International Journal of Pharmaceutics 386 (2010) 1–5

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Targeted antifungal delivery system: β-Glucosidase sensitive nystatin–star poly(ethylene glycol) conjugate

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ARTICLE INFO

Article history:
Received 8 September 2009
Received in revised form 15 October 2009
Accepted 17 October 2009
Available online 27 October 2009

Keywords:
Nystatin
Star poly(ethylene glycol)
Drug polymer conjugates
β-Glucosidase sensitivity
Controlled release *in vitro*

ABSTRACT

A new targeted intravenous conjugate of nystatin with pentaerythritol poly(ethylene glycol)ether has been prepared and characterised (NY_4–sPEG, M = 25 160). The conjugate contains a β-D-glucopyranoside molecular switch sensitive to β-glucosidases (E.C.3.2.1.21), which are specifically present in the enzyme outfit of fungal pathogens. The investigated conjugate is stable under *in vitro* conditions for 24 h (solution of phosphate buffer pH = 7.4). Spectrophotometrically controlled releasing of nystatin in model medium containing β-glucosidase ((*Aspergillus niger*) 2 mg/mL, 66.6 units/g; pH 7.4, 2×10^{-2} M), reported decomposition half-life of conjugate $\tau_{1/2} = (88 \pm 2)$ s. This implies that releasing of nystatin is controlled only enzymatically.

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1. Introduction

Nystatin (NY) and amphotericin B (AMB) are two structurally similar polyene macrolide antibiotics characterised by a potent broad-spectrum antifungal action (Bolard, 1986). Nystatin (Fig. 1) was isolated from *Streptomyces noursei* (Hazen and Brown, 1951) and represents superior activity *in vitro* and *in vivo* against fungi including *Aspergillus fumigatus*, *Candida albicans*, *Coccidioides immitis*, *Cryptococcus neoformans* and *Histoplasma capsulatum* (Hazen and Brown, 1950). Nystatin exhibits also significant antifungal activity against AMB resistant strains. However, nystatin itself (Mycostatin®) is poorly absorbed from gastrointestinal tract and its parenteral administration is limited especially by its toxicity and harmful infusion therefore. That is why its earlier applications were restricted only to topical use (Richardson and Warnock, 1997). In the last decade, new forms of NY were intensively investigated. The new parenteral liposomal formulation of NY, with significantly reduced toxicity and therapeutic activity demonstrated on experimental models has been developed (Alonso-Vargas et al., 2000; Hamill, 2003). This form of NY (Nyotran®) is currently in the late phase of clinical trials (Mohr et al., 2008; Tao, 2008).

In our previous papers (Sedlák et al., 2001, 2007a,b, 2008) we described preparation of a number of covalent conjugates of AMB with substituted poly(ethylene glycols) (PEG) with the aim to increase therapeutic index of AMB. We prepared and characterised two types of targeted conjugates of AMB with PEG. The first way of targeted antifungal delivery makes use of locally lowered pH value at the site attacked by fungal pathogen like in the case of the pH-sensitive nystatin liposomes (Nasti et al., 2006). The conjugates of AMB containing a pH-sensitive imine bond were prepared and characterised (Sedlák et al., 2007a,b). The second type of targeted AMB conjugate is derivate contained four AMB molecules covalently linked to sPEG by means of a β-D-glucopyranoside molecular switch sensitive to β-glucosidases (E.C.3.2.1.21) (Sedlák et al., 2008). On the basis of *in vitro* tests it can be presumed that after intravenous application of the polymeric conjugates the active antimycotic drug will be released in targeted way, i.e. only at the site of the tissue attacked by fungal pathogen, which – in contrast to healthy human tissue – contains β-glucosidases.

The aim of this study is to prepare and characterise a new analogous conjugate of nystatin which would release NY in the same way, i.e. in targeted way only at the site of the attacked organ. The suggestion of the conjugate structure (NY_4–sPEG) (Fig. 1) was inspired – as in the previous case (AMB_4–sPEG) – by the finding that many parasitic fungal pathogens, e.g., from genus *Aspergillus*, *Candida* or *Trichosporon* contain in their enzymatic outfit specific hydrolases β-glucosidases (E.C.3.2.1.21) (Li et al., 2001). β-Glucosidases are not present in healthy human tissue, they are only found in some intestinal bacteria. This finding was made use of earlier in the case of some peroral prodrugs in colonic delivery (Hiyarama and Uekama, 2007). It can be presumed that the system of targeted transport of nystatin that we suggest will also be applicable as an active system destroying fungal pathogens that are able to survive in phagocytic cells (Briones

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doi:10.1016/j.ijpharm.2009.10.034

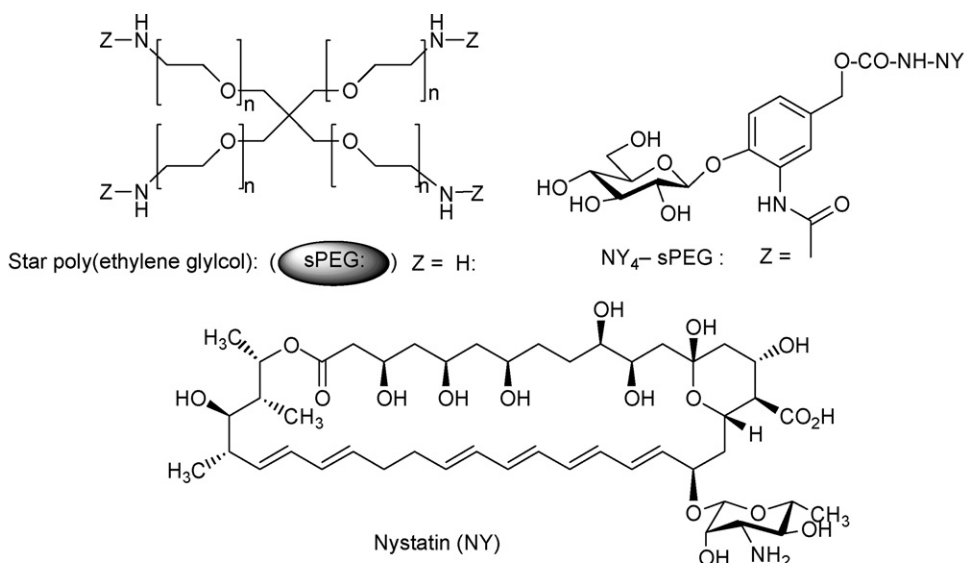


Fig. 1. Structure of β-glucosidase-sensitive nystatin–star poly(ethylene glycol) conjugate (NY_4–sPEG).

et al., 2008). The basic structural unit of the link NY_4–sPEG is formed by a β-glucosidic bond of substituted molecule of phenyl-β-D-glucopyranoside. With regard to its biocompatibility and binding capacity we have chosen for the polymeric carrier the star poly(ethylene glycol) [pentaerythritol poly(ethylene glycol)ether, M = 20 000] (sPEG) (Sedlák, 2005; Fichter et al., 2008). In contrast to the previous conjugate AMB_4–sPEG, the glucosidic switch in the conjugate NY_4–sPEG is linked by means of substituted urea. Enzymatic hydrolysis of the β-glucosidic bond should produce glucose, and the subsequent 1,6-elimination reaction (Sedlák et al., 2009) of labile carbamate link gives the carbamic acid of nystatin, which is hydrolysed to free nystatin very rapidly. The remaining molecule of polymeric carrier with covalently linked fragment of substituted 4-hydroxybenzyl alcohol is excreted from the organism (Scheme 1).

2. Experimental

2.1. Materials

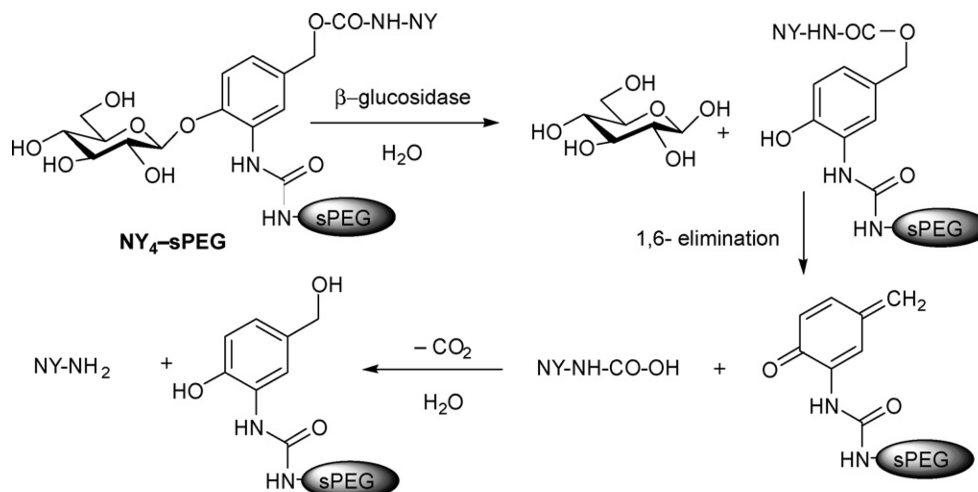
Unless otherwise stated pentaerythritol poly(ethylene glycol)ether (M = 20 000) was obtained from JenKem Technology USA. Nystatin and other chemicals and solvents were obtained from Fluka or Aldrich and used without further purification.

2.2. NMR

The ^{1}H NMR spectra were calibrated with respect to the middle signal in the multiplet of solvent (δ = 2.55). The ^{13}C NMR spectra were measured in standard way using broad-band proton decoupling and/or pulse sequence APT. The ^{13}C NMR spectra were calibrated with respect to the middle signal in the multiplet of solvent (δ = 39.6). The pulse sequence *gs* ^{1}H–^{13}C HMQC was measured with the CH interaction of 145 Hz.

2.3. HPLC

Releasing analysis was performed using a Shimadzu (Kyoto, Japan) HPLC system consisting of two Model LC-10ADvp pumps, a Model SPD-M10Avp UV/vis spectrophotometric detector, a Model RID-10A refractive index detector, a DGU-14A degasser, a CTO-10ASvp column oven and a SLC-10Avp system controller at 25 °C. Separation was achieved using a column PL aquagel – OH 30 8 μm, 300 mm × 7.5 mm (Polymer Laboratories, Shropshire, United King-



Scheme 1. Principle of nystatin release form NY_4–sPEG.

dom), preceded by a guard column PL aquagel – OH Guard 8 μm, 50 mm × 7.5 mm (Polymer Laboratories, Shropshire, Great Britane). Elution was carried with methanol:water (40:60, v:v) at a flow-rate of 1 mL/min. The mobile phase was filtered through a 0.45 μm Hydrophilic Polypropylene Membrane Filter (Pall Corporation, Ann Argot, Michigan, USA). The injection volume was set to 20 μl. The detection wavelength was 293, 305 and 320 nm.

The purity of conjugate NY_4–sPEG was estimated by HPLC using LiChroCART® 125 mm × 4 mm column packed with LiChrospher® 100 RP-18e 5 μm (Merck) and eluted with mobile phase of acetonitrile with 20 mM chelaton II.

2.4. GPC

Gel permeation chromatography was used for the estimation of M_w of the polymers **3–5** and conjugate NY_4–sPEG. The HPLC device was identical with purity determination instead following parameters: HEMA-BIO columns (hydrophilic modification HEMA-Gel, particle size 10 μm L, porosity 40/100/300/1000) at 25 °C using an RI detector and UV/vis detector. Redistilled water (pH 7.1) was used as the eluent. The columns were calibrated with a series of standard sPEGs of various molecular weights (JenKem Technology USA).

2.5. Microanalyses

The microanalyses were performed on an apparatus of FISONS Instruments, EA 1108 CHN.

2.6. Stability and kinetics measurements

The stability of NY_4–sPEG conjugate was monitored spectrophotometrically using an HP UV/vis 8453 Diode Array apparatus and 1 cm closable quartz cell in the cell holder kept at the constant temperature of 37 °C. The cell was charged with 2 mL phosphate buffer added from a pipette (pH 7.4; 2×10^{-2} M), and after reaching the said temperature, 15 μL methanolic solution of NY_4–sPEG conjugate was injected, so that the final concentration of the substrate was about 3×10^{-6} M. The enzymatic hydrolysis of NY_4–sPEG was monitored by the same methodology; however, the used solution was β-glucosidase (2 mg/mL) (E.C.3.2.1.21; Fluka; 66.6 units/g; *Aspergillus niger*) in phosphate buffer (pH 7.4, 2×10^{-2} M). The measured absorbance–time (*A*–*t*) dependence was used to calculate the half-life of enzymatic hydrolysis ($\tau_{1/2} = \ln 2/k_{obs}$) and the observed rate constant (k_{obs} (s^{-1})): $k_{obs}\tau = \ln \Delta A + \text{const.}$, where $\Delta A = (A_\infty - A_t)$.

2.7. Synthesis

1-(4-*tert*-Butyldimethylsilanyloxymethyl-2-nitrophenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside (**1**): A solution of 1-(4-hydroxymethyl-2-nitrophenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside (Sedlák et al., 2008) (1 g; 2 mmol), *tert*-butyldimethylsilyl chloride (0.9 g; 6 mmol) and imidazole (0.82 g; 12 mmol) in dry dimethylformamide (20 mL) was stirred at room temperature. After 96 h, the solution was evaporated, the residue was mixed with water and filtered, the filter cake was washed with water (3× 15 mL) and dried. Yield 1.2 g (97%); m.p. 184–186 °C, $[\alpha]_{20}{}^{D} = -10.0°$ (0.5 g/100 mL, $CHCl_3$). ^1H NMR (500.13 MHz, DMSO-d_6) δ: 0.08 (s, 6H); 0.89 (s, 9H); 1.96 (s, 3H); 2.00 (s, 3H); 2.01 (s, 3H); 2.02 (s, 3H); 4.13 (dd, 1H, *J* = 2 Hz; 12,5 Hz); 4.21 (m, 1H); 4.27 (m, 1H); 4.73 (s, 2H); 5.03 (t, 1H, *J* = 9.7 Hz); 5.07 (dd, 1H, *J* = 8.25 Hz; 9.7 Hz); 5.39 (t, 1H, *J* = 9.5 Hz); 5.63 (d, 1H, *J* = 8 Hz); 7.40 (d, 1H, *J* = 8.5 Hz); 7.61 (dd, 1H, *J* = 1.5 Hz; 8.5 Hz); 7.78 (d, 1H, *J* = 1.5 Hz). ^{13}C NMR (125.76 MHz, DMSO-d_6) δ: −5.5; 18.2; 20.4; 20.5; 25.7; 61.6; 63.2; 67.9; 70.4; 72.1; 72.2; 100.3; 119.9; 122.3; 130.8; 137.9; 141.2; 147.8; 169.2; 169.3; 170.1; 170.4. Anal. calcd for $C_{21}H_{25}NO_{13}$ (446): C, 52.84; H, 6.41; N, 2.28. Found: C, 52.96; H, 6.33; N, 2.53.

1-(2-Amino-4-*tert*-butyldimethylsilanyloxymethylphenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside (**2**): Glucopyranoside (**1**) (1.5 g; 2.44 mmol) was dissolved in ethyl acetate (250 mL), and PtO_2 (0.1 g; 0.4 mmol) was added to the solution. The mixture was hydrogenated using a slight overpressure of hydrogen (ca 5 kPa) at room temperature. The course of the reduction was monitored by means of TLC (Silica gel Merck, ethyl acetate, $R_F = 0.35$; detection with the Ehrlich reagent). After the reduction, the solution was decanted, filtered, evaporated until dry, and the residue was recrystallised from a mixture of hexane and diethyl ether. Yield 1.35 g (95%); m.p. 122–124 °C, $[\alpha]_{20}{}^{D} = -14.4°$ (0.5 g/100 mL, $CHCl_3$). ^1H NMR (500.13 MHz, DMSO-d_6) δ: 0.02 (s, 6H); 0.85 (s, 9H); 1.94 (s, 3H); 1.97 (s, 3H); 1.98 (s, 3H); 2.01 (s, 3H); 4.04 (d, 1H, *J* = 11 Hz); 4.17 (m, 2H); 4.48 (s, 2H); 4.54 (s, 2H); 4.96 (t, 1H, *J* = 9.5 Hz); 5.02 (dd, 1H, *J* = 8 Hz; 9.5 Hz); 5.26 (d, 1H, *J* = 8 Hz); 5.37 (t, 1H, *J* = 9,5 Hz); 6.44 (d, 1H, *J* = 8 Hz); 6.61 (s, 1H); 6.81 (d, 1H, *J* = 8 Hz). ^{13}C NMR (125.76 MHz, DMSO-d_6) δ: −5.1; 18.2; 20.5; 20.6; 20.7; 20.8; 26.0; 61.8; 64.4; 68.3; 70.9; 71.2; 71.9; 99.0; 113.1; 114.4; 115.7; 136.7; 138.2; 142.6; 169.7; 169.9; 170.0; 170.3. Anal. calcd for $C_{21}H_{25}NO_{13}$ (416): C, 55.56; H, 7.08; N, 2.40. Found: C, 52.76; H, 6.74; N, 2.69.

Polymer **3**: A cooled solution (−20 °C) of glucopyranoside (**2**) (300 mg; 0.51 mmol) and trioctylamine (218 mg; 0.62 mmol) in CH_2Cl_2 (10 mL) was treated with a solution of triphosgene (91.5 mg; 0.31 mmol) in CH_2Cl_2 (10 mL) added dropwise. After 3 h refluxing, the solvent was removed and the residue was distilled together with CH_2Cl_2 (4× 10 mL). The residue was mixed with a solution of s-PEG-NH_2 (1.285 g; 0.06 mmol) in CH_2Cl_2 (30 mL), and the mixture was stirred 24 h at room temperature. After concentrating, the solution was precipitated with diethyl ether (300 mL), and the precipitate was recrystallised from propan-2-ol. Yield 1.2 g (89%). ^1H NMR (500.13 MHz, $CDCl_3$) δ: 0.09 (s, 6H); 0.91 (s, 9H); 2.00–2.15 (m, 12H); 3.20–3.75 (m, 488H); 4.15–4.21 (m, 2H); 4.58 (m, 1H); 4.64–4.68 (m, 2H); 5.08–5.12 (m, 2H); 5.20–5.28 (m, 2H); 6.88–6.89 (m, 1H); 6.94–6.99 (m, 1H); 7.29–7.31 (m, 1H); 8.14 (s; 1H); 8.29 (s, 1H). Anal. calcd for $C_{1021}H_{1980}N_8O_{500}Si_4$ (22 483): C, 54.54; H, 8.88; N, 0.50. Found: C, 54.74; H, 8.93; N, 0.35. $M_w/M_n = 1.26$.

Polymer **4**: A cooled solution (0 °C) of polymer **3** (700 mg; 0.03 mmol) in CH_2Cl_2 (12 mL) was treated with TBAF (16 mg; 0.06 mmol). After 2 h, aqueous saturated solution of NH_4Cl (50 mL) was added at room temperature, and the mixture was extracted with CH_2Cl_2 (8× 50 mL). Combined organic portions were dried and concentrated and precipitated with diethyl ether (200 mL). The crude product was recrystallised from propan-2-ol. Yield 0.6 g (91%). ^1H NMR (500.13 MHz, $CDCl_3$) δ: 2.01–2.15 (m, 12H); 3.20–3.80 (m, 493H); 3.98–4.02 (m, 1H); 4.13–4.21 (m, 1H); 4.25–4.33 (m, 1H); 5.51–5.65 (m, 2H); 5.92–5.94 (m, 1H); 5.05–5.14 (m, 1H); 5.20–5.36 (m, 2H); 5.60–5.63 (m, 1H); 6.91–6.96 (m, 1H); 7.01–7.05 (m, 1H); 7.29–7.31 (m, 1H); 8.17 (s; 1H); 8.30 (s, 1H). Anal. calcd for $C_{997}H_{1924}N_8O_{500}$ (22 026): C, 54.37; H, 8.80; N, 0.51. Found: C, 54.61; H, 8.90; N, 0.40. $M_w/M_n = 1.22$.

Polymer **5**: A solution of polymer **4** (620 mg; 0.03 mmol) and 4-nitrophenyl chloroformate (300 mg; 1.5 mmol) in toluene (35 mL) was kept at the temperature of 50 °C. After 72 h, the toluene was distilled off, and the residue was mixed in diethyl ether (250 mL), filtered and washed with diethyl ether (10× 50 mL). After drying, the intermediate (ca 0.5 g) was dissolved in dimethylformamide (6 mL) with an addition of nystatin (84 mg, 0.09 mmol), 4-*N*,*N*-dimethylaminopyridine (6 mg; 0.05 mmol) and trioctylamine (175 mg; 0.5 mmol). The mixture was stirred 6 days at room temperature under argon atmosphere without access of light radiation. The crude polymer was precipitated from diethyl ether and recrystallised from propan-2-ol. Yield 500 mg

(65%). UV/vis (CH_3OH): λ_{max} (nm) 294; 307; 322. Anal. calcd for $C_{1189}H_{2216}N_{12}O_{572}$ (25 834): C, 55.28; H, 8.65; N, 0.65. Found: C, 55.62; H, 8.81; N, 0.49. M_w/M_n = 1.19.

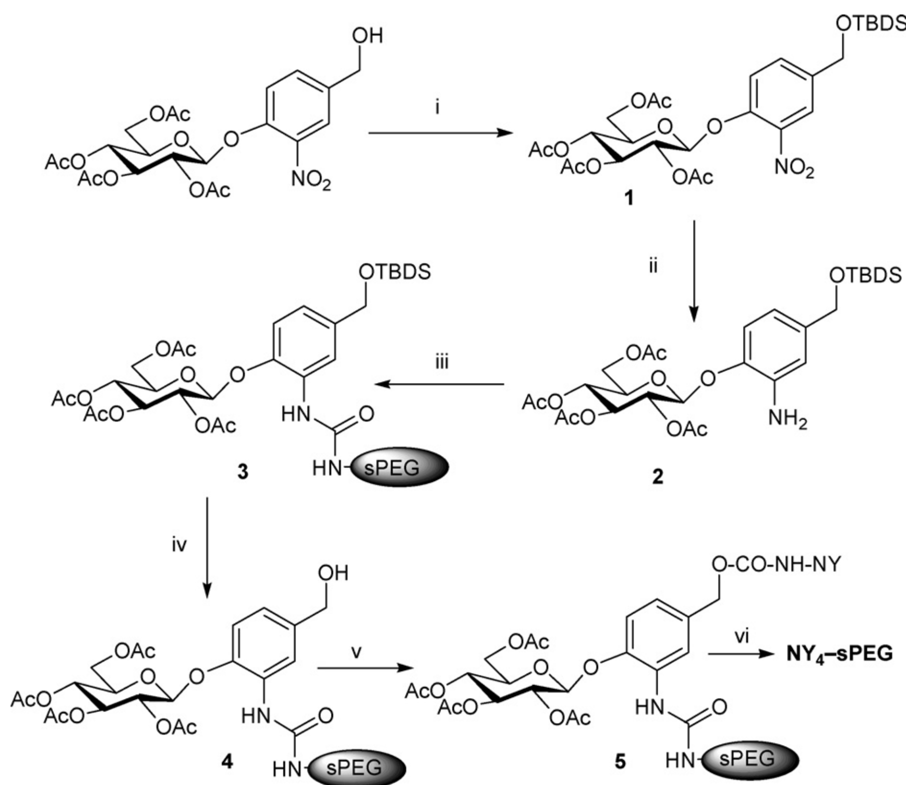
Conjugate NY_4–sPEG: A mixture of polymer **5** (350 mg; 0.014 mmol) and KCN (9 mg; 0.14 mmol) in methanol (15 mL) was stirred at the temperature of 25 °C. After 5 h, Amberlite IR-120 H ion resin (1.5 g) was added to the mixture. The ion exchanger was removed after 10 min by decanting, and the methanol solvent was distilled off under reduced pressure at the temperature of 35 °C. Yield 300 mg (86%). UV/vis (CH_3OH): λ_{max} (nm) 294; 307; 322. Anal. calcd for $C_{1177}H_{2184}N_{12}O_{556}$ (25 402): C, 55.65; H, 8.67; N, 0.66. Found: C, 55.83; H, 8.90; N, 0.48. M_w/M_n = 1.28.

3. Results and discussion

In contrast to the earlier described conjugate AMB_4–sPEG (Sedlák et al., 2008), the synthesis of conjugate NY_4–sPEG was carried out by another alternative method (Scheme 2). In the first reaction step, the hydroxyl group of 1-(4-hydroxymethyl-2-nitrophenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside was protected by *tert*-butyldimethylsilyl group to form 1-(4-*tert*-butyldimethylsilanyloxymethyl-2-nitrophenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside (**1**). In the second step, glucopyranoside **1** was reduced in the presence of the Adams catalyst, which led to formation of 1-(2-amino-4-*tert*-butyldimethylsilanyloxymethylphenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside (**2**). Verification of β-configuration of glucopyranosides **1** and **2** was carried out by the methodology of hetero-correlated ^1H–^{13}C HMQC 2D NMR spectra (Duus et al., 2000). In the case of nitro derivative **1**, the anomeric carbon atom exhibits the shift of 100.3 ppm, which corresponds with the doublet of anomeric hydrogen atom at 5.63 ppm with the coupling constant of 8 Hz. Amino derivative **2** has its anomeric carbon atom at 99.0 ppm, which corresponds with the doublet of anomeric hydrogen atom at 5.26 ppm (8 Hz). The values of coupling constant of the anomeric hydrogen atom with respect to the vicinal hydrogen atom indicate that the two atoms are in axial positions, i.e. the glucose fragment in both derivatives **1** and **2** assumes β-configuration [β-$^4C_1$(D)].

In this case glucopyranoside **2** was attached to the polymeric carrier by means of the urea residue. First, aminoglucopyranoside **2** was submitted to a reaction with triphosgene, and then the isocyanate formed reacted with pentaerythritol tetraaminopoly(ethylene glycol) (sPEG–NH_2) to give polymer **3**. As compared with the previous way of attaching the glucopyranoside fragment by means of carbamate link (65 °C/100 h), this way of attaching can be performed under very mild conditions (25 °C/24 h), the conversion of terminal amino groups of the polymer being quantitative. Subsequent deprotection of the hydroxymethyl groups gave polymer **4**, whose free hydroxyl groups were activated by means of 4-nitrophenyl chloroformate. In the following reaction step, four molecules of NY were attached through carbamate links to give polymer **5**. The final deprotection of acetylated hydroxyl groups in the glucose fragment of polymer **5** was achieved by reaction with catalytic amount of potassium cyanide, which was quantitatively removed after the reaction by means of an ion exchanger (Scheme 2).

The deprotection course was monitored by means of ^1H NMR spectroscopy: the untransparent spectrum of polymer **5** showed distinctly only the multiplet of CH_3-acetyl groups at 2.02 ppm, which was gradually disappearing. The final conjugate NY_4–sPEG was characterised by microanalysis; GPC was used for determination of M_w, and HPLC was adopted for determination of purity of the conjugate; the content of free NY was below 1 mol%. Furthermore, by means of UV/vis spectroscopy (typical maxima corresponding to polyene system of NY (O'Neil et al., 2001)) it was proved that



Scheme 2. Synthesis of NY_4–sPEG; reagents and conditions; (i) *tert*-butyldimethylsilyl chloride (TBDSCl)/imidazole/DMF, 25 °C/96 h; (ii) PtO_2/H_2/ethyl acetate, 25 °C/3 h; (iii): (a) triphosgene/CH_2Cl_2, reflux/3 h and (b) sPEG–NH_2/CH_2Cl_2, 25 °C/24 h; (iv) tetrabutylammonium fluoride/methanol, 25 °C/24 h; (v): (a) 4-nitrophenyl chloroformate/toluene, 50 °C/72 h and (b) 4 NY/DMF, 25 °C/144 h; vi: (a) KCN/methanol, 25 °C/5 h and (b) Amberlite 120-H/methanol, 25 °C/10 min.

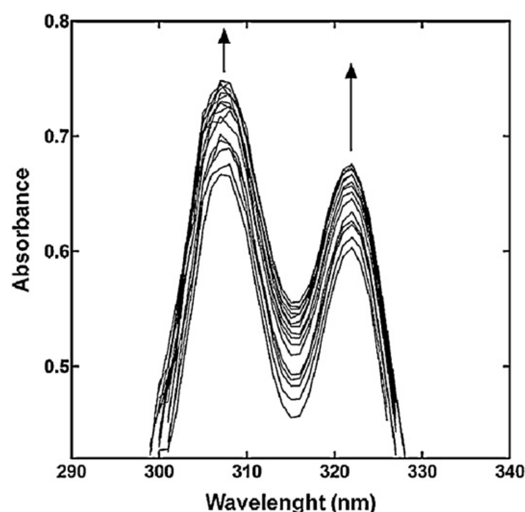


Fig. 2. Record of time change of spectrum for enzymatic hydrolysis of conjugate NY_4–sPEG (5×10^{-6} M) catalysed by β-glucosidase (2 mg/mL; 66.6 units/g) in phosphate buffer (pH = 7.4, 2×10^{-2} M) at 37 °C.

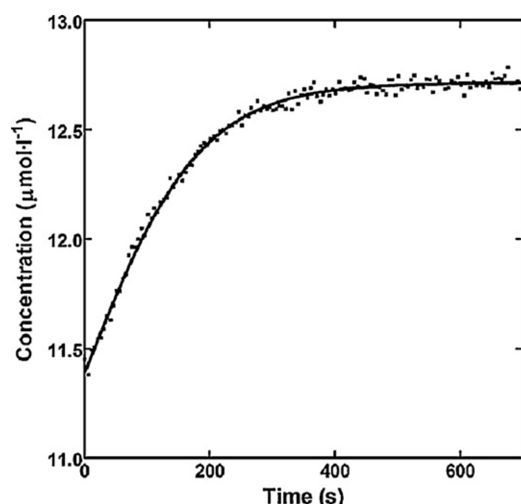


Fig. 3. Kinetic concentration (μmol l^{-1})–time (s) dependence followed at the wavelength of 322 nm [$\tau_{1/2}$ = (88 ± 2) s].

the content of NY in the conjugate corresponds to the molar ratio of 1:4 (sPEG/NY). The prepared conjugate is very well soluble in water and forms finely opalescent solutions.

Next part of research dealt with preliminary *in vitro* tests in various media of the conjugate prepared by us. First, we studied stability of the conjugate in phosphate buffer. Monitoring of time changes in UV/vis spectra showed that in phosphate buffer solutions (pH = 7.4) no visible spectral changes occur during 24 h. For a model medium imitating the tissue attacked by fungal pathogen we chose β-glucosidase (E.C.3.2.1.21) (*A. niger*) in phosphate buffer (pH = 7.4). Spectrophotometric monitoring of time change of spectral record of the conjugate showed an increase in the absorbance at 307 nm and 322 nm (Fig. 2), which is characteristic for the spectrum of nystatin. The rate of releasing of NY under the experimental conditions described obeys the kinetic equation of pseudo-first order, the half-life of NY release being $\tau_{1/2}$ = (88 ± 2) s (Fig. 3). The release of nystatin from the conjugate was also confirmed by means of HPLC. The results given show that the behaviour of conjugate NY_4–sPEG in the presence of buffered solution of β-glucosidase corresponds to Scheme 1.

4. Conclusions

With regard to increasingly appearing resistance of fungal strains against AMB (Hamill, 2003), a new promising intravenous liposomal drug form of nystatin was developed (Nyotran®) which is in late phase clinical trials at present. In this study we have prepared another alternative intravenous drug form—a new targeted polymeric conjugate. Under *in vitro* conditions it was confirmed that selective release of NY from the conjugate occurs only enzymatically, i.e. by action of β-glucosidase. The results of this preliminary study indicate that NY release should take place in a targeted way, at the site of tissue attacked by fungal pathogen containing β-glucosidases. However, appropriateness of practical application will necessitate further experimental studies on animal models and their critical evaluation.

Acknowledgment

The authors acknowledge the financial support provided by the Czech Ministry of Education, Youth and Sports, Project No. MSM 002 162 7501.

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