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Synthesis, nanosizing and in vitro drug release of a novel anti-HIV polymeric prodrug: Chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate

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ABSTRACT

A novel approach to improve the antiviral efficacy of nucleoside reverse transcriptase inhibitors (NRTIs) and reduce their side effects was developed by constructing a nanosized NRTI monophosphate-polymer conjugate using d4T as a model NRTI. Firstly, a novel chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate with a phosphoramidate linkage was efficiently synthesized through Atherton–Todd reaction under mild conditions. The anti-HIV activity and cytotoxicity of the polymeric conjugate were evaluated in MT4 cell line. Then the conjugate nanoparticles were prepared by the process of ionotropic gelation between TPP and chitosan-d4T conjugate to improve their delivery to viral reservoirs, and their physicochemical properties were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS) techniques and X-ray diffraction (XRD). In vitro drug release studies in pH 1.1 and pH 7.4 suggested that both chitosan-d4T conjugate and its nanoparticles prefer to release d4T 5′-(*O*-isopropyl) monophosphate than free d4T for prolonged periods, which resulted in the enhancement of anti-HIV selectivity of the polymeric conjugate relative to free d4T due to bypassing the metabolic bottleneck of monophosphorylation. Additionally, the crosslinked conjugate nanoparticles can prevent the coupled drug from leaking out of the nanoparticles before entering the target viral reservoirs and provide a mild sustained release of d4T 5′-(*O*-isopropyl) monophosphate without the burst release. The results suggested that this kind of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nano-prodrugs may be used as a targeting and sustained polymeric prodrugs for improving therapy efficacy and reducing side effects in antiretroviral treatment.

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) such as zidovudine (AZT), didanosine (ddI), lamivudine (3TC) and stavudine (d4T) have been widely used in the treatment of human immunodeficiency virus (HIV) infection. To exert their antiviral activity, NRTIs are activated by cellular kinases to finally form the corresponding 5′-triphosphate derivatives, which can efficiently terminate intracellular synthesis of the viral DNA from its RNA by competing with natural nucleotides as a substrate for reverse transcriptase in infected cells.[1–3] Although NRTIs have successfully extended the lifespan of millions of HIV-infected patients throughout the world in the past decades, these NRTIs are still far from ideal for clinic use at the present time, since some undesirable adverse effects such as neutropenia, peripheral neuropathy and drug resistance are associated with them.[4,5] Besides, the frequent administration of NRTIs in relatively high doses also results in HIV-patient incompliance.[6]

A number of methods have been developed to improve the antiviral efficacy of NRTIs and reduce their side effects based on the consideration of bypassing the metabolic bottleneck, improving cellular uptake efficacy, and targeting viral reservoirs, etc.[6–8] For instance, it was reported that the rate-limiting step for the intracellular generation of the bioactive metabolite of d4T, d4T-triphosphate, seems to be the conversion of d4T to its monophosphate derivative by nucleoside kinases. Accordingly, aryl phosphoramidate derivatives of d4T exhibited enhanced antiviral activity and selectivity since they can bypass the rate-limiting step of monophosphorylation, and have better membrane permeability resulting from the presence of the lipophilic group.[9,10]

Except developing novel nucleoside analogue inhibitors of the reverse transcriptase by chemical modification, the design of drug delivery systems (DDS) for NRTIs has been proven to be an efficient approach to overcome the drawbacks of NRTIs, such as limited stability, first pass metabolism and systemic toxicity.[6] For instance, drug-polymer conjugates as a special type of DDS have attracted considerable attention due to their particular therapeutic

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properties, such as prolonged half-life, enhanced bioavailability, and often targeting to specific cells, tissues or organs by attaching a homing device.[11] G. Giammona et al. reported the polymer-drug conjugate of PHEA-5′-*O*-succinylzidovudine with a prolonged duration of activity,[12] and K. C. Chimalakonda et al. synthesized the macromolecular prodrug of 3TC-dextran for selective antiviral delivery to the liver.[13] On the other hand, nanosized DDS is a very promising way of improving drug bioavailability.[14] Enhanced delivery and prolonged residence of NRTIs in monocytes/macrophages, central nervous system (CNS) and the gastrointestinal tract could be observed for nanoparticles or nanocapsules containing NRTIs.[6,15–17]

In this paper, we describe a combined strategy to improve the antiviral efficacy of NRTIs and reduce their side effects by constructing a nanosized NRTI monophosphate-polymer conjugate using d4T as a model drug. Chitosan, a well-known abundant natural polysaccharide mainly composed of 2-amino-2-deoxy-β-D-glucopyranose (D-glucosamine) residues was chosen as the polymeric vehicle since it is biodegradable, biocompatible, nontoxic, and easy to form nanoparticles.[18,19] Firstly, a novel chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate prodrug with a phosphoramide linkage between glucosamine and nucleoside monophosphate was synthesized through Atherton–Todd reaction under the mild conditions based on our previous work.[20,21] The anti-HIV activity and cytotoxicity of the polymeric conjugate were evaluated in MT4 cell line. Further, chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles prepared by ionically crosslinking with tripolyphosphate (TPP),[22] which could be used as a sustained polymeric nano-drug targeting to viral reservoirs. The drug release characteristics of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate and nanoparticles have also been investigated in vitro.

2. Results and discussion

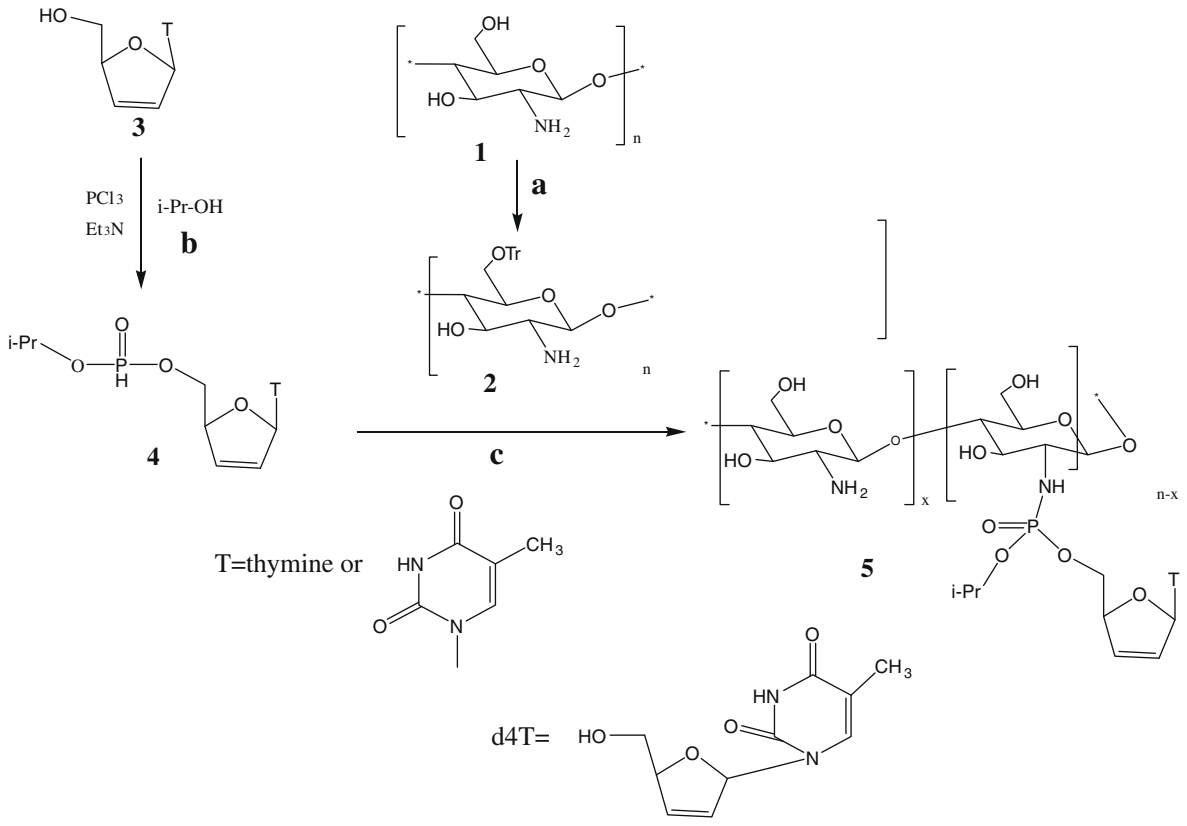
2.1. Synthesis and characterization of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate

A novel polymeric prodrug: chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate was efficiently synthesized through Atherton–Todd reaction under mild conditions (Scheme 1). The ^{1}H NMR and ^{13}C NMR spectra of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate have been shown in our previous work.[21] The degree of substitution (DS) of d4T monophosphate moiety was estimated by ^{1}H NMR spectra and confirmed by phosphorus content determination based on the ICP-AES method, was equal to 17.0% (corresponding to a content of d4T of 17.5% w/w).[21]

The results suggested 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 Cs-Tr resulted from steric hindrance of both chitosan and d4T group. It's also worth notice that various polymer phosphoramidate bioconjugates with drugs, peptides, proteins or other biologically active components could be also successfully constructed through Antherton–Todd reaction.

2.2. Anti-HIV activity and cytotoxicity of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate

The antiviral activity of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate and the parent nucleoside d4T was evaluated against HIV-1 in MT4 cells and summarized in Table 1. The polymeric conjugate inhibited virus infection with an IC_{50}



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.

(concentration required to reduce virus replication by 50% in culture assays) of 0.027 μg/ml, which was approximately 13-fold less potent than d4T (IC_{50} value of 0.002 μg/ml) against HIV-1 in culture assays. However, as the conjugate was much less toxic than d4T (CC_{50} (concentration required to inhibit cell growth by 50%) values of 658.6 and 30 μg/ml for the conjugate and d4T, respectively), the overall selectivity index (SI) for the conjugate (CC_{50}/IC_{50}) is superior to that of d4T. It is indicated that the synthesized chitosan-d4T conjugate prodrug with a phosphoramide linkage has remarkable anti-HIV activity with rather low cytotoxicity. The results might be ascribed to the sustained release of d4T 5′-(*O*-isopropyl) monophosphate from the conjugate, which can bypass the rate-limiting step of nucleoside phosphorylation and have better membrane permeability resulting from the presence of the lipophilic group.[21]

2.3. Preparation and characterization of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles

Although the synthesized water-soluble chitosan-d4T conjugate can achieve a good approach to control drug concentration in plasma and to improve the retention of the drug in the body by sustained-releasing d4T 5′-(*O*-isopropyl) monophosphate, it has restricted access to the viral reservoirs, such as monocytes/macrophages, the brain and the gastrointestinal tract. Thus, chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles were prepared by ionically crosslinking with TPP to enhance their delivery to the viral reservoirs. Additionally, d4T-loaded chitosan nanoparticles were also prepared by the same method for comparison.

The formation of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles occurs spontaneously upon incorporation negatively charged TPP into chitosan derivative (DS = 17.0%) solution due to the complexation between oppositely charged species.[19,22] The formation process of complexes is very simple and mild, avoiding the possible toxicity of chemical reagents and other undesirable effects. As shown in Figure 1(a), the conjugate nanoparticles appeared spherical shape with the diameter ranged between 100 and 200 nm, while Figure 1(b) illustrated that d4T-loaded chitosan nanoparticles formed under the same conditions had similar spherical structure but particle size smaller than 90 nm.

Table 2 exhibited particle size, zeta potential and drug loading capacity of chitosan-d4T conjugate nanoparticles and d4T-loaded chitosan nanoparticles. The results demonstrated that chitosan-d4T conjugate nanoparticles had a higher LC value than d4T-loaded chitosan nanoparticles under the conditions used. It is also noteworthy that hydrodynamic diameter of the nanoparticles measured by DLS was larger than the size estimated from TEM, since chitosan-based nanoparticles dispersed in an aqueous phase for the DLS experiments had high swelling capacity, while the TEM experiments were performed in dry samples. Though chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles and d4T-loaded chitosan nanoparticles were formed by the same complexation mechanism mainly based on the interaction between $-NH_3^+$ of chitosan and TPP, chemical modification of chitosan would greatly affected the size and surface properties of the obtained nanoparticles. It could be seen that chitosan-d4T conjugate nanoparticles with larger diameter had a positive zeta potential equal to ~17 mV, while d4T-loaded chitosan nanoparticles with smaller size had about 47 mV zeta potential. In comparison with the zeta potential of the blank chitosan/TPP nanoparticles reported about 30–50 mV,[26] the formation of phosphoramidate linkage of the chitosan-d4T conjugate resulted in decrease of the zeta potential value of nanoparticles.

Moreover, in order to identify the physical state and crystallinity of d4T in polymeric conjugate or crosslinked nanoparticles, the XRD spectra of chitosan, chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (DS = 17.0%), chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles and d4T-loaded chitosan nanoparticles are presented in Figure 2, and the XRD pattern of pure d4T is shown in Figure 3. As can be seen, the original chitosan powder showed two major broad crystalline peaks at 2θ of around 10.0° and 19.8°, respectively. While the diffraction peaks of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate were not recorded at the same position. The peak at 2θ of around 10.0° disappeared and instead new peaks at 2θ of 23.6°, 26.3° and 35.6° with low intensity could be observed. These new peaks were

Table 1
Anti-HIV activity and cytotoxicity of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate

Compound	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (DS = 17.0%)	0.027	658.6	24,393
d4T	0.002	30	15,000
d4T 5′-(*O*-Isopropyl) monophosphate	0.0008	104	130,000

Table 2
Physicochemical characteristics of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles and d4T-loaded chitosan nanoparticles

Nanoparticles	Particle size* (nm)	Polydispersity index*	Zeta potential* (mV)	LC (%)
Chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles	228.9	0.273	+17.23	13.1
d4T-Loaded chitosan nanoparticles	166.8	0.297	+46.75	4.63

* Data measured by DLS.

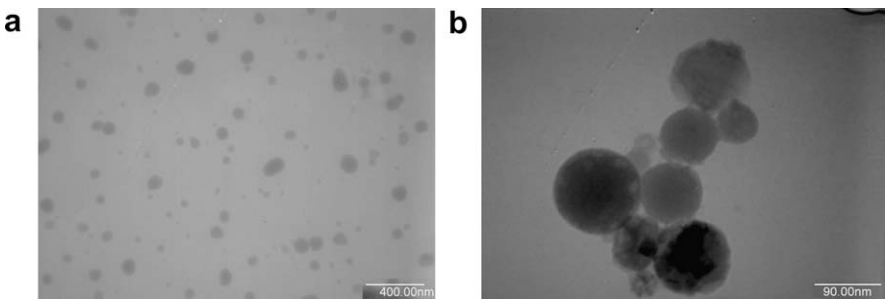


Figure 1. Transmission electron microscopy (TEM) micrographs of nanoparticles: (a) chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles, and (b) d4T-loaded chitosan nanoparticles.

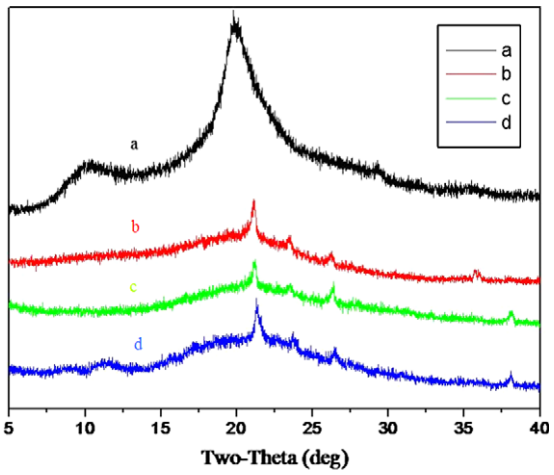


Figure 2. The XRD spectra of (a) chitosan, (b) chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate, (c) chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles, and (d) d4T-loaded chitosan nanoparticles.

also obvious in the chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles except the peak at $2\theta = 35.6°$ shifted to 38.1°. These new peaks may be attributed to a polymorph structure transformation when the initial crystal form in not thermodynamically stable and transform to the most stable form due to the attachment of d4T 5′-(*O*-isopropyl) monophosphate to chitosan through a phosphoramidate linkage. In addition, the relative intensity and position of XRD peaks of d4T-loaded chitosan nanoparticles were quite similar with those of the conjugate nanoparticles, but different from the simple addition of those of chitosan and d4T, which indicated that the crystal form of loaded d4T was also affected by the chitosan nanoparticles matrix.

2.4. In vitro release studies

In order to obtain some preliminary information about the potential use of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (DS = 17.0%) and its nanoparticles for antiretroviral treatment, the polymeric conjugate and its nanoparticles were subjected to in vitro hydrolysis studies in buffer solutions at pH 1.1 (simulated gastric juice) and pH 7.4 (extracellular fluids). The results of drug release were determined by HPLC and shown in Figures 4 and 5, respectively. In addition, the study of d4T releasing

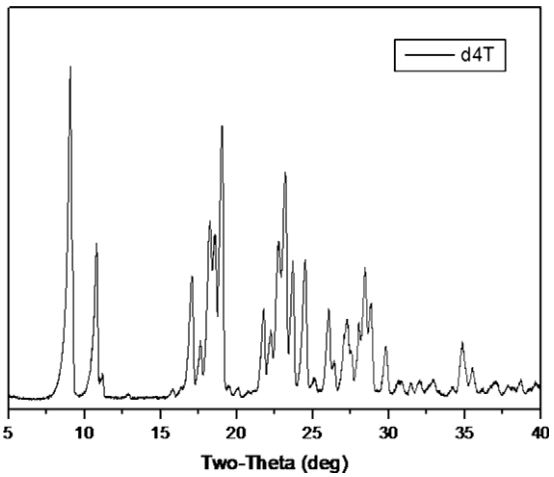


Figure 3. The XRD spectra of d4T.

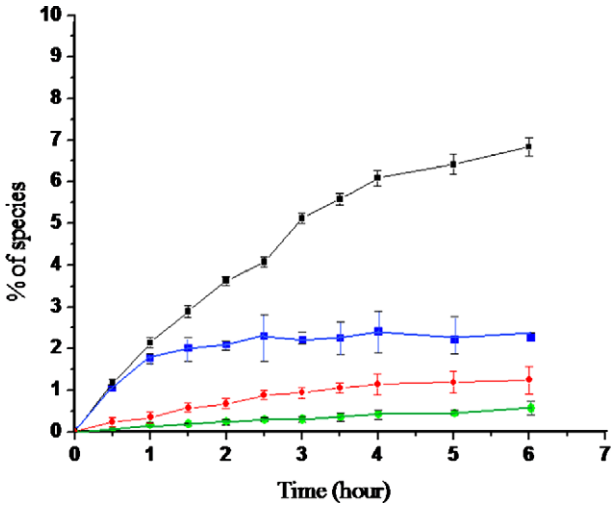


Figure 4. Release of d4T 5′-(*O*-isopropyl) monophosphate (■), d4T (●) from chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate and d4T 5′-(*O*-isopropyl) monophosphate (■), d4T (●) from chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles at pH 1.1 buffer solution at 37.0 ± 0.1 °C.

from d4T-loaded nanoparticles was also investigated for comparison (shown in Fig. 6).

It could be seen that both d4T monophosphate derivative and free d4T were released from chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate under used experimental conditions for a prolonged manner, and the amount of d4T 5′-(*O*-isopropyl) monophosphate was greater than that of free d4T. From the hydrolysis profile at pH 1.1, the starting hydrolysis rate of d4T 5′-(*O*-isopropyl) monophosphate and d4T from the polymeric conjugate were calculated to be 2.14%/h and 0.34%/h, respectively, and after 6 h about 6.84% of d4T 5′-(*O*-isopropyl) monophosphate and only 1.24% of free d4T were released. As for the hydrolysis at pH 7.4, approximately 7.27% of d4T 5′-(*O*-isopropyl) 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. These results confirmed that the phosphoramidate bond between chitosan and d4T 5′-(*O*-isopropyl) monophosphate has higher hydrolytic activity than d4T 5′-(*O*-isopropyl) monophosphate, and the chitosan-*O*-isopropyl-5′-*O*-d4T

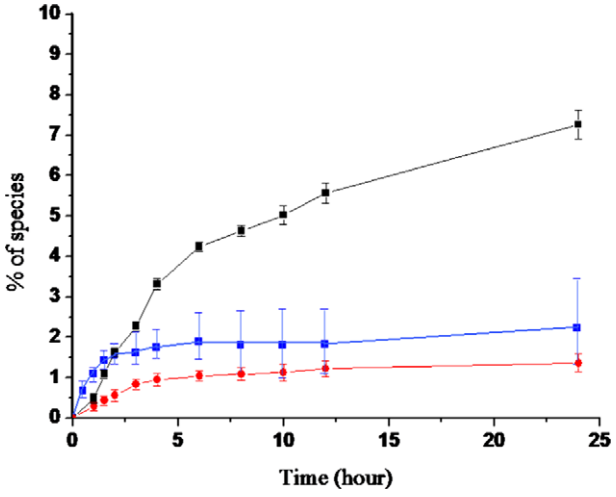


Figure 5. Release of d4T 5′-(*O*-isopropyl) monophosphate (■), d4T (●) from chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate and d4T 5′-(*O*-isopropyl) monophosphate (■) from chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles at pH 7.4 buffer solution at 37.0 ± 0.1 °C.

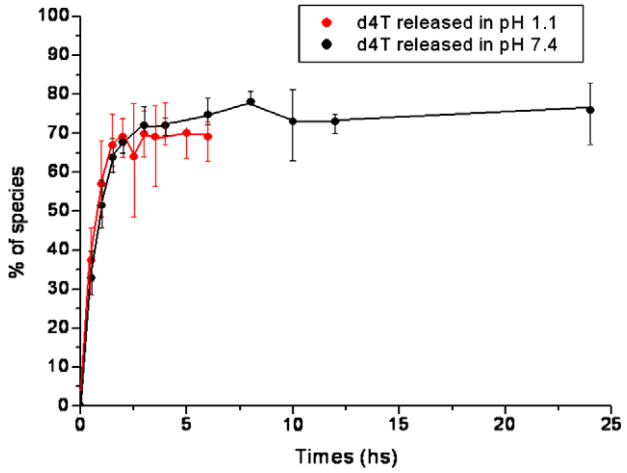


Figure 6. Release of d4T from d4T-loaded chitosan nanoparticles at pH 1.1 (●) and pH 7.4 (●) buffer solutions at 37.0 ± 0.1 °C.

monophosphate conjugate prefers to release d4T 5′-(*O*-isopropyl) monophosphate than free d4T. Thus, d4T 5′-(*O*-isopropyl) monophosphate could be considered as the major product released from the polymeric conjugate under both acidic and neutral conditions compared with little amount of free d4T (as shown in Fig. 7). Since the antiretroviral activity of nucleoside analogues may be more critically dependent upon the rate-limiting step of their 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 conjugate was able to bypass the rate-limiting step of monophosphorylation to improve therapy efficacy and reduce side effects.

As for the conjugate nanoparticles, it could be seen that at pH 1.1 both d4T 5′-(*O*-isopropyl) monophosphate and d4T were released with quite mild releasing rate compared with non-crosslinked chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate. The total released amount of d4T 5′-(*O*-isopropyl) monophosphate or d4T from the conjugate nanoparticles was less than half of released amount from the non-crosslinked conjugate within 6 h. However, approximately 2.22% of d4T 5′-(*O*-isopropyl) monophosphate was released from the conjugate nanoparticles within 24 h, whereas free d4T was hardly detected at pH 7.4. The results suggested that chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate can be protected from hydrolysis by forming the crosslinked conjugate nanoparticles, and confirmed the capacity of the conjugate nanoparticles to mainly release d4T 5′-(*O*-isopropyl) monophosphate, which was prevented from leaking out of the nanoparticles before entering the viral reservoirs.

By comparison, the d4T-loaded nanoparticles exhibited a releasing profile with two stages of an initial burst release period followed by a period of slower release, as shown in Figure 6. Approximately 65% of the drug content released in the initial period of burst release for 2 h both at pH 1.1 and at pH 7.4, which associated with the dissolution of drug adsorbed or located close to the surface of nanoparticles. Later the rate of release fell as the dominant mechanism changed to diffusion through the swollen rubbery chitosan matrix. Comparing data of the d4T-loaded nanoparticles, the crosslinked conjugate nanoparticles could avoid the burst release effect to provide a quite mild release rate. These results suggested that the drug release from the conjugate nanoparticles may involve three steps. First, water penetrates into the particulate system, which causes swelling of the matrix; second, a major amount of d4T monophosphate derivative with a little amount of free d4T are released from polymer backbone due to cleavage of chemical bond; the third step is the diffusion of drug from the swollen rubbery matrix.[19] It should be noticed that the bond cleavage step is the rate-limiting step in this controlled release process. Therefore, the conjugate nanoparticles with the capacity of targeting the viral reservoirs can provide a mild and gentle sustained release of d4T 5′-(*O*-isopropyl) monophosphate without the burst release.

3. Conclusion

A novel water-soluble chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate with DS value up to 17.0% was efficiently synthesized through Atherton–Todd reaction under mild conditions. The anti-HIV activity and cytotoxicity of the polymeric conjugate evaluated in MT4 cell line exhibited that the resulting polymeric conjugate has remarkable anti-HIV effect and rather low cytotoxicity. In vitro drug release studies at pH 1.1 and pH 7.4 indicated the polymeric conjugate prodrug prefers to release the d4T 5′-(*O*-isopropyl) monophosphate than free d4T for a prolonged period. This may lead to the enhancement of anti-HIV selectivity of the polymeric conjugate relative to the parent nucleoside analogue owing to the effect of bypassing the rate-limiting step of monophosphorylation mediated by thymidine kinase.

Aiming to enhance the delivery to viral reservoirs of HIV, chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparti-

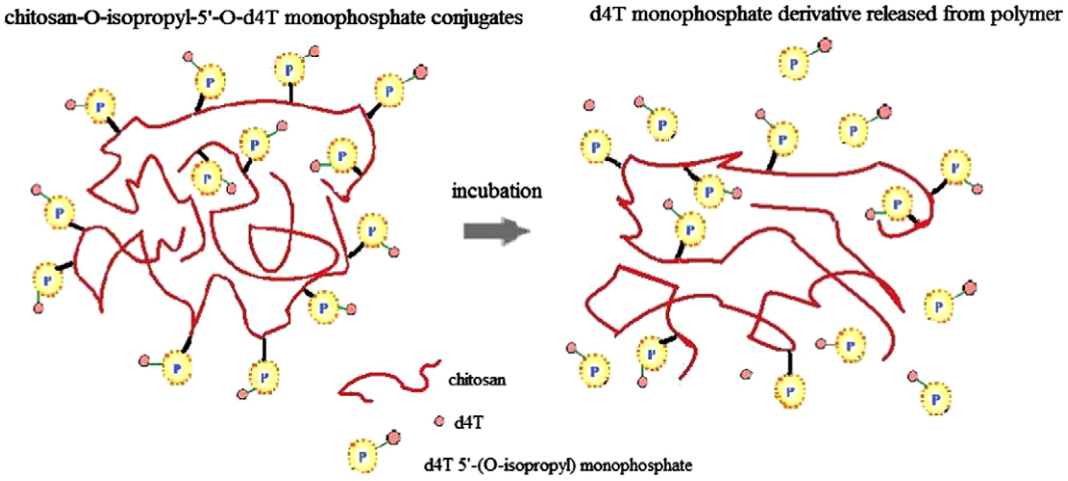


Figure 7. Schematic representation of drug release from polymeric conjugate.

cles were prepared by the process of ionotropic gelation using TPP. Comparing with the data of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate and d4T-loaded nanoparticles, in vitro drug release studies revealed that the crosslinked conjugate nanoparticles can prevent the coupled drug from leaking out of the nanoparticles in blood circulation and provide a mild sustained release of d4T 5′-(*O*-isopropyl) monophosphate without the burst release. Further research on in vitro and in vivo biological activities of this kind of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nano-prodrugs could lead to their applications in antiretroviral treatment.

4. Experimental

Chitosan (low molecular weight) was purchased from Sigma–Aldrich Chemicals Co, USA. Deacetylation degree of 88% was determined by ^{1}H NMR. 6-*O*-trity1 chitosan was firstly synthesized from chitosan in three steps, *N*-phthaloylation, 6-*O*-trityl protection and de-phthaloylation, according to the known procedure reported by Nishimura et al.[23] D4T was purchased from Yingjie Biotechnology Company (China), and dried at elevated temperature in vacuum. Triethylamine, tetrachloromethane and sodium tripolyphosphate (TPP) were purchased from Beijing Chemical Reagents Company (China). All other solvents were commercially available reagents of analytical grade, dried and purified by distillation before using. The chemical structures of the synthesized chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate were analyzed by ^{31}P, ^{1}H NMR in 2% (v/v) CD_3COOD/D_2O and ^{13}C NMR in 5% (v/v) CF_3COOD/D_2O at 600 MHz using a Bruker AV600 NMR instrument. Besides, the phosphorus content determination of the conjugate was performed by the ICP-AES (Atomic Emission Spectrometry, JY ULTIMA, France) method, thus the corresponding content of d4T was obtained. The *Mw* and *Mw*/*Mn* of the polymeric conjugate were measured by GPC (Waters 515-410, Column: Ultrahydrogel 250, Solvent: 0.1 M NaCl, 40 °C, Flow rate: 1.0 mL/min, Standards: PEO).

4.1. Synthesis of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate

The chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate was prepared as described in a previous study.[21] All experiments involving water-sensitive compounds were conducted under dry conditions. Briefly, *O*-isopropyl-5′-H-phosphonate of d4T was synthesized by using phosphorus trichloride as phosphorylation reagent according to the method reported by X. B. Sun et al.[24] 1.32 g (4 mmol) of *O*-isopropyl-5′-*H*-phosphonate of d4T 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 water-soluble target product, chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate (69 mg). ^{31}P NMR (263 K, 2% CD_3COOD/D_2O): δ = 8.09, 8.27 (–NH–P–); ^{1}H NMR (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 (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). *Mw* = 26.8 k, *Mw*/*Mn* = 1.63.

4.2. In vitro assays of anti-HIV activity and cytotoxicity of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate

MT4 cells, a human T4-positive cell line were infected with HIV-1 at the multiplicity (MOI) of 0.1, and HIV-infected MT4 cells were incubated for 1.5 h at 37 °C in a 5% CO_2 incubator. Subsequently, cells were cultured in 96-well microtiter plates in the presence of various concentrations of conjugate and aliquots of culture supernatants were removed from the wells on the fifth day after infection for p24 antigen assays. The applied p24 enzyme immunoassay (EIA) was the unmodified kinetic which utilizes a murine mAb to HIV core protein coated onto microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells. The 50% inhibition concentration (IC_{50}) was determined from the concentration–response curve using the median effect method.

Cytotoxicity of the conjugate was evaluated in parallel with antiviral activity using the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method. It was based on the viability of mock-infected cells, as monitored by the MTT method. The 50% cytotoxic concentration (CC_{50}) was determined from the concentration–response curve using the median effect method.

4.3. Preparation of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles

Chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles were prepared according to the procedure reported by Calvo et al. based on the ionic gelation of chitosan with TPP polyanions.[25,26] Briefly, 30 mg of the synthesized polymer-d4T conjugate was dissolved in 2% acetic aqueous solution at 1 mg/ml. Under magnetic stirring at room temperature, 10 mg TPP dissolved in 13 ml aqueous solution was added dropwise into the conjugate solution, and stirred continuously for 1 h to stabilize the nanoparticles through the electrostatic interaction with TPP. Then the suspension of nanoparticles dialyzed against deionized water and passed through a syringe filter (pore size 0.45 μm) prior to lyophilization. The drug loading capacity (LC) of the conjugate nanoparticles was calculated from the content of d4T coupled to chitosan.

For comparison, d4T-loaded chitosan nanoparticles were also prepared by premixing d4T with chitosan acetic aqueous solution at weight content of 18% and then following the above procedure at the same chitosan to TPP weight ratio of 3:1. The drug loading capacity (LC) of d4T-loaded chitosan nanoparticles was determined by the separation of nanoparticles from the aqueous medium containing non-associated d4T by centrifugation at 16,000 rpm for 30 min. The amount of free d4T in the supernatant was measured by HPLC. Each experiment was performed in triplicate. The LC value was calculated from (Eq. 1), which indicated below:

$$LC = (A - B)/C \times 100\% \quad (1)$$

A is the total amount of drug, B is the amount of free drug in supernatant, and C is the weight of nanoparticles.

4.4. Physicochemical characterization of nanoparticles

For measurement of particle size, zeta potential and polydispersity (size distribution) of freshly prepared chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate nanoparticles and d4T-loaded chitosan nanoparticles, Zetaplus (Brookhaven, USA) was used which is based on the Dynamic Light Scattering (DLS) techniques. All DLS measurements were done with a wavelength of 633 nm

at 25 °C with an angle detection of 90°. For zeta potential measurements, samples were diluted with 0.1 mM KCl and measured in the automatic mode.

Morphological characteristics of the nanoparticles were observed by Transmission Electron Microscope (TEM, Hitachi, H-800, Japan). One drop of freshly made nanoparticles solution was placed on 300 mesh copper grids coated with carbon film, allowing to sitting until dried. The physical form (crystalline or amorphous) of the lyophilized samples was determined by X-ray diffraction (XRD) over the range 2θ from 5° to 40°, a Rigaku *D*/max 2500 diffractometer (Japan) with Bragg–Brentano geometry (θ, 2θ) and Ni-filtered Cu Ka radiation.

4.5. In vitro drug release studies

In vitro release studies of chitosan-*O*-isopropyl-5′-*O*-d4T monophosphate conjugate, conjugate nanoparticles and d4T-loaded chitosan nanoparticles were carried out 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 samples were suspended in equal volumes of preheated buffer solutions at each different value, 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 product, and confirmed the compounds by ESI-MS.[12] Each experiment was repeated in triplicate.

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