Namazi *et al.*, ***BioImpacts,*** 2014, 4(4), 175-182
doi: 10.15171/bi.2014.010
http://bi.tbzmed.ac.ir/

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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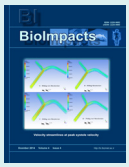
Article Info

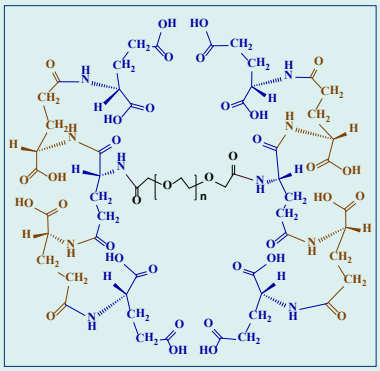


Article Type:
Research Article

Article History:
Received: 16 Mar. 2014
Revised: 03 May 2014
Accepted: 08 June 2014
ePublished: 22 Nov. 2014

Keywords:
Dendrimer
Poly (ethylene glycol)
Glutamic acid dimethyl ester
Drug-delivery

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 (G_1-COOH) and second generation (G_2-COOH) of dendrimer compounds. Thermal behavior of the synthesized dendrimers was investigated using DSC.

Results: The desired generations G_1-COOH, G_2-COOH and G_3-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.[1-5] The preparation of dendrimers with the divergent[1,6,7] and convergent[8] 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,[9] thermal action,[10] and molecular encapsulation,[11,12] 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.[13-16] Dendrimers have been examined for many uses, such as encapsulation of guest molecules,[4,17] or as nanoscale catalysts,[18] in biological recognition,[19] micelle mimics,[20] gene delivery[5] 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.[6, 27,28] These characteristics provide peptide dendrimers with worthy biodegradability and biocompatibility.[29] The semiglobular or globular topology of these compounds gives them a remarkable property of presenting themselves as multiple-armed macromolecular scaffoldings that have found utilizations in the design of vaccines, diagnostic compounds,[30] artificial enzymes[31] and biocompatible surfactants.[32] The ABA triblock linear-dendritic

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copolymers having B as the linear block and A as the dendritic block is one of the dendritic-linear copolymer hybrids. Previously, Park *et al* synthesized some of ABA triblock copolymers having polyethylene as the A block and poly (lysine) as the B blocks through a divergent method.[33] The synthesized compounds were used for transformation of DNA. One of the most characteristic properties of linear-dendritic copolymers is self-assembling in aqueous condition that is shown as the encouraging compounds for many new applications. In specific, based on PEG, linear dendritic copolymers have kinds of potential utilizations in the fields of cell mimic systems, chemical based sensors, hydrogels and drug delivery agents.[34,35] The external functional groups of peptide dendrimer can not only immobilize targeting portions but also link antitumor drugs with pH-sensitive bonds.[36]

Here, 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.[37,38] These advantages make it possible to reduce the toxicity with increasing the biocompatibility of dendrimers. Therefore, PEG could be used in biomedical and pharmaceutical utilizations.[34,39] The synthesis of different generations (G) of dendrimers containing citric acid and PEG as the core was previously published from our group.[6,40] 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.[41]

Materials and methods

Materials

PEG 600 diacid (acid number 175, 96–98%) purchased from Fluka was dried over Na_2SO_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 an LEO 906 microscope working at 100 KV for measuring of diameters G_1-(COOH) and G_2-(COOH).

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_2Cl_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_2Cl_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_2 in PG), 3.59-3.7(30 H, CH_2O in PEG), 3.9-4 (4H, O$C\underline{H}_2$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 viscose precipitate, then dried under vacuum at 25 °C for 12 h, yield 55%. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.9-2.4 (m, 8H, β-CH_2 and γ-CH_2 in PG), 3.4-3.6 (30 H, CH_2O in PEG), 3.58 (s, 12H, Me in ester group of PG), 3.9-4.1 (4H, O-CH_2-CO 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), 2400-3400 (ν, COO-H), 1714 (ν, acid C=O), 1662 (ν, amide C=O), 1094 (ν, C-O).

Synthesis of G_2-(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

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%. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.9-2.26 (m, 24H, β-CH_2 and γ-CH_2 in PG), 3.4-3.6 (30 H, CH_2O in PEG), 3.54-3.58 (s, 24H, Me in ester group of PG), 4 (4H, O-CH_2-CO in PEG), 4.35 (m, 6H, α-CH_2 in PG), 7.6-7.8 (d, 6H, NH-amide).

Deprotection of G_2-(COOMe)

G_2-(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 H_2O (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 G_3-(COOMe)

To a solution of G_2-(COOH) (1 g, 9.77×0^{-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 CH_2Cl_2. Then it was filtered off and reprecipitated in diethyl ether, and dried under vacuum at 40 °C as the red viscose, yield 20%. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.9-2.26 (m, 36H, β-CH_2 and γ-CH_2 in PG), 3.4-3.6 (30 H, CH_2O in PEG), 3.6-3.7 (s, 18H, Me in ester group of PG), 4 (4H, O-CH_2-CO in PEG), 4.5 (m, 9H, α-CH_2 in PG), 7.9-8.1 (d, 9H, NH-amide), 9.4-9.5 (5H, acid group of PG).

Preparation of G_1-(COOH)/NLX complex

For the preparation of G_1-(COOH)/NLX complex, first the dendrimer was dissolved in DMF and solution was refluxed with a solution of drug (excess of NLX) in 20 ml 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 G_1-(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 G_2-(COOH), the compound G_1-(COOMe) was deprotected. Deprotection of the terminal acid groups was achieved by hydrolysis with NaOH in MeOH/H_2O. Compound G_2-(COOH) was prepared the same procedure that used for synthesis of G_1-(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 (G_3-(COOH)) was also prepared through reaction between glutamic acid dimethyl ester salt and activated G_2-(COOH) by DCC. The ^{1}H NMR spectrum of G_1-(COOH) which shows multiplet peaks at 1.9-2.4 ppm for the β and γ-CH_2 protons (8H), 4.6–4.7 ppm for the α-CH protons of glutamic acid (2H), the protons of PEG (–OCH_2CH_2O–) at 3.6–3.8 ppm, 3.6-3.72 ppm for the protons of methyl ester (s, 12H), 3.9–4 ppm (–$COCH_2O$–) and 7.3 ppm (OCNH) (d, 2H) could be determined, also the chemical shift at 7.2 ppm is the peak of $CDCl_3$. 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 G_1-(COOH) which shows carbonyl 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 ^{1}H 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).

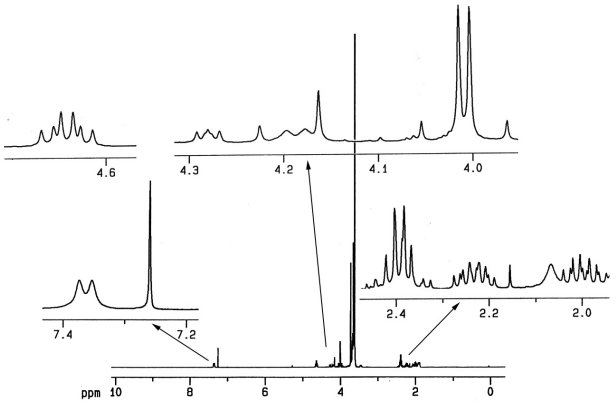

Fig. 1. ^{1}H NMR spectrum of G_1-(COOMe) in $CDCl_3$

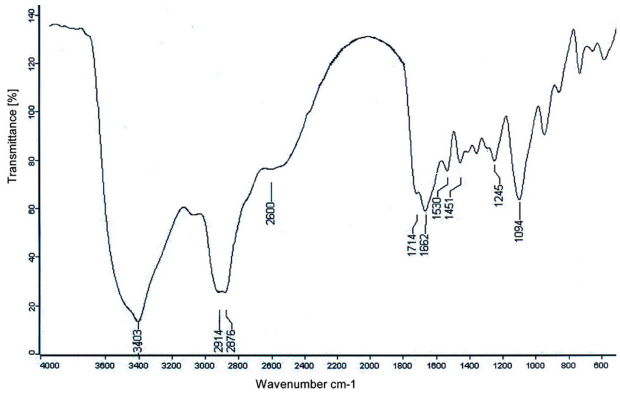

Fig. 2. FT-IR spectra of G_1-(COOH)

The ^{1}H NMR spectrum of G_2-(COOH) shows multiplet peaks at 1.9-2.26 ppm for the β and γ-CH_2 protons (m, 24H), 3.4-3.6 ppm for the protons of PEG (O-CH_2CH_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 β and γ-CH_2 protons, 4.5–4.7 ppm for the α-CH protons of glutamic acid, the protons of PEG (–OCH_2CH_2O–) at 3.6–3.8 ppm, 3.6-3.72 ppm for the protons of methyl ester, 3.9–4.2 ppm (–$COCH_2O$–) 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).

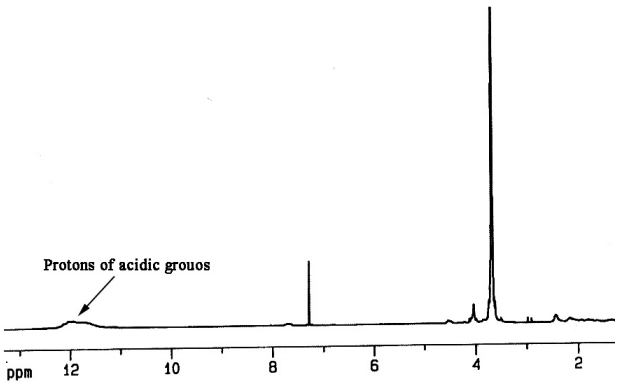

Fig. 3. ^{1}H NMR spectrum of G_1-(COOH) in $CDCl_3$

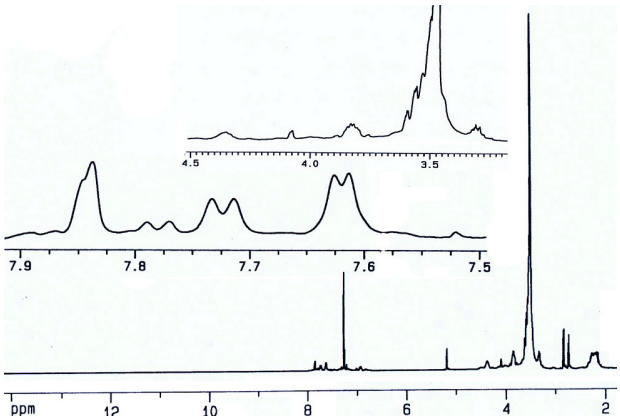

Fig. 4. ^{1}H NMR spectrum of G_2-(COOMe) in $CDCl_3$

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.

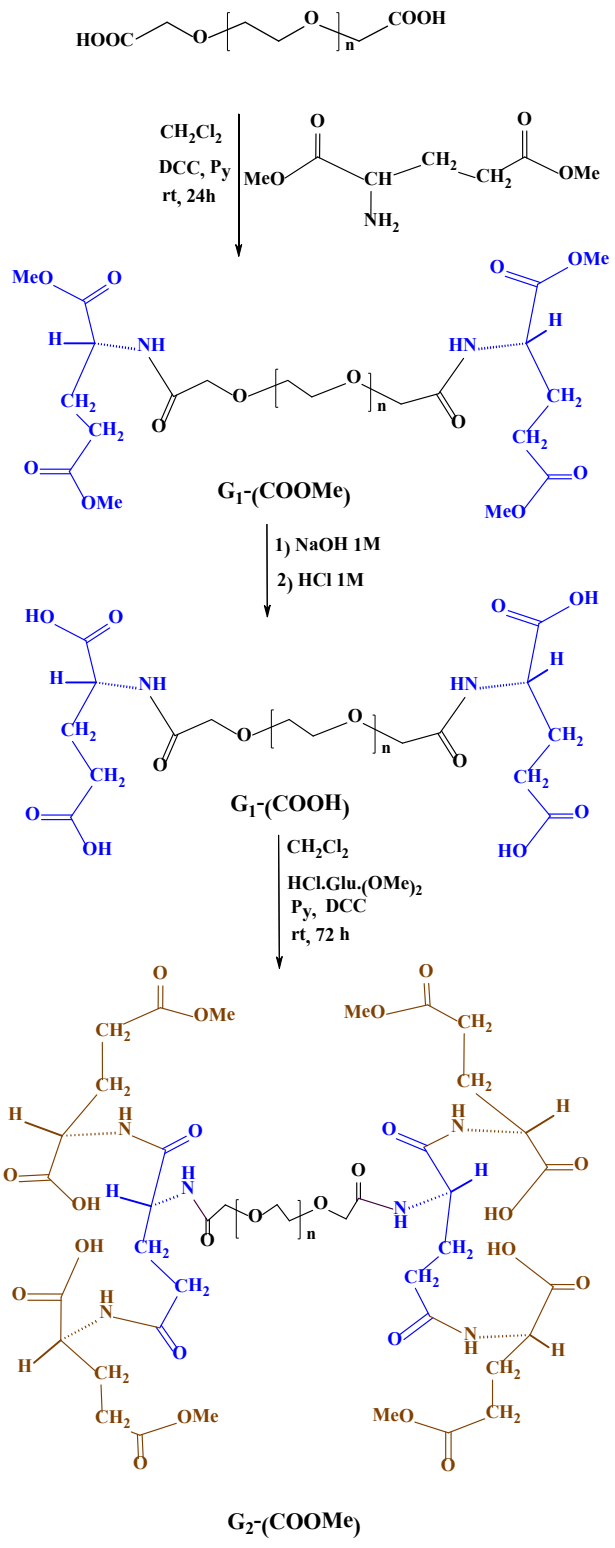

Fig. 5. Reaction scheme for the synthesis of G_1-(COOH) and G_2-(COOMe).

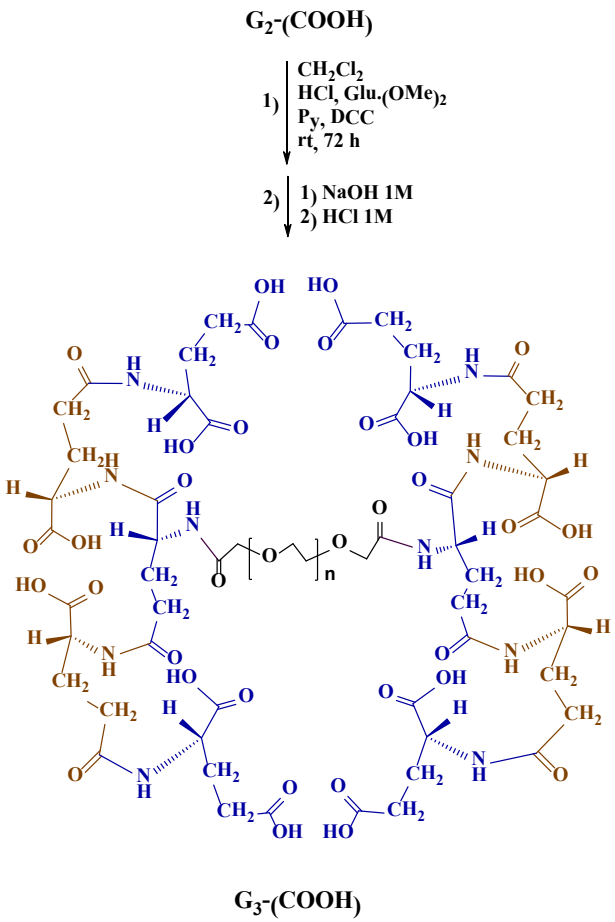

Fig. 6. Reaction scheme for the synthesis of G_3-(COOH).

Thermal properties

DSC was performed to examin 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.[4,6]

In order to investigate the drug delivery potentials of obtained compounds, the naltrexone (NLX) was loaded

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

Sample	G_1-(COOMe)			G_1-(COOH)			G_2-(COOMe)		
Solvents	Soluble	Insoluble	Swell	Soluble	Insoluble	Swell	Soluble	Insoluble	Swell
ichloromethane	+	-	-	-	-	+	+	-	-
DMF	+	-	-	+	-	-	+	-	-
THF	-	+	-	-	+	-	-	+	-
Methanol	-	+	-	-	-	+	-	+	-
Ethanol	-	+	-	-	-	+	-	+	-
Methanol-H_2O	-	-	+	+	-	-	-	-	+
Eehanol-H_2O	-	-	+	+	-	-	-	-	+
Chloroform	+	-	-	-	-	+	+	-	-

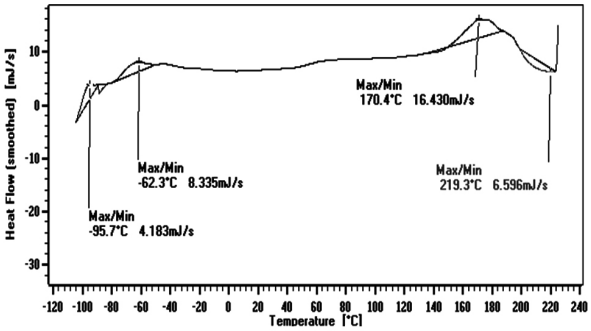

Fig. 7. The DSC curve of G_1-(COOH)

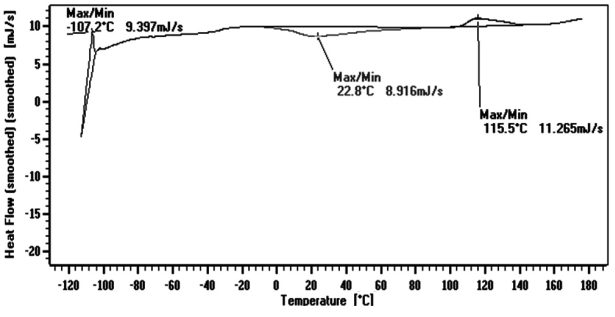

Fig. 8. The DSC curve of G_2-(COOH)

into the G_1-(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 G_1-(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 quantity of trapped drug molecules in drug/dendrimer complex was defined with calculation method. The weight of dendrimers before and after complexation (drug/dendrimer complex) was determined; their difference was the amount of trapped drugs in the dendritic compound. The calculation for determination of the quantity of NLX in drug/G_1-(COOH) complex is as follows:

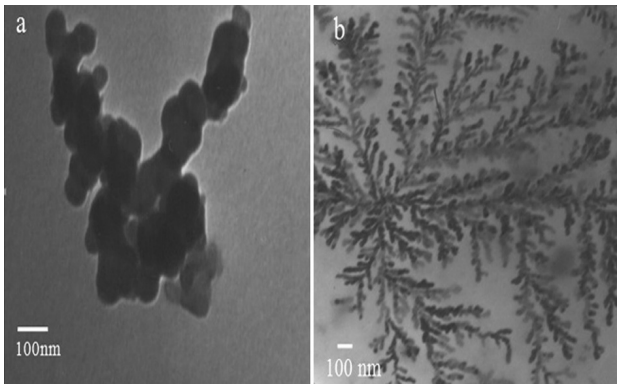

Fig. 9. TEM image and size of G_1-(COOH) and G_2-(COOH)

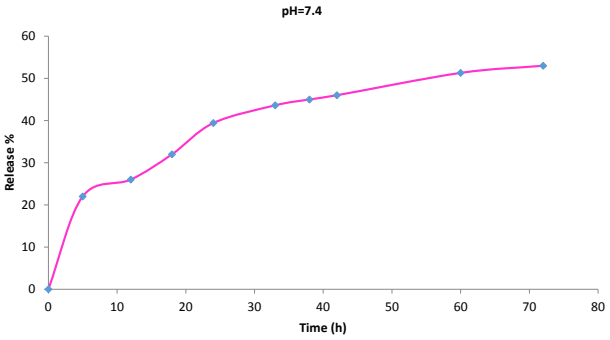

Fig. 10. Release curve of NLX from G_1-(COOH)/NLX (pH 7.4, 37 °C).

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 (drug)
4.4×10^{-4} mol (dendrimer)
mol% of NLX in G_1-(COOH)/NLX complex = ? 100
The mol% of NLX in G_1-(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, λ_{max} 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 G_1-COOH, G_2-COOH and G_3-COOH (F 1 and 2) were prepared through divergent method using PEG diacid 600 as a central compound. All ^{1}H NMR chemical shifts and FT-IR data were in agreement with the projected structure of these compounds. The DSC curves of G_1-(COOH) and G_2-(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 G_1-COOH and G_2-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.

Acknowledgements

Authors greatly acknowledge the Research Center for Pharmaceutical Nanonotechnology (RCPN), Tabriz University of Medical Science and the University of Tabriz for the financial supports of this work.

Ethical issues

It is not applicable here.

Competing interests

The authors report no competing interests.

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