Journal of Applied Polymer Science

Fabrication of galactosylated chitosan–5-fluorouracil acetic acid based nanoparticles for controlled drug delivery

Cui-Yun Yu,[1,2,*] Na-Mei Li,[1,*] Sa Yang,[1] Qian Ning,[1] Can Huang,[1] Wen Huang,[1] Zi-Ning He,[1] Dong-Xiu He,[1] Xiang-Wen Tan,[1] Li-Chun Sun[1,3]

[1]Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, University of South China, Hengyang 421001, China

[2]Learning Key Laboratory for Pharmacoproteomics of Hunan Province, Institute of Pharmacy and Pharmacology, University of South China, Hengyang 421001, China

[3]Department of Medicine, Tulane University Health Sciences Center, New Orleans, Louisiana 70112

[*]The first two authors contributed equally to this work.

Correspondence to: C.-Y. Yu (E-mail: yucuiyunusc@hotmail.com) and L. C. Sun (E-mail: lsun@tulane.edu)

ABSTRACT: In this study, a novel type of macromolecular prodrug, *N*-galactosylated chitosan (GC)−5-fluorouracil acetic acid (FUA) conjugate based nanoparticles, was designed and synthesized as a carrier for hepatocellular carcinoma drug delivery. The GC–FUA nanoparticles were produced by an ionic crosslinking method based on the modified ionic gelation of tripolyphosphate with GC–FUA. The structure of the as-prepared GC–FUA was characterized by Fourier transform infrared and ^{1}H-NMR analyses. The average particle size of the GC–FUA nanoparticles was 160.1 nm, and their drug-loading content was 21.22 ± 2.7% ($n = 3$). In comparison with that of the freshly prepared nanoparticles, this value became larger after 7 days because of the aggregation of the GC–FUA nanoparticles. An *in vitro* drug-release study showed that the GC–FUA nanoparticles displayed a sustained-release profile compared to 5-fluorouracil-loaded GC nanoparticles. All of the results suggest that the GC–FUA nanoparticles may have great potential for anti-liver-cancer applications. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42625.

KEYWORDS: biomaterials; biomedical applications; drug-delivery systems; nanostructured polymers

Received 15 February 2015; accepted 15 June 2015

DOI: 10.1002/app.42625

INTRODUCTION

Hepatocellular carcinoma is one of the most common malignancies in the world,[1] with an estimated 750,000 new cases and 700,000 deaths occurring worldwide in 2008.[2] Chemotherapy remains the main treatment for advanced or recurrent hepatocellular carcinoma. The disadvantages of most of the existing chemotherapeutic drugs include a low bioavailability, poor selectivity and intrinsic or acquired drug resistance, affects on healthy cells, and immunosuppression that can cause complications and even patient death.[3] For example, 5-fluorouracil (5-FU) is a drug widely used in cancer chemotherapy, alone or in combination. It is used extensively for treating various types of solid tumors, such as those present in cancers of the liver, stomach, intestine, colon, pancreas, ovary, and breast. 5-FU is a cytotoxic drug, which exhibits its activity by interfering with nucleic acid synthesis, inhibiting DNA synthesis, and eventually halting cell growth.[4] 5-FU has a short plasma half-life of 10–20 min and can be rapidly metabolized; this means administration by lengthy, high-dose intravenous injection or infusion is required to maintain therapeutic blood levels.[5] In addition, its clinical application is limited by unwanted side effects, such as gastrointestinal reactions, myelosuppression, alopecia, and ataxia. To minimize side effects, many efforts have been made to produce a drug-carrier system with stability, long-term circulation in the blood, and targeting of the site of action; these efforts have included polymeric nanoparticles,[6] liposomes,[7] micelles,[8] and nanogels.[9] In our laboratory, we previously prepared a polymeric pectin-based nanoparticle as a drug-delivery system. It showed a higher cytotoxicity toward the cancer cells (HepG2) and a longer half-life in the circulatory system. However, it had the disadvantage of burst release.[10] According to previous literature, it has been demonstrated that macromolecular prodrugs, with small-molecule anticancer drugs bonded to the polymer matrix, can effectively overcome the disadvantage of small-molecular anticancer drugs and have long-term circulation, sustained release, and active targeting ability.[11–13] In addition, the nanoparticles have the advantage of an enhanced permeability and retention effect, which result from their size; this makes

them able to reach tumor tissues passively in comparison to normal tissues.[14] Therefore, the emergence of a novel, sustained-release nanoparticle drug-delivery system for 5-FU is of significance for bench-to-bedside translation and clinical applications.

Chitosan (CS) is a naturally occurring polysaccharide with a low toxicity. It is biodegradable and has biocompatible characteristics.[15] For many decades, a large variety of CS derivatives with high solubility and enhanced permeation have been investigated for applications in drug and gene delivery, tissue engineering, and wound healing.[16–19] *N*-Galactosylated chitosan (GC) is a galactose ligand with CS modifications to the molecular structure.[20] Many studies have shown that the asialoglycoprotein receptor (ASGPR) is a surface receptor found on the membrane of hepatocytes facing the sinusoids with specificity for galactose or acetyl galactosamine ligands. Each hepatocyte contains approximately 2 million binding sites for ASGPR.[21] Therefore, the binding of the galactose ligand or acetyl galactosamine with ASGPR can induce liver-targeted transfer and higher growth inhibition. Also, according to previous studies, 5-FU could be conjugated to CS by chemical bonds.[22]

In this study, a novel macromolecular prodrug [GC–5-fluorouracil acetic acid (FUA) nanoparticles] was synthesized as a drug-delivery system, and its chemical structure was characterized through Fourier transform infrared (FTIR) spectroscopy and ^{1}H-NMR. Size distribution was measured by Nano ZS. Scanning electron microscopy (SEM) was used to visualize the particle morphology and surface properties of the nanoparticles. The *in vitro* release behaviors were investigated further.

EXPERIMENTAL

Materials

5-FU was purchased from Sigma-Aldrich. CS (medium molecular weight = 5×10^5, degree of deacetylation = 96%) was provided by Zhejiang Golden-Shell Pharmaceutical Co., Ltd. (China) and was used without further purification. Lactobionic acid (LA; i.e., 4-*O*-*β*-D-galactopyranosyl-D-gluconic acid) was obtained from J&K Chemical (Logan, UT). *N*-Hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide hydrochloride (EDC), and *N,N,N′,N′*-tetramethylethylenediamine were purchased from Fluka (Spain). All other chemical solvents and reagents were used without further purification.

Synthesis of FUA

KOH (2.56 g, 45 mmol) and 5-FU (1.34 g, 10 mmol) were dissolved in a clean flask; then, 5 mL of an aqueous solution of bromoacetic acid (1.7 g, 18 mmol) was added at a temperature of 40°C with smooth stirring. The reaction mixture was stirred at 60°C for 5 h, cooled by an ice bath, and adjusted to pH 5.5 with hydrochloric acid (HCl) after being filtered through a membrane. The crude product was recrystallized by water, and 1.53 g of FUA was obtained as a white solid.

Synthesis of GC

The coupling of CS with LA was performed with EDC and NHS by an active ester intermediate, as previously reported.[23] Briefly, 2.3 g of LA was dissolved in 50 mL of an *N,N,N′,N′*-tetramethylethylenediamine/HCl buffer solution (10 m*M,* pH 4.7) with stirring. The carboxyl group of LA was activated with a mixture of EDC (0.6 g) and NHS (0.14 g). Subsequently, 2.2 g of CS was added to the activated LA solution with stirring and allowed to react for 72 h at room temperature. The resulting product was dialyzed with a dialysis bag (molecular weight cutoff = 8000–10,000) against distilled water for 4 days. Finally, GC was obtained by freeze drying in a Labconco freeze-drying system and kept *in vacuo* at room temperature before use. The synthesis of GC was confirmed by FTIR spectroscopy and ^{1}H-NMR.

Synthesis of GC–FUA

FUA (50 mg) was dissolved in 25 mL of an HCl solution (50 mmol/L) with stirring. EDC (0.14 g) and NHS (0.04 g) were then added and stirred for 1 h. After that, GC (50 mg) was dropped into the solution and stirred for 72 h at room temperature. Finally, the reaction solution was put into a dialysis bag and dialyzed against distilled water for 3 days and then freeze-dried to obtain GC–FUA.

FTIR Spectroscopy and ^{1}H-NMR Spectroscopy

FTIR spectra were obtain with a Thermo Nicolet Corp. FTIR spectrometer. Dried samples were ground with KBr powder and compressed into pellets for FTIR examination. ^{1}H-NMR spectra were obtained with a Varian 600-MHz spectrometer (Varian). The samples were dissolved in a mixture of 10 μL of CF_3COOD and 500 μL of D_2O at a concentration of about 10 mg/mL for use in ^{1}H-NMR testing.

Preparation of the GC–FUA Nanoparticles

GC–FUA nanoparticles were produced by an ionic crosslinking method[24] [based on a modified ionic gelation of tripolyphosphate (TPP) with GC]. Briefly, GC–FUA (with GC as the control group) was dissolved in a 1% aqueous acetic acid solution for a final concentration of 2 mg/mL with stirring, and the pH was adjusted to 5 with 1 *M* NaOH and 1 *M* HCl. TPP was dissolved in H_2O at a concentration of 1 mg/mL and then adjusted to pH 8. The GC–FUA and TPP solutions need to be sterilized by filtration through 0.45- and 0.22-μm filters, respectively. The nanoparticles were spontaneously obtained upon the addition of 1 mL of a TPP aqueous solution to 4 mL of the GC solution (at a GC–FUA to TPP weight ratio of 12 : 1) under constant stirring at room temperature. The product was kept at room temperature for 30 min to assess further particle formation. The final product was kept at 4°C.

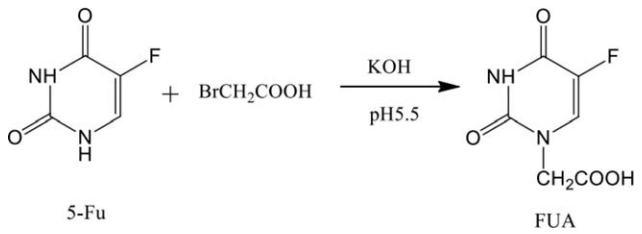
Determination of Drug-Loading Content

After the preparation of the GC–FUA nanoparticles, the aqueous phase was collected by centrifugation, and the drug content in the aqueous phase was determined as the drug loss. The drug concentration of the nanoparticles was determined in triplicate by a Shimadzu UV-1750 ultraviolet–visible spectrophotometer at 273 nm. The drug-loading content was calculated as follows:

$$\text{Drug-loading content}(\%) = \frac{\text{Weight of the drug in the nanoparticles}}{\text{Weight of the nanoparticles}} \times 100\%$$

Characterization of the GC–FUA Nanoparticles

The average size and size distribution (polydispersity index) of the GC–FUA nanoparticles were measured with a Zetasizer nanoparticle analyzer (Nano ZS, Malvern Instruments).



Scheme 1. FUA synthesis.

The morphology of the GC–FUA nanoparticles was observed by SEM (Hitachi S-4800, Hitachi, Ltd., Japan). Before observation, the sample was sputter-coated with gold.

In Vitro Release Study

The freshly prepared GC–FUA nanoparticles were put in a dialysis bag, then immersed in 30 mL of a phosphate-buffered saline solution (pH 7.4), and shaken in a water bath at 37°C. At predetermined intervals, 3-mL samples were removed from the release medium, and then, the same volume and temperature of fresh phosphate-buffered saline solution was added to the release medium. Then, the sample's drug concentration was determined through the measurement of the absorbance at 273 nm in an ultraviolet–visible spectrophotometer (Shimadzu UV-1750). The results of the triplicate test data were used to calculate the accumulated drug release. In addition, the *in vitro* drug-release behavior was compared with that of the 5-FU loaded GC nanoparticles (the control group).

RESULTS AND DISCUSSION

Synthesis and Characterization of FUA

The synthetic procedure of the bromine acetic acid modified 5-FU prodrug is shown in Scheme 1. The bromine acetic acid was conjugated to 5-FU through a substitution reaction. Figure 1(a)

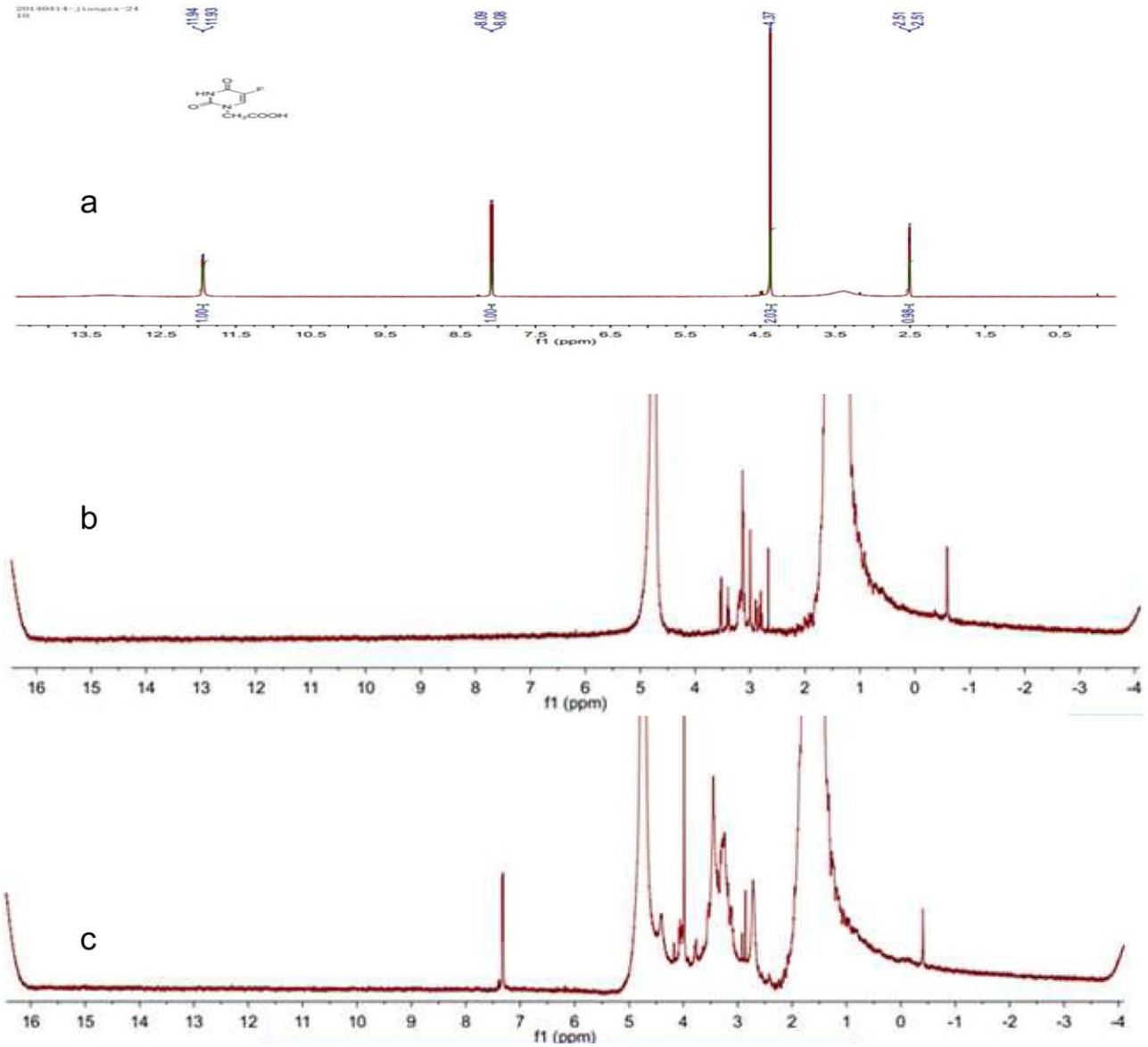
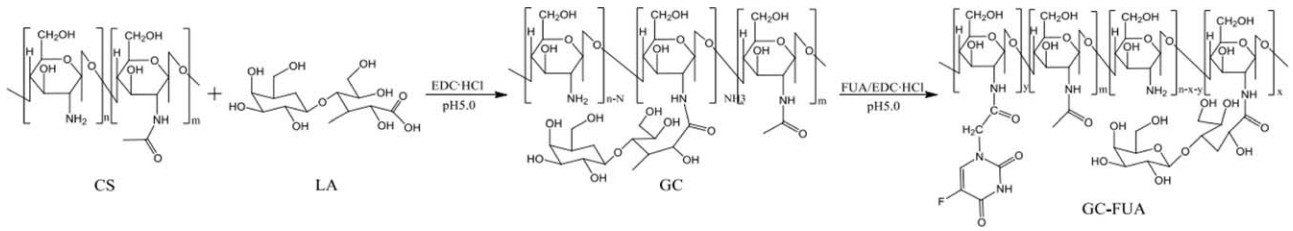


Figure 1. ^{1}H-NMR spectra of (a) FUA, (b) GC, and (c) GC–FUA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



Scheme 2. GC and GC–FUA synthesis.

shows the ^{1}H-NMR spectra of FUA. The proton assignment of FUA was as follows (D_2O/CF_3COOD, ppm): 4.37 (s, 2H, N—CH2), 8.09 (d, 1H, J = 5.2 Hz, 5-FU-H).

Synthesis and Characterization of GC and GC–FUA

The synthesis of GC and GC–FUA are shown in Scheme 2. In this reaction, the amide bond was formed between the carboxylic group of LA and the amine groups of CS first; then, the carboxylic group of FUA was reacted with the amine groups of GC to form amide links in the presence of the coupling agents EDC and NHS, which have been used extensively as chemical crosslinking agents in the field of biochemistry.[25–27]

The FTIR spectra of FUA, CS, GC, and GC–FUA are shown in Figure 2. FUA exhibited absorption bands at 3182 cm^{-1} (—COOH stretch), 1425 cm^{-1} (N_3—H stretch), 1377 cm^{-1} (—CH_2 stretch), and 987 cm^{-1} (—CF=CH— stretch); this proved that the bromine acetic acid was conjugated to 5-FU and FUA is obtained. CS exhibited absorption bands at 3446 cm^{-1} (O—H stretch overlapped with N—H stretch), 1658 cm^{-1} (amide I band, C—O stretch of acetyl group), and 1646 cm^{-1} (amide II band, N—H stretch). Compared to CS, the peaks of amides I and II for GC were different, being slightly shifted to 1641 and 1612 cm^{-1}, respectively. The peak at 3446 cm^{-1} for GC became wider; this indicated enhanced hydrogen bonding. The characteristic absorption band of GC–FUA was present at 987 cm^{-1} (—CF=CH— stretch) in the spectrum; this proved that FUA was coupled to the GC through chemical bonds. All these results were consistent with reported data.[28–30]

Figure 1(b,c) show the ^{1}H-NMR spectra of GC and GC–FUA. Compared with the spectra of GC, the proton signals at δs of 4.1 and 7.4 in the spectra of GC–FUA were attributed to the methylene peaks and the H-6 protons peaks in the FUA compound, which is shown in Figure 1(a). These results indicate that the FUA bonded to the GC successfully, and the GC–FUA conjugate was obtained.

Synthesis and Characterization of the GC–FUA Nanoparticles

The reaction mechanism is shown in Scheme 3, and the interaction was mediated by the electrostatic forces between the protonated NH_3^+ groups and the negative charged residues in TPP.

The radius of freshly prepared nanoparticles based on GC–FUA was in the 100–300 nm range and had a normal distribution, and the mean particle size was about 160 nm, as shown in Figure 3(A). After 7 days, the particle size of the GC–FUA nanoparticles became larger [average diameter = 548 nm; see Figure 3(B)]. The SEM images can be seen in Figure 4(A) and show that the GC–FUA particles were particulate matter of nanoscale size. The dried nanoparticles had a much smaller size compared with swollen nanoparticles in the aqueous phase; this was in good agreement with our previous results.[31,32] The differences in size between the dried and swollen nanoparticles may have resulted from the huge hydrodynamic volume of the nanoparticles. Figure 4(B) reveals the formation of large particles; this indicates that the GC–FUA nanoparticles aggregated easily in the aqueous phase. This may have been because GC–FUA had a large number of NH_3^+, and amide bonds could be formed between one or more GC–FUA chains when it interacted with TPP. In addition, in the GC–FUA chain, intramolecular and intermolecular hydrogen bonds were be formed by function of the hydroxyl and amino groups. These results were in accordance with our previous studies.[33] Therefore, freshly prepared GC–FUA nanoparticles were used for further *in vitro* study because of their stability with time and aggregation issue.

Drug-Loading Content

Ultraviolet analyses showed that the drug-loading content of the GC–FUA nanoparticles was 21.22 ± 2.7% ($n = 3$).

In Vitro Release Study

The *in vitro* drug-release study of the freshly prepared GC–FUA nanoparticles was performed in phosphate-buffered saline (pH 7.4, 37°C). The ester linkage between conjugate and FUA was easily hydrolyzed.[34] The maximum absorption wavelength of the GC–FUA dialysate was 273 nm; this was consistent with the maximum absorption wavelength of FUA. So, FUA was released

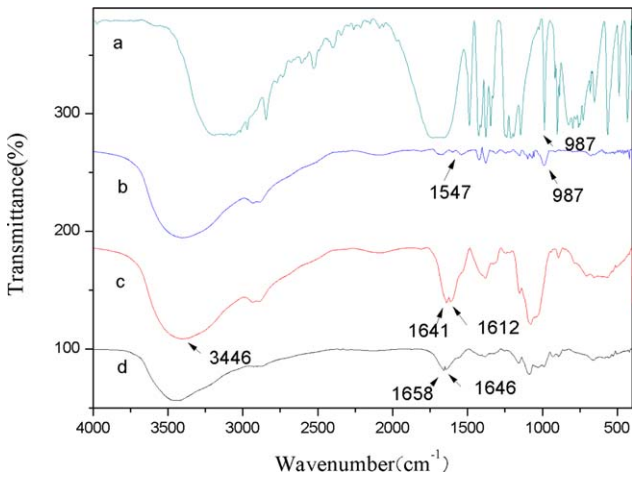
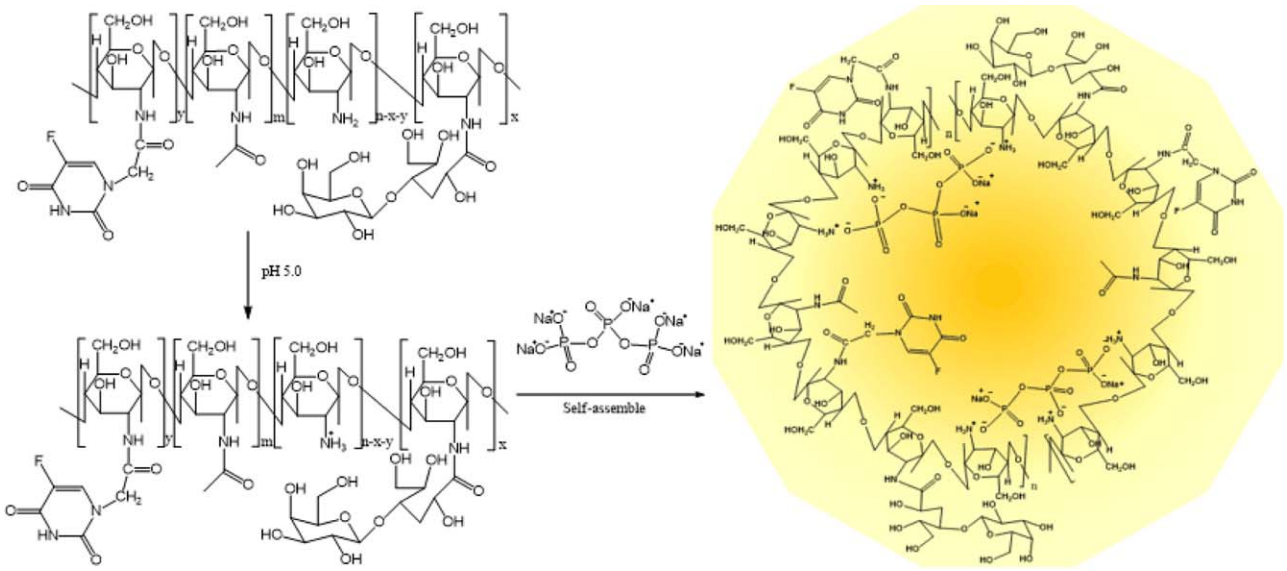


Figure 2. FTIR spectra of (a) FUA, (b) GC–FUA, (c) GC, and (d) CS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



Scheme 3. Self-assembled nanoparticles based on GC–FUA and TPP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

from the GC–FUA nanoparticles. The linear standard curve of FUA was determined to be $y = 0.0533x + 0.0129$ ($R^2 = 0.9986$), and the linear standard curve of 5-FU was determined to be $y = 0.0476x + 0.0114$ ($R^2 = 0.9991$), where x is the concentration and y is the absorbance.

As shown in Figure 5, the control group, 5-FU-loaded GC nanoparticles, almost achieved 100% drug release within 4 h. In contrast, rapid release was observed in the GC–FUA nanoparticles from 0 to 2 h, with a cumulative release percentage of 47%, which was presumably due to the diffusion of the surface FUA into the solution. A sustained slow release was recorded between 4 and 8 h, with a cumulative release percentage of 51.94–61.63%. This indicated that the GC–FUA nanoparticles had a prolonged release for 4–8 h. After 48 h, the release reached a plateau, with a cumulative release percentage of 96.4% at day 7. The results indicated that the GC–FUA nanoparticles, developed by a chemical synthesis method to integrate FUA, was more stable than conventional nanocarriers which encapsulated drug physically. Thus afforded a sustained slow-release profile.

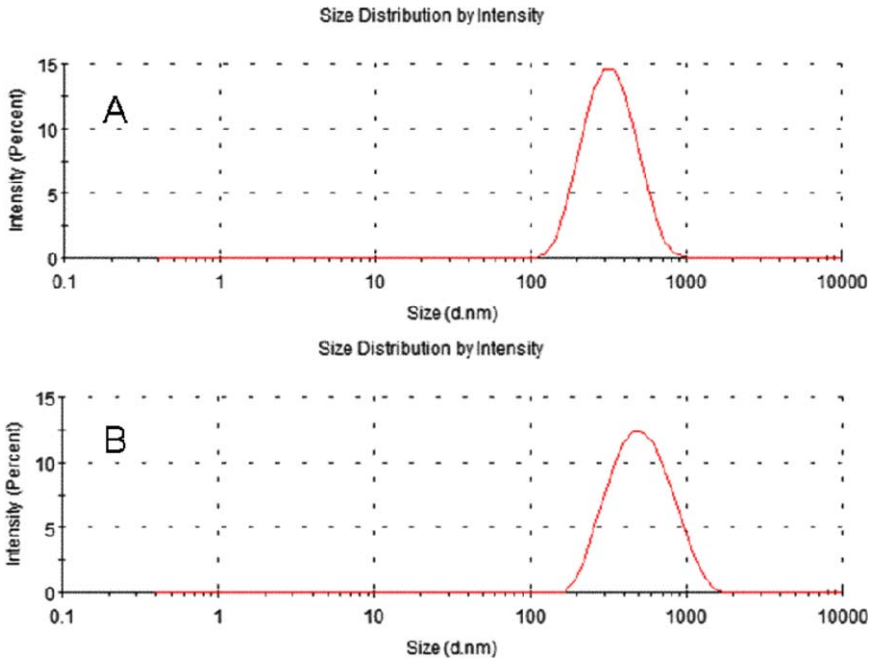


Figure 3. Particle sizes of the GC–FUA nanoparticles: (A) freshly prepared GC–FUA nanoparticles (average diameter = 160 nm) and (B) GC–FUA nanoparticles after static settlement for 7 days (average diameter = 548 nm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

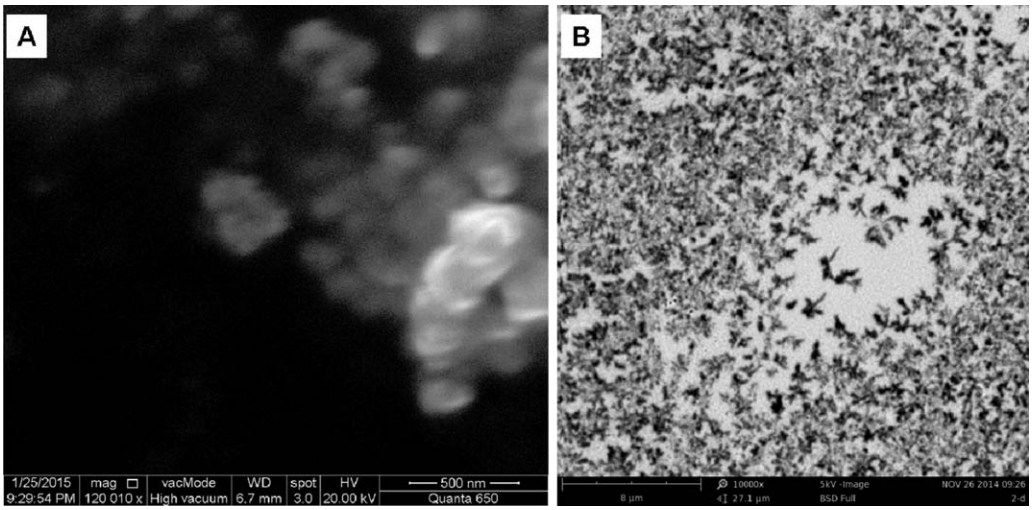


Figure 4. SEM images of the GC–FUA nanoparticles: (A) freshly prepared GC–FUA nanoparticles and (B) GC–FUA nanoparticles after static settlement for 7 days.

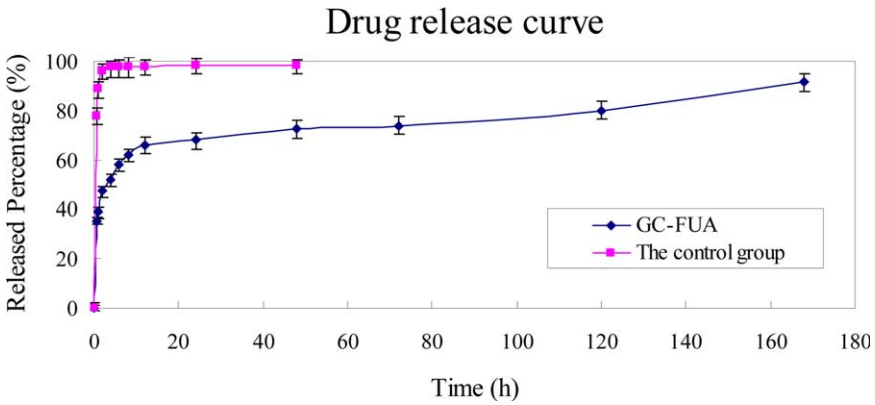


Figure 5. Release profile of the freshly prepared GC–FUA nanoparticles and 5-FU-loaded GC nanoparticles as the control group in phosphate-buffered saline (pH7.4, $n = 3$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

A novel formulation, GC–FUA nanoparticles with chemically conjugated 5-FU, was successfully designed and developed. The preparation conditions were mild and simple. The chemical structure and morphology were investigated by a combination of analytic techniques, including FTIR spectroscopy, ^{1}H-NMR, and SEM. The *in vitro* drug-release study showed that the resulting GC–FUA nanoparticles displayed a slow-release ability compared with the 5-FU loaded GC nanoparticles. Further in-depth studies will be carried out to improve the stability of the nanoparticles and to investigate the biological toxicity, targeting capacity, *in vivo* pharmacokinetics, and biodistribution of GC–FUA nanoparticles for treating liver cancer.

ACKNOWLEDGMENTS

Financial support from the National Natural Science Foundation of China (contract grant numbers 81471777 and 81102409), the Department of Science and Technology of Hunan Province (contract grant numbers 2014GK3082 and 2012FJ2016), and the Natural Science Foundation of Hunan province (contract grant number 13JJ6096) is acknowledged. Support from the Innovation Team of Antitumor Drugs (contract grant number NHCXTD05) and the Young Talent Program of the University of South China was given to one of the authors (C.-Y.Y.). Support from the Health Department of Hunan Province 225 Talent Project and the Construct Program of the Key Discipline in Hunan Province is also gratefully acknowledged.

REFERENCES

1. Gordon-Weeks, A. N.; Snaith, A.; Petrinic, T.; Friend, P. J.; Burls, A.; Silva, M. A. *Br. J. Surg.* **2011**, *98*, 1201.
2. Shimizu, M.; Shirakami, Y.; Imai, K.; Takai, K.; Moriwaki, H. *J. Carcinog.* **2012**, *11*, 1.
3. Nussbaumer, S.; Bonnabry, P.; Veuthey, J. L.; Fleury-Souverain, S. *Talanta* **2011**, *85*, 2265.
4. Burns, E. R.; Beland, S. S. *Cancer Lett.* **1983**, *20*, 235.
5. Cheng, M.; He, B.; Wan, T.; Zhu, W.; Han, J.; Zha, B.; Chen, H.; Yang, F.; Li, Q.; Wang, W.; Xu, H.; Ye, T. *PLoS One* **2012**, *7*, e47115.
6. Kawaguchi, H. *Prog. Polym. Sci.* **2000**, *25*, 1171.

7. Francis, C. S. *J. Controlled Release* **2004**, *96*, 353.
8. Sutton, D.; Nasongkla, N.; Blanco, E.; Gao, J. *Pharm. Res.* **2007**, *24*, 1029.
9. Jung, K. O.; Ray, D.; Daniel, J. S.; Krzysztof, M. *Prog. Polym. Sci.* **2008**, *33*, 448.
10. Yu, C. Y.; Wang, Y. M.; Li, N. M.; Liu, G. S.; Yang, S.; Tang, G. T.; He, D. X.; Tan, X. W.; Wei, H. *Mol. Pharm.* **2014**, *11*, 638.
11. Onishi, H.; Machida, Y. *Molecules* **2008**, *13*, 2136.
12. Huang, Y.; Park, Y. S.; Wang, J.; Moon, C.; Kwon, Y. M.; Chung, H. S.; Park, Y. J.; Yang, V. C. *Curr. Pharm. Des.* **2010**, *16*, 2369.
13. Li, M.; Liang, Z.; Sun, X.; Gong, T.; Zhang, Z. *PLoS One* **2014**, *9*, e112888.
14. Anitha, A.; Sreeranganathan, M.; Chennazhi, K. P.; Lakshmanan, V. K.; Jayakumar, R. *Eur. J. Pharm. Biopharm.* **2014**, *88*, 238.
15. Zhang, C.; Cheng, Y.; Qu, G. W.; Wu, X. L.; Ding, Y.; Cheng, Z. H.; Yu, L. L.; Ping, Q. N. *Carbohydr. Polym.* **2008**, *72*, 390.
16. Larionova, N. I.; Zubaerova, D. K.; Guranda, D. T.; Pechyonkin, M. A.; Balabushevich, N. G. *Polymer* **2009**, *75*, 724.
17. Weecharangsan, W.; Opanasopit, P.; Ngawhirunpat, T.; Apirakaramwong, A.; Rojanarata, T.; Ruktanonchai, U.; Lee, R. *J. Int. J. Pharm.* **2008**, *348*, 161.
18. Mansouri, S.; Cuie, Y.; Winnik, F.; Shi, Q.; Lavigne, P.; Benderdour, M.; Beaumont, E.; Fernandes, J. C. *Biomaterials* **2006**, *27*, 2060.
19. Kim, J. Y.; Choi, W. I.; Kim, Y. H.; Tae, G.; Lee, S.-Y.; Kim, K.; Kwon, I. C. *J. Controlled Release* **2010**, *147*, 109.
20. Yang, W.; Mou, T.; Guo, W.; Jing, H.; Peng, C.; Zhang, X.; Ma, Y.; Liu, B. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4840.
21. Fallon, R. J.; Danaher, M.; Saxena, A. *J. Biol. Chem.* **1994**, *269*, 26626.
22. Liang, M. H.; Ma, Z.; Wang, Z. Y.; Shen, Z. R. *Chin. Pharm. J.* **2011**, *46*, 1677.
23. Park, I. K.; Yang, J.; Jeong, H. J.; Bom, H. S.; Harada, I.; Akaike, T.; Kim, S. I.; Cho, C. S. *Biomaterials* **2003**, *24*, 2331.
24. Calvo, P.; Remunan-Lopez, C.; Vila-Jato, J. L.; Alonso, M. J. *J. Appl. Polym. Sci.* **1997**, *63*, 125.
25. Olde Damink, L. H.; Dijkstra, P. J.; Van Luyn, M. J.; Van Wachem, P. B.; Nieuwenhis, P.; Peijen, J. *Biomaterials* **1996**, *17*, 679.
26. Grabarek, Z.; Gergely, J. *Anal. Biochem.* **1990**, *185*, 131.
27. Timkovich, R. *Anal. Biochem.* **1977**, *79*, 135.
28. Zhang, C.; Ping, Q. N.; Ding, Y. *J. Appl. Polym. Sci.* **2005**, *97*, 2161.
29. Peniche, C.; Argüelles-Monal, W.; Davidenko, N.; Sastre, R.; Gallardo, A.; San Román, J. *Biomaterials* **1999**, *20*, 1869.
30. Song, B.; Zhang, W.; Peng, R.; Huang, J.; Nie, T.; Li, Y.; Jiang, Q.; Gao, R. *Colloids Surf. B* **2009**, *70*, 181.
31. Yu, C. Y.; Cao, H.; Zhang, X. C.; Zhou, F. Z.; Cheng, S. X.; Zhang, X. Z.; Zhuo, R. X. *Langmuir* **2009**, *25*, 11720.
32. Yu, C. Y.; Jia, L. H.; Yin, B. C.; Zhang, X. Z.; Cheng, S. X.; Zhuo, R. X. *J. Phys. Chem. C* **2008**, *112*, 16774.
33. Farid, M. M.; Hathout, R. M.; Fawzy, M.; Abou-Aisha, K. *Colloids Surf. B* **2014**, *123*, 930.
34. Goy, R. C.; Britto, D. D.; Assis, O. B. G. *Polím. Ciência Tecnol.* **2009**, *19*, 241.