Self-Aggregates of Cholic Acid Hydrazide–Dextran Conjugates as Drug Carriers

Qingguo Xu, Xubo Yuan, Jin Chang

Institute of Nanobiotechnology, School of Materials Science and Engineering, Tianjin University, Tianjin 300072, People's Republic of China

Received 21 January 2004; accepted 13 July 2004
DOI 10.1002/app.21198
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Cholic acid hydrazide–dextran conjugates (CAH–DEX's) with stable acryl hydrazone linkages were synthesized from cholic acid hydrazide and poly(aldehyde dextran) and were characterized by Fourier transform infrared (FTIR) spectroscopy, ^{1}H-NMR, and surface tension measurements. The conjugates developed a lower critical aggregation concentration, which was determined by 1,6-diphenyl-1,3,5-hexatriene dye solubilization methods, 1.41 and 2.10×10^{-2} mg/mL for CAH–DEX 9.0 and CAH–DEX 6.5, respectively. A hydrophobic drug, indomethacin (IN), was physically entrapped inside the self-aggregates, and the IN-loaded self-aggregates were analyzed with a dynamic light-scattering system, transmission electron microscopy, and atomic force microscopy. The maximum loading of IN reached 29.9% of the CAH–DEX self-aggregates, which suggested a high loading efficiency of 51.2%. The size of the self-aggregates increased when the drug was entrapped. IN was released from CAH–DEX self-aggregates at pH 4 much slower than at pH 7.4, and in pH 4 media, the release profile was pseudo-zero-order in kinetic terms for up to 14 days. There was almost no change in the FTIR spectra of the CAH–DEX's, which were incubated in buffers of pH 7.4 and pH 4 for 24 h, which indicated that acryl hydrazone was considerably resistant to hydrolysis. J Appl Polym Sci 95: 487–493, 2005

Key words: conjugated polymer; drug delivery systems; colloids; cholic acid; dextran

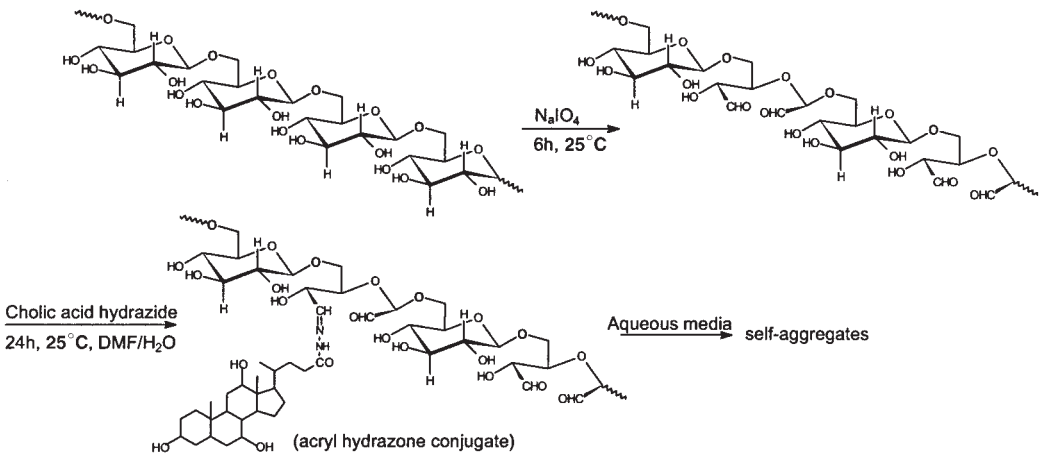
INTRODUCTION

The colloidal delivery system is a potential carrier because it may reduce unwanted toxic side effects, prolong blood circulation time, and reduce uptake by the reticuloendothelial system.[1,2] Among colloidal delivery systems, the self-assembly or aggregation of amphiphilic polymers has attracted increasing interest with respect to its biological importance and pharmaceutical or biotechnological applications.[3,4] Recently, the self-aggregates of hydrophobized water-soluble polysaccharides with cholesterol[4–6] or bile acids[7] have been extensively studied. Hydrophobized polysaccharides form stable and monodisperse self-aggregates by intramolecular and/or intermolecular hydrophobic interactions.[4]

Cholic acid is one of the major bile acids that are synthesized from cholesterol in the liver, stored in the gall bladder, and released in the small intestine. The bile acids can solubilize hydrophobic substances and thus help in the digestion of fats by the formation of micellar self-aggregates.[8] The amphiphilic properties of cholic acid make it an interesting material for the preparation of biocompatible materials for biomedical and pharmaceutical uses.[9–14] Dextran (DEX) is a biomedical material well known for its biocompatibility, biodegradability, and low toxicity. Coupling antitumor agents to DEX provides advantages in drug solubilization, stabilization, localization, and controlled release.[15,16] Hydrazide anticancer agents have been conjugated to poly(aldehyde dextran) with acryl hydrozone linkages. Under mildly acidic conditions, the hydrazide can be attached to aldehyde efficiently, and the acryl hydrazones are more resistant to hydrolysis and do not require reduction for stabilization.[17] Nichifor and coworkers[7,18] synthesized bile-acid-modified DEX's by covalently coupling bile acids to DEX through ester linkages and investigated the aggregation behavior of bile-acid-modified DEX's in water. However, the esterification was not very easy and required the use of a catalyst and coupling reagents.

In this study, we synthesized cholic acid hydrazide–dextran conjugates (CAH–DEX's) with acryl hydrazone linkages and prepared polymeric self-aggregates as carriers for water-insoluble drugs. Because cholic acid can self-associate in water and form micelles, we expected that the CAH–DEX would form a self-assembly structure in water. When cholic acid is part of hydrophilic DEX, the resulting amphiphilic polymer

Correspondence to: J. Chang (jinchang@tju.edu.cn).
Contract grant sponsor: National Nature Science Foundation; contract grant number: 50373033.
Contract grant sponsor: National Basic Science Research and Development Program (973); contract grant number: 2001CCC01400.



Scheme 1 Grafting of cholic acid to poly(aldehyde dextran).

might exhibit a better compatibility with biological systems.[19] The hydrophobic inner cores of the CAH–DEX self-aggregates could act as reservoirs of hydrophobic drugs, and the hydrophilic shell has the ability to stabilize and control the release of drugs. Indomethacin (IN) was incorporated into the self-aggregates as a hydrophobic drug, and its release from the CAH–DEX self-aggregates was investigated *in vitro*.

EXPERIMENTAL

Materials

DEX (average molecular weight = 40 kDa) was purchased from Amersham Biosciences (Uppsala, Sweden). Cholic acid methyl ester and IN were provided by Sigma Chemical Co. (St. Louis, MO). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co. Sodium periodate and hydrazine hydrate were supplied by Shanghai Chemical Co. (Shang, China). All of the other reagents were analytical grade.

Synthesis of cholic acid hydrazide (CAH)

CAH was synthesized by the method reported by Cortese.[20] Cholic acid methyl ester (5 g) was dissolved in 5 mL of ethanol; then, 2 mL of hydrazine hydrate was added, and the mixture was refluxed for 24 h. The system was diluted with 10 mL of hot 95% ethanol (65°C) and adjusted to 350 mL with boiling water. After the mixture was stored at 0°C for 24 h, the CAH was filtered off, washed thoroughly, and then freeze-dried. The yield was 82%, and the melting point was 188–189°C.

Synthesis of CAH–DEX conjugates

Poly(aldehyde dextran) was prepared by the oxidation of DEX according to a method reported in the literature.[21] We obtained white powder after lyophilization. The aldehyde content in the polymer, determined by a potentiometric titration method,[22] was 29%.

Scheme 1 shows the conjugation of CAH and poly(aldehyde dextran). Poly(aldehyde dextran) (100 mg) was dissolved in 4 mL of water; 26 and 39 mg of CAH (saccharide unit/cholic acid mol/mol = 10 and 15%, respectively) was dissolved in 8 mL of dimethylformamide, and then, the solutions were mixed. The mixture was adjusted to pH 4, stirred at room temperature for 24 h, and then poured into a large amount of methanol; the precipitate was separated by filtration. The resultant polymer conjugates were purified by a thorough washing with methanol and were then vacuum-dried at room temperature. Fourier transform infrared (FTIR; Bio-Rad FTS 135, Tokyo, Japan) and ^{1}H-NMR (Varian UMTY plus400, Palo Alto, CA) spectroscopy measurement were used to confirm the formation of the CAH–DEX conjugates.

The amount of cholic acid covalently bound to DEX was determined spectrophotometrically after the reaction with sulfuric acid as reported.[18] The degree of substitution (DS) was calculated with the following formula:

$$\mathrm{DS} = \frac{c/M_{\mathrm{CAH}}}{(m-c)/162} \times 100\ (\mathrm{mol}\%)$$

where c is the content in the bile acid (g), m is the amount of the polymer (g), M_{CAH} is the molecular mass of the CAH residue, and 162 is the molecular mass of the glucopyranosidic unit.

Critical aggregation concentration (CAC)

The CAC was determined by dye solubilization methods described previously.[23,24] A CAH–DEX solution of high concentration was prepared by the dissolution of a certain amount of polymer in water with sonica-

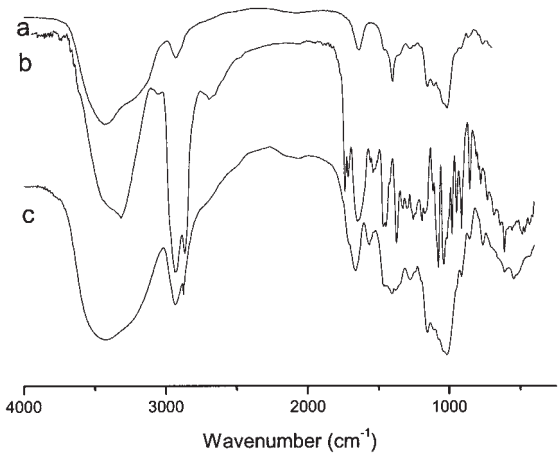


Figure 1 FTIR spectra of (a) poly(aldehyde dextran), (b) CAH, and (c) CAH–DEX.

tion. A series of solutions with different concentrations were prepared by the dilution of the original solution. DPH (10 μL, 0.4 m*M*) was added to the solutions, and the solutions were stored in the dark for 6 h. The reference sample was prepared the same way. The absorbance at 356 nm was measured on a Gary Eclipse fluorescence spectrophotometer (Varian Australian Pty., Ltd.). The values were plotted against the concentration of the polymer and the crossing point of the extrapolated straight line was the CAC.

Surface tension measurement

The surface tension of the solutions was measured with the Wilhelmy method on a Dataphysics DCAT-21 dynamic contact-angle analyzer (Bad Vilbel, Germany). The solutions with different concentrations were prepared by the dilution of a solution whose concentration was much higher than CAC. A platinum plate was used in the Wilhelmy procedure, and it was heated in a gas flame before each measurement. Surface tension measurements were taken at 25°C, and each value of the surface tension was obtained from the average of three measurements. The surface tension values were plotted against the concentration of the solutions.

Preparation of the polymeric self-aggregates and drug loading

The CAH–DEX self-aggregates were prepared with a diafiltration method.[12,13] A certain amount of polymer was dissolved in dimethyl sulfoxide (DMSO), and the solution was dialyzed against distilled water with a dialysis membrane with a molecular weight cutoff of 12,000 g/mol to form the polymeric self-aggregates. The medium was replaced every 3 h for 1 day. The resultant solution was sonicated for 3 min at 30 W with a probe-type sonifier and was then freeze-dried.

IN-loaded polymeric self-aggregates were prepared as follows: 50 mg of IN was dissolved in dimethylformamide to form a homogeneous solution, which was added to 100 mL of a 0.1% solution of CAH–DEX self-aggregates with stirring and stored for 24 h at room temperature. Subsequently, sonication was applied to decrease large self-aggregates,[25] and in ice-bath conditions, the drug-loaded self-aggregate solution was sonicated with a probe-type sonifier at 30 W for 2 min. The solution was centrifuged at 16,500 rpm for 15 min at 4°C. The precipitate was freeze-dried.

The particle size distribution of the CAH–DEX self-aggregates was measured on 90 Plus/BI-MAS multiangle particle sizing instrument (Brookhaven Instruments Co., Holtsville, NY) on the basis of dynamic light scattering. Transmission electron microscopy (TEM; Jeol 100CX-II, Tokyo, Japan) and atomic force microscopy (AFM; Nanoscope Ilia, Digital Instruments, Buffalo, NY) were used to characterize the shape and morphology of the self-aggregates.

To evaluate drug loading, a freeze-dried sample of the IN-loaded polymeric self-aggregates was suspended in methanol and vigorously stirred for 2 h; then, the sample was sonicated for 15 min in an ice-water bath. The resulting solution was then centrifuged at 4000 rpm, and the supernatant was taken for the measurement of the drug concentration with an ultraviolet spectrophotometer (Shimadzu, UV-2450/2550PC, Kyoto, Japan) at 320 nm.

In vitro drug release study

IN-loaded micelles (5 mg) and phosphate buffer saline (PBS; 1 mL, 0.1*M*, pH 7.4) were placed in a dialysis membrane (molecular weight cutoff = 2000 g/mol). Then, the dialysis membrane was introduced into a vial with 10 mL of PBS, and the media was stirred at 37°C and 75 rpm. The entire medium was removed and replaced with the same amount of fresh PBS at predeter-

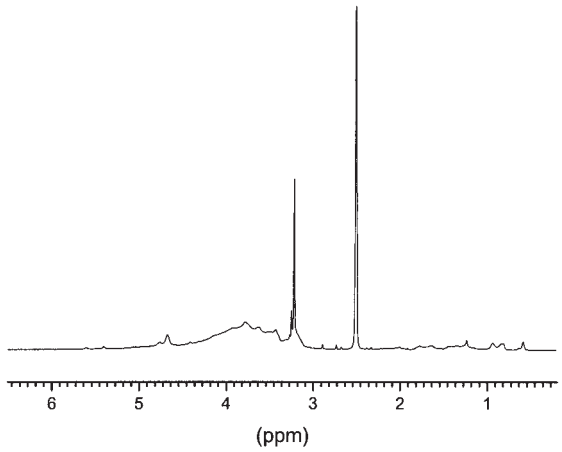


Figure 2 ^{1}H-NMR spectra of CAH–DEX.

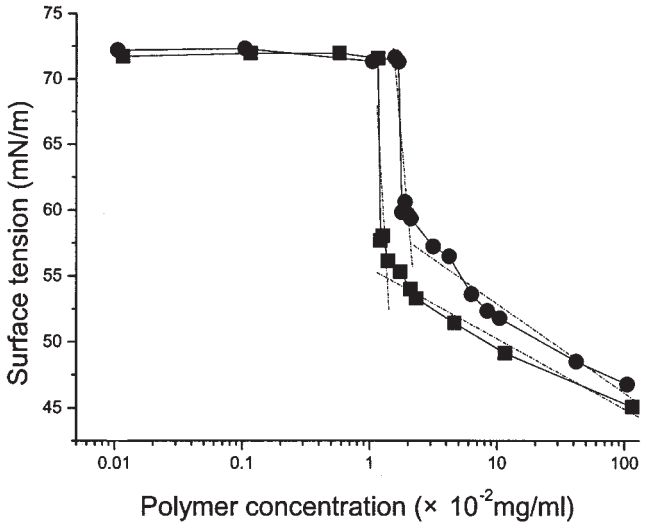


Figure 3 Surface tension curves of (●) CAH–DEX 6.5 and (■) CAH–DEX 9.0.

mined intervals. The amount of IN released from the micelles was determined with the ultraviolet spectrophotometer at 320 nm. Parallel experiments were developed in acetate buffer solution (0.1*M*, pH 4) with similar methods to examine the influence of pH on the drug release profiles.

RESULTS AND DISCUSSION

Synthesis and characterization of CAH–DEX's

The CAH–DEX conjugates were synthesized, as illustrated in Scheme 1, by the reaction of CAH and poly(aldehyde dextran). Figure 1 shows the FTIR spectra of poly(aldehyde dextran), CAH, and CAH–DEX. As shown in Figure 1(c), the absorbance bands at 1662 and 1565 cm^{-1} were assigned to the C═N and amide bending (CO—NH) in acryl hydrazone, respectively. Meanwhile, the image contained new peaks of C—H stretching at 2934 and 2875 cm^{-1} due to the cholic acid steroid skeleton. No peaks appeared at 1632 cm^{-1} (—NH_2), and the decrease in peaks at 1724 cm^{-1} of aldehyde showed that the cholic acid was attached to poly(aldehyde dextran) with covalent linkages.

The reaction of conjugation between cholic acid hydrazine and poly(aldehyde dextran) easily proceeded at room temperature and in aqueous conditions, unlike the esterification method. The DS's of the two aggregates were 6.5 and 9.0%, respectively. The two conjugates were termed CAH–DEX 6.5 and CAH–DEX 9.0. respectively.

The CAH–DEX's were soluble in DMSO but insoluble in D_2O. Figure 2 shows the ^{1}H-NMR spectra of CAH–DEX 6.5 in DMSO-d_6. The signal of the proton of cholic acid appeared at approximately 0.5–1.0 ppm, the signal of the proton of DEX was observed at about 3.2 ppm, and the signal of the proton of acryl hydrazone was observed at 5.4 ppm. No signals assigned to the cholic acid moiety at 0.50–1.0 ppm were observed when CAH–DEX was dispersed in D_2O, which suggested that CAH–DEX in water adopted an orientation in which the CAH existed in hydrophobic domains separated from hydrophilic DEX.[24] Surrounded by hydrophilic DEX, CAH developed limited molecular motion. CAH–DEX is amphiphilic in nature, and water is not a good solvent for cholic acid, but it can dissolve DEX very well. So the conjugates self-assembled or self-aggregated into multicore self-aggregates. The CAC of CAH–DEX's greatly depended on their DS. DPH was preferentially partitioned into the hydrophobic cores of the polymeric self-aggregates, which resulted in an increase in absorbance of DPH. The abrupt increase in the absorbance of DPH perfectly reflected the formation of the aggregates.[25] The CAC was determined to be 1.41 and 2.10×10^{-2} mg/mL for CAH–DEX 9.0 and CAH–DEX 6.5, respectively. The values of both conjugates were much lower than that of cholic acid, which was about 1.2 mg/mL. The lower CAC of the polymeric self-aggregates is an important property of amphiphilic polymers, which suggests the stability of the polymeric self-aggregates in dilute conditions.

The formation and characteristics of CAH–DEX self-aggregates were studied by surface tension measurements, dynamic light scattering, TEM, and AFM. The

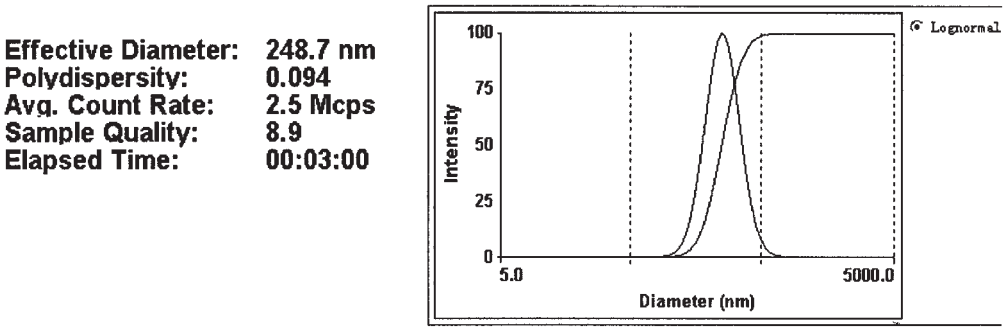


Figure 4 Image of the nanoparticle size distribution of CAH–DEX 9.0.

TABLE I
Characteristics of IN-Loaded CAH–DEX Self-Aggregates

DS of CAH–DEX (%)	d_1 (nm)	d_2 (nm)	Drug content (%)	Loading efficiency (%)
6.5	226.1 (0.025)	324.9 (0.156)	29.9	51.2
9.0	204.4 (0.120)	248.7 (0.094)	17.9	36.0

d_1 = effective diameter (polydispersity) of CAH–DEX self-aggregates from laser light scattering; d_2 = effective diameter (polydispersity) of IN-loaded CAH–DEX self-aggregates from laser light scattering.

surface tension values of the polymers abruptly decreased in the vicinity of the CAC with increasing concentration of the polymer, as shown by the surface tension curves (Fig. 3). Because of the formation of macromolecular self-aggregates, the hydrophobic parts tended to partition into the hydrophobic cores, which were surrounded by hydrophilic shells. In the solution, the solute was decreased greatly, so the surface tension decreased accordingly. From this measurement, we also calculated the CAC values to be about 1.36 and 2.00 $\times$ 10^{-2} mg/mL for the two conjugates. The surface tension measurements also confirmed the formation of the polymeric self-aggregates. These were very similar to results determined by the DPH dye solubilization methods.

The self-aggregates in aqueous media formed a relatively narrow particle size distribution (Fig. 4). When the hydrophobic drug IN was loaded, the size of the self-aggregates grew larger to some degree. The particle size increased on incorporation of the drug (Table I). Figure 5 shows the TEM image of IN-loaded CAH–DEX 6.5 self-aggregates. These self-aggregates were almost spherical in shape and about 60 nm in size. These results also indicated that amphiphilic polymeric conjugates could form nanoparticles spherical in shape with core–shell structures. These were much smaller than those observed in the dynamic light-scattering measurements, which resulted from the obvious swelling performed by the hydrophilic polymer shells of the self-aggregates in aqueous media. We also observed the morphology of IN-loaded CAH–DEX self-aggregates by AFM (Fig. 6); these had a spherical shape and ranged in size from 50 to 70 nm. These results coincided with those obtained from TEM observations.

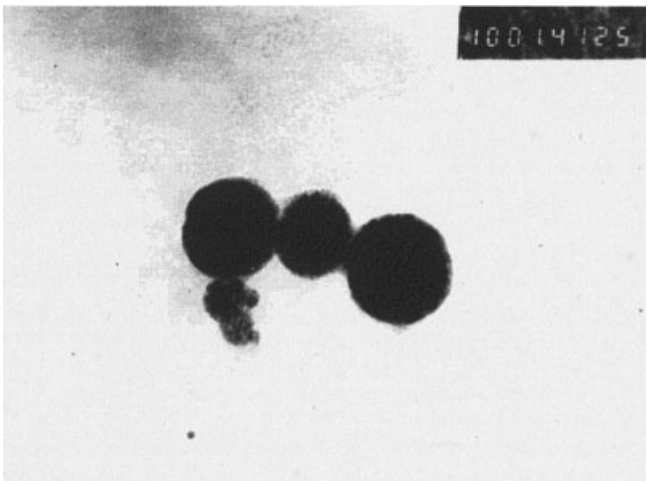


Figure 5 TEM image (100,000×) of IN-loaded CAH–DEX 6.5 self-aggregates.

Drug loading and release studies *in vitro*

The calculated IN loading content was 29.9 and 17.9 wt % for the self-aggregates CAH–DEX 6.5 and CAH–DEX9.0, respectively. The hydrophobic drug physically entrapped into the amphiphilic polymer self-aggregates was controlled by intramolecular and/or intermolecular interactions and the value of hydrophilic/lipophilic balance. For the CAH–DEX self-aggregates, CAH–DEX 9.0 formed a much more rigid core and restricted the motion of IN from the outer aqueous phase into hydrophobic domains, which resulted from hydrophobic interactions.[11] Therefore, CAH–DEX 9.0 developed a lower drug loading efficiency at high DS's.

To study the drug release behavior, we dispersed IN-loaded self-aggregates in buffer solutions. The release profiles of the hydrophobic drug from the self-aggregates into the surrounding aqueous phase are shown in Figures 7 and 8.

As shown in Figure 7, physically entrapped IN was released from the self-aggregates for about 2 days at pH 7.4. However, the IN-loaded CAH–DEX self-aggregates developed a very slow release profile at pH 4, and the release profile was pseudo-zero-order in kinetic terms for up to 14 days, as shown in Figure 8. The solubility of IN in water was greatly influenced by pH. In acetate buffer of pH 4, the solubility was 0.00417 mg/mL, whereas in PBS of pH 7.4, it increased to 0.511 mg/mL. Therefore, IN released from CAH–DEX self-aggregates faster at pH 7.4 than at pH 4, which resulted from faster IN diffusion in CAH–DEX self-aggregates in pH 7.4 media. The acryl hydrazone was sufficiently stable in aqueous media. We observed almost no change in the FTIR images of the CAH–DEX conjugates after incubation in PBS at pH 7.4 and pH 4 for 24 h. The acryl

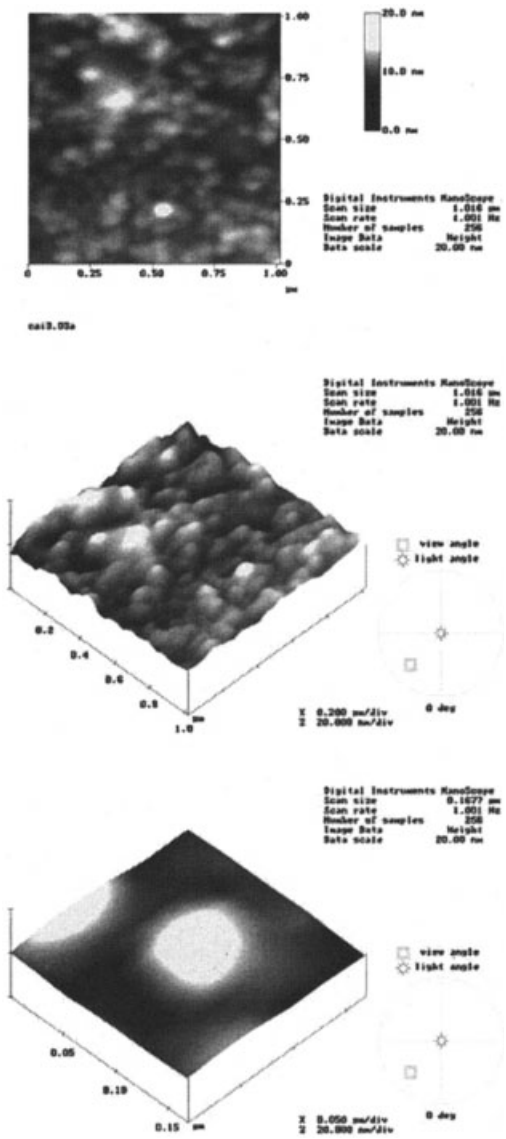


Figure 6 AFM images of IN-loaded CAH–DEX 6.5 self-aggregates.

hydrazone generated from aldehyde and hydrazide under reversible reaction conditions in acidic aqueous media were considerably more resistant to hydrolysis,[17] so sustained release of water-insoluble drugs from CAH–DEX self-aggregates can be expected. The drug release was controlled by the passive diffusion of drugs from the self-aggregates to the media. The slow release of the drug could have resulted from the solid-like hydrophobic cores of the CAH–DEX self-aggregates, which were constituted by cholic acid moieties.[11] The slow drug release from the CAH–DEX self-aggregates will allow use in parenteral delivery, where long circulation of the carriers may lead to increased drug accumulation at sites of solid tumors and localized drug release.

Under both conditions (pH 7.4 and pH 4), the drug was released from conjugates with a high DS more slowly than from conjugates with a low DS. It is probable

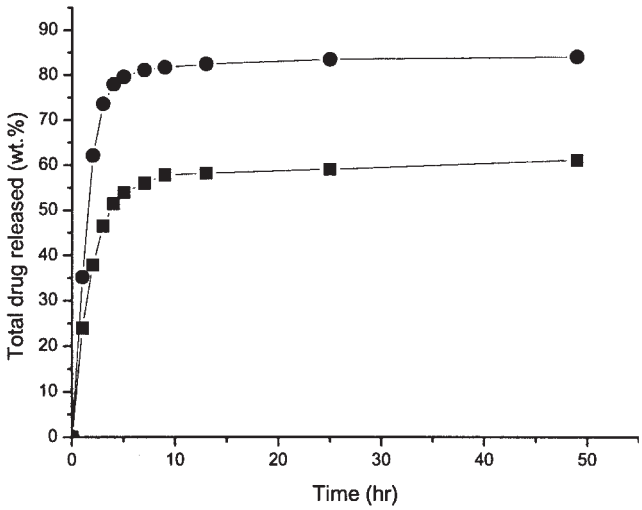


Figure 7 IN release from (●) CAH–DEX 6.5 and (■) CAH–DEX 9.0 self-aggregates in PBS (0.1*M*, pH 7.4, 37°C).

that the polymeric self-aggregates had rigid and hydrophobic cores due to cholic acid moieties[10] and that the cores were more rigid with increasing DS. Therefore, the molecular motion of IN was strongly restricted, and slower release behavior from the cores was observed for CAH–DEX 9.0. Meanwhile, IN diffused from the inner cores of CAH–DEX self-aggregates down its chemical concentration gradient. The greater the concentration gradient of the substance is, the faster its rate of diffusion will be across the carriers. Because the drug content in the IN-loaded CAH–DEX 6.5 self-aggregates was higher than that of CAH–DEX 9.0, the IN concentration gradient in CAH–DEX 6.5 was greater. So IN was released from the self-aggregates more rapidly for the CAH–DEX 6.5 self-aggregates.

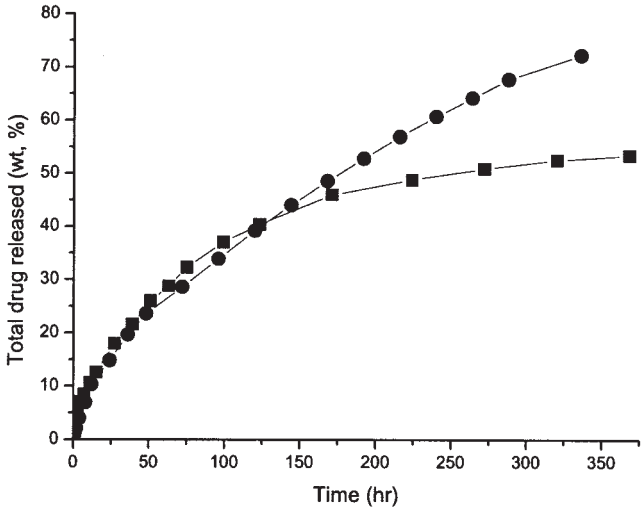


Figure 8 IN release from (●) CAH–DEX 6.5 and (■) CAH–DEX 9.0 self-aggregates in acetate buffer solution (0.1*M*, pH 4, 37°C).

The content of aldehyde groups on the surface of the self-aggregates was measured by potentiometric titration[26] and was estimated to be 0.156 mmol/g for the IN-loaded CAH–DEX 6.5 self-aggregates, which was adequate for the attachment of targeting agents to the particle surface.

CONCLUSIONS

CAH–DEX conjugates with acryl hydrazone linkages were synthesized from poly(aldehyde dextran) and CAH. CAH–DEX was amphiphilic in nature and formed stable self-aggregates in aqueous media. The conjugates developed lower CACs, about 1.41 and 2.10 $\times$ 10^{-2} mg/mL for CAH–DEX 9.0 and CAH–DEX 6.5, respectively. The self-aggregates could act as carriers for hydrophobic drugs, and AFM and TEM studies showed that their shape was spherical and that the size was about several nanometers. IN was released from CAH–DEX self-aggregates at pH 4 much slower than at pH 7.4, and at pH 4, the release profile was pseudo-zero-order in kinetic terms for up to 14 days. The hydrolytic properties and the stability of acryl hydrazone can provide many advantages, such as sustained drug delivery, and the aldehyde groups on the surface of the drug-loaded self-aggregates are desirable sites for the attachment of targeting agents. Doxorubicin-loaded CAH–DEX self-aggregates with monoclonal antibodies are currently under study for therapeutic targeting.

References

1. Kakizawa, Y.; Kataoka, K. Adv Drug Delivery Rev 2002, 54, 203.
2. Adams, M. L.; Lavasanifar, A.; Kwon, G. S. J Pharm Sci 2003, 92, 1343.
3. Davis, S. S. Pharm Technol 1981, 5, 71.
4. Akiyoshi, K.; Deguchi, S.; Tajima, H.; Nishikawa, T.; Sunamoto, J. Macromolecules 1997, 30, 857.
5. Taniguchi, I.; Akiyoshi, K.; Sunamoto, J. Macromol Chem Phys 1999, 200, 1386.
6. Tantguchi, I.; Akiyoshi, K.; Sunamoto, J. Macromol Chem Phys 1999, 200, 1554.
7. Nichifor, M.; Lopes, A.; Carpov, A.; Melo, E. Macromolecules 1999, 32, 7078.
8. Hofmann, A. F.; Mekjian, H. S. In The Bile Acids: Chemistry, Physiology and Metabolism; Nair, P. P.; Kritchevsky, D., Eds.; Plenum: New York, 1971; p l03.
9. Zhu, X. X.; Nichifor, M. Acc Chem Res 2002, 35, 539.
10. Lee, K. Y.; Jo, W. H.; Kwon, I. C.; Kim, Y. H.; Jeong, S. Y. Macromolecules 1998, 31, 378.
11. Lee, K. Y.; Kim, J. H.; Kwon, I. C.; Jeong, S. Y. Colloid Polym Sci 2000, 278, 1216.
12. Kim, I. S.; Jeong, Y. I.; Cho, C. S.; Kim, S. H. Int J Pharm 2000, 205, 165.
13. Kim, I. S.; Kim, S. H. Int J Pharm 2001, 226, 23.
14. Kim, Y. H.; Gihm, S. H.; Park, C. R.; Lee, K. Y.; Kim, T. W.; Kwon, I. C.; Chung, H.; Jeong, S. Y. Bioconjugate Chem 2001, 12, 932.
15. Mehvar, R. J Controlled Release 2000, 69, 1.
16. Rensberger, K. L.; Hoganson, D. A.; Mehvar, R. Int J Pharm 2000, 207, 71.
17. Heindel, N. D.; Zhao, H.; Leiby, J.; VanDongen, J. M. Bioconjugate Chem 1990, 1, 77.
18. Nichifor, M.; Carpov, A. Eur Polym J 1999, 35, 2125.
19. Denlike, J. K.; Zhu, X. X.. Macromol Rapid Commun 1994, 15, 459.
20. Cortese, F. J Am Chem Soc 1937, 59, 2532.
21. Azzam, T.; Raskin, A.; Makovitzki, A.; Brem, H.; Vierling, P.; Lineal, M.; Domb, A. J. Macromolecules 2002, 35, 9947.
22. Zhao, H.; Heindal, N. D. Pharm Res 1991, 8, 400.
23. Jeong, B.; Lee, D. S.; Shon, J. I.; Bae, Y. H.; Kim, S. W. J Polym Sci Part A: Polym Chem 1999, 37, 751.
24. Hub, K. M.; Lee, K. Y.; Kwon, I. C.; Kim, Y. H.; Kim, C.; Jeong, S. Y. Langmuir 2000, 16, 10566.
25. Han, S. O.; Mahato, R. I.; Kim, S. W. Bioconjugate Chem 2001, 12, 337.
26. Zhang, X. M.; Yuan, C. H.; Sun, Z. H. Hua Xue Tong Bao (Chinese) 1989, 12, 27.