Fabrication of triblock ABA type peptide dendrimer based on glutamic acid dimethyl ester and PEG as a potential nano drug delivery agent

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Abstract

Introduction: Peptide dendrimers build up from amino acids and they simulate to artificial proteins with globular architecture. These characteristics furnish peptide dendrimers with best biodegradability and biocompatibility in drug delivery systems.

Methods: A barbell-like dendrimer from glutamic acid dimethyl ester-poly (ethylene glycol)-glutamic acid dimethyl ester as ABA-type triblock copolymer (PG-PEG-PG) was prepared with liquid-phase peptide synthesis via a divergent approach. PEG 600 diacid (PEG-A) and glutamic acid dimethyl ester were used as the core and the monomeric building blocks, respectively. Linear-dendritic copolymer was prepared in the presence of DCC in pyridine. Transmission electron microscope (TEM) was used for measuring the size of first generation (G₁-COOH) and second generation (G₂-COOH) of dendrimer compounds. Thermal behavior of the synthesized dendrimers was investigated using DSC.

Results: The desired generations G₁-COOH, G₂-COOH and G₃-COOH were prepared by divergent method using PEG diacid 600 as a core compound. The size range of the resulted particles was found to be 20-100 nm for various generations. The isolated dendrimer was examined as the drug-delivery agent and the controlled release was carried out for drug molecule in pH 7.4.

Conclusion: Based on the obtained results, the synthesized biocompatible dendrimers could potentially be utilized as a drug carrier agent.

Introduction

Forth generation of polymers known as “dendrimers” are distinct synthetic macromolecules which have very branched architecture, a globular shape, high density of selected modifiable functional groups at their ends and monodispersity that has fascinated very interests in late years. The preparation of dendrimers with the divergent⁴,⁵ and convergent⁶ methods have abundantly been studied and a broad diversity of dendritic macromolecules have been synthesized. In comparison to the traditional linear and branched polymers, dendrimers differ in the viscosity,⁷ thermal action,⁸ and molecular encapsulation,⁹ because dendrimers have well determined size with a unique structure, high degree of molecular sameness and monodispersity, and a large number of controllable end functional groups.¹⁰-¹⁶

Dendrimers have been examined for many uses, such as encapsulation of guest molecules,¹⁷ or as nanoscale catalysts,¹⁸ in biological recognition,¹⁹ micelle mimics,²⁰ gene delivery¹ and as chemical sensors.²¹ These properties of dendrimers make them very suitable candidates to be assessed as vehicles for drug delivery.²²-²⁶

Amino acid based peptide dendrimers appear like artificial proteins with globular architecture.²⁷ The characteristics provide peptide dendrimers with worthy biodegradability and biocompatibility.²⁸ The semiglobular or globular topology of these compounds gives them a remarkable property of presenting themselves as multiple-armed macromolecular scaffolds that have found utilisations in the design of vaccines, diagnostic compounds,²⁹ artificial enzymes³⁰ and biocompatible surfactants.³¹ The ABA triblock linear-dendritic...
copolymer that contains citric acid and PEG as the core could be used in biomedical and pharmaceutical applications. PEG is composed of naturally occurring L-glutamic acids linked together through amide bonds. In this work, we report the preparation and characterization of poly-(L-glutamic acid) (PG) dendrimers with PEG as a core. Contrary to other artificial polymers that have been evaluated in clinical studies, PG is unique in that it is composed of naturally occurring L-glutamic acids linked together through amide bonds. PG is highly anionic in biological condition and biodegradable polymer. PEG is provided with superior biological and physicochemical characteristics, including hydrophilicity, high solubility in water and organic solvents, without toxicity, non-immunogenicity and improved biocompatibility. These advantages make it possible to reduce the toxicity with increasing the biocompatibility of dendrimers. Therefore, PEG could be used in biomedical and pharmaceutical applications. The synthesis of different generations (G) of dendrimers containing citric acid and PEG as the core was previously published from our group. Here, we report the preparation of a few new glutamic acid based dendrimers and PEG that contain glutamic acid residues on their end groups. The functionalized dendrimers with anionic glutamic acid are appropriate as building blocks for the electrostatic self-assembly for contemporary supramolecular architectures.

**Materials and methods**

**Materials**

PGE 600 diacid (acid number 175, 96–98%) purchased from Fluka was dried over Na$_2$SO$_4$. Glutamic acid dimethyl ester salt (from Merck, Germany) and pyridine was purified with refluxing over NaOH for 2 h and then distilled. N,N-Dicyclohexylcarbodiimide (DCC) was obtained from Merck (Darmstadt, Germany). Solvents were purified by common methods. N,N-Dimethylformamide (DMF) was distilled from CaH$_2$ on reduced pressure and then stored over molecular sieves (4 Å). Naltrexone (NLX) hydrochloride was obtained from Sigma (Sigma-Aldrich) and neutralized with NaOH 0.25M. Other reagents and solvents obtained from Merck (Darmstadt, Germany) and applied without purification.

**Instrumental measurements**

$^1$H NMR spectra were recorded on NMR 400 MHz Brucker in DMSO-d$_6$ and CDCl$_3$ solvents with tetramethylsilane (TMS) for the internal reference. The FT-IR spectra were obtained on a Shimadzu spectrometer Model FT-IR-8101M. The UV absorption spectra were recorded using 1700 Shimadzu spectrophotometer. Transmission electron microscopy (TEM) pictures were recorded on a LEO 906 microscope working at 100 KV for measuring of diameters $G_n$-(COOH) and $G_n$-(COO).

**Synthesis of glutamic acid dimethyl ester dendrimer with PEG-A as the core ($G_1$-(COOH))**

The PEG-A (1g, 1.67 mmol) was suspended in a three-necked round-bottom flask in freshly distilled CH$_2$Cl$_2$ (50 mL), and the flask was flushed with argon. Glutamic acid dimethyl ester salt as excess (1.1 g, 1.25 equiv.) reagent was added in one portion, to the solution. DCC (1 g, 1.5 equiv.) in 15 mL dry CH$_2$Cl$_2$ was added as a coupling agent at 0 °C and stirred for additional 30 min. Dry pyridine (4 mL) was added to this solution within 15 min. The solution was stirred at room temperature, for an additional period of time (24-72 h), depending on the generation number. The white crystalline precipitate (dicyclohexylurea) was filtered off. To decompose unreacted DCC, the mixture was treated with glacial acetic acid (10 mL) for 1 h at room temperature. The additional precipitate was filtered off, and the solution was placed in a 1 L separating funnel. It was washed with i) water 20 mL, ii) aqueous NaOH 1N 20 mL and iii) water 40 mL. The organic phase was collected, dried over MgSO$_4$, and its volume was reduced to 20 mL by rotary evaporation. The product was precipitated in diethyl ether and dried under vacuum at 25 °C for 24 h, and purified compound was obtained as an amorphous, yield 67%. $^1$H NMR (400 MHz, CDCl$_3$, δ, ppm): 1.95-2.42 (m, 8H, β-CH$_2$ and γ-CH$_3$ in PG), 3.59-3.7 (4H, OC$_2$H$_3$ in PEG), 3.9-4 (4H, OCH$_2$C=O in PEG), 4.61-4.66 (m, 2H, α-CH$_2$ in PG), 7.35-7.37(d, 2H, NH-amide).

**Deprotection of $G_1$-(COOMe)**

Hydrolysis: A dendritic $G_1$-(COOMe) (2 g) terminated with methyl ester groups was suspended in MeOH (30 mL) and NaOH 1 M (11 mL) was added with stirring; hence hydrolysis occurred within 5 h. Ten milliliters of water were added to the mixture. Carboxyl-terminated dendrimers of the first generations were precipitated by the addition of HCl when hydrolysis was completed. Addition of HCl 1 M (13 mL) to pH 3 gave a yellow viscoso precipitate, then dried under vacuum at 25 °C for 12 h, yield 55%. $^1$H NMR (400 MHz, CDCl$_3$, δ, ppm): 1.9-2.44 (m, 8H, β-CH$_2$ and γ-CH$_3$ in PG), 3.4-3.6 (30 H, CH$_2$O in PEG), 3.58 (s, 12H, Me in ester group of PG), 3.9-4.1 (4H, O-CH$_2$C=O in PEG), 4.5 (m, 2H, α-CH$_2$ in PG), 7.2 (2H, NH-amide). FT-IR (KBr, cm$^{-1}$): 2876 (ν, C–H), 2340-3400 (ν, COO–H), 1714 (ν, acid C=O), 1662 (ν, amide C=O), 1094 (ν, C–O).

**Synthesis of $G_1$-(COOMe)**

Argon inlet was added to the solution of $G_1$-COOH (2.4 g, 2.8 mmol) in dry DMF (15 mL) with reflux condenser, and stirred. Dry pyridine (0.1 mL) was added to the solution during 15 min and reaction was stirred vigorously for 10 min. A solution of DCC (2.28 g, 4×2.8 mmol) in 10 mL dry...
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DMF was added at 0 °C, then a solution of glutamic acid dimethyl ester salt (2.37 g, 4×2.8 mmol) in 10 mL DMF and triethylamine (2 mL) were added. The mixture was stirred at 0 °C for 1 h then at room temperature for 72 h under argon. The solution was filtered off and was placed at 5 °C for 24 h, then solution was filtered off. The product was precipitated in diethyl ether and dried under vacuum at 25 °C for 24 h and finally the design compound was obtained as the yellow oil, yield 40%. ¹H NMR (400 MHz, CDCl₃, δ, ppm): 1.9-2.26 (m, 24H, β-CH₂ and γ-CH₂ in PG), 3.4-3.6 (30 H, CH₂ in ester group of PG), 3.5-3.58 (s, 24H, Me in ester group of PG), 4 (4H, O-CH₂-CO in PEG), 4.35 (m, 6H, α-CH in PG), 7.4-7.7 (d, 6H, NH-amide).

Deprotection of G₃₋(COOMe)

G₃₋(COOMe) (2.2 g, 1.9 mmol) reacted to the mixture of NaOH 1 M (20 mL) and MeOH (30 mL), which resulted in a dark-red solution and stirred at 25 °C for 12 h. Then MeOH was evaporated in vacuum and the residue was diluted with H₂O (10 mL). Addition of HCl 1 M (20 mL) to pH 3.0 resulted in a clear red viscose precipitate, and the product was dried under vacuum at 25 °C for 24 h as the bright red oil, yield 45%.

Synthesis of G₃₋(COOMe)

To a solution of G₃₋(COOH) (1 g, 9.77×10⁻⁴ mol) in 15 mL dry DMF, dry pyridine (0.1 mL) was added and stirred vigorously for 10 min. A solution of DCC (1.59 g, 7.6×10⁻³ mol) in 10 mL dry DMF was added to mixture at 0 °C and reaction was stirred for 20 min. Then a solution of glutamic acid dimethyl ester salt (1.65 g, 7.6×10⁻³ mol) in 10 mL DMF and triethylamine (2.5 mL) were added and stirred at 0 °C for 1 h, then at room temperature for 72 h under argon. The solution was filtered off and placed at 5 °C for 24 h and again filtered off. The obtained product was precipitated in diethyl ether and then dissolved in CH₂Cl₂. Then it was filtered off and reprecipitated in diethyl ether, and dried under vacuum at 40 °C as the red viscose, yield 20%. ¹H NMR (400 MHz, CDCl₃, δ, ppm): 1.9-2.26 (m, 36H, β-CH₂ and γ-CH₂ in PG), 3.4-3.6 (30 H, CH₂O in PEG), 3.6-3.7 (s, 18H, Me in ester group of PG), 4 (4H, O-CH₂-CO in PEG), 5.5-5.8 (d, 9H, α-CH₂ in PG), 7.9-8.1 (d, 5H, acid group of PG).

Preparation of G₃₋(COOH)/NLX complex

For the preparation of G₃₋(COOH)/NLX complex, first the dendrimer was dissolved in DMF and solution was refluxed with a solution of drug (excess of NLX) in THF. The mixture was stirred for 2 h at 35-45 °C and the complex was precipitated in n-hexane and then dissolved in water, filtered and precipitated in diethyl ether. The resultant compound was dried in a vacuum oven for 3 h at 35 °C.

Results

The first generation of dendrimer G₁₋(COOH) was prepared by the reaction of PEG-A with glutamic acid dimethyl ester salt and DCC as a coupling agent condensation in dichloromethane as the solvent. For synthesis of G₁₋(COOH), the compound G₁₋(COOMe) was deprotected. Deprotection of the terminal acid groups was achieved by hydrolysis with NaOH in MeOH/H₂O. Compound G₁₋(COOH) was prepared the same procedure that used for synthesis of G₃₋(COOH) in DMF solvent. The reaction time required for the coupling had to be extended to 24 hours, 3 days, and 4 days for the dendrimers of generations 1, 2, and 3, respectively. The third generation (G₃₋(COOH)) was also prepared through reaction between glutamic acid dimethyl ester salt and activated G₁₋(COOH) by DCC. The ¹H NMR spectrum of G₁₋(COOH) which shows multiplet peaks at 1.9-2.4 ppm for the β and γ-CH₂ protons (8H), 4.6-4.7 ppm for the α-CH protons of glutamic acid (2H), the protons of PEG (–OH, CH(OH)₂) at 3.6-3.8 ppm, 3.6-3.72 ppm for the protons of methyl ester (s, 12H), 3.9–4 ppm (–COCH₂O–) and 7.3 ppm (OCNH) (d, 2H) could be determined, also the chemical shift at 7.2 ppm is the peak of CDCl₃. The integral ratio of aliphatic protons of PEG to the resonances of all glutamic β and γ protons at 1.9-2.4 ppm is 7 (comparing to 7 as a theoretical calculation) as shown in Fig. 1.

The FT-IR spectrum of G₁₋(COOH) which shows carboxyl groups of acid at 1714 cm⁻¹, carbonyl groups of amide at 1662 cm⁻¹ and wide peak at 2400-3500 cm⁻¹ was related to the terminal carboxyl groups of dendrimer (Fig. 2). Also elimination of peaks ¹H NMR at 3.6 and 3.7 ppm was related to the methyl esters and the appearance of acidic protons peaks at 12 ppm is evidence of the deprotection of ester groups (Fig. 3).

![Fig. 1. ¹H NMR spectrum of G₁₋(COOMe) in CDCl₃](image1)

![Fig. 2. FT-IR spectra of G₁₋(COOH)](image2)
The $^1$H NMR spectrum of $G_2$-(COOH) shows multiplet peaks at 1.9–2.26 ppm for the $\beta$ and $\gamma$-CH$_2$ protons (m, 24H), 3.4–3.6 ppm for the protons of PEG (O-CH$_2$CH$_2$O), 3.54–3.58 ppm for the protons of methyl ester (s, 24H) and 7.6–7.8 ppm for the amid protons (d, 6H) (Fig. 4).

The divergent approach (Fig. 5 and 6) requires building the dendrimer from its center to the outward. The principal disadvantage of this method is related to the incomplete reactions at dendrimer peripheral groups, and makes the defect that occurs with each new generation. The chromatographic method for the purification of final dendrimer compound is not easy because other products often show similar physical behaviors to those of the designed dendrimer. Therefore, higher generations of divergently prepared dendrimers usually have a certain degree of structural imperfection.

The $^1$H NMR spectrum of $G_3$-(COOH) which shows multiplet peaks at 2–2.5 ppm for the $\beta$ and $\gamma$-CH$_2$ protons, 4.5–4.7 ppm for the $\alpha$-CH protons of glutamic acid, the protons of PEG (–OCH$_2$CH$_2$O–) at 3.6–3.8 ppm, 3.6–3.72 ppm for the protons of methyl ester, 3.9–4.2 ppm (–COCH$_2$O–) and 7.8–8.2 ppm (OCNH) can be recognized. The wide peak in 9.4–9.5 ppm indicates that not all the carboxylic acid groups have reacted. However, only three carboxylic acid groups have reacted (in comparing rations).

Table 1 indicates the solubility property of $G_1$-COOH and $G_2$-COOH in common organic solvents. Dendrimers terminated by multiple glutamic carboxyls are soluble in water; however, dendrimers terminated by multiple glutamic methyl esters are swelling.

![Fig. 3. $^1$H NMR spectrum of $G_2$-(COOH) in CDCl$_3$](image)

![Fig. 4. $^1$H NMR spectrum of $G_2$-(COOMe) in CDCl$_3$](image)

![Fig. 5. Reaction scheme for the synthesis of $G_1$-(COOH) and $G_2$-(COOMe).](image)
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**Thermal properties**

DSC was performed to examine the thermal stability of \(G_1-(COOH)\) and \(G_2-(COOH)\) compounds. DSC curve of \(G_1-(COOH)\) gave two endothermic peaks at -62.3 °C and 164.3 °C and an exothermic peak at 210 °C (Fig. 7). The DSC of \(G_2-(COOH)\) gave an endothermic peak at 115.5 °C and an exothermic peak at 22.8 °C (Fig. 8).

**Investigation of morphology of \(G_1-(COOH)\) and \(G_2-(COOH)\) compounds with TEM**

The morphologies of \(G_1-(COOH)\) and \(G_2-(COOH)\) compounds were investigated with TEM, as shown in Fig. 9. The spherical particle’s diameter is ~20-100 nm. TEM established the self-assembled shapes of structures and the global sizes. Indeed, as shown in Figs. 9a and 9b, \(G_1-(COOH)\) and \(G_2-(COOH)\) clearly show uniform sizes with a spherical shape corresponding to hard spheres.

**Drug loading and release**

The characteristic structure and unique properties of dendrimers make these compounds so excellent candidates for examining them as drug carriers. Dendrimers are applied as drug-delivery agents in two ways: first, the drug molecule could be physically entrapped inside the cavity of dendrimers; second, the drug molecule could be covalently linked onto surface or other functional groups to obtain dendrimer–drug conjugates. In order to investigate the drug delivery potentials of obtained compounds, the naltrexone (NLX) was loaded

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**Table 1.** The solubility behavior of \(G_1-(COOMe)\) and \(G_2-(COOH)\) in common organic solvents: soluble (+) and insoluble (−)

| Sample          | \(G_1-(COOMe)\) | \(G_1-(COOH)\) | \(G_2-(COOMe)\) | \(G_2-(COOH)\) |
|-----------------|-----------------|----------------|-----------------|----------------|
| Solvent         | Soluble         | Insoluble      | Swell           | Soluble        | Insoluble      | Swell           | Soluble         | Insoluble      | Swell           |
| Dichloromethane | +               | -              | -               | +              | -              | -               | +               | -              | -               |
| DMF             | +               | -              | -               | +              | -              | -               | +               | -              | -               |
| THF             | -               | +              | -               | -              | +              | -               | -              | +              | -               |
| Methanol        | -               | +              | -               | -              | +              | -               | -              | +              | -               |
| Ethanol         | -               | +              | -               | -              | +              | -               | -              | +              | -               |
| Methanol-H₂O    | -               | +              | +               | -              | -              | -               | -              | +              | -               |
| Ethanol-H₂O     | -               | +              | +               | -              | -              | -               | -              | +              | -               |
| Chloroform      | +               | -              | -               | -              | +              | -               | +              | -              | -               |

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**Fig. 6.** Reaction scheme for the synthesis of \(G_3-(COOH)\).

**Fig. 7.** The DSC curve of \(G_1-(COOH)\).

**Fig. 8.** The DSC curve of \(G_2-(COOH)\).
into the G₁-(COOH) using solvent and non-solvent method. For preparation of drug-loaded dendrimer, first dendrimeric compound with excess amount of NLX drug was dissolved in a suitable solvent and the obtained complex was precipitated using a nonsolvent in order to separate the nontrapped drug. For further purification of complex it was dissolved in a solvent and then precipitated in a nonsolvent. This process was done for several times using solvent and nonsolvent systems, to reach the optimized pure dendrimer/drug complex. After purification of drug/dendrimer complex, the obtained compound was dried in vacuum.

The released drug from the dendrimer/drug was determined at 37 °C by a cellophane membrane dialysis bag. The dendrimer/drug was poured into aqueous buffered solution (pH 7.4). The mixture was conducted into a cellophane membrane dialysis bag. The amount of released drug was analyzed using a UV spectrophotometer. Release curve of NLX from G₁-(COOH)/NLX (pH 7.4, 37 °C) was shown in Fig. 10. Drug loading and release was investigated only at pH 7.4 to evaluate the potential of isolated compound as the drug delivery agent.

The calculation for determination of the quantity of NLX in drug/G₁-(COOH) complex is as follows:

Weight of dendrimer after complexation = X
Weight of dendrimer before complexation = Y
X−Y = Weight of drug
X= 0.562 g, Y= 0.529 g (equal to 3.72×10⁻⁴ mol of dendrimer)

0.562−0.529= 0.033 g (weight of NLX in the complex)
9.7×10⁻⁴ mol (dendrimer)
4.4×10⁻⁴ mol (dendrimer)

mol% of NLX in G₁-(COOH)/NLX complex = ? 100
The mol% of NLX in G₁-(COOH)/NLX complex: 22.04 mol%.

The data of UV from complexes determined the presence of drug in the obtained complex. The UV detection for defining the quantity of drug inside the complex was examined at 282 nm. The drug content and the percentage of encapsulation efficiency was 22.04%. In vitro release behavior of NLX from the NLX/dendrimer complexes was examined in buffered solution at pH 7.4, λmax 282, and 37 °C. UV absorbance measurements were performed for the characterization of NLX concentration in the complex solution.

Discussion

A synthetic technique was used based on DCC-promoted formation of an amide bond between the carboxyl and amino groups of L-glutamic acid. This chemistry is mainly well conventional for peptide synthesis and has been used for the synthesis of different dendrimers containing amide bonds. Compounds G₁-COOH, G₂-COOH and G₂-COOH (F 1 and 2) were prepared through divergent method using PEG diacid 600 as a central compound. All 1H NMR chemical shifts and FT-IR data were in agreement with the projected structure of these compounds. The DSC curves of G₁-(COOH) and G₂-(COOH) have shown the endothermic peaks probably attributed to phase transition, and the exothermic peaks attributed to the thermal decomposition of the compound.

The TEM analysis showed that G₁-COOH and G₂-COOH compounds have spherical shape with small sizes of the nanoparticles. Uniform size and distribution are important properties that can affect the intracellular trafficking. In vitro release of NLX from dendrimer was investigated. As shown in Fig. 10, almost 2% of the NLX was released in the first 10 h. The initial burst release of NLX might be attributed to NLX molecules located on the exterior of the dendrimer. This was followed by a sustained release period, which could be due to encapsulation of NLX within the dendrimer. The release rate of drug molecule determined that the release outline depends on various types of interactions between dendrimer and drug molecule and depends on pH. Also, the results showed that the PG-PEG-PG dendrimers can be used for sustained release of NLX. Therefore, all the obtained results confirmed that the PG-PEG-PG biodegradable glutamic acid dendrimers are potential candidates as effective drug carriers due to their relative stability in aqueous solution and their capability in drug encapsulation and release behaviors.
Conclusion
A new class of biocompatible dendrimers with PEG core and glutamic acid branches was successfully synthesized using divergent method. Glutamic acid and PEG were selected for their low toxicity, biocompatibility and their better aqueous solubility, that extensively made them suitable for application in drug formulations. Complexes of the prepared dendrimers with NLX molecule were developed. The obtained results showed that the encapsulation/interaction of NLX into/with dendrimers cause sustained release of the drug in vitro conditions. Also, the obtained data demonstrated that the synthesized dendrimers could be applied for sustained release delivery of NLX. Therefore, all our findings showed that the glutamic acid dendrimers with PEG core are potential for an efficient drug carrier system from pharmaceutical point of view because of their relative stability in aqueous solution and their ability in drug encapsulation and release properties.

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Ethical issues
It is not applicable here.

Competing interests
The authors report no competing interests.

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