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# Synthesis and characterization of ramose tetralactosyl-lysyl-chitosan-5-fluorouracil and its in vitro release

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**Abstract** In order to improve drug loading and achieve a good release effect, this paper adopts the ramose method, choosing chitosan as the carrier and 5-fluorouracil (5-Fu) as a model drug. Ramose chitosan-lysyl-5-Fu(3) and ramose tetralactosyl-lysyl-chitosan-5-Fu(6) were synthesized successfully, then the in vitro release of (6) was researched. The results show that the drug loading of (3) and (6) are 9.17 and 1.63% (w/w), respectively. The in vitro release behavior of (6) in pH 7.4 phosphate buffer solution and pH 1.2 HCl–KCl solution were studied. The zero order release time that (6) maintains in alkaline and acidic media are 64 and 24 h, and the total release by 184 h are 71.97 and 82.34%, respectively. The performance is smooth throughout the whole stage of release, and the concentration of cumulative release is lower in the alkaline environment than in the acidic environment over the same time.

**Keywords** Chitosan · Lysine · Lactose acid · Ramose · 5-Fluorouracil

## Introduction

To date, polysaccharides have been widely used in pharmaceutical technology as first choice excipients for production of traditional formulations. Their peculiar physicochemical and biological properties have been exploited to develop macromolecular prodrugs which can exhibit favorable biopharmaceutical properties and enforce the therapeutic performance of the parent drugs. These polymers display, in fact, high biocompatibility and biodegradability, multiple insertion

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points, and biological properties that can be advantageously exploited in drug delivery [1]. Compared with most polysaccharides, chitosan is a better drug carrier.

Currently, chitosan, or chitosan derivatives, as a carrier to carry 5-Fu mostly uses an encapsulated form. It has recently been prepared as nanoparticles or microspheres, which can improve the targeting performance, but the greatest disadvantage is instability release. Generally speaking, the reason for a burst release phenomenon is that the mechanical strength of in the encapsulated microcapsule or drug-containing gel is not strong enough, causing rapid leakage of in the drug, so we have begun to research chemical-bonded drugs [2]. Microspheres or nanoparticles can only achieve passive targeting. Refined targeting selectivity and high delivery efficiency are two equally important goals in the development of drug delivery systems [3]. So it is a good idea for us to choose a drug carrier with an active targeting group. The galactose group can be recognised by liver cell surface asialoglycoprotein (ASGP) receptors. ASGP receptors participate in the hepatic metabolism of serum proteins and can recognize a glycoprotein with galactose residues on the terminal position of the saccharide chain [4], so the compounds based on in the galactose group generate a target property. Glycobiology studies have shown that there are many target chambers on the surface of asialoglycoprotein receptors which can identify galactosyl. In the recognition process, a number of galactosyls combining with receptor proteins can generate synergy, which enhances the targeting and binding force. The more ligand groups, the stronger the targeting and binding effect, which is known as the "aggregation effect" or "multivalent effect" [5, 6].

The successful synthesis of a water-soluble cationic graft copolymer, chitosan-graft-poly (L-lysine), has been reported, and this novel material would be a good candidate as a scaffold for cell culture and tissue engineering [7]. Liu et al. [8] prepared polyion complex micelles (PIC micelles) based on methoxy-poly-(ethylene glycol) (PEG)-graft-chitosan and lactose-conjugated PEG-graft-chitosan for liver-targeted delivery of diammonium glycyrrhizinate (DG).

This paper chose 5-Fu as a model drug, galactose as a targeting group, and we adopted the coupling method, hoping to synthesize the target compound to have both good targeting property and superior release performance.

## Experimental

### Apparatus

FTIR spectra were recorded with a Nicolet Avatar FT/IR-360 spectrophotometer (USA). UV–Vis absorption spectra were recorded with a TU-1901 dual beam UV–Vis spectrophotometer (Purkinje General Instrument, Beijing, China) between 200 and 800 nm. We also used HP6890/5973 Agilent 1,100 Liquid mass spectrometer, Varian Mercury-VX 400-type (400 MHz) NMR, FD-1A-type freezer Dryer, WRS-1-type melting point apparatus, dialysis bag, MWCO: 8,000–14,000; Beijing Dingguo Biotechnology.

## Reagents

Chitosan (DD = 98.87%, M$\eta$ = 411KDa), self-restraint. Bromoacetic acid (AR); Aladdin Reagent. L-lysine (BR); National Pharmaceutical Group Chemical Reagent. 5-Fu: 99%, J & K Chemical Reagent. Lactose acid (LA): 97%; J & K Chemical Reagent. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl): 98.5%; National Pharmaceutical Group Chemical Reagent. 1-Hydroxy-2,5-pyrrolidinedione (NHS): 98%; National Pharmaceutical Group Chemical Reagent. *N,N*-Dimethylpyridin-4-amine (DMAP): 99%, Aladdin Reagent. All other chemicals were of analytical grade and used without further purification.

## Synthesis

### *Synthesis of N-carboxymethyl-5-Fu (1)*

Synthesized according to Ref. [9]. The procedure is as follows: the KOH solid 1.010 g (18 mmol) was added to a stirred 5-Fu 0.52 g (4 mmol) in distilled water (2 mL), 50 °C constant magnetic stirring 0.5 h. Then, 0.8341 g (6 mmol) $BrCH_2COOH$ dissolved in distilled water (2 mL) was added dropwise to the mixture and the mixture was stirred for 3 h at reflux. In the whole process, a moderate amount of KOH solid was added portionwise to maintain the pH value at 13. The resultant solution was cooled to r.t. and then the pH was adjusted with concentrated HCl to 5.5. On being kept in the refrigerator for 4 h, if a white precipitate appeared, then it was filtrated and the filtrate acidified with concentrated HCl to pH 1, whereupon a white needle-like crystal appeared. It was again kept in the refrigerator, for 12 h, filtrated, and the filter cake washed with 0 °C water three times, and dried under vacuum for 48 h to yield a white needle-like crystal. yield: 0.5595 g (73%); mp: 274–276 °C; MS: 186.8 $[M-1]^-$.

### *Synthesis of lysyl-5-Fu (2)*

In a 100-mL round-bottom flask, 0.094 g (0.5 mmol) carboxymethyl-5-Fu was dissolved in 4 mL distilled water, then EDC·HCl 0.1438 g (0.75 mmol), NHS 0.0863 g (0.75 mmol) and DMAP 0.0122 g (0.1 mmol) dissolved in 5 mL distilled water were added to it and the mixture was stirred for 4 h at room temperature. After that, 0.0548 g (0.375 mmol) lysine was added to the mixture and stirred until dissolved, then the pH value was adjusted by $NaHCO_3$ solid powder to 7.5–8.0, and the mixture was stirred for 24 h at 25 °C [10]; MS: 487.2 $[M + 1]^+$.

### *Synthesis of ramose chitosan-lysyl-5-Fu (3)*

EDC·HCl 0.1100 g (0.57 mmol), NHS 0.0656 g (0.57 mmol), DMAP 0.0122 g (0.1 mmol) dissolved in 2 mL distilled water were added to (2), and the mixture was stirred for 24 h at 25 °C. Chitosan 0.2000 g (1.25 mmol free $NH_2$) dissolved in 8 mL 0.1 M HCl solution was added to activation solution (2), the reaction was stirred for 48 h at 25 °C, dialyzed against deionized water over 3 days, and the product freeze-dried.

Compound (3) is yellowish sponge-like solid. IR (KBr): 3,376 $cm^{-1}$ (–OH and –NH stretching vibration mixed); 1,697 $cm^{-1}$ (amide I with C=O stretching vibration); 1,616 $cm^{-1}$ (amide II with N–H deformation vibration); 1,308 $cm^{-1}$ (amide III with C–N stretching vibration); 1,245 $cm^{-1}$ (C–F stretching vibration); 1,152 $cm^{-1}$ (C–O–C asymmetric stretching vibration); 1,082 $cm^{-1}$ (C–O stretching vibration of chitosan C3). UV–Vis spectra showed a strong absorption peak at 269 nm.

*Synthesis of branched trimeric lysine (4)*

Lysine amino protection [11]: the Lysine 1.7520 g (12 mmol) was added to a stirred mixture of 3 mL dioxane and 3 mL distilled water at reflux, and then 6 mL 2 M NaOH solution was added to it. Under the ice bath, 26.2000 g (120 mmol) di-tert-butyl dicarbonate($(Boc)_2O$) was added dropwise to the mixture in 1.5 h, and the mixture was stirred for 1.5 h at room temperature. In the whole process, it is necessary to control the pH value at 9 with 2 M NaOH solution. The resulting solution was concentrated by a rotary evaporator at 75 °C, cooled to r.t. and then the pH was adjusted with concentrated HCl to 2. This residue was extracted with ethyl acetate (3 × 35 mL) and the extract was washed with saturated $Na_2SO_4$ solution (3 × 5 mL), and dried (anhydrous $Na_2SO_4$), evaporation of ethyl acetate under reduced pressure gave a light yellow viscous oil.

Synthesis of ramose trimeric lysine was according to Ref. [12]: the amino-protected lysine dissolved in distilled water (2 mL) was added to the mixture of EDC·HCl 0.8400 g (4.38 mmol), NHS 0.5050 g (4.38 mmol), and DMAP 0.0535 g (0.438 mmol) dissolved in distilled water (3 mL), and then the mixture was activated for 4 h at room temperature. After that, 0.3200 g (2.19 mmol) lysine dissolved in distilled water (2 mL) was added, then the pH value was adjusted by $NaHCO_3$ solid powder to 8, and the mixture was stirred for 24 h at 25 °C. without separation, Evaporation was under reduced pressure with drying in the vacuum. The dried material dispersed in 2 mL trifluoroaceticacid (TFA) was stirred for 2 h at room temperature. Similarly, without separation, evaporation was under reduced pressure and drying in the vacuum: MS: 403.2 $[M + 1]^+$.

*Synthesis of tetralactosyl-trimeric-lysine (5)*

Synthesis of (5) was according to Ref. [12]. Lactose acid 0.2150 g (0.6 mmol) dissolved in 2 mL distilled water was added to the mixture of EDC·HCl 0.1380 g (0.72 mmol), NHS 0.0829 g (0.72 mmol), and DMAP 0.0088 g (0.072 mmol). The mixture was activated for 4 h at room temperature. After that, 0.1200 g (0.3 mmol) (4) was added then the pH value was adjusted by $NaHCO_3$ solid powder to 7.5–8.0. The mixture was stirred for 48 h at room temperature, and used directly for the next reaction without separation.

*Synthesis of ramose tetralactosyl-lysyl-chitosan-5-Fu (6)*

The mixture of EDC·HCl 0.0900 g (0.45 mmol), NHS 0.0520 g (0.45 mmol), and DMAP 0.0100 g (0.090 mmol) was added to (5), and activated for 4 h at room

temperature. After that, 0.1000 g (3) dissolved in 0.1 M HCl solution was added, then the pH value was adjusted with TEA to 7.5–8.0, and the mixture was stirred for 48 h at room temperature. After being dialyzed against deionized water over 3 days, and freeze-dried, the product, compound (6) is a yellowish spongy solid. $^{1}$H NMR ($F_3$COOD-DMSO-$d_6$, 20 °C): 1.075 (lysyl C4**H**); 1.536 (lysyl C5**H**); 1.751 (lysyl $C_3$**H**); 2.735 (lactosyl C2O**H**); 3.033 (lysyl C6**H**); 3.246 (C6**H** of trimeric lysine which combine with lactosyl); 4.049 (5-Fu-C$\mathbf{H}_2$ (connected to C2 of lysyl)); 4.558 (5-FuC$\mathbf{H}_2$ (connected to C6 of lysyl); 4.735 (lactosyl pyran ring C$^{1}$**H**); 7.623 (lysyl C6N**H**CO); 8.058 (5-Fu ring C=C–**H**); 8.159 (N**H**CO in chitosan); 8.276 (C2N**H**CO of lysyl) ppm. IR(KBr disk): 3,366 $cm^{-1}$ (O–H and N–H stretching vibration); 2,978, 2,934, 2,869 $cm^{-1}$ (C–H stretching vibration of lysyl, lactosyl and chitosan); 1715 $cm^{-1}$ (C4=O stretching vibration of pyrimidine ring); 1,699 $cm^{-1}$ (C=O stretching vibration of amide I); 1,650 $cm^{-1}$ (N–H deformation vibration of amide II); 1,558, 1,539 $cm^{-1}$ (N–H deformation vibration of amino involving in lysyl and chitosan); 1,367 $cm^{-1}$ (C–N stretching vibration of amide III); 1,251 $cm^{-1}$ (C–F stretching vibration); 1,155 $cm^{-1}$ (C–O–C asymmetric stretching vibration); 1,081 $cm^{-1}$ (C3=O stretching vibration of chitosan ring) $cm^{-1}$. UV–Vis spectra show a strong absorption peak at 269 nm.

## Determination of drug loading

Using a certain amount of (2) and (6) dissolved in 10 mL distilled water, UV–Vis absorption spectra were scanned from 200 to 800 nm, and a strong absorption peak showed at 269 nm.

Configuring a series of standard solutions with carboxymethyl 5-Fu: 0 mg $L^{-1}$, 5 mg $L^{-1}$, 10 mg $L^{-1}$, 15 mg $L^{-1}$, and 20 mg $L^{-1}$, which were measured at 269 nm. according to this step, we got the standard curve and linear regression equation as follows:

$$\mathrm{Abs} = 0.03984\mathrm{C/mg\ \ L^{-1}} + 0.00861(\mathrm{R} = 0.9995) \quad (1)$$

Then, 15 mg (3) and (6) were dissolved in 10 mL water, respectively, and we filtered the suspended solid, measured absorbance at 269 nm, and calculated the drug loading by the equation as follows:

$$\text{Drug Loading (DL)} = \frac{\text{the content of } 5-\text{Fu in sample}}{\text{the amount of sample}} \times 100\% \quad (2)$$

## The in vitro release study

The in vitro release was carried out according to Ref. [13]. An amount of 25 mg (6) was suspended in PBS (5 mL, pH 7.4) and artificial gastric juice HCl-KCl (5 mL, pH 1.2) buffer solution in two dialysis bags, respectively. The dialysis bag was sealed and then slowly shaken (50 rpm) in 100 mL of PBS and HCl-KCl at 37 °C in a 250-mL Erlenmeyer flask. Aliquots of the solution outside the dialysis membrane (5 mL) were replaced with 5 mL of dialysis medium at various time intervals and tested at 269 nm by UV–Vis spectrophotometer.

## Results and discussion

The synthetic route of compound (6) is shown in Scheme 1.

In the synthesis of compound (1), pH is a key factor, the yield was greatly affected by the base, and the use of KOH increased the yield by up to 73%. Coupling of compound (1) and lysine was performed using EDC·HCl/NHS/DMAP in water at ambient temperature. The use of water as reaction medium has received considerable attention in the context of green chemistry for several reasons: (1) it is cheap, safe, and environmentally benign, (2) reactions in aqueous medium eliminate the additional efforts in making the substrates and reagents dry before use and thus reduce/eliminate the consumption of drying agents, energy, and time, and (3) the unique physical and chemical properties of water can be utilized to realize reactivity or selectivity that cannot be attained in organic solvents [14]. In pursuit of our recent efforts to develop environmentally friendly synthetic methodologies by carrying out reactions in water, very gratifyingly, the desired product was obtained in a easy manner.

In the synthetic process of target compound (6), protection of lysine was with $(Boc)_2O$, because it is easy to crystallize, and there is a certain stability. More importantly, it is easy to remove the Boc group. Generally speaking, removal of the Boc group is by using TFA at room temperature. We still use the coupling agent EDC for the acylation reaction, because its urea derivatives have a good water-soluble property, which is conducive to the final step of dialyzing.

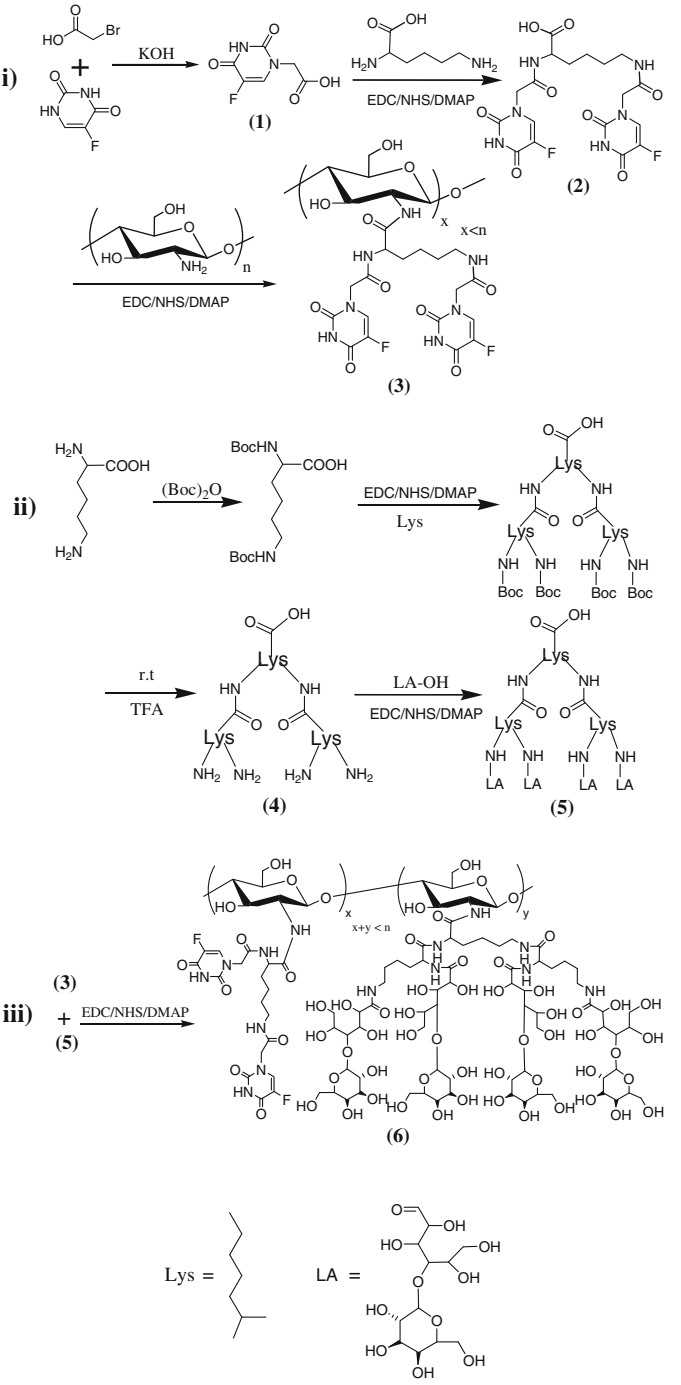
### Characterization

It can be seen from the IR of compound (3) that the absorption peaks of C4=O, C=C, and C–F are made obvious by introducing 5-Fu. Comparrf with chitosan, the obvious change is that the absorption peak of the amide group appeared in compound (3). There are three kinds of amino absorption peaks corresponding to the amino group in the structure. This can be very good at proving the existence of the ramose structure. Simultaneously, it also showed that the synthesis was successful.

Compared with IR of compound (6) and compound (3), the absorption peak of amide group and C4=O, C=C, and C–F in 5-Fu is obvious as shown before. But a strong absorption peak at 1,155 $cm^{-1}$ appeared, which was attributed to C–O–C asymmetric stretching vibration in the lactosyl group. We could judge from the IR of compound (6) that the lactosyl group was successfully introduced.

The UV–Vis spectra of compounds (3) and (6) showed that characteristic absorption peaks of 5-Fu ring all appeared, which showed that 5-Fu was successfully connected to chitosan. The drug loadings of compounds (3) and (6) are 9.17 and 1.63%, respectively.

$^1H$ NMR of compound (6) showed that 3.033 ppm is the chemical shift of C6-H after the acylation reaction between carboxymethyl 5-Fu and C6-$NH_2$ of lysine; 4.049 ppm is the chemical shift of 5-Fu-$CH_2$ after the acylation reaction between carboxymethyl 5-Fu and C2 -$NH_2$ of lysine; 4.558 ppm is the chemical shift of 5-Fu-$CH_2$ after the acylation reaction between carboxymethyl 5-Fu and C6-$NH_2$ of




**Scheme 1** The synthetic route of **(6)**

lysine. This is good at proving the successful synthesis of (2). The chemical shift of C6-H of lysine is 3.246 ppm after the acylation reaction between lactose acid and C6-$NH_2$ of lysine in trimeric lysine. However, the chemical shift of C6-H connected with chitosan will not appear at this place, which proved the existence of ramose structure. The chemical shift of 7.623, 8.159, and 8.159 ppm corresponded to three kinds of amide bonds respectively, one is two amide bonds of lysyl, the other is the formation between chitosan and lysine. In a word, the synthesis is so successful.

## The in vitro release behavior of compound (6)

As we all know, the pH of blood in human body is about 7.4, and pH of gastric juice is about 1.2, so we simulated the environment of these two fluids. The in vitro release property was tested by the standard method, and the result is shown in Fig. 1.

It can be seen from Fig. 1 that compound (6) shows good slow-release characteristics in alkaline environment. The time that maintains zero-order release is 64 h, and in this period, the accumulative release quantity reaches 54.65%; after 64 h, the release becomes slow, but still increases markedly, the accumulative release quantity reached 71.97% in 184 h. In the acidic medium, the accumulative release quantity is 82.34% in 184 h; the zero-order release time was maintained at 24 h. After that, the delivery rate become slow, but obviously also increases, and the rate of increase is much higher than that in the alkaline medium. The common feature is that the delivery rate become slow after the first fast release, which may be caused by hydrolysis balance. First of all, the hydrolysis rate is much faster than the delivery rate at pH 1.2. Secondly, in the acidic medium, the chitosan chain was hydrolyzed, which accelerated the diffusion of the drug. That is because the chitosan structure was considerably damaged after the modification with two large volumes of branching moleculars.

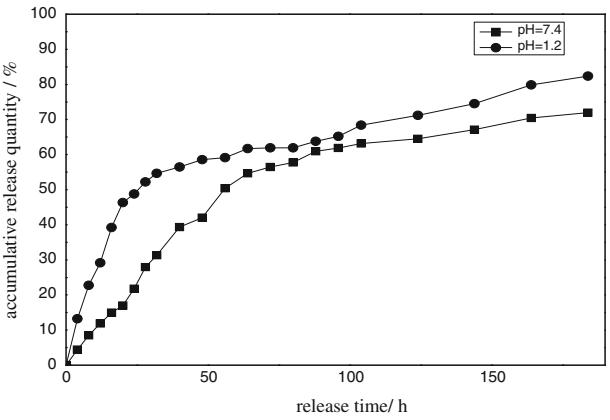



**Fig. 1** The in vitro release of (**6**)

## Conclusion

1) IR, $^{1}$H NMR, UV–Vis and LC/MS indicate successful synthesis of lysyl-5-Fu (2), ramose chitosan-lysyl-5-Fu(3), and ramose tetralactosyl-lysyl- chitosan-5-Fu(6).
2) The drug loading of ramose chitosan-lysyl-5-Fu(3) and ramose tetralactosyl-lysyl-chitosan-5-Fu(6) were 9.17 and 1.63% (w/w), respectively, which is determined by UV–Vis. On the other hand, we tested the behavior of the in vitro release in pH 7.4 PBS buffer solution and pH 1.2 HCl-KCl solution. The zero-order release time that compound (6) maintains in alkaline and acidic media were 64 and 24 h, and the total release by 184 h were 71.97 and 82.34%, respectively. The performance is smooth throughout the whole stage of release, and the concentration of cumulative release is lower in the alkaline environment than the acidic environment at the same time.

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