). The flow rate was 1.0 mL·min⁻¹, the detection wavelength was 306 nm and the sample injection volume was 20 µL.

**Preparation of PEG-Amino Acidyl-RES Conjugates** PEG-amino acidyl-RES conjugates were synthesized according to the route shown in Fig. 3. Selected amino acids were histidine, methionine, threonine, proline, lysine, glycine, alanine, phenylalanine, valine, leucine, isoleucine and tryptophan.

**Synthesis of PEG-Succinic Acid (Compound 5)** PEG2000 (compound 4) (20 g, 10 mmol) and succinic anhydride (2.5 g, 25 mmol) were dissolved in dry chloroform. Under stirring, 2 mL pyridine was added to the solution. The mixture was warmed to reflux for 36 h and then evaporated under vacuum to remove the solvent; the residue was dissolved in 60 mL of a saturated NaHCO₃ aqueous solution. The excess DCC in the filtrate was decomposed by 20 mL of a 10% acetic acid aqueous solution. The pH of filtrate was adjusted to 3 with a 2% hydrochloric acid aqueous solution. The mixture was kept at room temperature for 24 h. The filtrate was then extracted by dichloromethane (10 mL) and 10 mL 10% acetic acid aqueous solution was added into the filtrate to remove excess DCC. After agitating for 30 min, the mixture was steamed for a while and the organic phase was separated, washed off with saturated brine until neutral, dried by anhydrous sodium sulfate, filtered and condensed. The residue was recrystallized in anhydrous ether to obtain PEG2000 active ester 6 (3.13 g, 96.0%). 1H-NMR (500 MHz, ppm) δ: 2.71–3.03 (–CH₂ of succinyl and –CH₃ of NHS), 3.62–3.72 (–CH₂ of PEG), 4.27–4.29 (–CH₃ of PEG).

**Synthesis of PEG-Succinyl-lysine (Compound 7l)** Compound 5 (3 g, 1.36 mmol) was dissolved in 25 mL dichloromethane and cooled to 0°C. DCC (0.56 g, 2.71 mmol) and a precooled solution of NHS (0.32 g, 2.78 mmol, 5°C) in DMF (10 mL) were slowly added to the dichloromethane solution at room temperature, which was then agitated for 24 h. The obtained suspension was filtered, and 10 mL 10% acetic acid aqueous solution was added into the filtrate to remove excess DCC. After agitating for 30 min, the mixture was steamed for a while and the organic phase was separated, washed off with saturated brine until neutral, dried by anhydrous sodium sulfate, filtered and condensed. The residue was recrystallized in anhydrous ether to obtain PEG2002 active ester 6 (3.13 g, 96.0%). 1H-NMR (500 MHz, ppm) δ: 2.71–3.03 (–CH₂ of succinyl and –CH₃ of NHS), 3.62–3.72 (–CH₂ of PEG), 4.27–4.29 (–CH₃ of PEG).

**Synthesis of PEG-Succinyl-lysine (Compound 7j)** Compound 5 (3 g, 1.36 mmol) was dissolved in 25 mL dichloromethane and cooled to 0°C. DCC (0.56 g, 2.71 mmol) and a precooled solution of NHS (0.32 g, 2.78 mmol, 5°C) in DMF (10 mL) were slowly added to the dichloromethane solution at room temperature, which was then agitated for 24 h. The obtained suspension was filtered, and 10 mL 10% acetic acid aqueous solution was added into the filtrate to remove excess DCC. After agitating for 30 min, the mixture was steamed for a while and the organic phase was separated, washed off with saturated brine until neutral, dried by anhydrous sodium sulfate, filtered and condensed. The residue was recrystallized in anhydrous ether to obtain PEG2000 active ester 6 (3.13 g, 96.0%). 1H-NMR (500 MHz, ppm) δ: 2.71–3.03 (–CH₂ of succinyl and –CH₃ of NHS), 3.62–3.72 (–CH₂ of PEG), 4.27–4.29 (–CH₃ of PEG).

**Synthesis of PEG-Succinyl-lysine-RES (Compound 7i)** Compound 7j (0.5 g, 0.20 mmol) was dissolved in 15 mL dichloromethane and cooled to 0–5°C. A solution of RES (0.14 g, 0.61 mmol) in 5 mL DMF, DCC (0.15 g, 0.73 mmol) and DMAP (0.09 g, 0.74 mmol) was mixed under stirring at room temperature for 24 h. The suspension was filtered, and the pH of filtrate was adjusted to 3 with a 2% hydrochloric acid aqueous solution. The excess DCC in the filtrate was decomposed by 20 mL of a 10% acetic acid aqueous solution. The filtrate was then extracted by dichloromethane (10 mL×3), and the organic phase was washed with saturated brine, dried by sodium sulfate, filtered and condensed to remove dichloromethane. The residue was recrystallized with i-propanol to give 21 (0.29 g, 50%).

Lysine was replaced by other amino acids such as histidine, methionine, threonine, proline, glycine, alanine, phenylalanine, valine, leucine, isoleucine or tryptophan to prepare the relevant PEG-succinyl-amino acid (7a–k) with similar preparation methods and different yields (7a, 59%; 7b, 51%; 7c, 47%; 7d, 55%; 7e, 45%; 7f, 60%; 7g, 58%; 7h, 48%; 7i, 53%; 7j, 57%; 7k, 45%).

**Synthesis of PEG-Succinyl-lysine-RES (Compound 2j)** Compound 7j (0.5 g, 0.20 mmol) was dissolved in 15 mL dichloromethane and cooled to 0–5°C. A solution of RES (0.14 g, 0.61 mmol) in 5 mL DMF, DCC (0.15 g, 0.73 mmol) and DMAP (0.09 g, 0.74 mmol) was mixed under stirring at room temperature for 24 h. The suspension was filtered, and the pH of filtrate was adjusted to 3 with a 2% hydrochloric acid aqueous solution. The excess DCC in the filtrate was decomposed by 20 mL of a 10% acetic acid aqueous solution. The filtrate was then extracted by dichloromethane (10 mL×3), and the organic phase was washed with saturated brine, dried by sodium sulfate, filtered and condensed to remove dichloromethane. The residue was recrystallized with i-propanol to give 21 (0.29 g, 50%).

Lysine was replaced by other amino acids such as histidine, methionine, threonine, proline, glycine, alanine, phenylalanine, valine, leucine, isoleucine or tryptophan to prepare the relevant PEG-succinyl-amino acidyl-RES conjugates (2a–k) with similar preparation methods and different yields (2a, 63%; 2b, 60%; 2c, 60%; 2d, 52%; 2e, 45%; 2f, 42%; 2g, 51%; 2h, 41%; 2i, 55%; 2j, 50%; 2k, 56%).

**Synthesis of PEG-Acetic Acid (Compound 9)** PEG2000 (30 g, 0.015 mol) was dissolved in 300 mL toluene. A 60 mL
aliquot of toluene was removed by vacuum distillation and then naturally cooled to room temperature. A tert-butanol solution (30 mL) containing potassium tert-butoxide (5.21 g, 0.046 mol) was added into the toluene solution under stirring for 1 h. Ethyl bromoacetate (27.05 g, 0.16 mol) was added dropwise and refluxed for 24 h. The obtained suspension was filtered, and the filtrate was distilled under vacuum to remove tert-butanol, toluene and excess ethyl bromoacetate. The residue was dissolved in dichloromethane and recrystallized by anhydrous ether to give PEG2000-ethyl acetate (8, 30.46 g, 93.5%). $^1$H-NMR (500 MHz, ppm): $\delta$: 3.64–3.65 (–CH$_2$ of PEG), 4.19–4.22 (–CH$_3$ of acetyl).

Compound 8 was dissolved in 50 mL distilled water, and the pH of the solution was adjusted to 10 with 0.1 mol·L$^{-1}$ NaOH aqueous solution. In an ice water bath, the pH of the mixture was adjusted to 2 with 1% oxalic acid aqueous solution. The mixture was stirred at room temperature for 20 min. The mixture was extracted by dichloromethane (20 mL × 3), and the organic phase was washed off with brine until neutral, dried by anhydrous sodium sulfate, filtered and condensed.

The residue was recrystallized by anhydrous ether to obtain PEG2000-acetic acid (9, 10.41 mmol) was dissolved in 50 mL dichloromethane. DCC (2.41 g, 20.94 mmol NHS dissolved in 20 mL DMF) were added to the dichloromethane solution (30 mL) containing potassium tert-butoxide (5.21 g, 0.046 mol) and DMAP (0.05 g, 0.4 mmol) were added to the solution and reacted at room temperature for 24 h. The excess DCC was decomposed by 20 mL of a 10% acetic acid aqueous solution. The solution was adjusted with a 2% hydrochloric acid aqueous solution to a pH of 3. The mixture was then extracted by dichloromethane (10 mL × 3) and the organic phase was washed with brine until neutral, dried by anhydrous sodium sulfate, filtered and condensed under vacuum. The residue was recrystallized by i-propanol to obtain PEG2000-carboxymethyl-threonine-RES (3i, 0.36 g, 61%).

Threonine was replaced by other amino acids such as histidine, methionine, lysine, proline, glycine, alanine, phenylalanine, valine, leucine, isoleucine or tryptophan to prepare the relevant PEG-carboxymethyl-amino acidyl-RES conjugates (3a–h and 3j–l) with similar preparation methods and different yields (3a, 66%; 3b, 65%; 3c, 58%; 3d, 46%; 3e, 28%; 3f, 42%; 3g, 30%; 3h, 29%; 3i, 36%; 3j, 56%; 3k, 31%).

**Water Solubility**

The water solubility performances of two types of PEG-amino acidyl-RES conjugates were estimated by dissolving appropriate amounts of PEG-amino acidyl-RES conjugate in 1.0 mL of distilled water at 37±1°C for 24 h with continuous agitating. After centrifuged at 3000 rpm for 15 min, the supernatant was measured by HPLC to determine the maximum solubility of each PEG-amino acidyl-RES conjugate. The solubilities of PEG-amino acidyl-RES conjugates in water were determined by ultraviolet spectrophotometer. $^{[31]}$

**Free Dose Rate of RES in PEG-Amino Acidyl-RES Conjugates**

The polarity of conjugates increased with RES linking with PEG. No peaks corresponding to PEG-amino acidyl-RES conjugates appeared when a reversed phase column was used in the HPLC method. Therefore, the amount of RES detected by HPLC was that of free RES in conjugates.

A known amount of PEG-succinyl-amino acidyl-RES conjugate was weighed, dissolved in methanol and measured to a constant volume. The content of free RES was detected by HPLC. The peak area of free RES in the HPLC spectrum was determined using the calibration curve method to calculate the concentration. The free dose rate of RES in PEG-succinyl-amino acidyl-RES conjugate was calculated according to Eq. 1, where $FR$ stands for the free dose rate of RES in PEG-succinyl-amino acidyl-RES conjugate; $C$ is defined as the concentration of RES; $V$ is the volume of the PEG-succinyl-amino acidyl-RES conjugate solution; and $M$ is the mass of PEG-amino acidyl-RES conjugate.

$$FR = \frac{C \times V}{M} \times 100\% \quad (1)$$

The free dose rate of RES in PEG-carboxymethyl-amino acidyl-RES conjugates was determined using the same method.

**Loading Rate of RES in PEG-Amino Acidyl-RES Conjugates**

The content of RES in two types of PEG-amino acidyl-RES conjugates was determined by ultraviolet spectrophotometry. A known amount of PEG-succinyl-amino acidyl-RES conjugate was dissolved in ethanol and metered to a constant volume. The absorbance of the solution was determined using an ultraviolet spectrophotometer, and the data were evaluated using the calibration curve method to calculate...
the concentration corresponding to each absorbance. The loading rate \((LD)\) was calculated according to Eq. 2, where \(C_1\) is defined as the concentration of RES, \(M_1\) is the mass of PEG-succinyl-amino acidyl-RES conjugate and \(V_1\) is the metered volume of the solution.

\[
LD = \frac{C_1 \times V_1}{M_1} \times 100\% \tag{2}
\]

The loading rate of RES in PEG-carboxymethyl-amino acidyl-RES conjugates was determined using the same method.

**Controlled Release in Vitro of PEG-Amino Acidyl-RES Conjugates** The in vitro release performance of the prepared PEG-amino acidyl-RES conjugates was observed with or without lipase catalytic hydrolysis. In the in vitro release test of conjugates, the dynamic dialysis method was applied while simulating intestinal conditions (pH 7.4, phosphate buffered saline, PBS). The in vitro release test was conducted with an Intelligent Dissolution tester (RC2-8A, Tianjin University, China).

PEG-amino acidyl-RES conjugate (0.25 g), lipase (0.25 g) and PBS buffer solution (15 mL) were put into a dialytic bag and the bag was sealed. The bag was placed in a shaking basket, which was immersed in 60 mL PBS buffer solution at 37±0.5°C with continuous shaking. At pre-selected time intervals (0.5 h, 4.0 h, 8.0 h, 12.0 h, 36.0 h, 60.0 h, 72.0 h), 7.5 mL of release medium was taken out and the warmed equivalent fresh buffer solution (37±0.5°C) was added to maintain the system at a stable volume. The 7.5 mL of release medium that was withdrawn was then diluted to 50 mL via HPLC flowing system at a stable volume. The 7.5 mL of release medium that was withdrawn was then diluted to 50 mL via HPLC flowing phase, sonicated in an ultra-sonic bath, shaken for approximately 2 min and passed through a 0.45 µm membrane. The RES content was determined by HPLC and the accumulating release degree was calculated according to Eq. 3. The experiments were repeated three times.

The in vitro release of PEG-amino acidyl-RES conjugate without lipase catalytic hydrolysis was observed following the same method in the absence of lipase.

Data were evaluated using the calibration curve method to calculate concentrations corresponding to each time interval. The accumulated amount of RES released from the dialytic bag was obtained from Eq. 3, where \(RP\) stands for the amount of RES released from the dialytic bag at \(t\) (time) in buffers; \(C_t\) is defined as the concentration of RES at \(t\); \(M_2\) is the mass of PEG-amino acidyl-RES conjugate; and \(LD\) is the loading rate of RES in PEG-amino acidyl-RES conjugate.

\[
RP = \frac{C_t \times 60}{M_2 \times LD \times 10^\delta} \times 100\% \tag{3}
\]

**RESULTS AND DISCUSSION**

**Characterization of PEG–RES Conjugates** The prepared PEG-amino acidyl-RES conjugates were characterized by FT-IR, \(^1\)H-NMR, XRD and DSC.

The compounded spectra of FT-IR of PEG-amino acidyl-RES conjugates are shown in Fig. 2. As shown in Fig. 2, in the RES spectrum, there was a strong peak at 3302 cm\(^{-1}\) due to the three hydroxyl groups on RES. The peaks at 1607–1445 cm\(^{-1}\) can be attributed to skeleton vibration of the benzene ring and conjugated olefin, and the peak at 965 cm\(^{-1}\) is a typical absorption peak of \(trans\) olefin. In the PEG spectrum, the hydroxyl group peak at 3465 cm\(^{-1}\) is relatively weak, the peak at 2881 cm\(^{-1}\) can be attributed to methylene, and there was a moderate strong peak at 1114 cm\(^{-1}\) due to the existence of a C–O ether bond. In the spectra of prepared PEG conjugates (2, 3), enhanced peaks were observed at 3260–3400 cm\(^{-1}\) (–OH) and at 2865 cm\(^{-1}\) (–CH\(_2\)) because of linking with RES and methylenes from PEG, respectively. The peaks at 1736–1765 cm\(^{-1}\) were attributed to the characteristic absorptions of carbonyl groups and ester groups. From 1580 cm\(^{-1}\) to 1680 cm\(^{-1}\) there were strong amide peaks, and relatively strong absorptions of approximately 1110 cm\(^{-1}\) illustrated the existence of C–N bonds. The peaks at 1450–1600 cm\(^{-1}\) were attributed to the benzene ring and also indicate the presence of RES.

\(^1\)H-NMR (500 MHz, ppm, \(\delta\)) data of PEG conjugates are as follows.

- **2a** 2.56–2.74 (–CH\(_3\) of succinyl), 3.64 (–CH\(_2\) of PEG), 4.24 (–CH\(_3\) of PEG and chiral H of glycine), 6.38–7.45 (H from RES).
- **2b** 1.36–2.52 (H from tetrahydropyrrole), 2.56–2.78 (–CH\(_3\) of succinyl), 3.49–4.29 (–CH\(_2\) of PEG), 4.62–4.69 (2-H from tetrahydropyrrole), 6.54–7.47 (H from RES), 8.08 (H from amide).
- **2c** 1.20–1.58 (–CH\(_3\) from alanine), 2.52–2.77 (–CH\(_2\) of succinyl), 3.63–4.28 (–CH\(_2\) of PEG), 4.74–4.75 (chiral H from alanine), 6.34–7.45 (H from RES), 7.66 (H from amide).
- **2d** 0.92–0.98 (–CH\(_3\) from leucine), 1.62–1.73 (3-CH\(_3\) of leucine), 2.54–2.60 (–CH\(_2\) of succinyl), 3.64–4.23 (–CH\(_2\) of PEG), 4.74 (chiral H from leucine), 6.38–7.45 (H from RES), 7.75 (H from amide).
- **2e** 1.05 (–CH\(_3\) of valine), 2.54–2.73 (–CH\(_2\) of succinyl),
3.64–4.23 (–CH₂ of PEG), 4.74 (chiral H from valine), 6.24–7.47 (H from RES), 7.75 (H from amide).

2f 2.54–2.69 (–CH₂ of succinyl), 3.26 (H of benzyl), 3.64–4.27 (–CH₂ of PEG), 5.04 (chiral H from phenylanaline), 6.38–7.46 (H from RES).

2g 2.02 (–CH₃ of methionine), 2.11–2.14 (–CH₂ of methionine), 2.54–2.63 (–CH₂ of methionine, –CH₂ of succinyl), 3.64 (–CH₂ of PEG), 4.01–4.29 (chiral H from methionine and), 6.83–7.08 (H from RES).

2h 2.02 (–CH₃ of methionine), 2.11–2.14 (–CH₂ of methionine), 2.54–2.63 (–CH₂ of methionine, –CH₂ of succinyl), 3.64 (–CH₂ of PEG), 4.01–4.29 (chiral H from methionine and), 6.83–7.08 (H from RES).

2i 0.86–0.99 (5-CH₃ of isoleucine), 1.00–1.06 (3-CH₃ of isoleucine), 1.33–1.52 (4-CH₂ of isoleucine), 1.72–2.14 (3-CH of isoleucine), 2.55–2.69 (–CH₂ of succinyl), 3.64–4.28 (–CH₂ of PEG), 4.90–4.91 (chiral H from isoleucine), 7.90 (H from amide).

2j 1.20–1.38 (–CH₃ of threonine), 2.62–2.81 (–CH₂ of threonine), 3.63–4.28 (–CH₂ of PEG), 4.52–4.78 (methenyl of threonine), 6.38–7.43 (RES-H), 7.95 (H from amide).

2k 2.42–2.67 (–CH₂ of succinyl), 2.88–2.95 (C-3H of tryptophane), 3.62–4.28 (–CH₂ of PEG), 5.10 (C-2H), 6.47–7.61 (H of benzene ring from indole and RES), 7.80 (H from amide), 9.23–9.37 (–NH₂ from indole).

2l 1.20–2.47 (H of lysine), 2.59–2.68 (–CH₂ of succinyl), 3.64–4.28 (–CH₂ of PEG), 4.73 (C-2-H of lysine), 6.38–7.46 (H from RES), 8.02 (H from amide).

3a 1.21–1.32 (H of glycine), 3.64 (–CH₂ of PEG), 4.02–4.29 (–CH of glycine and –CH₂ of carboxymethyl), 4.57–4.82 (H from –OH of RES), 6.38–7.49 (H from RES), 7.79–8.01 (H from amide).

3b 1.19–2.06 (H from tetrahydropyrrole), 3.62 (–CH₂ of PEG), 3.96–4.30 (–CH₂ of carboxymethyl), 4.69 (H from tetrahydropyrrole), 6.50–7.58 (H from RES), 7.75 (H from amide).

3c 1.11–1.92 (–CH₃ from analine), 3.61–3.62 (–CH₂ of PEG), 3.90–4.27 (–CH₂ of carboxymethyl), 4.52 (H from –OH of RES), 6.49 (chiral H from analine), 7.00–7.28 (H from RES), 7.72 (H from amide).

3d 1.07–1.93 (–CH₃ of valine), 3.63 (–CH₂ of PEG), 3.93–4.21 (–CH₂ of carboxymethyl), 4.40 (chiral H from valine), 4.80–5.10 (–OH of RES), 6.38–7.59 (H from RES).

3e 1.11–1.38 (–CH₃ of valine), 3.64 (–CH₂ of PEG), 4.14–4.34 (–CH₂ of carboxymethyl), 4.40–4.42 (chiral H from valine), 4.80–5.17 (–OH of RES), 6.39–7.63 (H from RES).

3f 3.63 (–CH₃ of PEG), 3.91–4.29 (–CH₁ of carboxymethyl), 4.41 (chiral H from phenylalanine), 4.62–4.83 (–OH of RES), 6.38–7.63 (H from RES and benzene ring of phenylalanine).

3g 2.02 (–CH₃ of methionine), 2.11–2.14 (–CH₂ of methionine), 3.63 (–CH₂ of PEG), 3.91–4.36 (–CH₂ of methionine, –CH₂ of carboxymethyl, chiral H from methionine), 4.84–4.96 (–OH of RES), 6.36–7.46 (H from RES), 7.96–8.08 (H from amide).

3h 3.64 (–CH₂ of PEG), 3.92–4.17 (–CH₂ of carboxymethyl), 4.24–4.42 (chiral H from histidine), 4.65–4.90 (–OH of RES), 6.42–7.48 (H from imidazole ring and RES), 7.75 (H from amide).

3i 0.97–1.00 (5-CH₃ of isoleucine), 1.01–1.09 (3-CH₃ of isoleucine), 1.30–1.36 (4-CH₂ of isoleucine), 1.61–2.04 (3-CH of isoleucine), 3.64 (–CH₃ of PEG), 3.93–4.18 (–CH₁ of carboxymethyl), 4.20–4.42 (chiral H from isoleucine), 4.79–4.82 (–OH of RES), 6.39–7.49 (H from RES), 7.89–7.91 (H from amide).

3j 1.01–1.27 (–CH₃ of threonine), 3.63 (–CH₂ of PEG), 3.97–4.01 (–CH₂ of carboxymethyl), 4.09–4.40 (methenyl of threonine), 4.84–4.94 (–OH of RES), 6.37–7.47 (RES-H), 7.79–8.00 (H from amide).

3k 3.64 (–CH₂ of PEG), 3.97–4.41 (–CH₂ of carboxymethyl and C-3H of tryptophane), 4.71 (C-2H), 6.38–7.47 (H from indole and RES), 7.73–7.80 (H from amide).

3l 1.17–2.00 (–CH₃ of lysine), 2.52–2.67 (C-6-H of lysine), 3.64 (–CH₂ of PEG), 3.83–4.42 (–CH₂ of carboxymethyl and chiral H of lysine), 4.80–4.99 (–OH of RES), 6.39–7.65 (RES-H).

Differential scanning calorimetry can be used to observe water or solvent loss, the melting point of samples according to the endothermic performances, and they can also be used to determine if free RES exists in polymer conjugates. Figure 3 presents a diagram of DSC curves of PEG and RES in comparison with PEG-amino acidyl-RES conjugates. As shown in Fig. 3, the melting point endothermic peaks of RES and PEG appeared at 267.92°C and 57°C, respectively. In relative DSC curves of PEG conjugates, one endothermic peak appeared approximately 42–57°C due to solvent volatilization whereas no endothermic peaks appeared at temperatures between 200°C and 300°C. These findings clearly demonstrate that RES is linked with PEG through a connecting arm to form new compounds rather than existing in a free state.

X-Ray analysis was conducted to confirm the results of DSC analysis. The X-ray diffraction patterns are shown in

Fig. 3. DSC of PEG, RES and PEG Conjugates
Fig. 4 including that of RES, PEG2000 and PEG conjugates. The amount of PEG2000 was the same as that of the PEG conjugates, and the amount of RES was 12% of the amount of the PEG conjugate (determined by the maximum loading rate). As presented in Fig. 4, the diffraction pattern of RES shows a succession of sharp peaks at different angles due to its crystalline structure. Two marked peaks appeared at 19.34° and 23.5° in the diffraction spectrum of PEG2000. In comparison, the diffraction patterns of PEG conjugates exhibited no marked peaks for RES; however, two typical peaks analogous to PEG2000 were observed with minor differences due to PEG2000 having the highest molecular weight among the conjugates. Two PEG conjugate peaks on either side were observed to expand slightly. For example, the two peaks of PEG-succinyl-alanine-RES (2c) moved 0.22° and 0.16°, respectively. These results indicate the formation of new compounds.

Water Solubility, Free Dose Rate and Loading Rate of RES in PEG Conjugates

It has been demonstrated that PEG conjugates can significantly improve the solubility of drugs. The PEG conjugates prepared in this study have higher water dissolving capacities as well as water solubilities that are greater than 900 mg·mL⁻¹ (shown in Table 1). In contrast, the water solubility of RES is only 0.03 mg·mL⁻¹ (warming required). These results demonstrate that, under the same conditions, the water solubility of PEG conjugates is much better than that of RES.

The free dose rate and loading rate of RES in PEG-succinyl-amino acidyl conjugates were calculated, and the results are shown in Table 1. Remarkably differences in both the loading rate and the free dose rate of the conjugates may be caused by PEG wrapping pattern and spatial structure of the amino acid. Especially, PEG wrapping pattern could arise the activity decrease of terminal carboxyl groups resulting in the loading rate. In preparation of conjugates, excessive entanglement of PEG can cause difficulty in washing leading to the higher free dose rate.

Controlled Release in Vitro of Conjugates

To obtain preliminary information about the potential use of a PEG-amino acidyl-RES conjugate as a delivery system for a prolonged release of RES, tests of in vitro release were studied in a buffer solution of pH 7.4. Because lipase has a well-known ability to catalyze the hydrolysis of ester bonds, the release performance of conjugate was observed in the presence or absence of lipase and the results are shown in Fig. 5.

As shown in Fig. 5, all of the conjugates demonstrated satisfactory controlled release performance over a 72 h period regardless of the presence or absence of lipase. The release of PEG-succinyl-lysine-RES (2l) was the highest with lipase (99.1%) or without lipase (78.5%), whereas the release of PEG-carboxymethyl-proline-RES (3b) was the lowest with lipase (52.2%) or without lipase (29.6%). This difference in release performance may be caused (1) by the stability of the ester bond between the amino acid and RES or, in other words, by the hydrolyzing ability of the ester bond; or (2) by the function, wrapping pattern and spatial structure of the macromolecular carrier, which can affect the hydrolysis of some conjugates and RES release. Hydrolysis of certain PEG-succinyl-amino acidyl-resveratrol conjugates is faster than the corresponding PEG-carboxymethyl-amino acidyl-resveratrol conjugates, such as 2a, 2c, 2f, 2h, 2j and 2l; however, some carboxymethyl conjugates hydrolyze faster than their corre-

| Conjugates | Water solubility (mg·mL⁻¹) | Loading rate (%) | Free dose rate (%) |
|------------|----------------------------|------------------|--------------------|
| 2a         | >900                       | 2.11 ± 0.01      | 0.1322 ± 0.0010    |
| 2b         | >900                       | 9.06 ± 0.01      | 0.0651 ± 0.0008    |
| 2c         | >900                       | 11.05 ± 0.02     | 0.9304 ± 0.0005    |
| 2d         | >900                       | 11.55 ± 0.01     | 0.2039 ± 0.0005    |
| 2e         | >900                       | 12.03 ± 0.03     | 0.3742 ± 0.0011    |
| 2f         | >900                       | 8.89 ± 0.02      | 0.0801 ± 0.0004    |
| 2g         | >900                       | 14.52 ± 0.01     | 0.7260 ± 0.0003    |
| 2h         | >900                       | 7.80 ± 0.01      | 0.5213 ± 0.0033    |
| 2i         | >900                       | 11.96 ± 0.03     | 0.0328 ± 0.0012    |
| 2j         | >900                       | 9.58 ± 0.01      | 0.5019 ± 0.0025    |
| 2k         | >900                       | 8.55 ± 0.02      | 0.7029 ± 0.0007    |
| 2l         | >900                       | 5.48 ± 0.01      | 0.3104 ± 0.0016    |
| 2m         | >900                       | 4.80 ± 0.02      | 0.1016 ± 0.0003    |
| 2n         | >900                       | 5.02 ± 0.04      | 0.5127 ± 0.0012    |
| 2o         | >900                       | 8.41 ± 0.06      | 0.4204 ± 0.0010    |
| 2p         | >900                       | 1.59 ± 0.07      | 0.1135 ± 0.0020    |
| 2q         | >900                       | 6.94 ± 0.02      | 0.2482 ± 0.0028    |
| 2r         | >900                       | 5.17 ± 0.02      | 0.1547 ± 0.0011    |
| 2s         | >900                       | 5.52 ± 0.03      | 0.0942 ± 0.0016    |
| 2t         | >900                       | 5.41 ± 0.05      | 0.5140 ± 0.0010    |
| 2u         | >900                       | 2.48 ± 0.04      | 0.0640 ± 0.0043    |
| 2v         | >900                       | 2.10 ± 0.04      | 0.1901 ± 0.0024    |
| 2w         | >900                       | 4.76 ± 0.01      | 0.3778 ± 0.0017    |
| 2x         | >900                       | 2.98 ± 0.01      | 0.3117 ± 0.0022    |

n=3.
sponding succinyl conjugates \((\text{3b, 3c, 3d, 3g, 3i, 3k})\). Hence, the structure of the amino acid linked with RES likely influenced the rate of hydrolysis and the release capacity rather than the linking arm. Based on these experimental results, the release of conjugates in the presence of lipase was found to be higher than in the absence of lipase, and the difference in release performance ranged from 2.2 to 27.1%. For example, 71.8% of RES was released from PEG-carboxymethyl-isoleucine-RES \((\text{3i})\) in the presence of lipase while 44.7% was released in the absence of lipase. Additionally, 36.5% of RES was released from PEG-carboxymethyl-valine-RES \((\text{3e})\) in the presence of lipase while 34.3% was released in the absence of lipase. For some conjugates, such as PEG-carboxymethyl-proline-RES \((\text{3b})\), the release of RES was lower than 30% without lipase. Meanwhile, we have also observed almost of resveratrol was released from PEG-carboxymethyl-amino acidyl conjugates at the time of 12h irrespective of the lipase treatment (Figs. 5c, d). This maybe caused by structure of the spacers. Spacers with shorter chain (carboxymethyl) most likely hydrolyze faster than that with longer chain (succinyl). Another reason could be the linking pattern between the spacers with PEG. Succinyl conjugates links with PEG through an ester bond but carboxymethyl conjugates links by an ether bond. Ether bond could be kept after hydrolysis while the ester bond between PEG and succinyl could be hydrolyzed. Double ester bonds of the succinyl conjugates could be hydrolyzed irrespective of which is broken first, if ester bond between PEG and succinyl is hydrolyzed first, release of RES from succinyl-RES still needs time. Therefore, in comparison, most of the succinyl conjugates take more time to release RES than the carboxymethyl conjugates. In summary, the release mechanism of conjugates is still so complex that needs more attention to explore.

CONCLUSION

Two types of novel PEG-amino acidyl-RES conjugates using 12 amino acids were prepared via the spacers of succinic acid/acetic acid. These conjugates are expected to improve the water solubility of RES and increase uptake of these essential amino acids with the release of RES. The conjugates were characterized by FT-IR, \(^1\)H-NMR, XRD and DSC. All of the PEG conjugates demonstrated a great ability to increase the solubility of RES up to 900 mg·mL\(^{-1}\). The \textit{in vitro} release behaviors of the conjugates were examined in a buffer solution, and the results demonstrated that more RES was released from PEG conjugates in the presence of lipase than in the absence of lipase within the same time period. Therefore, these novel PEG conjugates can greatly enhance the controlled release of RES and increase its half-life, thereby improving the pharmacokinetics of RES.

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