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Synthesis, Characterization, and Antifungal Activity of Nystatin—Gum Arabic Conjugates

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ABSTRACT: Nystatin, a polyene tetraene antibiotic widely used in the treatment of mycoses, was coupled with oxidized polysaccharide gum Arabic, by forming Schiff base structures between amine groups of antibiotics and aldehyde groups of modified carbohydrate. Imine conjugates synthesized in this way were reduced with sodium borohydride to secondary amines. Two imine and two amine conjugates were obtained with different nystatin content. The conjugates were characterized by UV–Vis, FTIR, ^{1}H NMR spectroscopy, and thermogravimetric analysis. Solubility in water, unlike nystatin, and significant activity against *Candida albicans* and *Aspergillus niger* with minimum inhibitory concentrations in range of 3.125–6.25 μg mL^{-1} and 6.25–25 μg mL^{-1}, respectively, indicate that the chemical integrity and the biological function of these compounds were retained. A comparison of stability of the conjugates in the dry form, solution and under different pH values showed that the conjugates exhibited better stability than pure drug. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: nystatin; polysaccharide; coupling; solubility; antifungal activity

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INTRODUCTION

Nystatin (Nys) is a polyene tetraene antibiotic frequently used in the treatment of mycoses in human and veterinary medicine. This drug is active against most pathogenic fungi, such as some *Candida* and *Cryptococcus* species and its use has greatly increased in recent years as a result of a higher frequency of fungal infections. The biological activity of Nys, as well as other polyene antibiotics, is based on impairment of cell membrane integrity by the formation of transmembrane pores due to interactions with sterols.[1] Because of its sensitivity to light, oxygen and heat, to retain its chemical structure and at the same time biological function, various dispersion of Nys, as well as pastilles, tablets, creams, and powder, have been used for oral or topical administration. The usage of liposome-incorporated Nys was also investigated[2] and recently its use in the form of an antifungal mucoadhesive film was examined.[3] The medical application of this antibiotic, however, was limited due to its side effects, namely toxicity and insolubility in water and other injectable solvents at room temperature.[4]

Polysaccharides are important class of biomacromolecules with a large variety of unique and, in most cases, complex chemical structures that affect their hydrophilic nature and biocompatibility. Many of them are used in different fields of medical application, for example, for modification of pharmaceuticals for controlled release and targeted delivery, as water-soluble drug carriers which increases drug solubility and stability, extends the activity and reduces side effects and drug toxicity.[5–7]

Gum Arabic (GA), produced by the *Acacia* tree, is a water soluble, highly branched complex polysaccharide, comprised mostly of galactose, arabinose, rhamnose, and glucuronic acid. There is also a very small amount of protein, which content ranges from 1 to 3% of the total gum, depending on the production area. Features such as nontoxicity, biodegradability, and biocompatibility make this polysaccharide suitable for use in various industries, as a food additive, in textile manufacture, pharmacy, medicine, cosmetics, and so forth.[8,9]

Periodate oxidation of polysaccharides containing vicinal diol structures is a convenient route for the synthesis of polymer–drug conjugates, especially, if the latter have an appropriate functionality, such as aliphatic amine groups. The resulting polyaldehydes react with amine compounds to give Schiff base

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structures or alkylamines if the coupling occurs in the presence of a reducing agent, for example, sodium borohydride. The coupling reaction should proceed under mild conditions so that biologically active compounds are not inactivated.

Oxidized GA has been used as a carrier for various drugs, such as primaquine,[10] ampicillin,[11] and amphotericin B,[12] mainly due to its availability and biodegradability. The degree of oxidation can be varied over a wide range by changing the periodate/polysaccharide ratio, that affects the amount of drug that can be bound.

In this work, the main objective was to synthesize and characterize nystatin-GA conjugates to provide some new aspects of its potential application, which was not performed so far.

EXPERIMENTAL

Oxidation of GA

GA from *Acacia* tree (Sigma) was oxidized using sodium *m*-periodate (Carlo Erba) as previously reported.[10] Two samples of the polysaccharide (500.0 mg each, 0.003 mol saccharide units) were dissolved in 116 and 290 mmol L^{-1} aqueous solutions of sodium *m*-periodate (5.0 mL), respectively. The oxidations were performed at room temperature in the dark, in capped glass test tubes (16×125 mm^2; Fisherbrand), with constant magnetic stirring.

Aliquots were taken periodically from each reaction mixture for determination of periodate titrimetrically.[13] After 6 h, samples of 20% and 50% oxidized GA were obtained and reactions were stopped by adding 1,2-ethanediol (1.0 mL) in each reaction mixture. Excess iodate ions were removed from reaction mixture by dialysis against deionized water more than 48 h with changes of water every 6 h, until the negative reaction to the iodate ions (test with 1 mol L^{-1} solution of silver nitrate). Dialysis tubing (Spectra/Por, MWCO 12000) was obtained from Spectrum Medical Industries, CA. Purified oxidized samples were frozen, lyophilized to dryness on the Christ Alpha 2-4 LD plus lyophilizer at 0.02 mbar and −30°C, and stored at 4°C until use in subsequent reactions. Typical yield was 78–82%.

Preparation of Nys–GA Imine Conjugates

Two lyophilized samples of GA, 20% and 50% oxidized (50.0 mg each) were dissolved separately in 5.0 mL of borate buffer (0.1 mol L^{-1}, pH 8.0) in capped glass test tubes. Nys (Fluka) was added in obtained solutions (15.0 and 50.0 mg, respectively). The coupling reaction was performed at 37°C for 24 h in the dark with constant stirring. After completion of the reaction, the conjugates were dialyzed against deionized water for ~72 h, in the darkness, with frequent changes of water (every 4 h) until the negative probe to the antibiotic in the surrounding water for dialysis (after concentrating the surrounding water for dialysis to 2–3 mL, we recorded UV–Vis spectra of those samples; dialysis was performed when there were no absorption peaks in this spectral range). Obtained solutions of conjugates (Nys–GA20 and Nys–GA50) were frozen, lyophilized to dryness and stored at 4°C until use.

During all the procedures that included Nys and its conjugates, the extent of photochemical decomposition was minimized by protecting from light.

Preparation of Nys–GA Amine Conjugates

Part of imine conjugates was reduced with aqueous solutions of sodium borohydride (1.5 mol $NaBH_4$/mol of saccharide unit) for 24 h at 4°C. Obtained solutions of reduced conjugates (redNys–GA20 and redNys–GA50) were dialyzed in the darkness for 48 h, and, after complete removal of excess reducing agent ions, they were frozen, lyophilized, and stored at 4°C until use.

Characterization of Conjugates

Instrumental Methods. The UV–Vis spectra were recorded on an UV–Vis spectrophotometer GBC Cintra 40. GA, Nys, and their conjugates were dissolved in solvent mixture of dimethyl sulfoxide (DMSO) and water in 1: 3 (v/v) ratio. Spectra were recorded in spectral range between 260 and 445 nm.

The FTIR spectra were obtained in solid state, using attenuated total reflectance sampling technique, on a Perkin-Elmer FTIR 1725X spectrophotometer at the wavenumbers between 400 and 4000 cm^{-1}.

^1H NMR spectra were recorded on a Varian Gemini 2000 instrument at 200 MHz using DMSO-d_6 as a solvent and tetramethylsilane as an external standard.

Nys–GA conjugates (1 mg/mL in DMSO) were analyzed by thin-layer chromatography (TLC) using Silicagel 60 plates (Merck). The solvent system was chloroform: methanol: ammonia, 4: 10: 1 (v/v/v). The spots were visualized under UV light or by spraying with 50% sulfuric acid solution and heating at 100°C for 5 min. Nys (as standard), GA and Nys–GA mixture, 1: 2 (w/w) were used for comparison.

Thermal stability of the samples was analyzed by thermogravimetry (TG) using SDT Q600 (TA Instruments) for simultaneous TG/differential thermal analysis analysis. Experiments were performed in the range from room temperature to 600°C in atmosphere of nitrogen. Heating rate was 20°C min^{-1}.

The weight average molar masses (MW) of oxidized, reduced GA, and amine conjugates (Nys–GA20 and Nys–GA50) were determined using gel permeation chromatography (GPC) on Sepharose CL-4B. Samples were dissolved in water at concentration of 1.0 mg mL^{-1}. The column (1.2×80 cm^2) was eluted with sodium chloride (0.05 mol L^{-1}) at a flow rate of 0.1 mL min^{-1}. Fractions (3.0 mL each) were collected and elution of each polymer was monitored with phenol-sulfuric acid.[14] Column was protected from light to avoid photochemical decomposition of conjugates. A standard curve was constructed using dextran standards (Fluka) of MW range between 5 and 500 kDa.

Determination of Conjugate Stability. Stability of conjugates was investigated under different conditions: in dry form, in solution, and at various pH values.

Amine and imine conjugates were kept as a lyophilized powder at 25°C in light-protected glass tubes for 6 months. Samples were taken after 1 week, and after 1, 3, and 6 months. The chemical stability of the conjugates was determined by measuring UV absorption at 307 nm and GPC, using samples freshly prepared at a concentration of 0.05 mg mL^{-1} in deionized water.

Solutions of conjugates and Nys at concentrations of 1.0 mg mL^{-1} in phosphate buffer (0.1 mol L^{-1}, pH 7.4) were incubated at 25°C in light-protected capped glass tubes for 7 days. Parallel experiments were performed with free Nys and Nys–GA mixture, 1 : 1 (w/w). During this period, samples were taken every 24 h and analyzed by UV–Vis spectrometry for Nys content and GPC for determination of MW.

Solutions of the imine and amine conjugates (1.0 mg mL^{-1}) in buffers with different pH values (0.5 mol L^{-1} sodium acetate buffer, pH 4.0 and 5.0; 0.5 mol L^{-1} phosphate buffer, pH 6.0, 7.0, and 8.0; 0.5 mol L^{-1} borate buffer, pH 9.0) were incubated at 25°C. Samples were taken after 24 h and the stability was determined by UV detection at 307 nm.

Antifungal Activity. Antifungal activity of the conjugates was determined by agar dilution method using Sabouraud dextrose agar.[15] Test microorganisms were *Candida albicans* ATCC 24433 and *Aspergillus niger* ATCC 16888. Initial concentrations of samples were made in mixture of DMSO and water in 1: 3 (v/v) ratio. The following dilutions of samples were prepared: 0.39–50 μg mL^{-1}. Pure Nys served for comparison, and solvent mixture as a control. Loopful of stationary-phase microbial cultures containing $\sim10^4$–10^5 cfu mL^{-1} were spread over the surface of the agar plates. The minimum inhibitory concentrations (MICs) were determined as the lowest concentrations of compounds preventing any visible growth after 48 h (*C. albicans*) or 72 h (*A. niger*) at 28°C.

Hemolytic Activity. Hemolytic activity of the conjugates was determined against fresh sheep erythrocytes. These cells were suspended in phosphate-buffered saline (PBS) at concentration of 2% (v/v) and were washed in the PBS by centrifugation (3000 rpm for 10 min). The hemolysis reaction was performed in the test tube containing 0.1 mL of the serially diluted Nys–GA imine and amine conjugates and 0.9 mL of the erythrocytes suspension. Suspension of erythrocytes in PBS was used as a blank. After incubation at 37°C for 1 h, centrifugation and total lysis of the remained erythrocytes in the pellet with deionized water, content of the released hemoglobin was measured spectroscopically at 560 nm. Percentage of hemolysis was determined on the basis of different hemoglobin content in erythrocytes treated with conjugates and blank.

RESULTS AND DISCUSSION

Synthesis of Nys–GA Conjugates

Imine and amine Nys–GA conjugates were prepared by coupling reaction between two samples of oxidized GA and Nys, as shown in Figure 1. GA [Figure 1(A)] was oxidized by sodium *m*-periodate in aqueous solution [Figure 1(B)]. Two samples with different degrees of oxidation were obtained, depending on periodate/polysaccharide ratio. The oxidized samples were purified from excess of iodate ions by dialysis and lyophilized.

The aldehyde groups in the oxidized samples of GA were coupled with the primary amine groups of Nys [Figure 1(E)] to form Schiff base [Figure 1(C)]. The coupling reaction was performed in borate buffer. The aldimines synthesized in this reaction and released from excess antibiotic molecules by dialysis were reduced to secondary amines with sodium borohydride

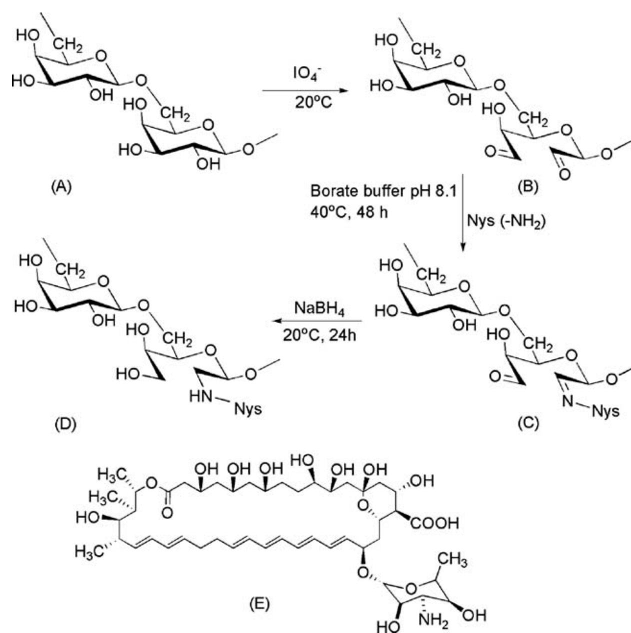

Figure 1. Schematic representation of coupling reaction of Nys with GA *via* imine bond: GA (A); dialdehyde GA (B); Nys–GA imine derivative (C); Nys–GA amine derivative (D); structure of the Nys (E).

[Figure 1(D)]. In this way, the unreacted aldehyde groups of the oxidized GA were reduced to the corresponding alcohols, which is important in preventing the possibility of further chemical bonding of the conjugates to biopolymers and other compounds *in vivo*.

To check the influence of the reaction conditions during conjugation on the free antibiotic, parallel testing with pure Nys, without polysaccharide, was performed.

Characterization of Conjugates

UV–Vis Spectroscopy. The UV–Vis spectra of the solutions of GA and oxidized GA (Figure 2) in solvent mixture of DMSO and water in 1: 3 (v/v) ratio showed no expressed absorptions, which was expected considering that polysaccharides, in general, do not contain groups that absorb in the UV–Vis spectral region. Essential changes in the UV spectra appeared as the result of coupling reactions of oxidized samples with the antibiotic. Synthesized products (Figure 2) showed in their spectra four distinctive peaks of different intensity with absorption maxima at 281 nm, 294 nm, 307 nm, and 323 nm. Comparing these data with the spectra of pure Nys[16] and sample of Nys that has been parallel subjected to the conjugation reaction conditions (Figure 2), no differences in the positions and relative intensities of the characteristic absorption peaks could be observed. The UV–Vis absorption spectra of the reduced conjugates with sodium borohydride showed the same characteristic peaks as the imine conjugates and the free Nys. Based on these results, it can be concluded that aldehyde groups of the oxidized polysaccharide coupled successfully with amine groups of the antibiotic. Retention of the UV absorption maxima indicated that Nys was in its native configuration, which was also proved for some other antibiotic–polysaccharide conjugates in the similar reaction conditions.[12]

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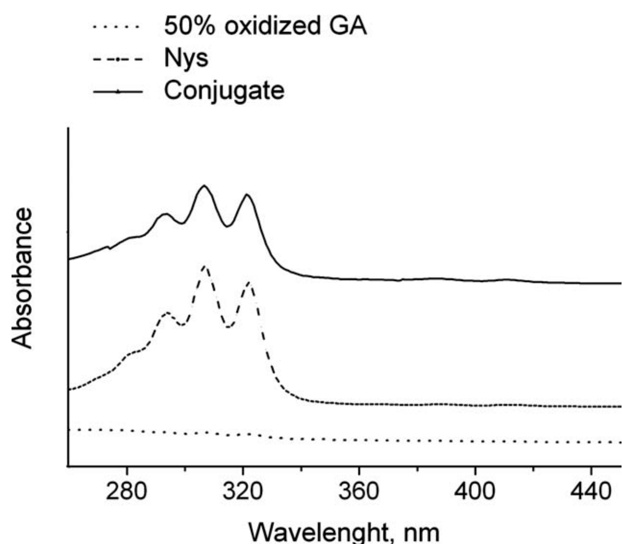

Figure 2. UV–Vis spectra of the solutions of: 50% oxidized GA (0.5 mg mL^{-1}), Nys (0.05 mg mL^{-1}), and Nys–GA50 (0.125 mg mL^{-1}) in solvent mixture of DMSO and water, 1: 3 (v/v).

FTIR Spectroscopy. The FTIR spectrum of native GA [Figure 3(A)] showed the characteristic bands for inter-OH and intra-OH stretching at 3000–3500 cm^{-1}, a band at 2910 cm^{-1} assigned to $—CH_2$ stretching, as well as bands at 1630–1600 cm^{-1} and broad band at 1400 cm^{-1} that were characteristic for —COO-asymmetric, and $—COO^-$ symmetric stretching vibrations, respectively. The bands at 1200–900 cm^{-1}, the finger prints of carbohydrates, gave evidence at 1020 cm^{-1} for —C—O stretching, absorption at 1150 cm^{-1} that indicated —C—O—C stretching was overlapped with former peak, and the absorption at 850 cm^{-1} was characteristic for the presence of beta glycosidic linkage.[17]

In the FTIR spectrum of 50% oxidized GA [Figure 3(B)], the characteristic absorption bands of dialdehyde polysaccharide appeared at 1730 and 880 cm^{-1}. The former sharp peak corresponded to the aldehyde symmetric vibrational band (carbonyl), and the latter indicated the hemiacetal structure between the aldehyde groups and neighboring hydroxyl groups.[18] In the 20% oxidized GA, the peak related to the aldehyde group is not visible (data not shown), which is consistent with previously published data.[19,20]

The FTIR spectrum of pure Nys [Figure 3(C)] was characterized by broadband with maxima at 3370 cm^{-1} which corresponded to —O—H and —N—H stretching, band at 2930 cm^{-1} assigned to symmetric and asymmetric —CH vibration of the $—CH_2$ group, absorption at 1703 cm^{-1} evidenced —C=O stretching of the ester group, bands at 1437, 1396, and 1010 cm^{-1} were characteristic of $—CH_3$, $—COO^-$, and polyene sequences, respectively.[21]

FTIR spectrum of the synthesized Nys–GA50 conjugate [Figure 3(D)] showed that the characteristic sharp absorption band of dialdehyde GA was reduced, which suggested that part of these groups was successfully coupled with Nys giving imine base, while part of free aldehyde groups remained on the oxidized polysaccharide after reaction with Nys. This finding is consistent with the results of determining degree of substitution from UV data, and it is reasonable because Nys [Figure 1(E)] is a relatively large molecule that, due to steric reasons, would not be able to react quantitatively with all free aldehyde groups in oxidized GA even to exclude conformational changes of polyaldehyde chain induced by different molecular interactions.[18] FTIR spectrum of the conjugate confirmed the presence of characteristic absorptions of Nys and GA, however, band of imine bond at 1580–1690 cm^{-1} was difficult to detect due to overlapping with characteristic absorption peaks of other groups in the same region.

FTIR spectrum of the synthesized redNys–GA50 conjugate [Figure 3(E)] showed two main characteristics which differed it from spectrum of imine conjugate and proved that reduction

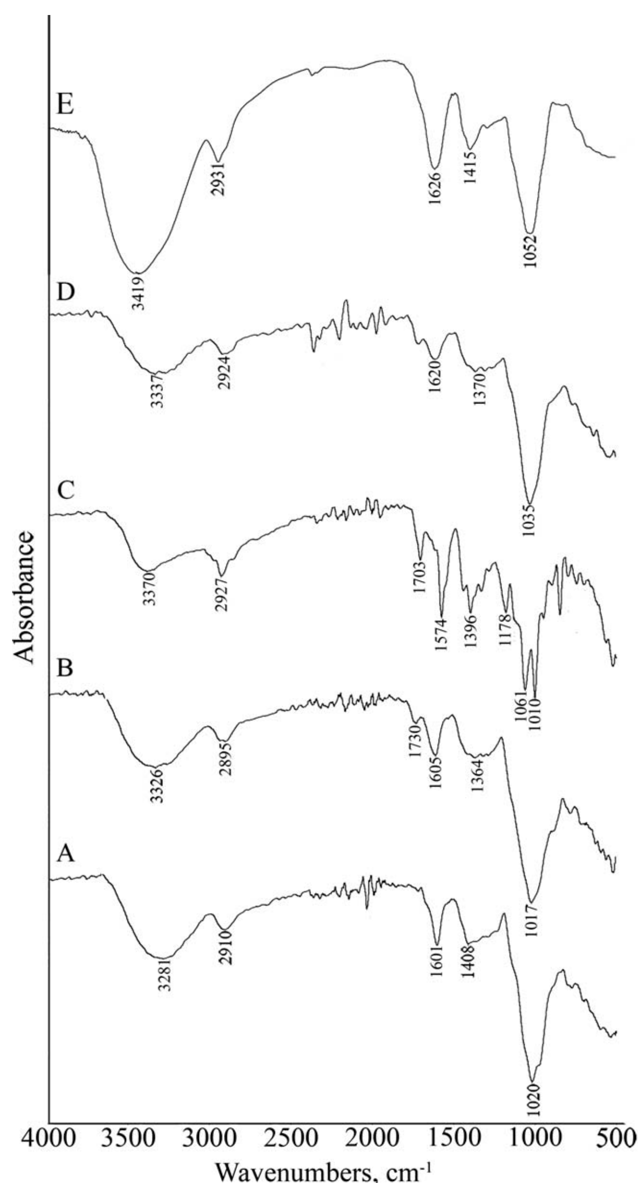

Figure 3. FTIR spectra of: GA (A), 50% oxidized GA (B), Nys (C), Nys–GA50 (D), and redNys–GA50 (E).

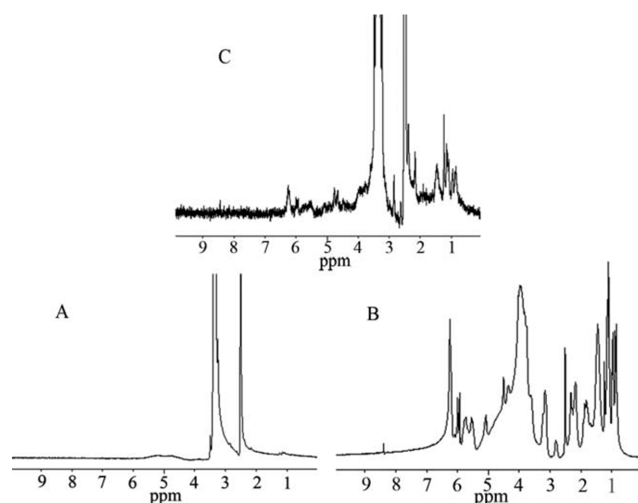

Figure 4. ^{1}H NMR spectra of the solutions of: GA (A), Nys (B), and Nys–GA50 (C) in DMSO-d_6.

was done successfully. Sharp absorption peak at 1626 cm^{-1} which corresponded to —N—H bending vibrations proved presence of amine group which was the result of reduction of imine group in Nys–GA50. Also, this spectrum showed characteristic broadband with maxima at 3419 cm^{-1} (due to —N—H and inter-OH and intra-OH stretching vibrations). In the case of primary amines, this peak should be splitted in two, due to symmetrical and asymmetrical —N—H stretching, but this is impossible to detect because of large number of hydroxyl groups in molecule that absorb in this region too.

^{1}H NMR Spectroscopy. Formation of the conjugates was further confirmed by their ^{1}H NMR spectral data (Figure 4).

The ^{1}H NMR spectrum of native polysaccharide GA [Figure 4(A)] showed characteristic chemical shifts of ^{1}H signals at 5.20–5.43 ppm related to α-D-GlcpA, α-D-Galp, terminal α-L-Araf and 3-α-L-Araf, in the region at 4.69–4.81 ppm ascribed to β-D-Galp and α-L-Rhamp and part of spectra at 4.38–4.48 ppm, which was assigned to β-D-GlcpA.[22,23]

GA is a complex heteropolysaccharide, which is known for its ability to produce viscous solutions. For this reason, the NMR spectrum of oxidized GA of satisfactory quality and good resolution of all signals (this is specifically related to signal of aldehyde group at 9.30 ppm) could not be observed.

To prove the formation of aldehyde groups, a mild controlled acid hydrolysis of GA according to the method of Defaye and Wong[24] and then oxidation under the same conditions as described in Experimental section for the sample of 50% degree of oxidation were performed. In ^{1}H NMR spectrum of that prepared GA (data not shown), several new peaks appeared at down field (above 5.50 ppm). These peaks are likely attributed to protons of hemiacetals formed during reaction of oxidation.[25,26] In addition to these signals, a peak at 9.30 ppm was observed too, indicating that —CHO groups were present after the oxidation.

^{1}H NMR spectrum of Nys [Figure 4(B)] showed dominant peaks related to the polyene part of the molecule (5.52–6.31 ppm), polyol segment (4.25–3.24), and hemiketal six-membered ring (4.51–1.16) as well as methylene region (2.40–1.40) and methyl groups (1.17–0.95).[27,28]

^{1}H NMR spectrum of Nys–GA50 is shown in Figure 4(C). Characteristic peaks of ^{1}H protons of Nys, as well as peaks of oxidized GA, could be observed. Apart from some overlapping signals, the part of the spectrum related to the polyene section of the antibiotic, as well as characteristic signals of sugar residues of GA, are particularly evident. Identical patterns of polyene regions (5.57–6.31 ppm) in the spectra of the pure antibiotic and conjugate were observed.

Thin-Layer Chromatography. TLC was used to confirm covalent bonding between Nys and GA (Figure 5). On developed plate, the conjugate appeared as a single spot at start line, without any traces of nonbonding free Nys. On contrary, in the lane containing the mixture of GA and Nys two distinctive spots could be observed, the first on start line corresponded to GA, and the second corresponded to Nys. Existence of one spot on TLC was evidence that all Nys was chemically bound in the synthesized conjugate.

Thermal Stability Analysis. Thermal stability of GA, oxidized GA, Nys and conjugates was analyzed by TG. Generally, degradation of native and oxidized samples occurred in two stages, as shown in Figure 6. The GA exhibited a similar behavior with a lost up to 74% of its mass in two steps.[29] In the first stage, that ranged between room temperature and 178°C, the mass loss was about 14%. This stage was attributed to the loss of adsorbed and hydrogen bound water. The second stage of weight loss started at 178°C and continued up to 489°C. There was about 50% of weight loss due to the degradation of the polysaccharide. The 50% oxidized GA started to degrade at a

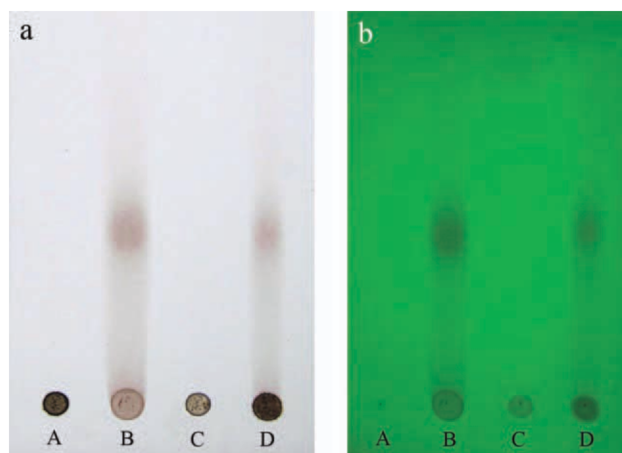

Figure 5. Photograph of thin-layer chromatogram of: GA (A); Nys (B); Nys–GA50 (C), and Nys–GA mixture, 1: 2 (w/w) (D) on: (a) Silicagel 60 plates and (b) Silicagel 60 F_{254} plates with ultraviolet indicator. Solvent system: chloroform: methanol: ammonia, 4: 10: 1 (v/v/v). Detection: by spraying with 50% sulfuric acid solution and heating at 100°C for 5 min (a) and by visualization under UV light (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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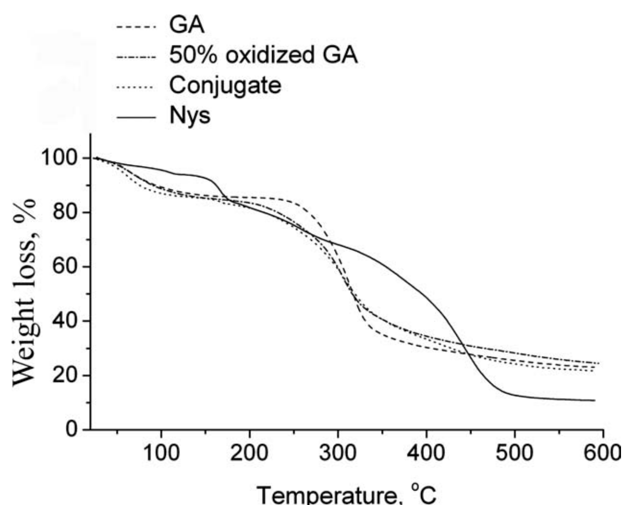

Figure 6. Thermograms of GA, 50% oxidized GA, Nys, and Nys–GA50.

lower temperature. During the first stage, which started from room temperature and continued up to 165°C, the mass loss was about 15% due to the elimination of water. In the second stage which ranged between 165 and 485°C, there was a loss of about 56% in weight due to decomposition of the monomer units. As can be seen, the oxidized samples were somewhat less stable than the parent polysaccharide. Similar effect was observed in the case of the linear polysaccharide too.[19]

Slightly reduced thermal stability of oxidized GA samples could be attributed to the opening of the sugar rings due to periodate oxidation, resulting in a polymer that allows free rotation of the β-glycosidic linkages. The imine and amine conjugates showed very similar TG curves and mass loss occurred in three stages: the first, up to 142°C when mass loss was about 15%, the second, up to 189°C (about 3% mass loss), and the third, up to 544°C, in which the mass loss was the largest, about 60%. These compounds showed improved thermal stability and degraded at a slower rate compared to the oxidized polysaccharide. This can indicate that the formation of imine and amine linkages with Nys improved stability, compared to the oxidized samples, which could be attributed to the covalent binding of Nys leading to changes in conformation of oxidized macromolecular chains. In addition, interactions of hydrogen bonds between Nys and oxidized glycan chains may occur.

Thermal analysis showed that free Nys is stable up to 100°C, whereas the oxidized polymer and conjugates lost about 8% of their mass up to 100°C. Nys looses mass in four steps: 6% up to 125°C, 13% up to 210°C, another 13% up to 303°C, and 57% up to 551°C (the total mass loss was 89%). These results are in agreement with the literature data for other antibiotic polyene structure, natamicin.[30]

MWs Determination. The molecular weights of the native GA and amine conjugates were determined by GPC. It was shown that MW of native GA was about 307 kDa. Amine conjugates of GA having degree of oxidation 20% and 50% had MWs of 370 and 359 kDa, respectively. The MWs of the amine conjugates were increased compared to corresponding polyols, indicating that these samples most likely tends to form hydrogen bonds between newly formed secondary amine group with the hydroxyl groups of conjugates.[11] The stability of the molecular weight, relative to native GA, can be explained by a (1,3) glycosidic bonds in backbone of GA that were not subjected to periodate oxidation and remained intact after this reaction. The periodate ion attacks vicinal diols promoting C—C bond breaking; such sites are located in the side chains. This fact is important for the stability of mass, unlike in the case of linear polysaccharides, for example, dextran, where the molecular weight decreased up to 30% as consequence of periodate oxidation.[26]

Stability of the Conjugates in Dry Form, in Solution and Under Different pH Values. Stability of amine and imine conjugates in dry (lyophilized) form and in solution was studied, as mentioned above. Each result is the average of two repeated experiments under the same conditions.

During the period of 6 months of the study, it was found that under appropriate storage of lyophilized conjugates, intensity of the UV absorption at 307 nm for each sample remained the same, compared with those at the start time of the experiment, showing that there was no degradation during storage. Also, other parameters such as MWs, antifungal, and hemolytic activity did not show noticeable change.

After 7 days of examining stability of the conjugates in solution, it was found that they were more stable than pure Nys (Figure 7). Their absorbance at 307 nm decreased to lesser extent and slowly compared with pure Nys, whose rapid absorption decrease indicated its inactivation. Nys–GA amine and imine conjugates lost about 3% and 10%, respectively, of their UV absorbance at 307 nm during 1 week of incubation, whereas the absorbance of the free Nys decreased about 65%. The decrease in absorbance of mixture Nys–GA, 1: 1 (w/w) was lower and delayed compared with free Nys, indicating that the presence of the polysaccharide stabilized this antibiotic in the solution, but

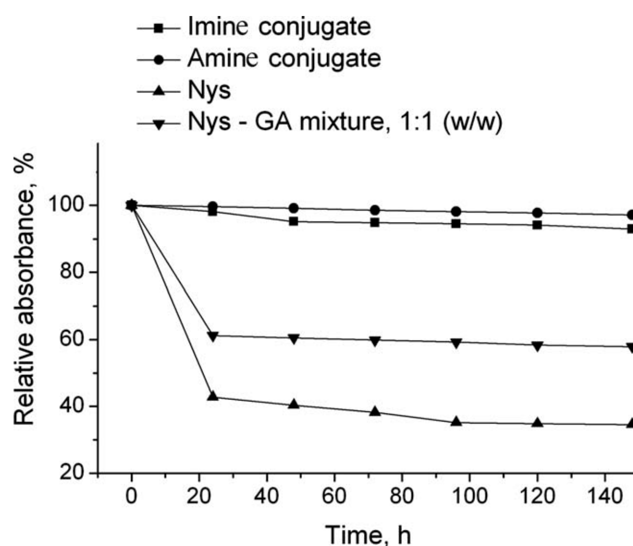

Figure 7. Stability of the aqueous solutions (1 mg mL^{-1}) of Nys–GA50, redNys–GA50, Nys and Nys–GA mixture, 1: 1 (w/w) while stored in the dark at 25°C.

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not for a long time. It was also observed that MWs of the conjugates increased about 20% after one week. Possible reason could be a change in conformation that affected the results of GPC.[31]

Study of the stability of the conjugates under different pH values showed that it is pH dependent, and that conjugates were more stable at acidic pH than the free Nys (Figure 8). Under alkaline conditions (pH 7–9) the conjugates were stable, especially the amine derivative. Imine conjugates lost about 9% of their absorbance at 307 nm during 24 h of incubation, amine conjugates about 6%, whereas free Nys lost about 32% at the same time. In all investigated conditions, the UV absorbance of conjugates changed less than in the case of free Nys, which demonstrated that coupling of Nys to the GA has positive effect on increasing its aqueous stability.

Antifungal Activity. The synthesized conjugates were evaluated against *C. albicans* and *A. niger* for their antifungal activity. The results are shown in Table I. The MIC for *C. albicans* was in the range of 3.125–6.25 μg mL^{-1} for both the imine and amine conjugates, whereas for *A. niger*, the MIC for both conjugates was in the range of 6.25–25 μg mL^{-1}. In general, conjugates were less active in comparison with pure Nys. This can be explained by the lower content of antibiotics in these compounds, in which some of the aldehyde groups did not chemically react with the amine group of Nys due to steric hindrance and the complex conformation of polymer chains in solution.

The imine and amine conjugates containing the polysaccharide with a higher degree of oxidation (50%) had a higher antifungal activity compared to those containing the less oxidized glycan (20%). These results were expected, considering the fact that the former have a larger number of sites for reaction with the antibiotic molecules, and thus a higher content of antibiotic. The UV absorbance of conjugates at 307 nm was correlated with its MIC against *C. albicans* and *A. niger*.

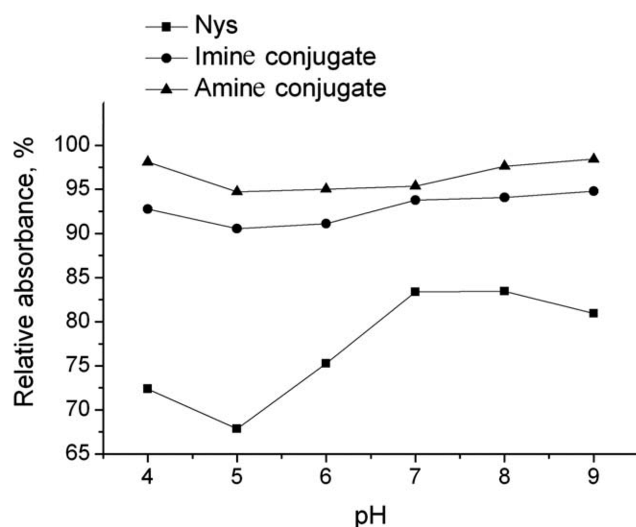

Figure 8. Stability of the solutions (1 mg mL^{-1}) of Nys–GA50, redNys–GA50, and Nys at different pHs. Stability was determined in relation to the initial UV absorption at 307 nm after 24 h of incubation in the dark at 25°C.

Table I. Antifungal Activity of Synthesized Conjugates

Sample	MIC (μg mL^{-1}) *C. albicans*	MIC (μg mL^{-1}) *A. niger*
Nys-GA20	6.25	25
Nys-GA50	3.125	6.25
redNys-GA20	6.25	25
redNys-GA50	3.125	12.5
Nys	1.56	6.25

Hemolytic Activity. The hemolytic potential of the synthesized conjugates was determined using fresh sheep erythrocytes. As reported previously,[32] the hemolytic activity of Nys is relative low in comparison with other polyene antibiotics. In the present experiments, Nys at a concentration of 10 μg mL^{-1} showed about 23% of hemolytic activity, whereas hemolytic activities of the conjugates at a concentration of 500 μg mL^{-1} for all samples were below 5%. This confirmed the fact that conjugation with polysaccharides reduces hemolytic activity of this class of antibiotics.[31]

CONCLUSIONS

In this study, conjugates of oxidized samples of GA and the polyene antibiotic nystatin were prepared. Increasing reactivity of the carbohydrate was achieved by the introduction of aldehyde groups into the polysaccharide chain using oxidative transformation by periodate reagent in aqueous solution. The degree of oxidation could be varied by changing the polysaccharide/periodate ratio.

The resulting polyaldehydo glycans with different degrees of oxidation were coupled with the amine groups of nystatin to give structures of Schiff's bases. The aldimines formed in the coupling reaction were reduced to stabile secondary amines with sodium borohydride. The formation of conjugates resulted in significantly increased stability in the different storage conditions and TG investigations, whereas antifungal activities were retained and hemolytic activity decreased, compared with free nystatin.

From the standpoint of potential application, the solubility of these compounds in water is an indication of their potential use in various formulations that would be nontoxic at physiological pH values. Thus, the binding of nystatin to the oxidized samples can be altered by varying the extent of oxidation of the polysaccharide, thereby changing the concentration of the antibiotic.

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