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International Journal of Pharmaceutics 245 (2002) 67–73

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Development of a polymeric nanoparticulate drug delivery system
In vitro characterization of nanoparticles based on sugar-containing conjugates

In-Sook Kim, Sung-Ho Kim *

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

Received 17 January 2002; accepted 18 June 2002

Abstract

This paper reports the synthesis of polymeric nanoparticles from a sugar-containing conjugate for liver-specific drug delivery. The conjugate was composed of lactobionic acid, diamine-terminated poly(ethylene glycol) and cholic acid (abbreviated as LEC). The conjugate was characterized by ^{1}H NMR and FT-IR spectroscopy measurements. In aqueous media, the conjugate can self-assemble to form core-shell type nanoparticles, and the formation of a core-shell structure was observed by fluorescence spectroscopy. The critical association concentration (CAC) of the LEC conjugate nanoparticles was determined from fluorescence excitation spectra to be 0.05 g/l. The LEC nanoparticles were mostly spherical with sizes ranging from 10 to 30 nm. Clonazepam (CNZ) was used as a model hydrophobic drug, and was incorporated into the hydrophobic core of the nanoparticles. CNZ was released more slowly at a higher drug loading due to drug crystallization.

Keywords: Polymeric nanoparticle; Sugar-containing conjugate; Liver-specific drug delivery

1. Introduction

Controlled drug delivery technology represents one of the frontier areas of science, which involves a multidisciplinary scientific approach. These delivery systems offer numerous advantages compared to conventional dosage forms. These include improved efficacy, reduced toxicity, and improved patient compliance and convenience. Such systems often use macromolecules as drug carriers. This field of pharmaceutical technology has grown and has diversified rapidly in recent years.

Among the different dosage forms reported, both nano- and microparticles have gained increasing importance, due to a tendency to accumulate in the inflamed areas of the body (Illum et al., 1989). Nano- and microparticles occupy a unique position in drug delivery technology due

* Corresponding author. Tel.: +82-62-230-6379; fax: +82-62-222-5414

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

0378-5173/02/$ - see front matter
PII: S0378-5173(02)00336-8

to their attractive properties. In particular, nanoparticles have several advantages in pharmaceutical applications. They are easily purified and sterilized. In addition, they offer drug targeting possibilities and a sustained release action (Allemann et al., 1993). Nanoparticles based on a core-shell structure or polymeric micelle present advantages in terms of lengthy circulation in the body, drug solubility, drug stability, and a high level of drug encapsulation (Yokoyama et al., 1991; Gref et al., 1994; Kwon et al., 1995).

For liver disease therapy, there has been growing interest in the area of liver-cell specific drug delivery systems in recent years. This may be due to a failure of other pharmacological approaches to the liver disease site as a result of the non-specific delivery mechanism towards the other organs and several side effects. A great deal of effort has been made to achieve an appropriate liver targeting of chemotherapeutic agents with liposomes (Kim and Han, 1995), microspheres (Kim et al., 1993), and drug-carrier molecule conjugates (Seymour et al., 1991). Among the liver-associated surface receptors, the asialoglycoprotein receptor (ASGP-R: galactose receptor) is well known to be present only on hepatocytes (Ashwell and Harford, 1982). It is also retained on several human hepatoma cell lines (Fallon and Schwartz, 1988). If a ligand binds to a galactose receptor, the ligand–receptor complex is rapidly internalized and the receptor recycles back to the surface (Ciechanover et al., 1983). Accordingly, the receptor shows a high binding capacity and efficient cellular uptake of galactosylated ligands. Therefore, designing a drug delivery system for galactose receptor-mediated endocytosis would be useful for targeting the hepatocyte/liver and hepatoma cells (Nishikawa et al., 1993; Goto et al., 1994).

In this study, core-shell type polymeric nanoparticles composed of cholic acid (CA) and diamine-terminated poly(ethylene glycol) (ATPEG) with a galactose moiety from lactobionic acid (abbreviated as LEC) was synthesized for a liver-specific drug delivery system. CA is a naturally occurring substance and acts as a drug incorporation site with hydrophobic characteristics. ATPEG is a modified PEG which has well defined biocompatibility, is non-toxic, and has non-immunogenic properties. It is known to prevent interactions with cells and proteins due to its hydrophilic nature (Wang et al., 1997). Because of the amphiphilicity of the LEC conjugate, core-shell type nanoparticles with a sugar moiety were prepared by a diafiltration method, and the physico-chemical characteristics were evaluated in vitro.

2. Materials and methods

2.1. Materials

ATPEG with an average molecular weight of 2000 was supplied by the Texaco Chem. Co. (Ballaire, TX). CA and *N*-hydroxysuccinimide (NHS) were purchased from the Sigma Chem. Co. (St. Louis, MO). Lactobionic acid (LA) and *N*,*N*′-dicyclohexyl carbodiimide (DCC) were obtained from the Aldrich Chem. Co. (Milwaukee, USA). The dialysis membranes with a molecular weight cutoff (MWCO) of 2000 g/mol were purchased from Spectra/Por^{TM} Membranes. Dimethyl sulfoxide (DMSO) and other chemicals were of reagent grade and used without further purification.

2.2. Synthesis of LEC conjugate

The LEC conjugate was prepared by a two-step coupling reaction of LA, ATPEG, and CA. Initially, the conjugation between LA and one ATPEG terminal was performed. LA (0.2 mmol), DCC (0.4 mmol), NHS (0.4 mmol), and ATPEG (0.2 mmol) were separately dissolved in DMSO. The DCC/DMSO solution was added to the LA/DMSO solution, and stirred for 30 min to activate the LA carboxyl group. The NHS/DMSO solution was added to the activated LA solution, and the reactions were conducted at room temperature for 12 h. In the reaction mixture, dicyclohexylurea (DCU) was formed, which was then filtered to remove DCU. The ATPEG/DMSO solution was then added into the reaction mixture, and stirred for 30 min to complete the conjugation of the activated LA and ATPEG. The solution was

placed in a dialysis membrane and dialyzed against distilled water for 7 days. The resulting solution in the dialysis membrane was freeze-dried, and a second conjugation between CA and the LA/ATPEG (abbreviated as LE) conjugate was carried out using the same procedure mentioned above. The freeze-dried LEC conjugate was stored in a refrigerator at 4 °C until needed.

2.3. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy (Magna IR 550, Nicolet) was used to confirm the synthesis of the LE and LEC conjugates.

2.4. ^{1}H NMR spectroscopy

The ^{1}H nuclear magnetic resonance (NMR) spectra were measured in DMSO-d_6 to confirm the synthesis of the LEC conjugate using a 600 MHz high resolution spectrometer (FT-NMR, AVANCE 600, Bruker, Germany).

2.5. Fluorescence spectroscopy

The LEC conjugate solution was prepared as follows: 20 mg of the LEC conjugate was dissolved in 5 ml of DMSO and dialyzed using a dialysis membrane against distilled water for 2 days. The resultant solution in the dialysis membrane was adjusted to various concentrations of the LEC conjugate. The critical association concentration (CAC) of the LEC conjugate was estimated to demonstrate the potential for nanoparticle formation by a spectrofluorophotometer (Shimadzu RF-5301 PC, Japan) using pyrene as a hydrophobic probe (Wilhelm et al., 1991; Kim et al., 2000a,b,c; Kim and Kim, 2001). The samples were prepared as follows: a known amount of pyrene dissolved in acetone was added to a series of 20 ml vials and the acetone was then evaporated. The pyrene concentration was then adjusted to give a final concentration of 6.0×10^{-7} M in 10 ml of various concentrations of the conjugate solution. The resulting solutions were heated for 3 h at 65 °C to equilibrate the pyrene and the nanoparticles. The mixture was then left to cool overnight at room temperature. The emission wavelength used for the excitation spectra was 390 nm. The excitation and emission bandwidths were 1.5 and 1.5 nm, respectively.

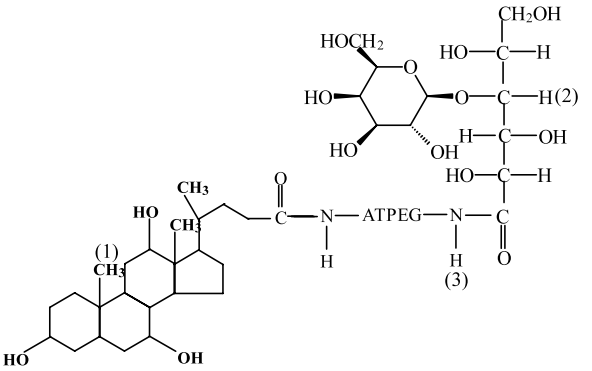

Fig. 1. Scheme of LEC conjugate.

2.6. Transmission electron microscope (TEM) measurement

The morphology of the polymeric nanoparticles was observed using a JEM-2000 FX II (JEOL, Japan) at 80 kV. A drop of polymeric nanoparticle suspension in water was placed on a copper grid coated with carbon film and dried at 20 °C. The specimen on the copper grid was negatively stained with 0.1% phosphotungstic acid.

2.7. Differential scanning calorimetry (DSC) measurement

The melting temperature (T_m) was measured with a Universal V2.4F (TA instruments) differential scanning calorimeter. The measurements were carried out at temperatures from room temperature to 300 °C under nitrogen at a scanning rate of 10 °C/min.

2.8. In vitro drug release studies

The release experiment was carried out as previously reported (Kim and Kim, 2001). Five milligrams of the clonazepam (CNZ)-loaded LEC core-shell type nanoparticles were suspended in 1 ml PBS and subsequently placed into a dialysis membrane (MWCO 2000 g/mol). The dialysis

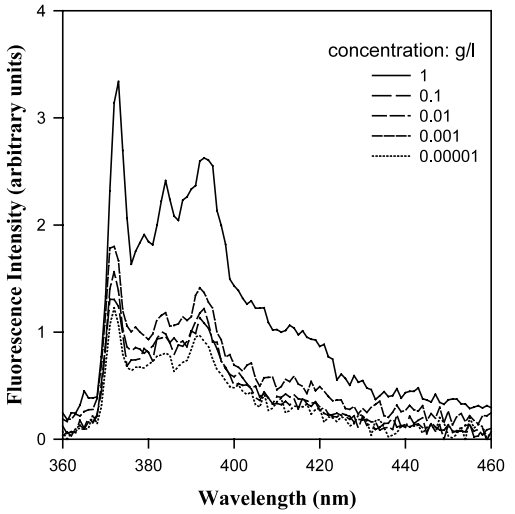

Fig. 2. Fluorescence emission spectra of pyrene/LEC against concentration of LEC in distilled water (excitation wavelength: 339 nm). [pyrene] = 6.0×10^{-7} M.

membrane was placed into a 20 ml bottle with 10 ml PBS and the media was stirred at 100 rpm at 37 °C. At set time intervals, the whole medium (10 ml) was taken and replaced with the same volume of fresh PBS. The concentration of the CNZ released in the PBS was determined by a UV spectrophotometer (Shimadzu UV-1201, Japan) at 306 nm.

3. Results and discussion

The synthesized LEC conjugate structure is represented schematically in Fig. 1. The FT-IR spectra provided evidence of LE and LEC conjugate synthesis. In the LE spectrum, the amide stretch and amide bend vibration was observed at 3335 and 1627 cm^{-1}, respectively. The LEC conjugate spectrum contained C–H stretch absorption bands at 2927 and 2851 cm^{-1} due to CA, and an amide bend at 1576 cm^{-1}. The LEC conjugate was also characterized by ^{1}H NMR spectroscopy as shown in the previous report (Kim et al., 2000a). The proton signals of (1), (2), and (3) occurred at approximately 0.5, 1.7, and 5.8 ppm, respectively.

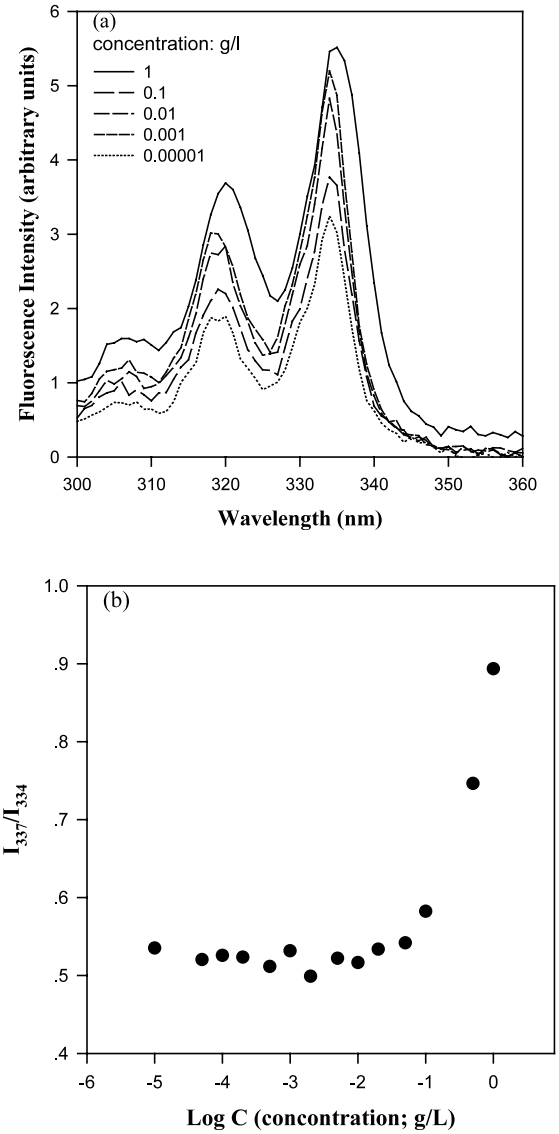

Fig. 3. Fluorescence excitation spectra of pyrene/LEC against concentration of LEC in distilled water (emission wavelength: 390 nm) (a) and plots of the intensity ratio I_{337}/I_{334} from pyrene excitation spectra vs. log C of the LEC conjugate in distilled water (b). [pyrene] = 6.0×10^{-7} M.

From the fluorescence spectroscopy study, nanoparticle formation was investigated and the results are shown in Figs. 2 and 3. In Fig. 2, the fluorescence emission spectrum of pyrene was shown at a fixed excitation wavelength of 339 nm against various LEC concentrations. The

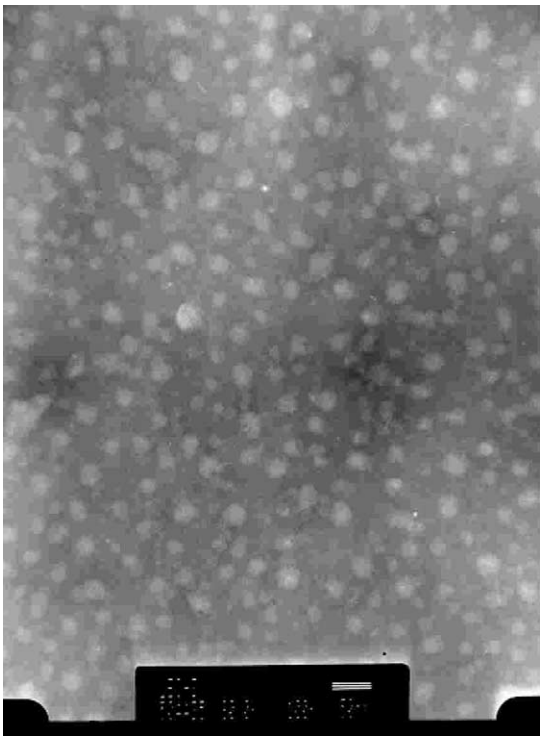

Fig. 4. TEM photograph of LEC nanoparticles.

fluorescence intensity increased with increasing LEC concentrations, which indicated the formation of self-assembled polymeric LEC nanoparticles in water such as block co-polymeric micelles (Wilhelm et al., 1991; Kim et al., 2000b) or polymeric conjugate nanoparticles (Kim et al., 2000c; Kim and Kim, 2001).

Fig. 3(a) shows the fluorescence excitation spectra of pyrene at various LEC conjugate concentrations. A red shift in the pyrene excitation spectrum was observed with increasing LEC concentration. It is thought that pyrene is preferentially partitioned into the hydrophobic core segment as previous report (Wilhelm et al., 1991; Kim et al., 2000b,c; Kim and Kim, 2001). The intensity ratio of I_{337}/I_{334} against the polymer concentration in the pyrene excitation spectrum is plotted in Fig. 3(b). This result shows that the ratio is almost flat at very low concentrations and rapidly increases at higher concentrations. The CAC value was 0.05 g/l, which was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the lower concentration points.

The morphologies of the LEC nanoparticles were observed by TEM, and are shown in Fig. 4.

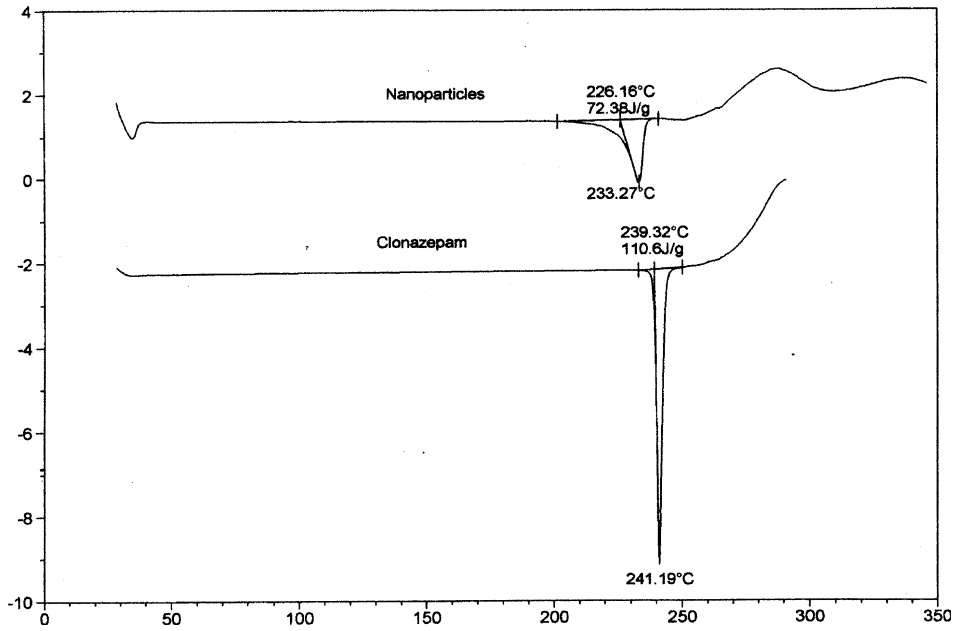

Fig. 5. DSC thermograms of CNZ and CNZ-loaded LEC nanoparticles.

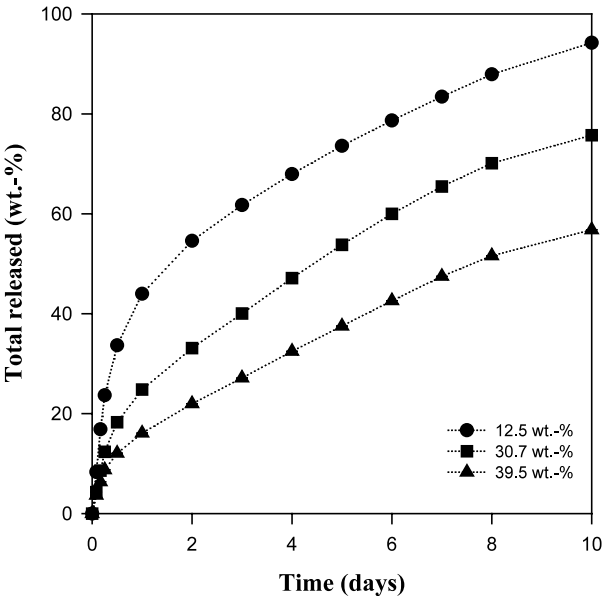

Fig. 6. CNZ relelase from LEC nanoparticles as a function of drug loading contents ($n = 3$).

The LEC nanoparticles were mostly spherical with sizes ranging from 10 to 30 nm.

Fig. 5 shows DSC thermograms of the CNZ and CNZ-loaded LEC nanoparticles. CNZ itself melts at 241 °C whereas the melting temperature of CNZ in the CNZ-loaded LEC nanoparticles was 233 °C, indicating that parts of the entrapped drug exists in a crystalline form.

The CNZ released from the LEC nanoparticles against the drug loading concentration is shown in Fig. 6. The figure suggests the higher the drug loading concentration, the slower the drug release. At a lower loading, CNZ is dispersed in the core portion whereas crystallization of the drug in the core occurs at higher drug loads. These results were supported by the DSC thermograms as shown in Fig. 5. As reported elsewhere, the crystallized drug should dissolve and diffuse to the outer aqueous phase more slowly than the dispersed drug (Jeong et al., 1998).

In conclusion, the core-shell structure of the LEC nanoparticles was characterized by fluorescence spectroscopy. The hydrophobic model drug was physically entrapped within the inner core of the structures by a hydrophobic interaction. The CNZ release kinetics is suggested to be dominantly governed by a diffusion mechanism from the inner core portion. The use of LEC nanoparticles for facilitating specific hepatic targeting of anti-cancer agents is currently under investigation.

Acknowledgements

This work was supported by Korea Research Foundation Grant (KRF-2001-015-FP0127).

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