Synthesis and Chemical Characterization of a Polymeric Prodrug for Prolonged Release of Nitrofurazone

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Summary: Chitosan, a natural polysaccharide obtained from partial or total deacetylation of chitin, is a linear copolymer composed of β(1→4) linked *N*-acetyl glucosamine and glucosamine monomer residues. Owing to its biological properties (such as biocompatibility, low toxicity, antimicrobial activity and haemostatic effect) chitosan has been widely exploited in the pharmaceutical industry to produce tablets, gels, nanoparticles and films. The aim of this study was to develop a polymeric prodrug for prolonged drug release of nitrofurazone (NF) on the skin, with chitosan as the carrier. The drug is conjugated to chitosan by means of ester bonds and the cleavage of these bonds by non-specific cutaneous esterases provides the prolonged release of NF. This prodrug could be formulated in films or gels to accelerate wound healing, by acting as a physical barrier that prevents both loss of natural skin humidity and secondary microbial contamination, as well as to treat skin injuries caused by microorganisms susceptible to NF, whose antimicrobial spectrum is added to that of chitosan.

Keywords: chitosan; nitrofurazone; prodrug; prolonged drug release; synthesis

Introduction

As we enter the twenty-first century, research at the interface of polymer chemistry and the biomedical sciences has led to the first nano-sized (5–100 nm) polymer-based pharmaceuticals, the 'polymer therapeutics'. Polymer therapeutics include rationally designed macromolecular drugs, polymer–drug and polymer–protein conjugates, polymeric micelles containing covalently bound drug and polyplexes for DNA delivery.[1]

Polymeric materials such as peptides, polysaccharides and other natural products have recently attracted attention as biodegradable drug carriers. They can optimize clinical drug delivery, minimize the undesirable drug properties and improve drug efficiency.[2] Besides this, macromolecular carriers are widely used to prolong drug release and consequently decrease its toxicity.[3] To be employed as drug carriers, polymers must be innocuous, biodegradable and non-immunogenic and the drug-polymer linkage should be stable until the drug is delivered to the target compartment,[4] especially if it taken systemically.

Chitosan, a linear polysaccharide composed of monomer residues of *N*-acetyl glucosamine and glucosamine,[5–7] is a widely-used carrier because of its free NH_2 groups, which provide a reaction center, either to modify or to bind the drug.[8] In this study, it was chosen as the polymeric carrier on account of its biocompatibility, biodegradability, non-toxi-

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city, bioadhesivity, adsorption capacity, film-forming ability and haemostatic effect on blood coagulation, besides its spectrum of action against bacteria, fungi and viruses.[5–14]

Nitrofurazone (NF), also called nitrofural, is the semicarbazone of nitrofuraldehyde.[15] Its spectrum of action includes microorganisms as diverse as *Staphylococcus aureus, Streptococcus* spp, *Escherichia coli, Clostridium perfringens, Aerobacter aerogenes* e *Proteus* spp.[16] Beyond these, it has been reported that NF shows activity against a great variety of Gram-positive and Gram-negative bacteria and other microbes, including strains of *Alcaligenes faecalis, Bacillus anthracis, Corynebacterium, Erysipelothrix insidiosa, Gardnerella vaginalis, Klebsiella pneumoniae, Moraxella lacunata, Neisseria, Paracolobactrum*(?), *Serratia marcescens, Salmonella and Shigella.*[20] Considering the diseases that these microorganisms can caused on the skin, NF could be used topically to treat a number of cutaneous conditions, such as boils, carbuncles, impetigo, folliculitis, erysipelas, various cutaneous abscesses[15–18] and second or third-degree burns,[16–17,19] and for prophylaxis against secondary infections of susceptible areas, such as surgical wounds.[16–17] It has also been shown to be active against *Trypanosoma cruzi*,[17] but its toxicity[20,21] has restricted its systemic use to clinically-monitored treatment of African Trypanosomiasis, also known as sleeping sickness.[16]

The aim of this study was to synthesize a prodrug of NF using chitosan as a polymeric carrier. This prodrug is intended to promote the prolonged release of NF by the action of cutaneous non-specific esterases, whose presence on the skin has already been demonstrated by several authors[22–25] and which are essential to catalyze the cleavage of the synthesized ester bonds and thus release the drug from its latent polymeric form. This polymeric prodrug could be formulated in films or gels to be employed in the treatment of topical cutaneous diseases caused by NF-susceptible microorganisms.

Experimental Part

Materials

Chitosan of medium molecular weight was purchased from Aldrich Chemical Company, with a degree of acetylation of 0.15 and a viscosimetric average molecular weight of 145 kDa. NF was synthesized by Prof. Leoberto Costa Tavares of Faculty of Pharmaceutical Sciences, University of São Paulo (Brazil). All other analytical grade reagents were supplied by Merck Chemicals (potassium carbonate, chloroform, methanol, acetic acid, tetrahydrofuran (THF), sulfuric acid and sodium hydroxide), Synth (formaldehyde) and Vetec (succinic anhydride). Intrinsic viscosity [η] of chitosan was measured on an AVS-350 viscometer (Schott-Geräte) coupled to an AVS-50 automatic dilution module. Chitosan and its polymeric derivatives were lyophilized in a Christ Alpha 1-2 to obtain the dry product. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ADPX 300 MHz spectrometer, using deuterated dimethyl sulfoxide (DMSO-d_6) to analyze NF derivatives and deuterated water (D_2O), heated between 50 to 70 °C, to analyze chitosan derivatives. The thermogravimetric (TG) and differential thermogravimetric (DTG) curves were obtained on a TGA-50 thermobalance from Shimadzu, the sample being heated from 25 to 600 °C at 10 °C.min^{-1} in an atmosphere of N_2 flowing at 50 mL.min^{-1}. Differential scanning calorimetry (DSC) analysis was carried out in a DSC-50 calorimeter cell from Shimadzu, with a N_2 atmosphere flowing at 100 mL.min^{-1}, a heating rate of 10 °C.min^{-1} and a temperature interval between 25 to 500 °C.

Synthesis of Hydroxymethylnitrofurazone (NFOH)[26–28]

NF (5 mmol) was allowed to react with 18 mL formaldehyde in a 5 mmol aqueous solution of potassium carbonate. The reaction mixture was stirred at room temperature for 48 hours, while being monitored by TLC (mobile phase of chloroform:methanol:acetic acid, 85:10:5, v/v/v). The sus-

pended product was then filtered and washed several times with methanol to remove excess unreacted formaldehyde.

Synthesis of Hydroxymethylnitrofurazone hemisuccinate (SCNFOH)[28]

NFOH (2 mmol) reacted with 2 mmol succinic anhydride in a medium composed of 10 mL THF and 2 drops of 20% aqueous sulfuric acid. The reaction was stirred at room temperature for 3 hours, while being monitored by TLC (mobile phase of chloroform:methanol:acetic acid, 85:10:5, v/v/v). After that, solvent was evaporated at 40 °C under vacuum. The product was washed several times with cooled purified water to remove excess unreacted succinic acid.

Synthesis of Polymeric Prodrug of Chitosan and Hydroxymethylnitrofurazone Hemisuccinate (Q-SCNFOH)[28]

Chitosan (2%) was dissolved in 1% aqueous acetic acid and 15 mL of this solution (equivalent to 1.86 mmol chitosan) was mixed with 0.31 mmol SCNFOH dissolved in 10 mL water with 2 drops of 20% aqueous sulfuric acid. The reaction mixture was stirred for 48 h at room temperature, protected from the light. After that, the reaction medium was dialyzed for 3 days against purified water in cellulose acetate membranes with a cutoff of 12 kDa for

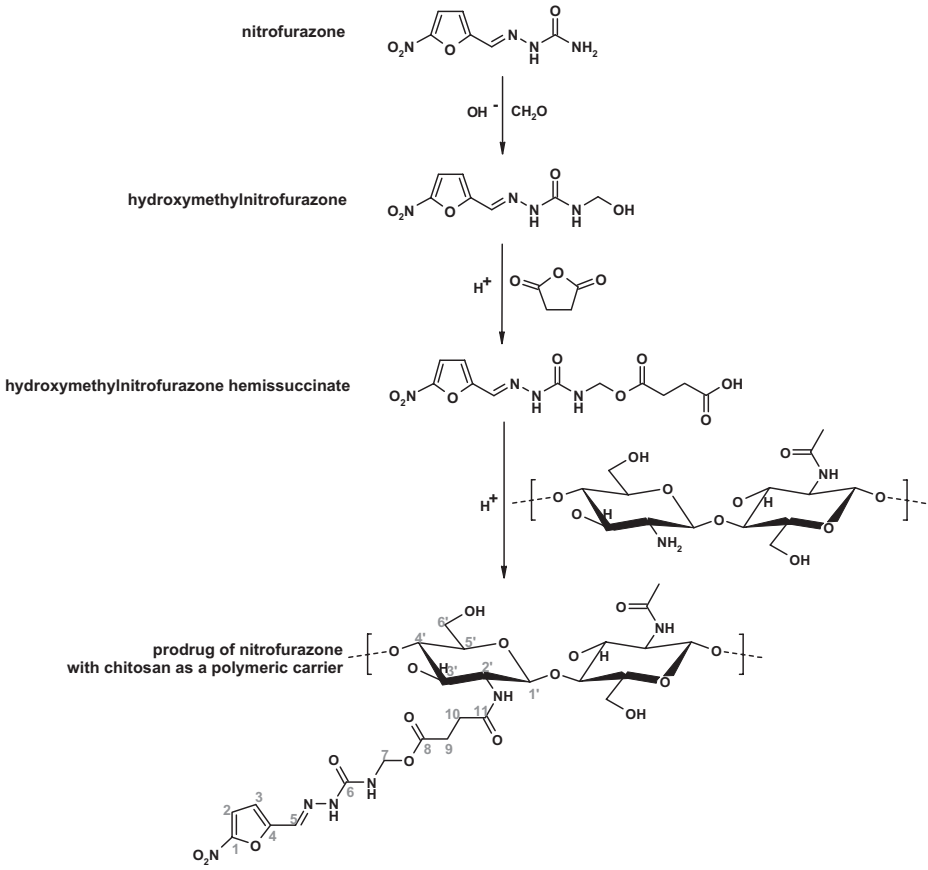

Figure 1.
Synthesis of the polymeric prodrug of NF, with chitosan as polymeric carrier.

3 days and freeze-dried to obtain the dry polymeric prodrug.

Results and Discussion

In order to promote the synthesis of the polymeric prodrug, NF was chemically modified to its carboxylic derivative (SCNFOH), which could acylate either free amino side-groups of deacetylated chitosan units or hydroxyl groups on chitosan (Figure 1). Ester or amide linkages between polymer and drug are not accidental and they are designed to be cleaved by cutaneous non-specific esterases. Prolonged delivery is ensured because there is more than one step to recovering the active form (NF) from the polymeric prodrug: (1) cleavage of the amide bond between SCNFOH and chitosan; (2) cleavage of the ester between succinic acid and NFOH and (3) chemical reversion (catalyzed under acidic conditions) of NFOH to NF. In this light, the designed polymeric compound could be classified as a triple prodrug, since it involves 3 steps to deliver the active molecule.

Regarding the drug activity, it is important to emphasize that NFOH has demonstrated therapeutic activity *per se* against *Trypanosoma cruzi*[26–27,29] and *Leishmania*.[28] La-Scalea and coworkers[30] have studied the reduction of the NFOH nitro group by cyclic voltammetry. Those authors concluded that, as with NF, the antimicrobial activity of NFOH might also be dependent on reduction of the nitro moiety. In this case, the delay or even inhibition of the acid-catalyzed rearrangement of NFOH to NF could help to prolong the drug delivery, so as to maintain the antimicrobial activity, since NFOH is also active and 4-fold less mutagenic than NF.[29] The

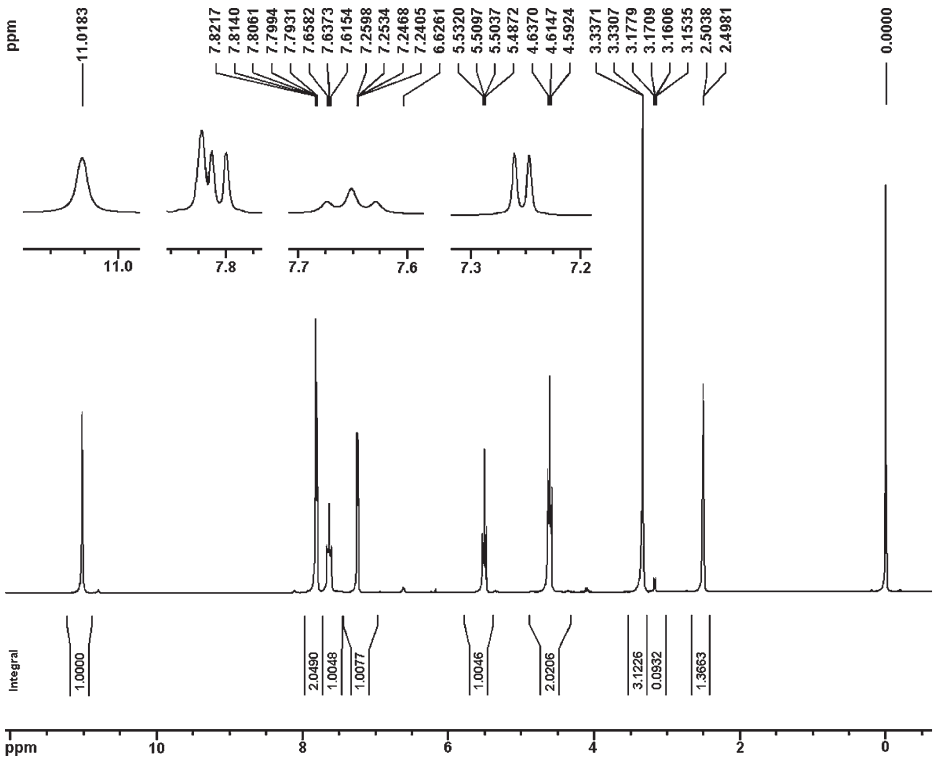

Figure 2.
^{1}H NMR spectrum (300 MHz, room temperature, DMSO-d6) of NFOH.

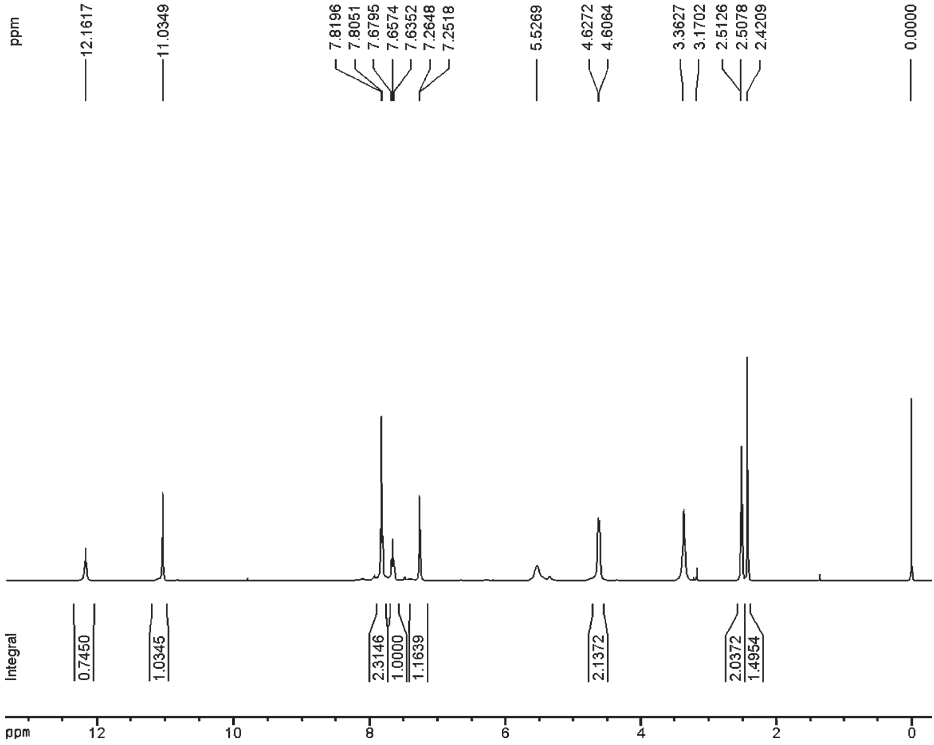

Figure 3.
^{1}H NMR spectrum (300 MHz, DMSO-d6) of SCNFOH.

newly discovered activity of NFOH against *Leishmania* suggests the topical use of this polymeric prodrug in the treatment of cutaneous and mucocutaneous forms of leishmaniasis.

All the synthetic products were characterized by ^{1}H and ^{13}C NMR, DSC and TG.

The hydroxymethylation of NF to NFOH was confirmed by the appearance

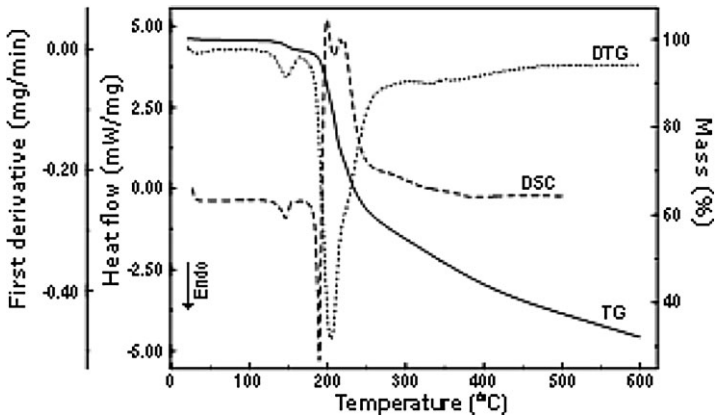

Figure 4.
TG/DTG and DSC curves (N_2 atmosphere, heating rate 10 °C.min^{-1}) for NFOH.

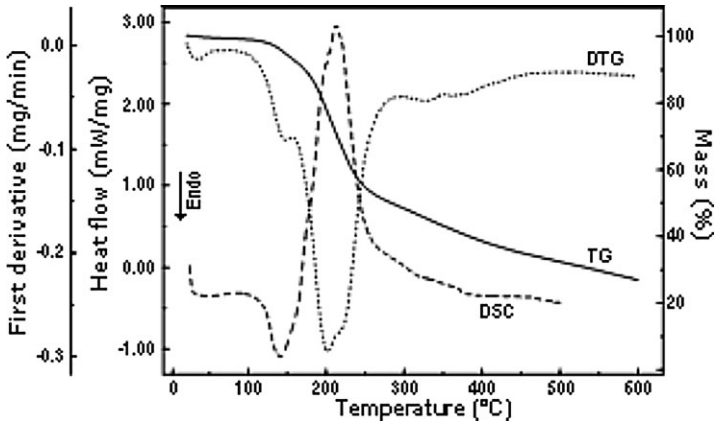

Figure 5.
TG/DTG and DSC curves (N_2 atmosphere, heating rate 10 °C.min^{-1}) for SCNFOH.

of two triplets at 4.61 and 5.51 ppm in the ^{1}H NMR spectrum (Figure 2). They are attributed, respectively, to the hydrogen atoms of the methylene (H-7) and hydroxyl group introduced in the NFOH structure. The occurrence of NF hydroxymethylation is reinforced because, in the NF spectrum (data not shown), protons of the terminal

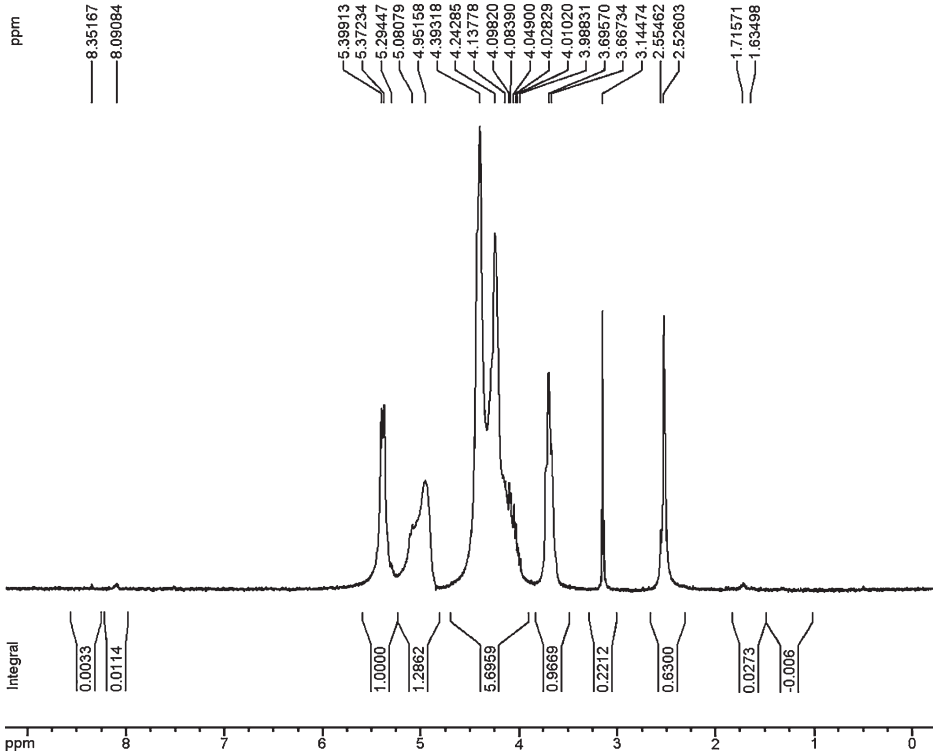

Figure 6.
^{1}H NMR spectrum (300 MHz, 50 °C, D_2O + HCl) of Q-SCNFOH.

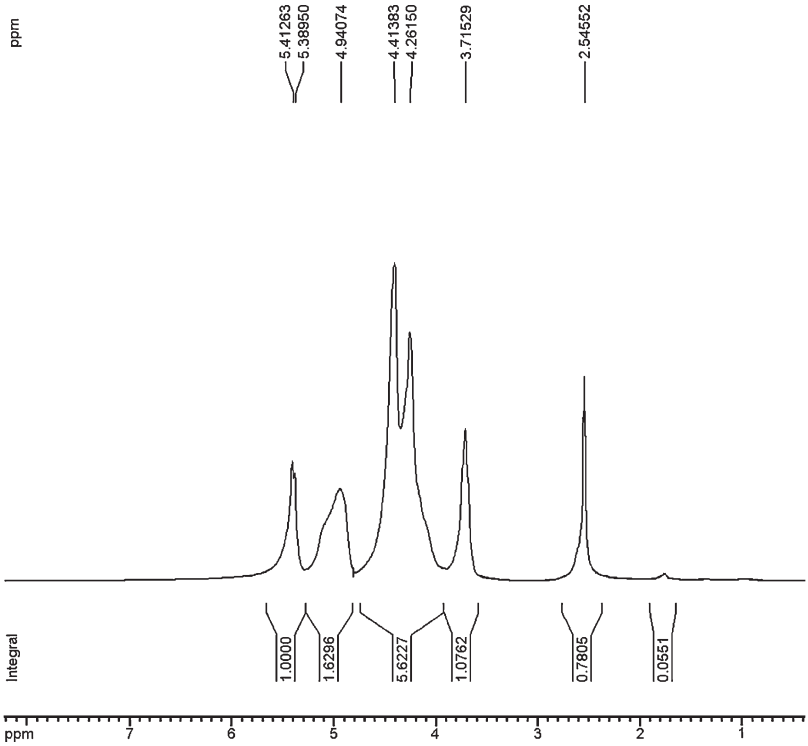

Figure 7.
^{1}H NMR spectrum (300 MHz, 70 °C, $D_2O + HCl$) of chitosan.

nitrogen appear as a singlet and, after substitution, the unreplaced hydrogen couples to H-7 protons as a triplet at 7.64 ppm.

The SCNFOH synthesis was confirmed by two new signals at 2.51 and 12.16 ppm (Figure 3), which correspond, respectively, to the methylene hydrogens (H-9 and H-10) of the succinic acid spacer and to the acidic proton of the terminal carboxyl group. The introduction of the carboxylic spacer into NFOH was also confirmed by the behavior of the H-7 hydrogens, which appear as a triplet in the NFOH spectrum (Figure 2) and a doublet in the SCNFOH spectrum (Figure 3), due to the influence of the new vicinity. However, the presence of the signal at 5.52 ppm in the SCNFOH spectrum, due to the hydroxyl group of unreacted NFOH, indicates that the SCNFOH isolation method needs to be optimized.

Figure 4 shows the superimposed DSC and TG/DTG curves for NFOH. The TG/DTG curves show that NFOH was thermally stable up to 120 °C and its thermal decomposition process involved two steps: the first, with a low mass loss ($\Delta m = 2.64\%$, $T_{peakDTG} = 147$ °C) between 120 and 170 °C, and the second, with a great mass loss ($\Delta m = 43.9\%$, $T_{peakDTG} = 205$ °C) between 170 and 310 °C. Beyond 310 °C, there was a gradual mass loss up to 600 °C, due to the elimination of the carbonized material previously formed. The DSC curve shows that an exothermic event ($\Delta H = 41.28\ J.g^{-1}$, $T_{peakDSC} = 146$ °C) occurred between 120 and 157 °C, which coincides with the first mass loss. The second mass loss observed on the TG/DTG curves corresponds to another three thermal events on the DSC curve, between 170 and 310 °C: one endothermic ($\Delta H = 165.7\ J.g^{-1}$, $T_{peakDSC} = 189$ °C) and two exothermic ($\Delta H = 1300\ J.g^{-1}$, $T_{peakDSC} =$ 199 and 217 °C). The thermal decomposition of NFOH showed a different behavior

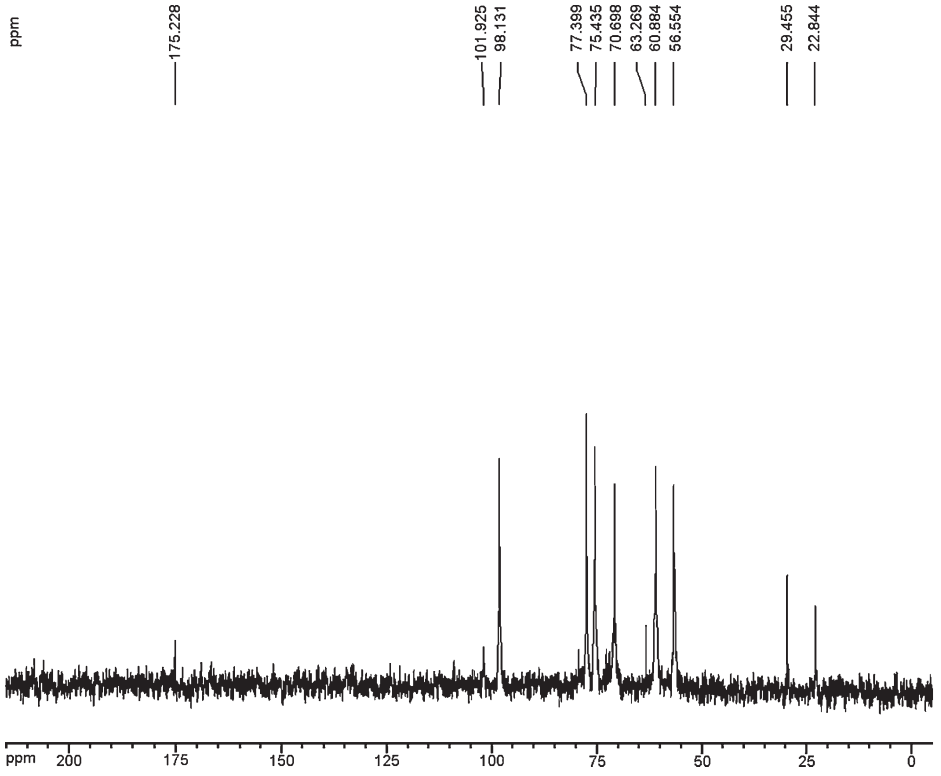

Figure 8.
^{13}C NMR spectrum (75 MHz, 50 °C, $D_2O + HCl$) of Q-SCNFOH.

from that of its precursor, NF (curve not shown), which decomposed in a single exothermic event ($\Delta H = 730\,J.g^{-1}$, $T_{peakDSC} = 245\,°C$) with a great mass loss ($\Delta m = 52.1\%$, $T_{peakDTG} = 245\,°C$). Besides, it was noted that the hydroxymethylation of NF caused a reduction in thermal stability of the derivative, NFOH, as the decomposition temperature dropped from 220 to 120 °C.

The curves of DSC and TG/DTG obtained for SCNFOH are plotted together in Figure 5. The first mass loss ($\Delta m = 1.05\%$, $T_{peakDTG} = 35\,°C$) corresponds to the elimination of moisture from the sample, since the product was washed with water during its isolation. The thermal decomposition of SCNFOH, as for NFOH, occurs in two steps: one between 95 and 160 °C, with a small mass loss ($\Delta m = 6.28\%$, $T_{peakDTG} = 147\,°C$), and the other between 160 and 295 °C, which consists of the superposition of three events, with a large mass loss ($\Delta m = 43.8\%$, $T_{peakDTG} = 202$, 211 and 220 °C). The DSC curve shows two events: (1) endothermic between 90 and 165 °C ($\Delta H = 154.3\,J.g^{-1}$, $T_{peakDSC} = 140\,°C$) and (2) exothermic between 165 and 300 °C ($\Delta H = 1060\,J.g^{-1}$, $T_{peakDSC} = 213\,°C$), which coincide respectively with the first and the second steps of mass loss. It was also noted that the introduction of the succinic spacer into NFOH resulted in a decrease in thermal stability of the prodrug from 120 to 90 °C. It is clear that subsequent derivatization of NF causes progressive loss of thermal stability, which should be observed in the reaction and analysis conditions.

There is strong evidence that the synthesis of the prodrug with chitosan as carrier really occurred. Comparing the ^1H NMR spectrum of the prodrug (Figure 6) with that of chitosan without substitution

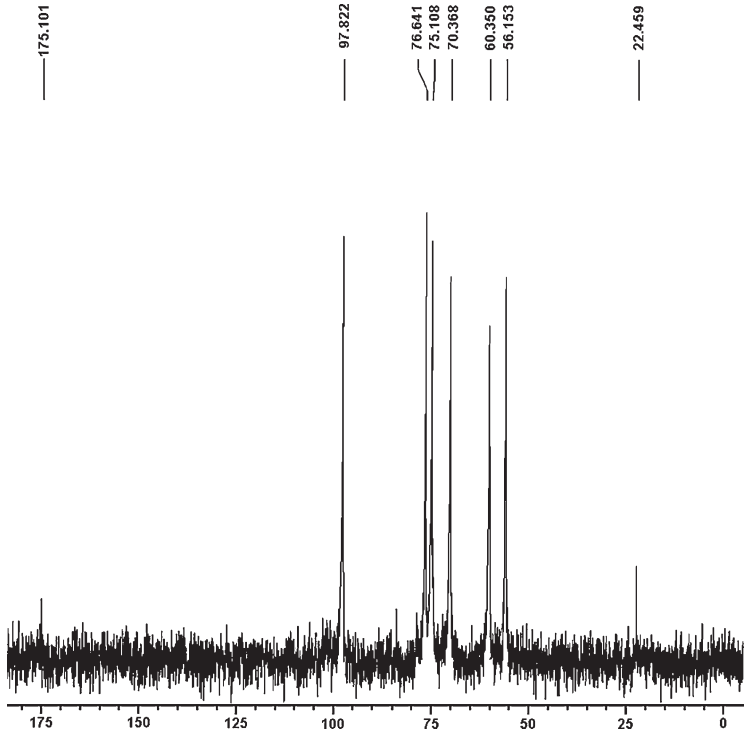

Figure 9.
^{13}C NMR spectrum (75 MHz, 70 °C, D_2O + HCl) of chitosan.

(Figure 7), a new singlet, which is attributed to methylene groups in the succinic spacer of SCNFOH, can be observed at 3.14, rather than at 2.42 ppm (Figure 3). This signal shift of almost 1 ppm to a lower region of the magnetic field is due to a difference in the measurement temperature. As H-1 of chitosan glucosamine is

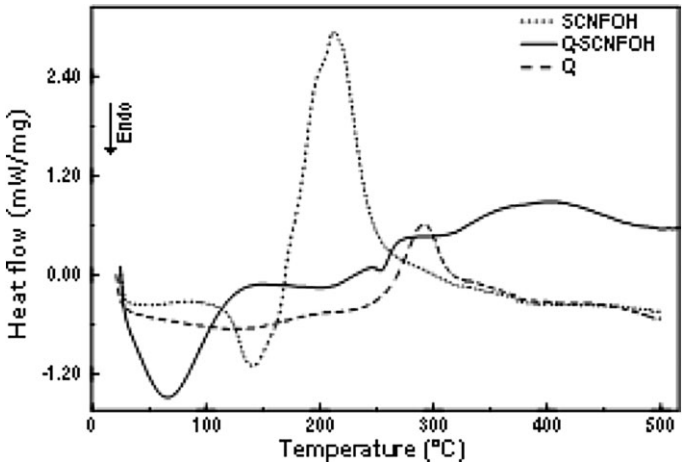

Figure 10.
Superposition of the DSC curves for SCNFOH, Q-SCNFOH and chitosan (Q) (N_2 atmosphere, heating rate 10 °C.min^{-1}).

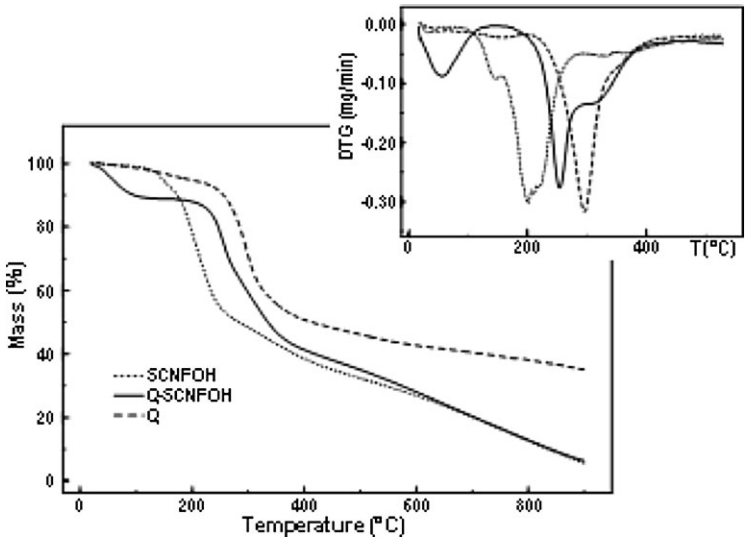

Figure 11.
Superposition of the TG/DTG curves for SCNFOH, Q-SCNFOH and chitosan (Q) (N_2 atmosphere, heating rate 10 °C.min^{-1}).

usually covered by the water signal, it is important to increase the temperature to shift all signals to lower magnetic field, thus allowing its visualization. However, since thermal analysis of NF derivatives indicated a stepwise fall in thermal stability, it was chosen to perform the prodrug NMR analysis at a lower temperature (50 °C) than that used for unmodified chitosan (70 °C), which explains the difference in the signal between the chitosan and polymeric prodrug spectra. The NFOH and SCNFOH spectra (Figure 2 and 3, respectively) were performed at room temperature, in view of their thermal instability and because it was unnecessary to raise the temperature since there was no chitosan in these samples.

Comparing ^{13}C NMR spectra of the polymeric prodrug (Figure 8) and chitosan (Figure 9), two new signals at 63 and 29 ppm can be observed in the former and these are attributed respectively to C_7 and $C_{9/10}$ of SCNFOH.

Figure 10 shows the superimposed DSC curves of SCNFOH, chitosan and the product Q-SCNFOH. The absence of thermal events characteristic of SCNFOH in the Q-SCNFOH DSC curve excludes the possibility of the new compound being a physical mixture of reagents. Moreover, the pattern of DSC curves shows that all samples are different species from each other.

The superimposed TG/DTG curves (Figure 11) show that Q-SCNFOH had more moisture than the unreacted chitosan. This is because the polymeric prodrug was lyophilized for its isolation, while the chitosan sample was analyzed as a film, after a drying step in a thermostatic chamber. According to the curves, the Q-SCNFOH was thermally less stable than chitosan. The polymeric prodrug decomposes in two exothermic events, while chitosan apparently decomposes in a single step at a higher temperature. In fact, the changes detected in Q-SCNFOH thermal behavior reinforce the conclusion that the desired prodrug was obtained, because it is hard to believe that unreacted SCNFOH remained adsorbed to chitosan after 4 days of dialysis and a final filtration step.

Conclusion

While the polymeric prodrug of NF with chitosan as the carrier has been synthesized

successfully, the methodology is still in the process of optimization to improve product stability and yield of the reactions. Further studies on NFOH stability and kinetics of NF release from the prodrug will be performed.

Acknowledgements: The authors would like to acknowledge CNPq and FAPESP for financial support and to thank PhD Maria Inês de Almeida Gonçalves for the execution of the NMR spectra.

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