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Novel synthesis and in vitro drug release of polymeric prodrug: Chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate

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ABSTRACT

A novel approach to synthesize chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate was developed. Chitosan-d4T monophosphate prodrug with a phosphoramidate linkage was efficiently synthesized through Atherton-Todd reaction. In vitro drug release studies in pH 1.1 and 7.4 indicated that chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate prefers to release the d4T 5′-(*O*-isopropyl)monophosphate than free d4T for a prolonged period. The results suggested that chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate may be used as a sustained polymeric prodrug for improving therapy efficacy and reducing side effects in antiretroviral treatment.

Polymer–drug conjugates have attracted considerable attention in drug delivery due to their particular therapeutic properties, such as prolonged half-life, enhanced bioavailability, lower immunogenicity and antigenicity, and often targeting to specific cells, tissues or organs.[1–3] A number of natural or synthetic polymers have been investigated as polymeric vehicles to improve therapy efficacy of the drug. In particularly, chitosan, a well-known abundant natural polysaccharide mainly composed of 2-amino-2-deoxy-β-D-glucopyranose (D-glucosamine) residues, has been used extensively to deliver drugs and other biologically active components, such as peptides, proteins and oligonucleotides owing to its favorable biological properties such as low toxicity, biocompatibility and biodegradability.[4–6] In addition, various chemical conjugation approaches have been developed to form the suitable polymer–drug conjugates, most of them involve the formation of ester or amide linkages between drug and polymeric backbone by direct coupling reactions or by incorporation of a spacer arm, and carbodiimides are commonly used as zero lengths cross-linkers to form amide or phosphoramidate linkage between a carboxylate or phosphate and an amine. For example, antitumor macromolecular prodrugs of *N*-succinyl-chitosan–mitomycin C (MMC) conjugates with a succinic spacer have been prepared via carbodiimide coupling reaction.[7]

D4T (as known as Stavudine) is an oral medication that is used for the treatment of infections with the human immunodeficiency virus (HIV). It is in a class of drugs called reverse transcriptase inhibitors which also include zidovudine (AZT) and lamivudine (3TC). These drugs block the reverse transcriptase enzyme which changes HIV's RNA into the form of DNA. For instance, d4T is converted within the body to its active form (d4T triphosphate) which is similar to thymidine triphosphate, a chemical that is required by the HIV virus to make new DNA. As a nucleoside analog reverse transcriptase inhibitor like AZT and 3TC, d4T also has remarkable therapeutic activity for the treatment of Hepatitis B virus (HBV) and other DNA-virus infections. However, all these nucleoside analog reverse transcriptase inhibitors have undesirable adverse effects such as neutropenia, peripheral neuropathy and drug resistance.[8,9] To improve their antiviral efficacy and reduce side effects, Giammona et al.[10] reported the polymer–drug conjugate of PHEA-5′-*O*-succinylzidovudine with a prolonged duration of activity, and Chimalakonda et al.[11] synthesized the macromolecular prodrug of 3TC-dextran via carbodiimide coupling reaction for selective antiviral delivery to the liver. However, the nucleoside drugs control-released from the reported polymer-nucleoside prodrugs do not exert antiviral activity directly, but are successively phosphorylated to the corresponding mono-, di- and triphosphates by cellular kinases to terminate the viral DNA elongation, and for d4T the rate-limiting step in the intracellular metabolism is their

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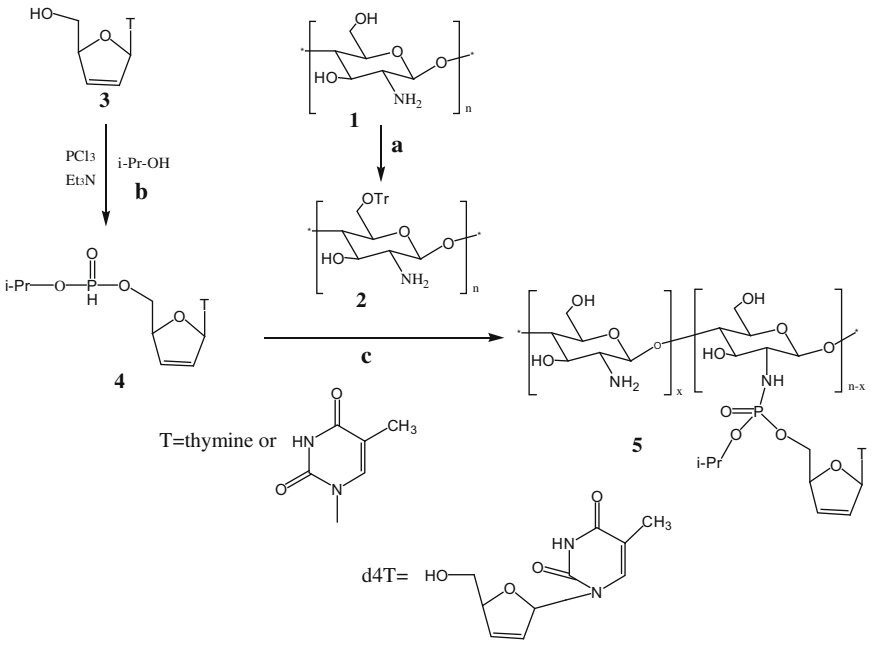
initial conversion to the 5′-*O*-monophosphate by nucleoside kinases and there is also a complicated regulatory mechanism involving positive or negative feedback.[12] On the other hand, many nucleoside phosphonate and phosphate prodrugs exhibited a remarkable antiviral activity since they can bypass the rate-limiting step of monophosphorylation, and have better membrane permeability resulting from the presence of the lipophilic group.[12–14] Sun et al.[15] reported the one-pot synthesis of asymmetrical *O*-alkyl-*H*-phosphonates of d4T, among them, *O*-isopropyl-5′-*H*-phosphonate of d4T is the most active for anti-HIV in clinical trials.[16] For these reasons, we describe a novel synthesis of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate prodrug with a phosphoramide linkage between glucosamine and nucleoside monophosphate through Atherton-Todd reaction under the mild condition and with reasonable degree of substitution, based on our previous work.[17] And the drug release behavior of obtained chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate in vitro have also been investigated.

General procedure for the preparation of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate **5** is shown in Scheme 1. Since Antherton-Todd reaction is an efficient method for the construction of phosphoramidates from H-phosphates under the mild conditions and with reasonable yield to our knowledge,[17,18] it was used to form a phosphoramidate linkage between d4T and chitosan instead of carbodiimide coupling reaction. Chitosan **1** with poor solubility in organic solvents was firstly modified into 6-*O*-triphenylmethy1 chitosan **2** according to Nishimura et al.,[19] and **2** with free amino group exhibited the excellent solubility in organic solvents such as DMA, pyridine, thus the further phosphorylation could be performed in homogeneous solution. While parent d4T **3** was modified to *O*-isopropyl-5′-*H*-phosphonate of d4T **4** using phosphorus trichloride as phosphorylation reagent according to the method reported by Sun et al.[15] The pure intermediate product **4** was obtained by silica gel chromatography (^{31}P NMR 8.62, 8.74 ppm). The following Antherton-Todd reaction of **4** with 6-*O*-trityl chitosan **2** in DMA with the help of Et_3N and CCl_4 under mild conditions yielded 6-*O*-trityl chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate, and the following removal of 6-*O*-triphenylmethy1 in formic acid led to chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate **5.** The pure product **5** was obtained after dialysis and lyophilization, and fully characterized by ^{31}P, ^{1}H and ^{13}C NMR spectra, and GPC (Column: Ultrahydrogel 250, Solvent: 0.1 M NaCl, 40 °C, Flow rate: 1.0 mL/min, Standards: PEO).[20] The degree of substitution (DS) of d4T monophosphate moiety was calculated by the amount ratio of H_{14} to H_2. The ^{1}H NMR and ^{13}C NMR spectra of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate **5** are shown in Figures 1 and 2, respectively. When the mole ratio of the *O*-isopropyl-5′-*H*-phosphonate of d4T **4** to trityl chitosan **2** was 10:1, the conjugate with M_w 26.8k and M_w/M_n 1.63 was obtained in 17.0% DS (corresponding to a content of d4T of 17.5% w/w) and 69% yield. Besides, the phosphorus content determination of the conjugate was performed by the ICP-AES (Atomic Emission Spectrometry) method, it was measured that the weight content of phosphorus was 2.5% corresponding to the content of d4T of 18.1% (w/w) and the d4T loading ratio (DS) of 17.6%. Comparing with the data of the amount of linked-drug and yield based on carbodiimide coupling reaction,[10,11] it's proved that Antherton-Todd reaction can provide an alternative approach for efficiently coupling nucleoside drugs to chitosan by forming a phosphoramidate linkage, although there exists the limitation of reactivity of H-phosphonate of d4T to trity1-chitosan resulted from steric hindrance of both chitosan and d4T group.

It's worth mentioning that polymeric conjugates with a phosphoramidate linkage between polymer backbone and drugs, peptides, proteins or other biologically active components could be also successfully constructed through Antherton-Todd reaction. The obtained chitosan phosphoramidate bioconjugates could be used in many biomedical applications such as drug/gene delivery systems because phosphorus is the indispensable and essential element in the human body and it can replace other linkers for the drug/gene delivery systems.[17]

In order to obtain some preliminary information about the potential use of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate **5** as a polymeric prodrug for antiretroviral treatment, the polymeric conjugate (DS = 17.0%) was subjected to in vitro hydrolysis studies in buffer solutions at pH 1.1 (simulated gastric juice)



Scheme 1. Synthetic route of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate. Reagents and conditions: (a) (i) phthalic anhydride, DMF, 130 °C, N_2, 8 h, 83%; (ii) TrCl, pyridine, 90 °C, N_2, 24 h, 86%; (iii)NH_2–NH_2· H_2O, 80 °C, N_2, 16 h, 81%; (b) (i) CH_2Cl_2, PCl_3, −30 °C, 2 h; (ii) rt, 6 h, (iii) *i*-Pr–OH, 0 °C, 2 h. (c) (i) 2, Et_3N, CCl_4, DMA, 0 °C, 24 h; (ii) HCOOH, rt, 0.5 h.

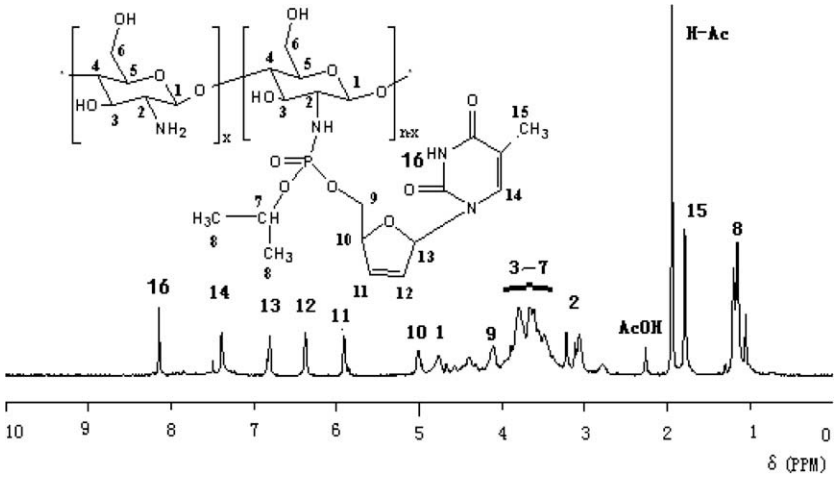


Figure 1. ^{1}H NMR spectra of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (DS = 17.0%) in 2% D_3CCOOD/D_2O at 293 K.

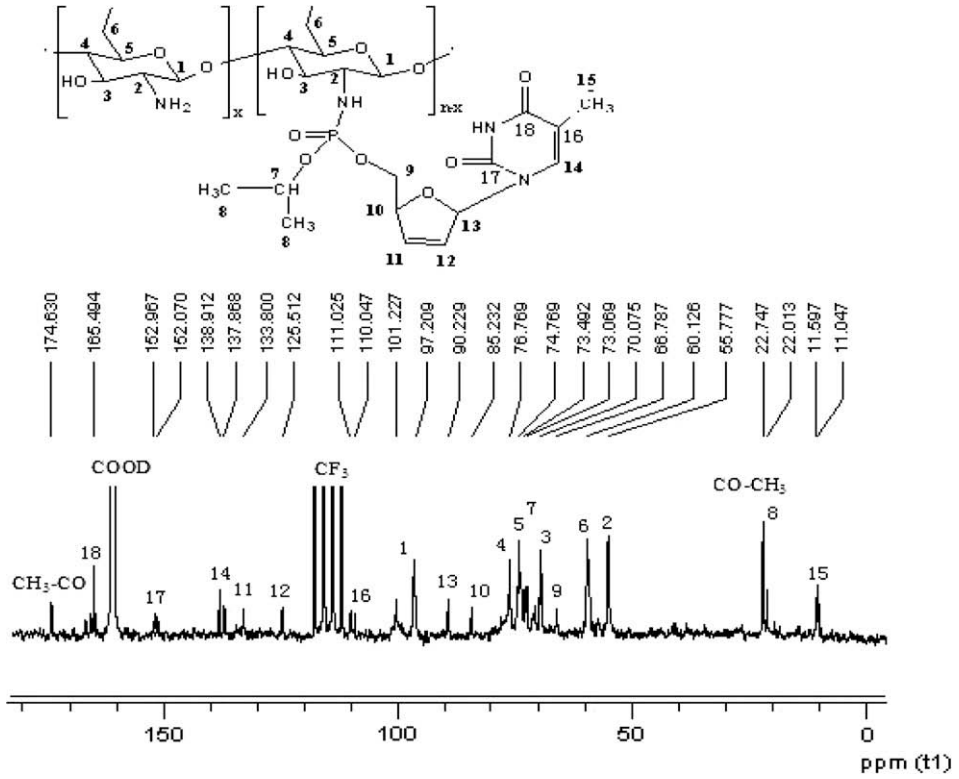


Figure 2. ^{13}C NMR spectra of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (DS = 17.0%) in 5% CF_3COOD /D_2O at 293 K.

and pH 7.4 (extracellular fluids), and the results of drug release were determined by HPLC[21] and shown in Figures 3 and 4, respectively. As can be seen, a major amount of d4T monophosphate derivative with a little amount of free d4T were released from chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate under used experimental conditions for a prolonged manner. From the hydrolysis profile at pH 1.1, about 6.84% of *O*-isopropyl-5′-*O*-d4T monophosphate was released from the polymeric conjugate within 6 h with a starting rate of 2.14%/h, while only 1.24% of free d4T was detected with a starting rate of 0.34%/h. As for the hydrolysis at pH 7.4, approximately 7.27% of *O*-isopropyl-5′-*O*-d4T monophosphate was released from the polymeric conjugate within 24 h with a starting rate of 1.09%/h, whereas about 1.36% of free d4T was detected with a starting rate of 0.29%/h. As comparison, hydrolysis investigations at pH 1.1 and 7.4 had been also carried out on *O*-isopropyl-5′-*O*-d4T monophosphate under the same experimental conditions used for the polymeric conjugates. The appearance of free d4T was not observed, which indicated *O*-isopropyl-5′-*O*-d4T monophosphate was not cleaved to release free d4T at least during the period of the experiment. The results suggested that the phosphoramidate bond between chitosan and *O*-isopropyl-5′-*O*-d4T monophosphate has higher hydrolytic activity than *O*-isopropyl-5′-*O*-d4T monophosphate, and the chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate prefers to release *O*-isopropyl-5′-*O*-d4T monophosphate than free d4T. Previous research[22–24] have already reported the hydrolysis mechanism of the similar phosphoramidate derivatives of d4T and the conversion of d4T monophosphate to d4T with a rational explanation. Refer to our

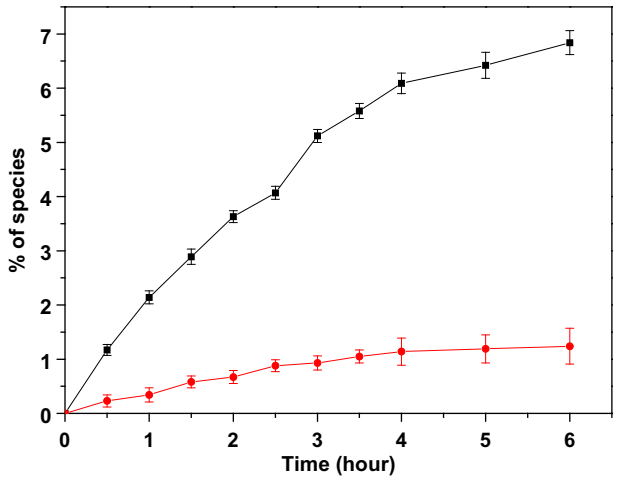


Figure 3. Release of d4T 5′-(*O*-isopropyl)monophosphate derivative (■) and d4T (●) from chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (DS = 17.0%) in pH 1.1 buffer solution at 37.0 ± 0.1 °C.

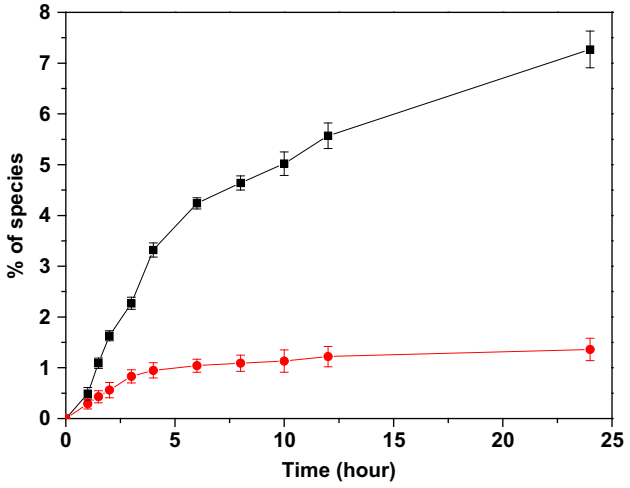


Figure 4. Release of d4T 5′-(*O*-isopropyl)monophosphate derivative (■) and d4T (●) from chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (DS = 17.0%) in pH 7.4 buffer solution at 37.0 ± 0.1 °C.

work, the P–N bond and 5′-O–P bond have cleaved under both acidic and neutral conditions, but only 1.36% of d4T was released in 24 h under neutral conditions and it can be considered as byproduct compared with 7.27% of d4T 5′-(*O*-isopropyl) monophosphate which was released from conjugate as major product. Since the antiretroviral activity of d4T may be more critically dependent upon the rate-limiting step of its conversion to the 5′-*O*-monophosphate by nucleoside kinases in the intracellular metabolism, a delayed release of the d4T 5′-(*O*-isopropyl) monophosphate from the chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate was able to bypass the rate-limiting step of monophosphorylation to improve therapy efficacy and reduce side effects. Further studies on the polymer-nucleoside monophosphate nano-prodrugs formed by self-assembly, their chemical stability and bioavailability in vitro and in vivo are in progress.

In summary, a novel and efficient synthesis method of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate was developed through Atherton-Todd reaction under mild conditions. This method is also suitable for synthesis of other polymer conjugates with a phosphoramide linkage between polymer backbone and biologically active components. In vitro drug release studies in pH 1.1 and 7.4 indicated the polymer prodrug can provide a sustained release of *O*-isopropyl-5′-*O*-d4T monophosphate in the blood circulation. Further understanding of in vitro and in vivo biological activities of this kind of polymer-nucleoside monophosphate prodrugs with a prolonged release period could lead to their applications in antiretroviral treatment.

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20. *Typical procedure for the synthesis of chitosan–O-isopropyl-5′-O-d4T monophosphate conjugate* **5**: *O*-isopropyl-5′-*H*-phosphonate of d4T **4** (1.32 g, 4 mmol) was dissolved in 10 mL dimethylacetamide (DMA), the solution was added dropwise to 6-*O*-trityl chitosan (160 mg, ~0.40 mmol of free NH_2) in a mixed solution of DMA (10 ml), triethylamine (1.76 ml) and tetrachloromethane (2 ml) in ice-water bath. After a 24 h stirring, the solution was filtered. The filtrate was added to EtOH (200 mL) and the precipitate formed was collected by centrifugation. Then the precipitate was dissolved in 10 mL of formic acid and was stirred for 0.5 h at room temperature. After removing formic acid by rotary evaporation, the residue was dissolved in 2% AcOH (20 mL), and then filtered. The filtrate was dialyzed with distilled water for 3 days and lyophilized to provide the target product, chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate **5** (69 mg).^{31}P NMR at 293 K (2% CD_3COOD/D_2O): δ = 8.09, 8.27 (–NH–P–); ^{1}H NMR at 293 K (2% CD_3COOD/D_2O): δ = 1.15, 1.20 (d, H_8), 1.79 (s, H_{15}), 3.07, 3.12 (s, H_2), 3.49–3.89 (m, H_3, H_4, H_5, H_6, H_7), 4.10 (br, H_9), 4.77 (br, H_1), 5.02 (s, H_{10}), 5.91 (s, H_{11}), 6.38 (s, H_{12}), 6.80 (s, H_{13}), 7.39 (s, H_{14}), 8.15 (s, H_{16}); ^{13}C NMR at 293 K (5% CF_3COOD/D_2O): δ = 11.6 (C_{15}), 22 (C_8), 22.7 (CO–CH_3), 55.8 (C_2), 60.1 (C_6), 66.8 (C_9), 70 (C_3), 73.5 (C_7), 74.8 (C_5), 76.8 (C_4), 85.2 (C_{10}), 90.2 (C_{13}), 97.2, 101.2 (C_1), 111 (C_{16}), 125.5 (C_{12}), 133.8 (C_{11}), 137.8, 138.9 (C_{14}), 152 (C_{17}), 165.5 (C_{18}), 174.6 (CH_3–CO).
21. Drug release studies. The drug release behaviors of chitosan–*O*-isopropyl-5′-*O*-d4T monophosphate conjugate were studied in buffer solutions at pH 1.1 (HCl, NaCl and glycine) and pH 7.4 (Na_2HPO_4, KH_2PO_4, NaCl). Equal known aliquots of adduct were dissolved in equal volumes of preheated buffer solutions at each different pH values, shaken in a shaking water bath at 37.0 ± 0.1 °C and sampled at predetermined intervals. Each sample was directly analyzed by HPLC (Detection wavelength: 263 nm, Analytical RP-18 Lichrospher column, Eluent: CH_3OH: 0.1% H_3PO_4 (20:80 v/v), Flow rate: 1.0 mL/min) monitoring the released amount of d4T moieties, including d4T and d4T monophosphate derivative. Each experiment was repeated in triplicate.
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