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International Journal of Pharmaceutics 226 (2001) 23–29

international journal of pharmaceutics

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Evaluation of polymeric nanoparticles composed of cholic acid and methoxy poly(ethylene glycol)

In-Sook Kim, Sung-Ho Kim *

Department of Biological Chemistry, College of Pharmacy, Chosun University, # 375 Seosuk-dong, Dong-gu, Kwangju 501-759, South Korea

Received 4 April 2001; accepted 14 May 2001

Abstract

Amphiphilic polymeric conjugate based on cholic acid (CA) as the hydrophobic component and methoxy poly(ethylene glycol) (MPEG) as the hydrophilic component was synthesized using a 1,1′-carbonyldiimidazole (CDI) mediated conjugation. Fluorescence spectroscopy measurements suggested that CA and MPEG (abbreviated as CE) conjugate was associated in water to form polymeric core-shell type nanoparticles with a critical association concentration (CAC) value of 0.063 g l^{-1}. From the transmission electron microscope (TEM) observation, CE nanoparticles were almost spherical with a size range of approximately 10–30 nm in the dried state, which was in agreement with the result from particle size measurement using photon correlation spectroscopy (PCS). Clonazepam (CNZ) was physically loaded into the CE nanoparticles with a 16.2 wt.% loading. CNZ release was pseudo zero-order in kinetic terms for up to 3 days. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Amphiphilic polymer; Polymeric conjugate; Cholic acid; Poly(ethylene glycol); Nanoparticle

1. Introduction

In recent decades, nanoparticles have been widely employed in various fields of life sciences such as separation technologies, histological studies, clinical diagnostic assays, and drug delivery systems (Kreuter, 1991; Leroux et al., 1995; Kubitschko et al., 1997; Schroeder et al., 1998; Demoy et al., 1999; De Jaeghere et al., 2000). The nanoparticles prepared from amphiphilic macromolecular materials consist of hydrophobic and hydrophilic part. The hydrophobic part forms the inner core of the nanoparticle structure, in which the active principle (drug or biologically active material) is dissolved, entrapped, or encapsulated. Hydrophilic part forms the hydrated outer shell, which is believed to play a key role in avoiding uptake by the reticuloendothelial system (RES; Gref et al., 1994). Advantages of the core–shell structure systems are the targeting ability, reduced toxic side effects, the solubilization of hydrophobic drugs, stable storage, long blood circulation, favorable biodistribution, and reduced reaction with RES (Yokoyama et al., 1990, 1991; Kwon et

* Corresponding author. Tel.: +82-62-2306379; fax: +82-62-2225414.

E-mail address: shkim@mail.chosun.ac.kr (S.-H. Kim).

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PII: S0378-5173(01)00756-6

al., 1993, 1994, 1995; Gref et al., 1994). These properties were applicable to amphiphilic polymeric conjugate composed of both hydrophobic and hydrophilic components.

We already reported a thermo-responsive core–shell type polymeric nanoparticle composed of poly(L-lactic acid) and poly(*N*-isopropylacrylamide) block copolymers, a targetable polymeric nanoparticle composed of cholic acid (CA) and poly(ethylene glycol) end-capped with a sugar moiety, and a novel hydrogel nanoparticles composed of dextran and poly(ethylene glycol) macromer (Kim et al., 2000a,b,c).

In this study, our interest was directed towards the formation of polymeric nanoparticles based on amphiphilic polymeric conjugates composed of CA and methoxy poly(ethylene glycol) (MPEG). This study involves the synthesis of CA and MPEG (abbreviated as CE) conjugate and the preparation of polymeric nanoparticles by the diafiltration method, which was performed as previously reported (Kim et al., 2000a,b,c).

CA is one of the major bile acids, the main product of cholesterol metabolism, and biologically, one of the most detergent-like molecules in the body. CA acts as a hydrophobic core and represents a potential drug incorporation site. MPEG is a modified poly(ethylene glycol) which has well defined biocompatibility, non-toxic, and non-immunogenic properties. And MPEG is known to prevent interactions with cells and proteins due to its hydrophilic nature (Lee et al., 1989).

The physicochemical characteristics of CE polymeric nanoparticles were investigated. To evaluate the CE nanoparticles as drug carriers, clonazepam (CNZ) was incorporated as a hydrophobic model drug and the release of CNZ was monitored in vitro.

2. Materials and methods

2.1. Materials

CA and MPEG with a molecular weight of 2000 were purchased from Sigma Chem. Co. (St. Louis, USA), 1,1′-carbonyldiimidazole (CDI) from the Aldrich Chemical Company Inc. (Milwaukee, USA). CNZ was obtained from ROCHE, Switzerland. Dimethyl formamide (DMF) and other chemicals were reagent grade and used without purification.

2.2. Synthesis of CE conjugate

CA (1 mmol), CDI (1.5 mmol), and MPEG (1 mmol) were individually dissolved in DMF, and the CDI/DMF solution was added to the CA/DMF solution. The mixture was stirred for 30 min at room temperature to activate the carboxyl group of the CA. MPEG/DMF solution was then added to the activated CA solution, and the mixture stirred at 60 °C for 48 h. The resultant solution was dialyzed against distilled water using dialysis membrane with a molecular weight cutoff (MWCO) 2000 g mol^{-1} (Spectra/Por™ Membranes) for 7 days and then the dialyzed solution was freeze-dried and the CE conjugate stored in a refrigerator at 4 °C until use.

Fourier transform-infrared (FT-IR) spectroscopy measurement (Nicolet, Manga IR 550) was used to confirm the synthesis of the CE conjugate.

2.3. Preparation of CE polymeric nanoparticles and drug loading

The formation of CE nanoparticles was carried out by the diafiltration method (Kim et al., 2000a,b,c). Briefly, 20 mg of CE was dissolved in 5 ml of DMF. To form polymeric nanoparticles, the solution was dialyzed against distilled water for 24 h using MWCO 2000 g mol^{-1} dialysis membrane. The solution was then either analyzed or freeze-dried.

CNZ-loaded polymeric nanoparticles were prepared as follows, 20 mg of CE conjugate was dissolved in 4 ml of DMF, and 20 mg of CNZ in 1 ml DMF were added to this solution. To form the polymeric nanoparticles and remove the free drug, the solution was dialyzed against distilled water for 24 h using MWCO 2000 g mol^{-1} dialysis membrane. The medium was replaced every 1 h for the first 3 h and every 3 h for 21 h, and then freeze-dried.

To evaluate the drug loading, a freeze-dried sample of CNZ-loaded CE polymeric nanoparticles was suspended in methanol, vigorously stirred for 2 h and sonicated for 15 min. The resultant solution was centrifuged at 3000 rpm for 20 min, and the supernatant was taken to measure the drug concentration using an ultra violet (UV) spectrophotometer (Shimadzu UV-1201, Japan) at 306 nm.

2.4. Fluorescence spectroscopy measurement

To investigate the fluorescence spectroscopy characteristics, the CE conjugate suspension was prepared as follows without drug, 20 mg of CE conjugate was dissolved in 5 ml of DMF and dialyzed against distilled water for 1 day using a MWCO 2000 g mol^{-1} dialysis membrane. The resultant solution was then adjusted to various CE conjugate concentrations.

To prove the potential of core–shell type nanoparticle formation and estimate the critical association concentration (CAC) of the CE conjugate, the fluorescence spectroscopy was measured with a spectrofluorophotometer (Shimadzu RF-5301 PC, Shimadzu Co. Ltd., Tokyo, Japan), using pyrene as a hydrophobic probe (Kalyanasundaram and Thomas, 1977; Wilhelm et al., 1991; Kim et al., 2000a,b,c). Sample suspensions were prepared by adding a known amount of pyrene in acetone to a series of 20 ml vials, and then removing the acetone by evaporation to a final pyrene concentration of 6.0×10^{-7} M. Various concentrations (10 ml) of CE conjugate suspension were then added to each vial and heated for 3 h at 65 °C to equilibrate the pyrene and the nanoparticles, and then left to cool overnight at room temperature. Excitation wavelength was 339 nm for emission spectra, and the emission bandwidth was 1.5 nm.

2.5. Transmission electron microscope (TEM) observation

The morphology of the polymeric nanoparticles was observed using a JEM-2000 FX II (Jeol, Japan) instrument. A drop of polymeric nanoparticle suspension was placed on a copper grid coated with carbon film and dried at 25 °C. The specimen on the copper grid was then negatively stained with 0.01% phosphotungstic acid. Observation was performed at 80 kV.

2.6. Photon correlation spectroscopy (PCS) measurement

PCS was measured with a Zetasizer 3000 (Malvern Instruments, UK) with a He–Ne laser beam at a wavelength of 633 nm at 25 °C at a scattering angle of 90°. The nanoparticle suspension was prepared by the diafiltration method, and the particle size was measured without filtering (concentration, 1 g l^{-1}).

2.7. In vitro release studies

Five milligram of CNZ-loaded CE polymeric nanoparticles and 1 ml of phosphate buffer saline (PBS; 0.1 M, pH 7.4) were placed in a dialysis membrane (MWCO 2000 g mol^{-1}), and the membrane was introduced into a vial with 10 ml of PBS. The medium was stirred at 100 rpm at 37 °C. At predetermined time intervals, the entire medium was removed and replaced with the same amount of fresh PBS. The amount of released CNZ from the CE nanoparticles was measured with an UV spectrophotometer at 306 nm.

3. Results and discussion

3.1. Analysis of CE conjugate

Fig. 1 shows the scheme of CDI-mediated conjugation of CA and MPEG. The CE conjugate was examined by FT-IR spectroscopy in Fig. 2. An absorption band at 3436 cm^{-1} was observed caused by the terminal hydroxyl group (Kalyanasundaram and Thomas, 1977; Deng et al., 1990). This band became weak in the CE conjugate due to CDI-mediated conjugation with CA. A strong absorption peak at 1113 cm^{-1} was observed due to the C–O of the ester. The band at 2888 cm^{-1} was attributed to C–H stretch and was present in both polymers (Andini et al., 1988).

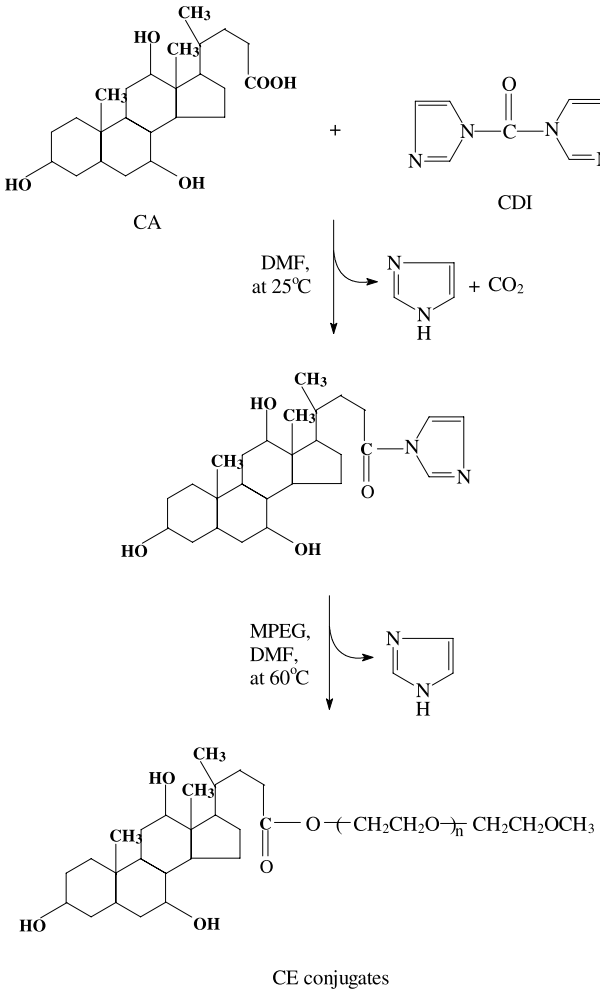

Fig. 1. Synthetic scheme of CE conjugate.

To investigate the core–shell structure nanoparticle formation of the CE conjugate, the fluorescence probe technique was used and the CAC was determined using pyrene as a hydrophobic probe (Kalyanasundaram and Thomas, 1977; Wilhelm et al., 1991; Kim et al., 2000a,b,c).

Fig. 3 shows the fluorescence emission spectra of pyrene against various concentrations of CE. The fluorescence intensity increased along with the concentration of CE, which indicated the formation of core–shell structure polymeric nanoparticles of CE in water. When pyrene was introduced into the core domain from a good solvent, it is thought that pyrene was preferentially solubilized into the nanoparticles.

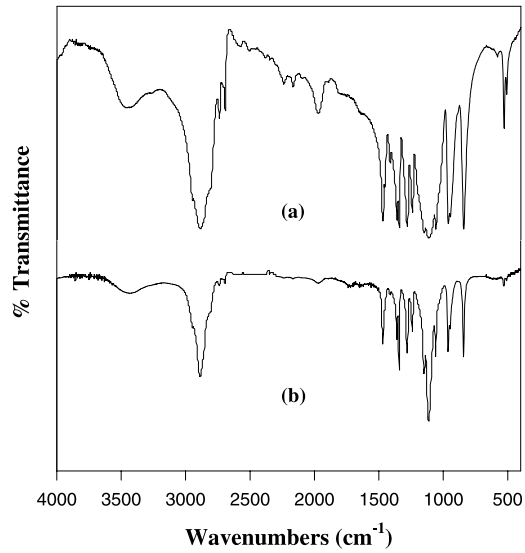

Fig. 2. FT-IR spectra of MPEG (a) and CE conjugate (b).

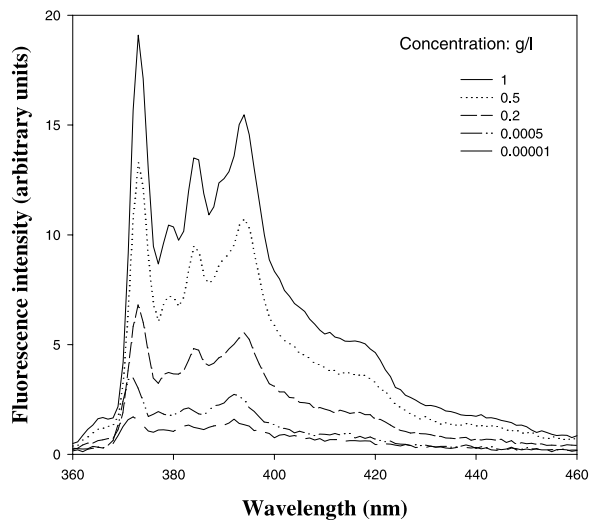

Fig. 3. Fluorescence emission spectra of pyrene/CE against concentration of CE in distilled water (excitation wavelength, 339 nm). [Pyrene] = 6.0×10^{-7} M.

In the excitation spectrum, a shift to the red was observed with increasing concentration of CE conjugate as shown in Fig. 4a. Such a shift was observed in the pyrene excitation spectrum in the study of nanoparticle formation of poly(L-lactic acid) and poly(*N*-isopropylacrylamide; Kim et al.,

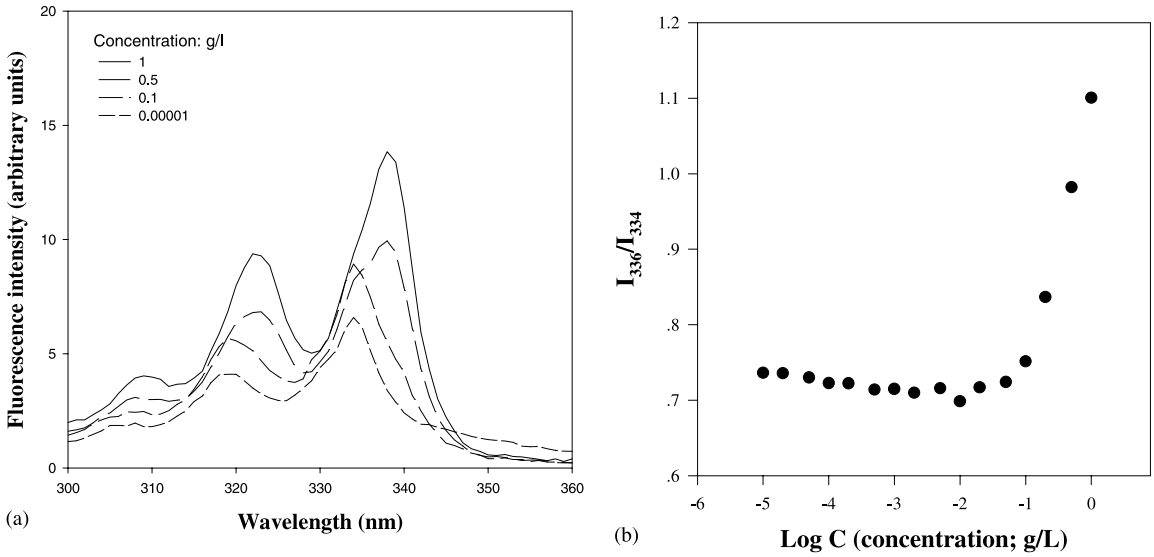

Fig. 4. Fluorescence excitation spectra of pyrene (6.0×10^{-7} M) against CE concentration in distilled water (emission wavelength, 390 nm) (a) and plots of the intensity ratio I_{336}/I_{334} from the pyrene excitation spectra vs. log C of CE conjugate in distilled water (b).

2000a). Plot of I_{336}/I_{334} versus log C is shown in Fig. 4b. This shows that the ratio was almost flat at lower concentrations, and rapidly increased at higher concentrations. The CAC was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentrations. The estimated CAC value was 0.063 g l^{-1}. From the study of the fluorescence probe measurements, it can be said that the CE can form core–shell type nanoparticles in water upon critical concentration (i.e. CAC) and has an amphiphilic nature.

Fig. 5 shows a TEM photograph of CE polymeric nanoparticles. The particles are almost spherical in shape and the sizes ranged from about 10 to 30 nm in diameter, which compares with the dimensions of viruses, and thus, may be able to penetrate the sinusoidal and fenestrated capillaries, that have pore sizes of approximately 100 nm. This result also indicated that amphiphilic polymeric conjugate can form spherical polymeric nanoparticles in the same manner as AB type block copolymers.

The particle size distribution of the CE suspension was measured by PCS measurement. CE conjugate formed a relatively narrow particle distribution of 22.3 ± 2.0 nm based on the number average with the polydispersity of 0.238 ± 0.02, and this result coincide with that obtained from TEM observations.

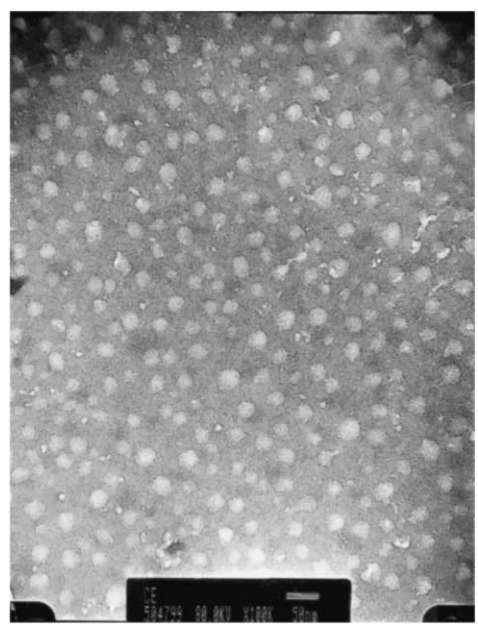

Fig. 5. TEM photograph of CE nanoparticles.

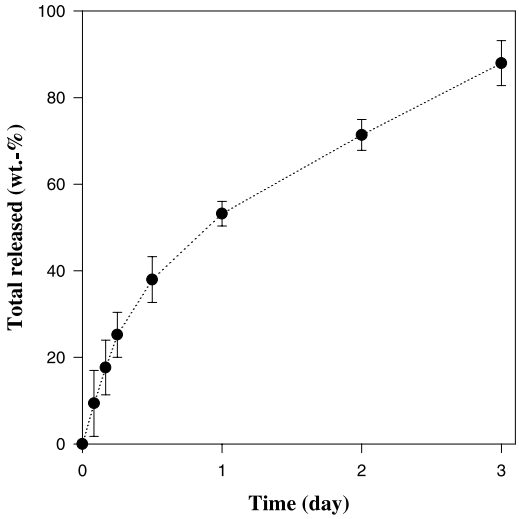

Fig. 6. CNZ release from CE nanoparticles in PBS (0.1 M, pH 7.4) at 37 °C ($n = 3$).

3.2. Drug loading and release studies in vitro

The drug loading content of CE nanoparticles was calculated as 16.2 wt.%. To study drug release behavior, CNZ-loaded nanoparticles of CE were simply redispersed in PBS (0.1 M, pH 7.4) without surfactant. The release kinetics of CNZ from the CE nanoparticles into the surrounding aqueous phase is shown in Fig. 6. The CNZ release from the CE polymeric nanoparticles showed pseudo zero-order kinetics over 3 day period.

In conclusion, the CE conjugate can form the core–shell structure in water, and shows nano-sized spherical shapes. CNZ was released from CE nanoparticles by pseudo zero-order kinetics. As a result, the CE nanoparticles can be used as appropriate vehicles for the drug delivery of hydrophobic drugs.

Acknowledgements

This study was supported by research funds from Research Center for Proteineous Materials, Chosun University, 2001.

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