Carbohydrate Polymers 79 (2010) 628–632

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis and drug release *in vitro* of porphyran carrying 5-Fluorouracil

Zhongshan Zhang [a,b], Quanbin Zhang [a,*], Jing Wang [a,b], Xuelian Shi [a,b], Jingjing Zhang [a,b], Houfang Song [a,b]

[a] *Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China*
[b] *Graduate University of the Chinese Academy of Sciences, Beijing 100049, China*

ARTICLE INFO

Article history:
Received 5 August 2009
Received in revised form 21 August 2009
Accepted 11 September 2009
Available online 18 September 2009

Keywords:
Porphyran
5-Fluorouracil
Drug release
In vitro

ABSTRACT

Porphyran, the sulfated polysaccharide from red algae *Porphyra haitanensis*, possesses excellent bioactivities, especially the immune activity. In order to provide a water-soluble macromolecule prodrug of 5-FU showing slow release of 5-FU, reducing side-effect, we employed porphyran as a drug carrier, and carried out fixation of 5-FU to porphyran at 6-position through acetyl spacer group *via* ester bond. The chemical characteristic and release behavior of 5-FU from the conjugate obtained were studied *in vitro* at 37 °C in three different medium. The results represented that the release mechanism of all the conjugates was a typical Fickian diffusion. However, further *in vivo* studies on animal models are necessary to establish the efficiency of the system.

1. Introduction

5-Fluorouracil (5-FU), which has been in use against cancer for about 40 years, acts in several ways, principally as a thymidylate synthase inhibitor (Bosch, Harbers, & Heidelberger, 1958; Bounous, Pageau, & Regoli, 1978; Myers, 1981). However, its anticancer activity is generally accompanied by undesirable side-effects (Hiller & Zhuk, 1967; Ouchi, Yuyama, & Vogl, 1985). In order to avoid the side-effect, much attention has been focused on the attachment of drug to polymeric backbone. This is because the drug released from the polymer increases the duration of drug activity due to slow release or of target directing function of polymeric drug in the body (Suzuki, Tokoro, Owaka, Suzuki, & Suzuk, 1986). Generally speaking, polymeric drugs could be used to overcome toxicity problems and to prolong the duration of anti-tumor activity. In addition, a possible affinity of the polymeric drug for tumor cells may be obtained. In previous studies, the chitosan-5FU and chitin-5FU conjugates were reported that they showed nontoxic, nonimmunogenetic and compatible characteristic (Ouchi, Banba, Fujimoto, & Hamamoto, 1989; Ouchi, Banba, Matsumoto, & Suzuki, 1989). And on the other hand, the appropriate hydrolysis rate of the covalent bonds between drugs and carrier polymer was desired for slow release of drugs from the carrier polymer.

Polysaccharide, made up of many monosaccharides joined together by glycosidic bonds, possess marked immunological properties ranging from nonspecific stimulation of host immune system, resulting in anti-tumor, anti-viral, and anti-infective effects, to antioxidant, anti-mutagenic or hematopoietic activity (Bohn & BeMiller, 1995). And thus, many polysaccharide-5FU conjugates have attracted attention in the search for controlled release or slow release materials. Porphyran, the sulfated polysaccharide comprising the hot-water soluble portion of cell wall, is the main component of red algae, *Porphyra haitanensis*. It contains disaccharide units consisting of 3-linked β-D-galactosyl residues alternating with 4-linked α-L-galactose, and some residues occur as the 6-sulfate (Gretz, McCandless, Aronson, & Sommerfeld, 1983). As the general polysaccharide, porphyran possesses excellent bioactivities, especially the immune activity.

In order to provide a water-soluble macromolecule prodrug of 5-FU showing slow release of 5-FU, reducing side-effect, we employed porphyran, which is water-soluble material as a drug carrier, and carried out fixation of 5-FU to porphyran at 6-position through acetyl spacer group *via* ester bond. The chemical characteristic and release behavior of 5-FU from the conjugate obtained were studied *in vitro* at 37 °C in three different medium.

2. Materials and methods

2.1. Materials

P. haitanensis, cultured near the coast of Putian County, Fujian Province, China, was collected in December 2003. Natural polysaccharide porphyran (P) was isolated from hot-water soluble extracts of *P. haitanensis* as described previously (Nishide, Ohno, Anzai, & Uchida, 1988). In brief, the alga was firstly treated with dilute formaldehyde solution, and then extracted with hot water. The low-molecular-weight porphyran (LP) was prepared with the method described earlier by Zhao, Zhang, Qi, and Zhang (2006).

* Corresponding author. Tel./fax: +86 532 82898703.
E-mail address: qbzhang@ms.qdio.ac.cn (Q. Zhang).

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.09.009

5-Fluorouracil was purchased from Beijing Chemical Reagents Company (Beijing, China) recrystallized from water, and dried in vacuum. Chloroacetic anhydride and 4-dimethylaminopyridine (DMAP) were purchased from Shanghai Medpep Co., Ltd. (Shanghai, China) and used as received. Methanol, chloroform, formamide (FA), ethanol triethylamine (TEA) and diethyl ether were purified by usual methods. Dialysis membranes were purchased by Spectrum Co., and molecular weight was cut off at 3600 Da.

2.2. Analytical methods

The chloracetyl content was determined by the modified hydroxylamine-ferric trichloride method (Notari & Munson, 1969).

Infrared spectrums were measured by a Nicolet Magna-Avatar 360 with KBr disks. The ultraviolet (UV) spectra were obtained on a 756MC spectrophotometer.

2.3. Synthesis of chloracetylated P and chloracetylated LP

Chloracetylated P was prepared by the modified method of Morgenstern and Kammer (1996). Two gram sample P and 80 mL FA were added in a three-necked flask with magnetic stirrer, to which 1.5 g DMAP (0.01 mol) as a catalyst was added and stirred for 10 min. And then 12 g chloroacetic anhydride (0.07 mol) was added in three batches and stirred at 70 °C for 4 h. The reaction solution was poured into 85% ethanol and the resultant precipitate was filtered and washed well with chloroform. After that, the precipitate was dissolved in distilled water and the solution was dialyzed against tap water for 48 h and distilled water for 24 h using 3600 Da MW cutoff dialysis membranes. The resultant was concentrated and lyophilized at room temperature in vacuum with the yield of 75.5% (Scheme 1). Chloracetylated LP was prepared with the same method (yield: 90.7%).

2.4. Synthesis of polymers with 5-FU terminal group

Chloracetylated P (0.5 g) was well dissolved in 10 mL FA. And meanwhile, 1.3 g 5-FU (0.01 mol) and 1.4 g TEA (0.01 mol) were dissolved in 20 mL FA and then this solution was added into the above solution of chloracetylated P. The mixture was stirred at 75 °C for 24–40 h. And then the reaction solution was poured into 85% ethanol and the resultant precipitate was filtered and washed well with chloroform and then diethyl ether. The product (P-5FU) was dried in vacuum at room temperature (Scheme 2) (Gao, Gu, & Ping, 2007). Chloracetylated LP was handled with the same method to give the conjugate (LP-5FU).

2.5. Determination of the content of 5-FU in the conjugate

It was proved that the synthesized polymer could be fully hydrolyzed in acid solution. Therefore, the drug contents were determined by releasing it in 0.1 mol/L HCl solution at 37 °C. The extent of hydrolysis was estimated from the amount of released 5-FU measured by UV spectra at 265 nm. When the absorbance was invariable, it was proved that the drug was completely released. Then compared with 5-FU standard curve, the content of 5-FU in the product were thus calculated.

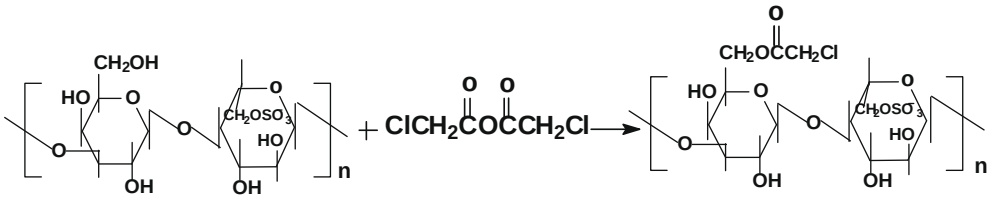
2.6. Release behavior of 5-FU from the conjugate

The release behavior of 5-FU from the conjugate was studied in batch experiments. The conjugate loaded 5-FU (100 mg) was placed in a sealed semi-permeable membrane and the latter was incubated in 100 mL of phosphate buffer, 0.1 M HCl and 0.01 M NaOH aqueous solution, respectively within a shaker at constant temperature (37 ± 0.5 °C). The medium were taken at selected time intervals and then put in a fresh release medium. The 5-FU concentration in the medium was measured compared with 5-FU standard curve within the corresponding release medium spectrophotometricly (Sirica & Woodman, 1971). The curves of cumulative percent drug release as a function of time for conjugates were plotted.

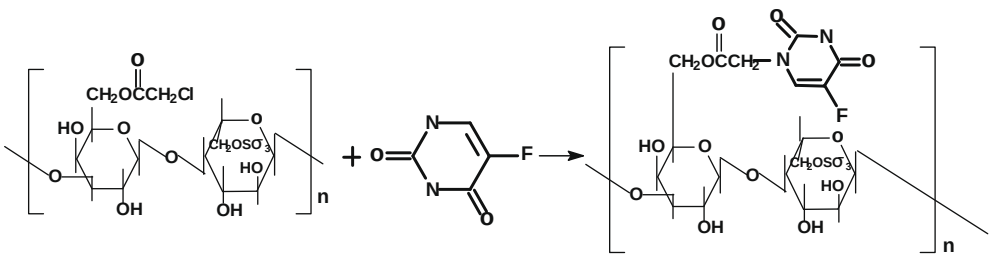
3. Results and discussion

3.1. Synthesis of chloracetylated P and chloracetylated LP

To facilitate the linking of porphyran to 5-FU via ester bond a spacer group chloracetyl was used. To introduce ester-reactive groups, porphyran was reacted with the heterobifunctional chloroacetic anhydride. The activated ester of chloroacetic anhydride couples to the porphyran terminal primary hydroxyl group to yield oxygen-linked chloracetate group (–OOCCH$_2$Cl). The P–OOCCH$_2$Cl synthesized was then reacted with water soluble 5-FU to get de-



Scheme 1. The preparation of chloracetylated P (or LP).



Scheme 2. The preparation of conjugates carrying 5-FU.

sired conjugate P–OOCCH$_2$–FU (P-5FU). The acetylation of the polysaccharide was a common reaction. In previous studies, chloroacetylation of polymers was conducted generally by using pyridine as a base and benzene or chloroacetic acid as a solvent (Aridoss, Balasubramanian, Parthiban, Ramachandran, & Kabilan, 2007; Jackson & Shirley, 1967; Steele & Anderson, 1972). However, when we carried out initially only poor yields were achieved due to the poor solubility of porphyran in the above solvent. Instead, when DMAP was used as basic catalyst and simultaneity FA as solvent, products were obtained along with the expected product and the yield of the product improved significantly. All the objective polymers were structurally confirmed by IR and UV spectrum, as shown in Figs. 1 and 2. Concretely, the IR absorption peaks of the chloracetylated P (the structure of LP was the same as P) included 3428, 2963, 1753 (C═O), 1648, 1412, 1320 (CH_2 for C–CH_2–Cl), 1259, 1202 (C–O–C), 1165 (C–O–C), 1073, 1038, 896, 773 (C–Cl) cm^{-1}. The UV spectra of P and chloracetylated P are illustrated in Fig. 2. It was obviously found that both two samples (d and e in Fig. 2) had no absorption at 220 and 320 nm.

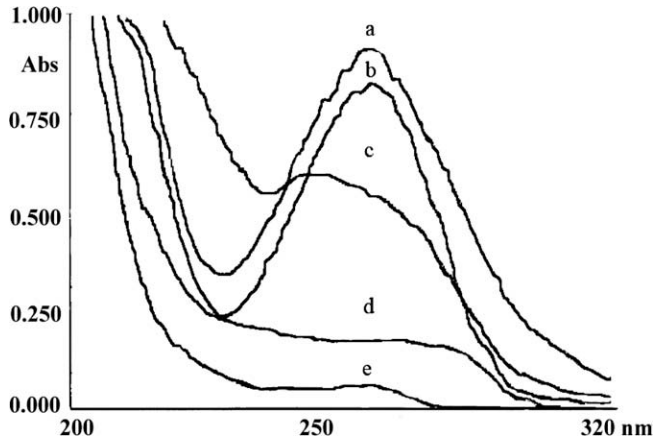


Fig. 2. The UV spectra of 5-FU (a), the mixture of 5-FU and porphyran (b), conjugate (c), chloracetylated P (or LP) (d) and porphyran (e).

3.2. Synthesis of polymers with 5-FU terminal group

The resultant polymeric prodrugs were composed of different molecular weight porphyran units as drug carrier and 5-FU unit as drug-active terminal group. It is generally considered that P or LP lacked the functional groups that can react with 5-FU. Therefore, a series of polymeric prodrugs were synthesized by two-step reaction of chloracetylated P or chloracetylated LP with 5-FU, as detailedly described in Scheme 2. In this research, we add DMSO into the reactive solution as a medium, which could avail to the reaction. Fig. 1 showed the IR absorption of P-5FU including 3428, 2963, 1715–1668 (OCO and C═O/5FU), 1648, 1412, 1320 (CH_2 for C–CH_2–Cl), 1259, 1202 (C–O–C), 1165 (C–O–C), 1073, 1038, 896, 814 (C–F) cm^{-1}. The peak at 773 cm^{-1}, assigned to C–Cl, had disappeared. The UV spectra in Fig. 2 indicated that the pure 5-FU (a) had the maximal absorption at 265 nm and the mixture of the 5-FU and porphyran (b) also had maximal absorption at 265 nm. But P-5FU (c) had maximal absorption at 258 nm, which was the sign of 5-FU coupled with polymer.

3.3. Chemical characterization of the products

In this study, we used two raw materials (P and LP) with different molecular weights (2.77×10^5 and 5.82×10^3 Da, respectively). Table 1 described Chemical characterization of the products. In the chloroacetylation reaction process, the Mw influenced the yield of chloracetylated product and the degree of chloracetyl. Because of the crinkle and steric hindrances of sugar chain and low solubility of reactant, the chloroacetylation reaction of P was more difficult than that of LP. So the results were that both the yield and chloracetyl content of P were obviously lower than that of LP.

In this research, we prepared three conjugates with different characterization, which were also shown in Table 1. From the table, it was noticed that the reactive condition was important to the yield of the product. In our studies, we found that the prolongation of reactive time and the decrease of molecular weight could improve the substituting degree of 5-FU (D5FU).

3.4. Release behavior of 5-FU from the conjugate

In order to evaluate the release behavior of 5-FU from conjugates, the hydrolysis of three samples were studied *in vitro* at 37 °C in various media. The ester bonds of these conjugates were found to be cleaved to give free 5-FU in NaOH solution and to give acetic 5-FU in HCl solution and phosphate buffer (Scheme 3). It was also found that the release rate of 5-FU from conjugates was strongly dependent on pH of the dissolution medium, the molecular weight and composition of the polymer employed (Zhang et al., 2005). The results were shown in Fig. 3, which exhibited the

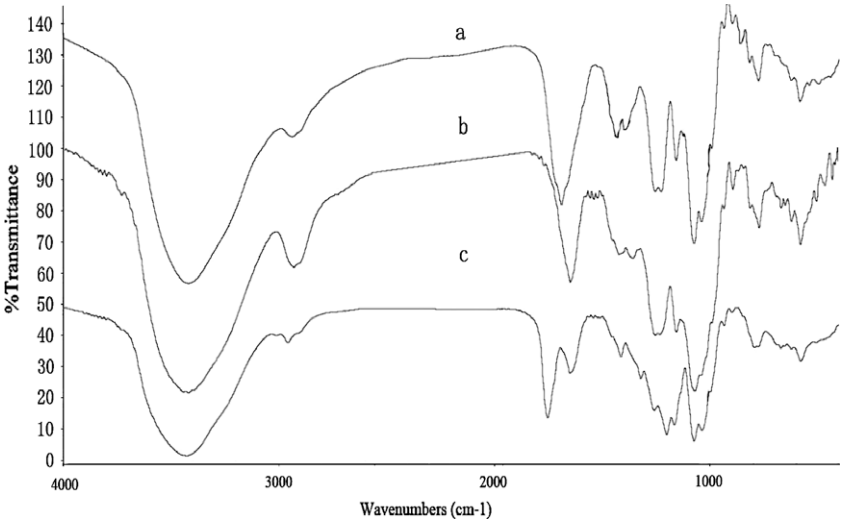
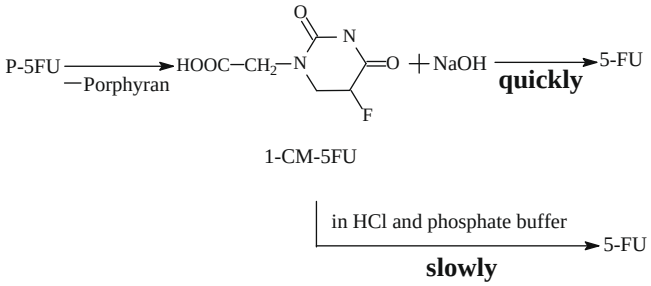


Fig. 1. The spectra of the conjugate (a), P (LP) (b) and chloracetylated P (LP) (c).

Table 1
The chemical characteristic of all the samples.

Samples	Reaction time (h)	Yield (%)	Chloracetyl[a] (%)	5-FU (%)
Chloracetylated P	4	75.52	4.45	–
Chloracetylated LP	4	90.74	18.87	–
P-5FU$_1$	24	80.45	–	3.62
P-5FU$_2$	40	73.20	–	6.57
LP-5FU	40	59.39	–	17.71

[a] The contents of chloracetyl in conjugates were not determined because of the disturbance of 5-FU.



Scheme 3. The hydrolytic mechanism of the conjugates in different mediums.

release profiles of the conjugates in phosphate buffer, 0.1 M HCl and 0.01 M NaOH aqueous solution, respectively.

As seen in Fig. 3, the release percents of three release profiles were significantly different at each time point. It was clear that the drug release durations of these conjugates were extremely correlated with the concentration of drug loading, the pH of media and molecular weight of the conjugates. The data indicated that the release is controlled not only by diffusion and interaction with the polymer chain, but also by the rate of degradation of the covalent linkages to the chains.

Fig. 3a showed 5-FU release profiles from three conjugates at 0.1 M HCl at 37 °C. The hydrolytic rate of the ester bonds was obtained in the following order: LP-5FU > P-5FU$_2$ > P-5FU$_1$. It was thought to be due to the fact that the more the number of 5-FU units introduced into the polymer, the more easily the chain scission could occur during hydrolytic degradation. And similarly, the hydrolytic rate in 0.01 M NaOH follows the sequence of content of 5-FU loading. However, in the phosphate buffer, the hydrolytic rate was in order: P-5FU$_2$ > LP-5FU > P-5FU$_1$. The reason was that when more 5-FU was introduced into the high-molecular-weight polymer backbones, the packing density of the copolymer chains decreased and led to a decrease in hydrophilicity. For a single conjugate, pH of the medium played an important role in the hydrolytic rate. As seen in Fig. 3, the hydrolytic rate of LP-5FU and P-5FU$_1$ in three medium was in order: NaOH > phosphate buffer > HCl. Scheme 3 described the hydrolytic mechanism of acetyl ester linking porphyran and 5-FU in three medium solutions. Firstly, the conjugate hydrolyzed to release 2-(5-fluorouracil-1-yl) acetic acid (1-CM-5FU), which was a dynamic balance. In the alkaline solution, NaOH could reacted with 1-CM-5FU to make the hydrolyze conducting completely, and finally gave free 5-FU quickly. However, in the neutral and acid solution, there was no reactant to break down the balance and ultimately gave more 1-CM-5FU and fewer free 5-FU slowly.

Molecular weight (MW) was a marked factor influencing the release of 5-FU. From the Fig. 3, we found that LP-5FU with low MW hydrolyzed much more quickly than other two conjugates with high MW. It was clear that the high solubility and low steric hindrance resulting from the low MW led to an increase in hydrophilicity.

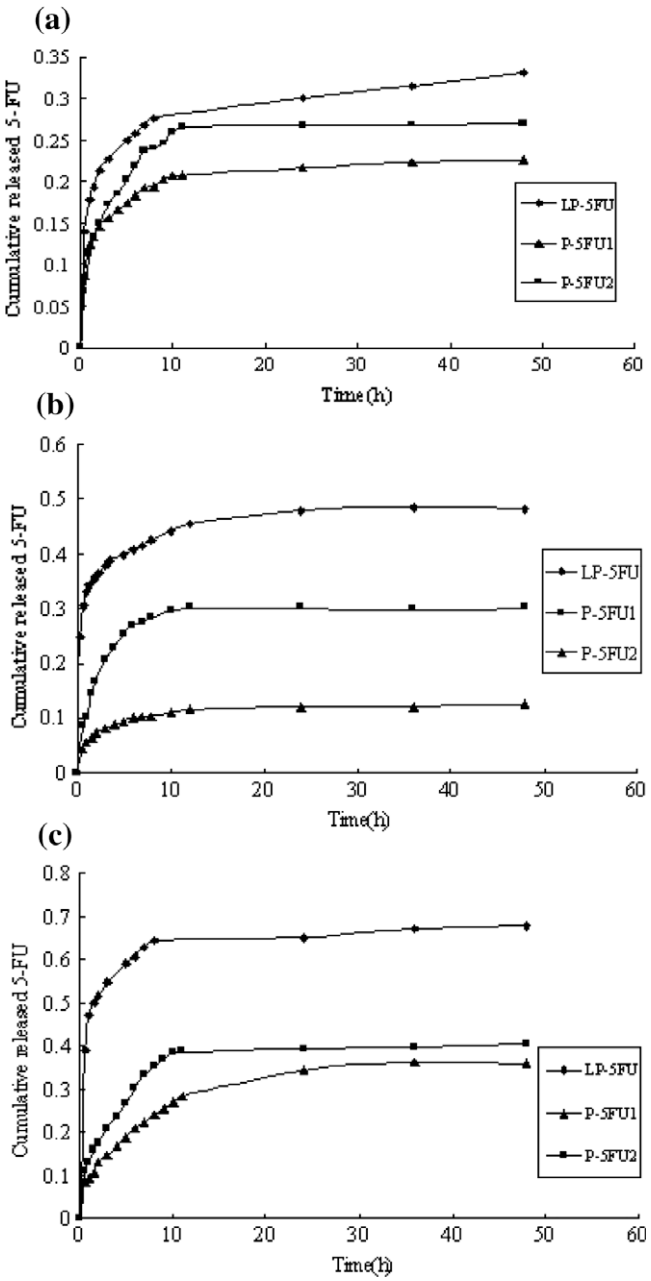


Fig. 3. The release profiles of three conjugates in different mediums.

3.5. Analysis of release data

5-FU release kinetics was analyzed by plotting the cumulative release data versus time and Ritger–Peppas' equation was utilized to illustrate the mechanism of drug release (Philip & Nicolas, 1987):

$$M_t/M_\infty = Kt^n, \quad (1)$$

where M_t/M_∞ represents the fractional drug release and t is the release time, K is a constant characteristic of the drug–polymer system and n is the characteristic exponent of release mechanism. All the release profiles tested in this paper were calculated by the equation and Table 2 listed the n values and regression coefficient (r) of the equation in three medium. From the table, no value of n was beyond 0.45 which is the threshold of n value between Fickian and non-Fickian mechanism. It indicated that all the release

Table 2
Release kinetics of 5-FU from three conjugates in different medium.

Mediums	Conjugates	Equations	n	r
0.1 M HCl	LP-5FU	$M_t/M_\infty = 31.84t^{0.1464}$	0.1464	0.9801
	P-5FU$_1$	$M_t/M_\infty = 11.83t^{0.4403}$	0.4403	0.9879
	P-5FU$_2$	$M_t/M_\infty = 5.65t^{0.3089}$	0.3089	0.9950
Phosphate buffer	LP-5FU	$M_t/M_\infty = 17.34t^{0.2312}$	0.2312	0.9913
	P-5FU$_1$	$M_t/M_\infty = 11.63t^{0.2536}$	0.2536	0.9871
	P-5FU$_2$	$M_t/M_\infty = 10.50t^{0.4063}$	0.4063	0.9874
0.01 M NaOH	LP-5FU	$M_t/M_\infty = 45.66t^{0.1649}$	0.1649	0.9919
	P-5FU$_1$	$M_t/M_\infty = 9.75t^{0.4246}$	0.4246	0.9890
	P-5FU$_2$	$M_t/M_\infty = 13.53t^{0.4421}$	0.4421	0.9937

mechanism of the conjugate is a typical Fickian diffusion and consistent with those result of experiments.

4. Conclusion

In this studies, we prepared three conjugates linking porphyran with 5-FU and investigated their release mechanisms. The contents of 5-FU loading were different due to the different reactive condition. The drug release durations of these conjugates were extremely correlated with the concentration of drug loading, the pH of media and molecular weight of the conjugates. The results represented that the release mechanism of all the conjugates was a typical Fickian diffusion. However, further *in vivo* studies on animal models are necessary to establish the efficiency of the system.

Acknowledgements

This work was supported by the Department of Science and Technology of Shandong Province (2007BS07004). We are grateful to Prof. Zuhong Xu and Prof. Zhien Li for their help in this experiment.

References

Aridoss, G., Balasubramanian, S., Parthiban, P., Ramachandran, R., & Kabilan, S. (2007). Synthesis and antimicrobial activities of N-chloroacetyl-2,6-diarylpiperidin-4-ones. *Medicinal Chemistry Research, 16*, 188–204.

Bohn, J. A., & BeMiller, J. N. (1995). (1 → 3)-β-D-Glucans as biological response modifiers: a review of structure–functional activity relationships. *Carbohydrate Polymers, 28*, 3–14.

Bosch, L., Harbers, E., & Heidelberger, C. (1958). Studies on fluorinated pyrimidines. V. Effects on nucleic acid metabolism in vitro. *Cancer Research, 18*, 335–343.

Bounous, G., Pageau, R., & Regoli, P. (1978). Enhanced 5-fluorouracil mortality in rats eating defined formula diets. *International Journal of Clinical Pharmacology and Biopharmacy, 16*, 265–267.

Gao, H., Gu, Y. Q., & Ping, Q. N. (2007). The implantable 5-fluorouracil-loaded poly (L-lactic acid) fibers prepared by wet-spinning from suspension. *Journal of Controlled Release, 118*, 325–332.

Gretz, M. R., McCandless, E. L., Aronson, J. M., & Sommerfeld, M. R. (1983). The galactan sulphates of the conchocelis phases of *Porphyra leucosticta* and *Bangia atropurpurea*. *Journal of Experimental Botany, 34*, 705–711.

Hiller, S. A., Zhuk, R. A., & Lidak, M. Y. (1967). Pyrimidine nucleoside analogues. I. N_1-(a-furanidyl)derivatives of natural pyrimidine bases and their antimetabolites. *Doklady Akademii Nauk SSSR, 176*, 332–335.

Jackson, T. G., & Shirley, D. A. (1967). Friedel–Crafts acetylation and chloroacetylation of the benzophenothiazines. *The Journal of Organic Chemistry, 32*, 1190–1194.

Morgenstern, B., & Kammer, H. W. (1996). Solvation in cellulose–LiCl–DMAc solutions. *Trends in Polymer Science, 4*, 87–92.

Myers, C. E. (1981). The pharmacology of the fluoropyrimidines. *Pharmacological Reviews, 33*, 1–15.

Nishide, E., Ohno, M., Anzai, H., & Uchida, N. (1988). Extraction of porphyran from *Porphyra yezoensis* Ueda F. Narawaensis Miura. *Nippon Suisan Gakkaishi, 54*, 2189–2194.

Notari, R. E., & Munson, J. W. (1969). Hydroxamic acids. I. Factors affecting the stability of the hydroxamic acid–iron complex. *Journal of Pharmaceutical Sciences, 58*, 1060–1064.

Ouchi, T., Yuyama, H., & Vogl, O. (1985). Synthesis of poly(ethylene glycol)-bound 3-(5-fluorouracil-1-yl)propanoic acid, its hydrolysis reactivity and antitumor activity. *Die Makromolekulare Chemie. Rapid Communications, 6*, 815–819.

Ouchi, T., Banba, T., Fujimoto, M., & Hamamoto, S. (1989). Synthesis and antitumor activity of chitosan carrying 5-fluorouracils. *Macromolecular Chemistry and Physics, 190*, 1817–1822.

Ouchi, T., Banba, T., Matsumoto, S., & Suzuki, M. (1989). Antitumor activity of chitosan and chitin immobilized 5-fluorouracils through hexamethylene spacers via carbamoyl bonds. *Journal of Bioactive and Compatible Polymers, 4*, 362–371.

Philip, L. R., & Nicolas, A. P. (1987). A simple equation for description of solute release. I. Fickian and non-Fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. *Journal of Controlled Release, 5*, 23–36.

Sirica, A. E., & Woodman, R. J. (1971). Selective aggregation of L1210 leukaemia cells by the polycation chitosan. *Journal of the National Cancer Institute, 47*, 377–381.

Steele, J. W., & Anderson, W. K. (1972). Chloroacetylation of 1-azaphenothiazine. *Canadian Journal of Chemistry, 50*, 1026–1029.

Suzuki, K., Tokoro, A., Owaka, Y., Suzuki, S., & Suzuk, M. (1986). Effect of N-acetyl chitooligosaccharides on activation of phagocytes. *Microbiology and Immunology, 30*, 777–787.

Zhang, X. F., Li, Y. X., Chen, X. S., Wang, X. H., Xu, X. Y., & Jing, X. B. (2005). Synthesis and characterization of the paclitaxel/MPEG-PLA block copolymer conjugate. *Biomaterials, 26*, 2121–2128.

Zhao, T. T., Zhang, Q. B., Qi, H. M., & Zhang, H. (2006). Degradation of porphyran from *Porphyra haitanensis* and the antioxidant activities of the degraded porphyrans with different molecular weight. *International Journal of Biological Macromolecules, 38*, 45–50.