Nystatin–Dextran Conjugates: Synthesis and Characterization

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SYNOPSIS

The coupling of nystatin (Nys), a water-insoluble antifungal agent, to dextran via an imine or amine bond was systematically investigated. Dextran was first oxidized to dialdehyde dextran using potassium periodate, purified from the oxidizing agent, and reacted with Nys to form the Schiff base. The Schiff base was reduced to the amine using borohydride. All reactions took place in water. The purification of the oxidized dextran from the oxidizing agent was essential to prevent oxidative degradation of Nys at the coupling step. The effects on the coupling yield of the following factors: dextran molecular weight, degree of oxidation (aldehyde content), Nys to dextran ratio, temperature, and reaction pH were studied. A 95% coupling yield was obtained at the optimized coupling conditions: pH 8.9 ± 0.1, 50% degree of oxidation, and initial ratio of Nys to dialdehyde dextran 1 : 2.5. In all experiments, dextran was decreased in molecular weight during the oxidation step. Both imine and amine forms of Nys–dextran conjugates were soluble in water and exhibited improved stability in aqueous solutions as compared to the unbound drug. The conjugates showed comparable minimum inhibitory concentration (MIC) values against *Candida albicans* and *Cryptococcus neoformans*. The conjugates were about 25 times less toxic than free Nys after a single injection in mice. © 1996 John Wiley & Sons, Inc.

Keywords: Nystatin • dextran conjugates • *Candida* • polymer conjugates • antifungal • oxidized dextran

INTRODUCTION

Life-threatening fungal infections have become increasingly widespread, especially among immunocompromised patients, such as those undergoing cancer treatment or transplantation and those with AIDS. The current treatment of severe systemic fungal infections is inadequate, due to limited availability of effective parenteral drug formulations and the appearance of new opportunistic fungal infections resistant to the marketed drugs. The pattern of new or newly resistant species emerging in response to widespread and prolonged drug treatment renders the development of new effective parenteral antifungal drug delivery systems highly important.[1]

Nystatin (Nys), a tetraene-diene antifungal agent, possesses a broad spectrum of activity. Problems associated with its insolubility in injectable solvents and high toxicity in iv injections have precluded its systemic administration.[1,2] Nys is unstable in aqueous medium and is sensitive to oxidation and heat.[3-6] Various dispersions of Nys have been used for oral and topical administration with beneficial effect.[7-10] Encapsulation of Nys in liposomes[11,12] reduced toxicity, improved the survival of mice infected with *Candida albicans*, and provided a means for intravenous administration of Nys that could make it an active systemic antifungal agent. However, major technical difficulties associated with Nys leakage from liposomes, Nys chemical integrity, and liposome batch-to-batch reproducibility still need to be solved to allow applicative development of a pharmaceutical intravenous dosage form. Thus,

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there is a need for an IV injectable nontoxic delivery system for Nys for optimal treatment of serious fungal diseases.

The potential use of soluble macromolecular prodrugs as a mean of achieving targeted drug delivery has attracted considerable interest in recent years.[13-17] Besides drug targeting, other important objectives may be achieved by using soluble drug carriers, including enhancement of drug solubility, stability of the therapeutic agent, prolonged circulation life-time, extended duration of activity, and reduction of side-effects and drug toxicity. The latter objectives may be obtained by employing macromolecular transport groups without any apparent specificity for discrete cell-surface receptors. Numerous reviews have described the potential application of proteins, polypeptides, polysaccharides, and synthetic polymers as high molecular weight carriers for various therapeutically active compounds.[13-17]

Oxidized dextran has been used as a conjugate carrier for various proteins,[18-20] antibiotics, and some other low molecular weight drugs,[21,22] mainly due to their inertness, biodegradability, and simplicity of preparation. In addition, the molecular weight of the activated carrier and the pH of coupling can be changed over a wide range to produce drug conjugates with various prolongation effects. Oxidation degree of the polymer can be varied to a large extent, thus leading to various amounts of bound drug. The properties of preparations can also be regulated by choice of the appropriate polymer : drug ratio.[18,19,23] In several reports, the excess oxidizing agent was not removed from dialdehyde dextran (DAD), thus oxidizing susceptible drugs, such as Nys during the coupling reaction.

We report here the synthesis, characterization, and stability of Nys–dextran conjugates in order to provide aqueous-base injectable Nys formulations that possess improved antifungal activity with reduced toxicity.

MATERIALS AND METHODS

Materials

Nys powder (USP XXII, 5269 IU/mg) was a gift from Taro Pharmaceutics (Haifa, Israel). Dextran (MW 9300, 18,000, 40,000, 74,000), potassium periodate, sodium borohydride, and fluorescamine were purchased from Sigma (St. Louis, MO). All other reagents and solvents were analytical grade.

Instrumentation

Infrared (IR) spectroscopy (Anelect Instruments FT-IR model fx-6160) was performed using polymer dispersion in Nujol cast between two NaCl plates. Ultraviolet (UV) spectroscopy was performed using a Kontron Instruments Uvikon model 930. Molecular weight distribution of the dextran derivatives was estimated by high-pressure and low-pressure gel permeation chromatography (GPC) systems. The first consisted of a Spectra Physics (Darmstadt, Germany) P1000 pump with UV detection (Applied Bioscience 759A Absorbency UV detector) at 254 nm, a Rheodyne (Coatati, CA) injection valve with a 20 μL loop, and a Spectra Physics Data Jet integrator. Samples were eluted with double-distilled water (DDW) through a Bio-Sil R SEC-125 HPLC Gel Filtration Column 300 × 7.5 mm (Japan), at a flow rate of 1 mL/min.

The low-pressure GPC system comprised a Sephadex G-75 Column (a total volume of 55 mL and a void volume of 19 mL) and DDW as eluent. Molecular weights of the eluted dextran derivatives was estimated using dextran standards of MW range between 5000 and 110,000. Fluorescence was measured using Spectrofluorimeter Jasco FB-770 (Japan Spectroscopic Co.). ^{1}H-NMR spectra were obtained on a Varian 300 MHz spectrometer using D_2O as solvent and TMS as external standard.

Aldehyde content in DAD was determined by an iodometric method, DAD was reacted with excess of iodine/potassium iodide solution and the unreacted iodine was reverse titrated by sodium thiosulphate. The aldehyde content in DAD was calculated from the amount of reacted iodine. Total dextran content was determined by the anthrone method.[24]

Preparation of Dialdehyde Dextran (DAD)

DAD was prepared from the reaction of dextran with potassium periodate and purified by anion-exchange chromatography. In a typical experiment, dextran of average MW 40,000 (1 g, 0.0599 mol glucose units) was dissolved in 20.0 mL of DDW, potassium periodate (1.275 g, 0.0554 mol) was added, and the mixture was stirred at room temperature for 2 h until potassium periodate was completely dissolved. The DAD formed was separated from excess periodate and reaction by-products by applying it through a column (6 × 80 mm, V_o = 2.0 mL) filled with Dowex-1 in the acetate form. Dowex-acetate was obtained by the pre-treatment of the commercial anion exchanger with aqueous 1*M* acetic acid solution. The

purified DAD at a concentration of 50.0 mg/mL exhibited a degree of oxidation in the range of 50%, as determined by the iodometric method. DAD with 5–50% degree of oxidation were obtained from reaction with a proportional amount of periodate under similar conditions.

Conjugation of DAD and Nys

Nys was conjugated to oxidized dextran under various reaction conditions. Oxidized dextrans with a degree of oxidation between 5 and 50% were prepared from dextrans of a molecular weight ranged between 9300 and 74,000. The conjugation reactions were conducted in buffer solutions of pH range between 7.0 and 10.0 at 4 and 37°C for 2–48 h under constant stirring. Dialdehyde dextran with a concentration range of 10–35 mg/ml was reacted with 4–20 mg/mL Nys. The imine conjugate was purified by dialysis through a 12,000 molecular weight cut-off dialysis tubing against deionized water for 24 or 48 h at 4°C and lyophilized to dryness. The amine derivative was obtained by reduction of the imine conjugate before purification by adding 1.2–4 molar excess of sodium borohydride to the conjugation solution at 4°C for 30 or 60 min with stirring. The amine conjugate was purified by dialysis through a 12,000 molecular weight cut-off dialysis tubing, as described above, and stored in dry form following lyophilization. Sterile conjugates were obtained by sterile filtration through a 0.2 μm sterile filter (Schleicher & Schuell, Dassel, Germany) followed by lyophilization under sterile conditions. The sterility of the solutions was assessed using the Bactec 46 apparatus (Johnson Laboratories, Towson, MD) measuring radioactive carbon dioxide quantitatively in Bactec culture vials inoculated with sample tests.

In a typical experiment, 20.0 mL of purified DAD solution (25 mg/mL) was mixed with an equal volume of 0.2*M* borate buffer solution pH 9.1, and 400.0 mg of Nys was added to the polymer solution (10 mg/mL). The pH value of the mixture was maintained at pH 8.9 ± 0.1 for 16 h at 37°C. The crude conjugate was dialyzed against DDW for 30 h at 4°C using molecular porous membrane tubing with MW cutoff 12,000 followed by centrifugation for 10 min at 2000 rpm and lyophilization. The lyophilized light-yellow product (925 mg, 67% yield) contained 35% of Nys (87% yield) as evaluated by UV absorption at 416 nm.

Nys content was determined from the reaction of aqueous solutions of Nys (2 mL) with 0.01% acetone solution of fluorescamine (1 mL). Nys concentrations ranging from 0.2–2000 μg/mL in 0.2*M* borate buffer pH 8.0 at room temperature. Emission spectra were recorded at 430–570 nm and 390 nm excitation.[25]

Stability of Nys and Nys–dextran conjugates in various pH solutions was determined by UV absorption at 416 nm. Solutions of Nys in DMSO : buffer 75 : 25 and Nys–dextran conjugates in phosphate buffer at a pH range 3–9 (0.3 mg Nys/mL) were stored at 25°C for 20 h. The stability was determined relative to the initial UV absorption at 416 nm. The heat stability was determined using drug solutions (0.3 mg Nys/mL) stored at 4, 37, and 56°C.

Effectiveness of Nys Conjugates

The minimal inhibitory concentration (MIC) values of Nys and its conjugates were determined against *Candida albicans* CBS 562 (reference type strain) and *Cryptoccocus neoformans* B-3501 according to the agar dilution method using yeast nitrogen base medium (Difco).[26] MIC for both fungi were determined using Nys and Nys–dextran with Nys equivalent in concentration increments of 5, 10, 20, 40, and 60 μg/mL.

RESULTS AND DISCUSSION

Coupling Reaction

Imine and amine Nys–dextran derivatives were prepared by the reaction of DAD and Nys, as described in Figure 1. Dextran was oxidized to DAD using potassium periodate and purified by ion exchange

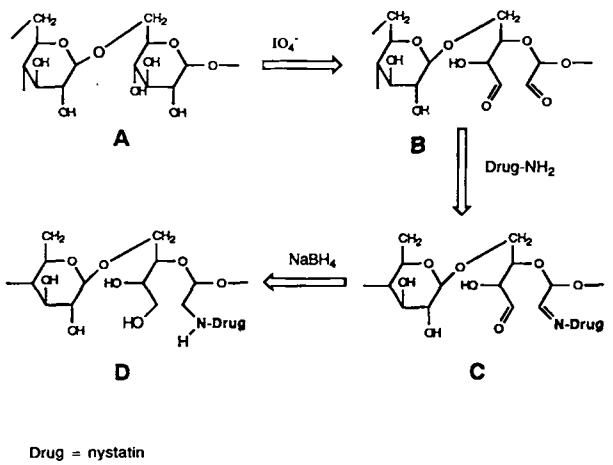

Figure 1. Schematic representation of binding of Nys to dextran via imine or amine bonds: (A) dextran; (B) dialdehyde dextran, DAD; (C) Nys–dextran imine conjugate; (D) Nys–dextran amine conjugate.

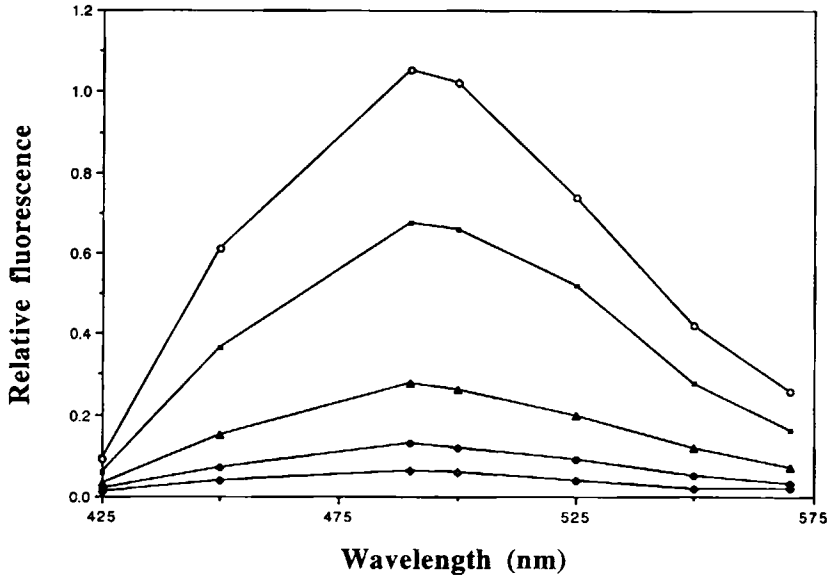

Figure 2. Typical differential emission spectra for several concentrations of free Nys using the fluorimetric method: (-⊖-) 2.5 μg/mL, (—♦—) 5.0 μg/mL, (— △ —) 10.0 μg/mL, (-■-) 25.0 μg/mL, (— ○ —) 50.0 μg/mL.

chromatography before the conjugation step. The purification of DAD from periodate oxidation reagent was necessary to prevent degradation of Nys during the coupling reaction. The DAD derivative was reacted with Nys to form an azomethene bond (Schiff bases) with the primary amino group of Nys. The drug–dextran azomethene bond was then reduced to the amine bond using sodium borohydride. The unreacted aldehyde groups on the DAD are reduced to the corresponding alcohols, which prevent further binding of the Nys–dextran conjugate to proteins and body components *in vivo*.

The conjugation yield was evaluated from the concentration of Nys before and after the reaction with DAD using spectrafluorimetry which is based on the specific reaction of fluorescamine with the primary amine groups in free Nys.[25] Nys concentrations from 0.05 to 0.2 mg/mL were accurately determined using this method. Typical differential emission spectra curves for fluorescamine derivatives of Nys are shown in Figure 2. The control probe curve (without Nys) was automatically subtracted. The pH does not significantly affect the fluorescence of Nys. The optimal Nys determination conditions were 0.2*M* borate buffer with a pH between 7 and 8. The method is rapid, simple, highly reproducible, and can be easily applied for *in vitro* determination of drug release from a polymer matrix, emulsion,

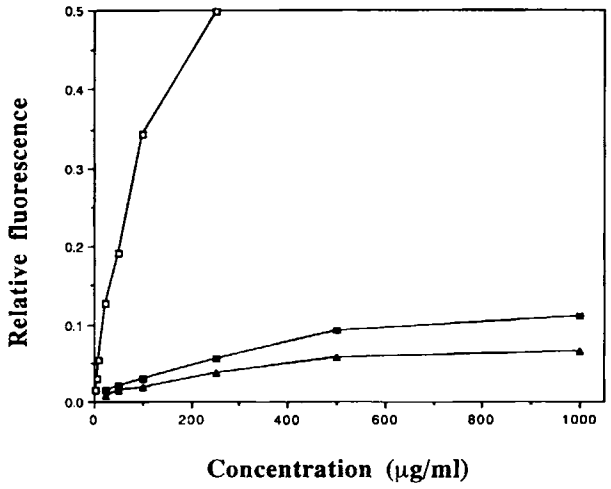

Figure 3. Fluorescence of Nys and Nys–dextran conjugates after reaction with fluorescamine: (□) Nys, (■) Nys imine conjugate, (▲) Nys amine conjugate.

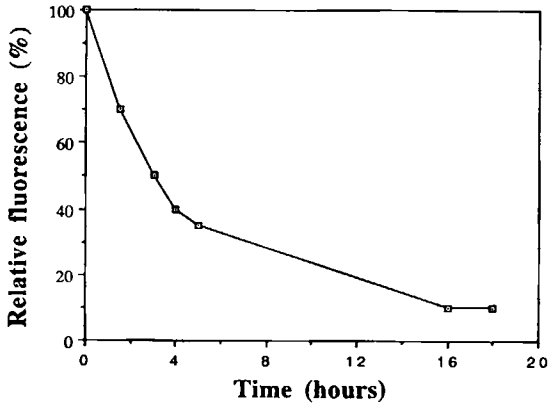

Figure 4. Nys coupling to dialdehyde dextran as a function of time followed by the disappearance of Nys from the reaction mixture using the fluorimetric method.

Table I. Reaction Conditions and Yield for Nys Coupling to Dialdehyde Dextran (DAD) under Various Conditions

		Coupling Conditions						
Dextran Initial MW	DAD[a] MW	Percentage of Oxidation	pH	Time (h)	Temperature (°C)	DAD (mg/mL)	Nys (mg/mL)	Yield[b] (%)
9300	9000	10	7.0	2	20	35	5	3
9300	6000	50	8.9	16	37	20	10	90
18,000	18,000	5	7.5	6	4	20	10	5
18,000	15,000	12	8.0	24	20	35	15	20
18,000	12,000	25	8.5	16	37	20	10	50
18,000	9000	50	8.9	16	37	35	10	95
40,000	15,000	25	9.5	2	4	35	20	70
40,000	20,000	50	8.5	10	20	10	10	50
40,000	14,000	50	8.9	20	37	25	10	95
74,000	23,000	25	10.0	4	4	25	15	60
74,000	20,000	50	8.9	16	37	25	5	95

[a] DAD with various degrees of oxidation were obtained by oxidation of dextran under various conditions (not shown). The oxidized dextrans were used for Nys counpling at various conditions.
[b] The coupling yield is determined as the % of bound Nys from the initial reacted Nys.

liposomes, etc. The amine conjugated Nys–dextran possesses a significantly lower fluorescence than intact Nys at a wide range of concentrations (Fig. 3). This method can be used to follow the conversion of Nys to Nys–conjugate. The decrease in Nys concentration during the coupling reaction, which represents the amount of Nys reacted with aldehyde dextran is shown in Figure 4. The coupling reaction was followed by measuring Nys concentration in the reaction mixture using the fluorimetry method. Alternatively, the concentration of active Nys was determined by UV measurements. A typical UV spectrum of Nys is shown in Figure 5. The UV max for Nys are 290, 307, and 322 nm, however, the absorbance at 400–420 nm is characteristic for active Nys. The UV absorbance of Nys at 416 nm was correlated with its MIC against *C. albicans*. Accordingly, degraded Nys that did not show UV absorbance at 416 nm, did not show antifungal activity. To emphasize the importance of purification of DAD from periodate before the addition of Nys for the coupling reaction, unpurified DAD was reacted with Nys and the UV absorbance and MIC of the conjugation product were determined. No UV absorbance at above 350 nm and no antifungal activity (all fungi remain alive) were found for the Nys conjugate prepared from unpurified DAD.

Table I illustrates the influence of various factors on the conjugation yield of water-soluble Nys–dextrans. Oxidized dextrans had lower MW compared to the starting nonoxidized dextrans. Coupling yields of up to 95% of the reacted Nys was obtained. The coupling yield was mostly affected by the reaction pH. The highest coupling yield was achieved when Nys at a concentration of 10 mg/mL was reacted with DAD of 50% degree of oxidation at a concentration of 25 mg/mL for 16 h at 37°C in a borate buffer solution pH 8.9 ± 0.1. Despite the extensive degradation of the polymer during oxidation, the purified Nys–dextran conjugates had a defined peak of a narrow molecular weight distribution with no low molecular weight fractions or free Nys.

The lyophilized conjugates were readily soluble in water at concentrations up to 100 mg/mL and were insoluble in DMSO or methanol, in contrast to the low solubility of free Nys in water (0.3 mg/mL), and its high solubility in DMSO (30 mg/mL) and in methanol (1 mg/mL).[27]

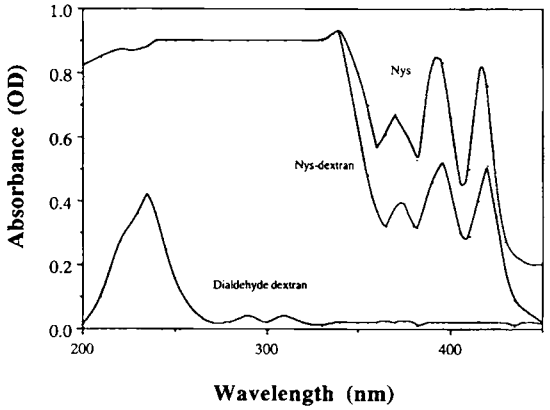

Figure 5. Typical UV spectra of dialdehyde dextran, Nys in DMSO/water 75 : 25, and Nys–dextran conjugates in water.

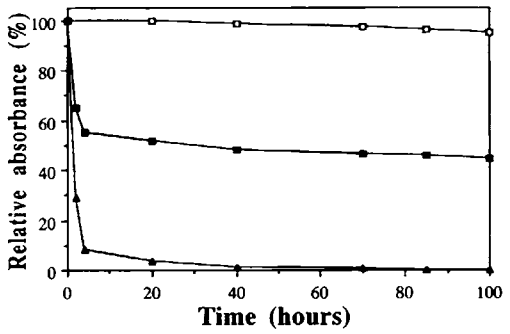

Figure 6. Stability of Nys–dextran in phosphate buffer stored at 37°C: (□) Nys imine conjugate, (■) free Nys solution containing dextran, (▲) free Nys. Stability was determined by UV absorbance at 416 nm with drug concentration of 0.3 mg/ml.

The imine and amine Nys–dextran conjugates were characterized by UV, ^{1}H-NMR, and IR. Typical UV spectra of the conjugates and Nys in DMSO : water are shown in Figure 5. The conjugates have similar spectra to that of Nys with slight shifts in peak wavelengths at 416 and 380 nm which correspond to the Nys chromophore. The peak absorbance at 416 nm is characteristic of active Nys, it was thus useful for the determination of the activity of Nys. The main UV peak absorbances for Nys and Nys conjugates as determined in water–(a) Nys 416, 394, 322, 307, 292, and 232 nm; (b) for the unreduced imine derivative: 418, 393, 321, 307, 289, and 233; and (c) for the amine-reduced form: 416, 394, 322, 307, 285, and 233 nm. UV calibration curves at 416 nm were made for the quantitative analysis of Nys and Nys conjugates. Nys was determined in DMSO and showed linearity between 0.06 and 2 mg/mL drug concentration. Linearity in water was between 0.06 and 0.25 mg/mL, Nys conjugates in water showed linearity up to 5 mg/mL.

The IR and ^{1}H-NMR spectra of Nys–dextran conjugates were compared to that of the free Nys and unreacted dextran. Both the ^{1}H-NMR and IR spectra of Nys–dextran conjugates contained the typical chemical shifts corresponding to both Nys and dextran (data not shown).

Stability Studies

The stability of both imine and amine Nys conjugates were determined in various solutions and at various temperatures. The Nys inactivation under various conditions has been reported.[3,6]

The UV spectra can be used to monitor the stability of Nys derivatives in solutions. As described above, the Nys UV absorption at 416 nm is characteristic for active Nys, reduction in this peak size corresponds to the decrease in active Nys concentration. The peaks at the maximum absorption (300–320 nm) exist for both active and degraded Nys. Thus, UV at 300–320 nm can not be used for monitoring Nys stability. Heat-degraded Nys and Nys–dextran conjugates (56°C for 24 h) had negligible absorbance above 350 nm.

The conjugates were found to be stable in aqueous solutions for 100 h at 4 and 37°C. The stability of the synthesized conjugate in comparison to the unbound Nys (in the presence and absence of dextran) in aqueous solutions (0.3 mg Nys/mL, at 37°C) was studied by monitoring the UV absorbance at 416 nm, which corresponds to intact Nys. The results presented in Figure 6 prove that Nys–DAD conjugate practically retained its UV absorbance after 100 h of storage. The solution of free Nys and the mixture of Nys with dextran were rapidly degraded, with the later being more stable. The presence of dextran in the solution stabilized the free Nys.

The stability in aqueous solutions at various pH values (4–9) was studied. The stability was determined by comparing the UV absorbance at 416 nm at time zero and after 20 h. The stability is pH dependent; under basic conditions (pH 7–8) both conjugates were stable, with the amine derivative being more stable. The conjugates were more stable at acidic pH than the free Nys (Fig. 7). There was a 34% decrease in UV absorbance at pH 5 after 20 h for Nys, while during this period the amine conjugate

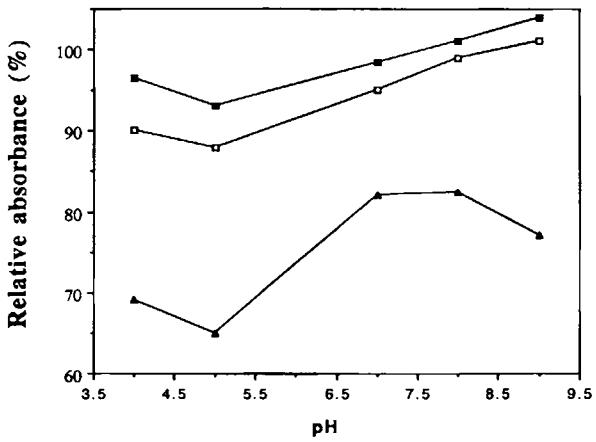

Figure 7. Stability of Nys and Nys–dextran conjugates at various pH solutions: (□) Nys imine conjugate, (■) Nys amine conjugate, (▲) free Nys. Stability was of a 0.3 mg/ml solutions in DMSO : buffer (75 : 25) for Nys and buffer solutions for the conjugates. The stability was determined following the reduction in the UV absorbance at 416 nm after 20 h of storage.

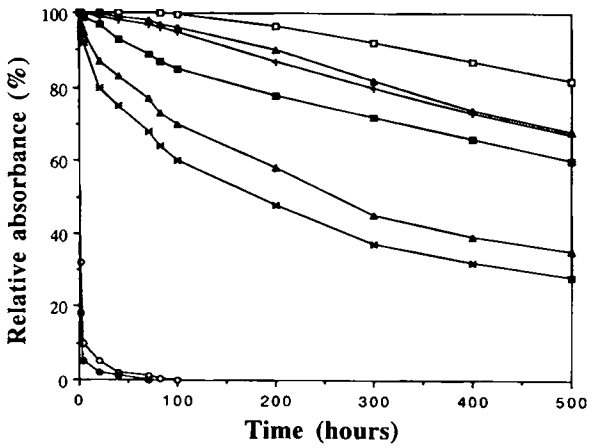

Figure 8. Stability of Nys conjugates as compared to free Nys in water solutions under various temperatures: (□) Nys amine conjugate at 4°C, (△) Nys amine conjugate at 37°C, (▲) Nys amine conjugate at 56°C, (■) Nys imine conjugate at 4°C, (+) Nys imine conjugate at 37°C, (×) Nys imine conjugate at 56°C, (O) free Nys at 4°C, (●) free Nys at 37°C. Stability of solutions in deionized water (0.3 mg/mL) stored at 4, 37, and 56°C was determined by following the UV absorbance at 416 nm.

lost 8% and the imine derivative 11%. Incubation at above pH 7 decreased the absorbance for free Nys, while the conjugates did not change. These results corroborate our hypothesis that coupling Nys to DAD markedly increases its aqueous stability.

The stability of Nys conjugates under various temperatures in water solutions was determined. Because of the low aqueous solubility of Nys, concentrations of 0.3 mg Nys equiv/mL were used. The solutions were stored at 4, 37, and 56°C, and the stability was detected by following the UV absorbance at 416 nm. The results presented in Figure 8 show a rapid deterioration of the free Nys, whereas the Nys conjugates were significantly more stable, with the amine conjugate being more stable. Both Nys–dextran conjugates practically retained their UV absorbance after 100 h storage. When exposed to higher temperature (56°C), the conjugates underwent degradation, more significant for the imine derivative. When stored under dry conditions protected from light and oxygen, the free Nys and its conjugates were stable for at least 6 months.

Antifungal Activity of Nys Conjugates

The *in vitro* antifungal activity measured by MIC of *Candida albicans* and *Cryptococcus neoformans* as representative fungi revealed that the conjugates are similarly active to the unbound drug. The MIC of the imine and amine conjugates of *C. albicans* and *Cr. neoformans* were 20 μg/mL and the MIC value of the free Nys was 10 μg/mL.

The polymer conjugates exhibited decreased toxicity (data not shown). No toxic signs were observed following a single-bolus IV injection in BALB/c male mice (weighing 20 g) at a concentration of 100 mg Nys/kg, while the maximal tolerated dose of free Nys was 4 mg/kg. The results of the detailed toxicity and efficacy studies will be published separately.

CONCLUSIONS

A method for producing a stable water-soluble polysaccharide conjugate of Nys is described. The drug was conjugated by a Schiff-base reaction with oxidized dextran. High conjugation yield of active Nys was obtained. The conjugates were highly water soluble and could be appropriately formulated for injection. The conjugates showed comparable MIC values against *Candida albicans* and *Cryptococcus neoformans*. The conjugates were about 25 times less toxic than free Nys after a single injection in mice.

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