J Incl Phenom Macrocycl Chem (2011) 71:87–93
DOI 10.1007/s10847-010-9905-2

ORIGINAL ARTICLE

Nystatin–polyethylene oxide conjugates with enhanced solubility in water

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Received: 16 September 2010 / Accepted: 11 November 2010 / Published online: 23 November 2010

Abstract Due to its broad-action spectra nystatin has been used for years for mucocutaneous candidosis, still its clinical use has been limited due to the lack of absorption by the oral route, and systemic side-effects and toxicity. In order to overcome low water solubility and high toxicity new conjugates nystatin–polyethylene oxide were synthesized and characterized from physico-chemical, as also from the controlled release pattern and antifungal efficacy point of view. To the knowledge of the authors this structure has not been reported previously in the literature and indicate an interesting release pattern as well as increased biological activity.

Keywords Nystatin · Sustained release · PEO

Introduction

Nystatin (Ny) is a polyene antifungal antibiotic characterized by a potent broad-spectrum antifungal action [1] including a wide range of pathogenic and nonpathogenic yeasts and fungi [2]. The first polyene drug to be identified, it was extracted from *Streptomyces noursei* and its action mechanism is based on binding the ergosterol from the structure of fungal cell membrane, resulting in fungal death [3]. Even Ny is active against a variety of fungal pathogens including *Candida, Aspergillus, Histoplasma,* and *Coccidioides* and has been used for years for mucocutaneous candidosis, clinical use has been limited due to the lack of absorption by the oral route, and systemic side-effects and toxicity, manifested by thrombophlebitis, fever, rigors and hemolytic anemia [4, 5]. Because of its toxicity nystatin cannot be given in sufficiently high doses to completely eradicate the fungal infections, amplifying the resistance phenomena described for this compound [6].

In order to overcome low water solubility and high toxicity many efforts have been devoted to the complexation of Ny with different compounds like cyclodextrins [7], liposomes [8] or with amphiphilic polymers or copolymers [9]. Together with liposomes, polymers are a promising class of transporters through the huge variety of structures and manipulability of their properties [8, 10] or the synthesis of new forms of Ny more soluble but less active than the common ones [11].

One of the most spectacular applications of the therapeutic systems is represented by those polymeric transporters of active substances, referred to as intelligent, stimulo-active or environment sensitive polymers; the interest for the study of such polymers increased exponentially in the last years. Such conjugated polymers are able to release in a controlled manner the conjugated bioactive agents in accordance with the smallest variations of environmental physical or chemical stimuli. Examples are the polymeric-drugs and polymeric-protein complexes; there are many success applications of the intelligent polymers-bimolecular systems in medicine and biotechnology [12]. Up to now not too much interest in complexation of Ny with polyethylene oxide was granted. Besides two brief reports related to the obtaining and FT-IR characterization of Ny polyethylene oxide (PEO) ointments [13], of β-glucosidase sensitive Ny star poly(ethylene glycol) conjugates [14] and pluronic block copolymers were described [15].

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PEO due to its low toxicity and high solubility in water PEO might be a good candidate for increasing the low solubility of Ny in water. This paper deals with the synthesis and characterization of the new conjugates PEO–Ny with increased solubility in water (Phase solubility studies) and with the ability to separate the Ny from the polymer chain in a controlled way at physiological pH at 37 °C. The biological activity of new obtained conjugates was also analyzed in order to evaluate the new conjugates as possible systems with controlled release for clinical applications.

Materials and methods

Materials

Nystatin kindly offered by "Antibiotica" Iasi and PEO 1,000 Da (Aldrich) were used as received without further purification. Double distilled water, 0, 1 N natrium hydroxide solution and acetone were used throughout the study. The artificial sputa (pH 7.2) was prepared by solving 1.47 g KCl, 0.19 g $KHPO_4$, 1.25 g $NaHCO_3$ and 0.52 g KSCN in 1 L distilled water.

Methods

Solubility studies

Solubility studies were carried out according to the Higuchi and Connors method [16]. PEO solutions of different concentrations ($0.5–40 \times 10^{-2}$ M) were added to a supersaturated solution of Ny in equal volumes and shaken at (37 ± 1 °C) for 24 h. After reaching equilibrium, the solutions were filtered. The absorbance of each solution containing different mole fraction of the drug and PEO was measured by UV at 306 nm and the concentration of Ny in each solution was determined with reference to a suitably constructed standard curve, after 1/100 dilution.

Preparation of the solid complex

Ny–PEO complexes were obtained by stirring a mixture of 1.4×10^{-5} M Ny and 0.9×10^{-5} M PEO (structural units) in acetone for 72 h, at room temperature. During the reaction, the vessel was maintained in dark in order to avoid the exposure of Ny to the light. During the reaction, the vessel was maintained in dark in order to avoid the exposure of Ny to the light. The initial dark yellow clear solution turned into yellowish slurry. The slurry was poured in cold water and the resulted precipitate was filtered and dried in vacuum. Yield 87%.

Determination of nystatin releasing from the complex

Ny releasing kinetics was established by suspending 23 mg Ny–PEO conjugates (containing 20 mg Ny—determined experimentally by measuring the absorbance of Ny) in vials containing 10 mL pH 7.2 artificial sputa and stirring the mixture for determinate interval of time varying from 5 to 300 min. After the end of the pre-established time each suspension was quickly filtered and the UV absorption of the corresponding supernatant was measured. Concentration of released Ny was determined spectrophotometrically at 310 nm with reference to a suitably constructed standard curve.

Physico-chemical characterization

UV measurements were performed on an Analytik Jena Specord 200 spectrophotometer. DSC data were obtained on a Perkin Elmer-Diamond device using samples of 2–6 mg with a heating rate of 10°C/min, in temperature range 30–330 °C, under nitrogen gas flow. DSC data also allowed us to calculate the inclusion ratio of PEO and Ny cavity using Eq.1.

$$\textbf{Inclusion ratio} = (\Delta \mathbf{H_1} \times \mathbf{100})/\Delta \mathbf{H_2} \quad (1)$$

where ΔH_1 represents drug discomposure enthalpy in the inclusion complex, ΔH_2 represents free drug discomposure enthalpy. ΔH_1 and ΔH_2 were obtained for the same amount of pure drug and complexed drug (2.5 mg).

X-ray diffraction patterns (XRDP) were obtained on a Bruker AXS D8 advance RX diffractometer.

Nuclear magnetic resonance studies (^{1}H-NMR) were performed in dimethylsulfoxide (DMSO) on a DRX 400 Bruker Advance equipment.

In vitro antifungal activity

In vitro antifungal activity was determined on *Candida albicans* ATCC 10231-yeast strain using pure and conjugated Ny solutions in DMSO used for inoculation on the yeast strain inocula. Yeast strain inocula were obtained using stock cultures, prepared by the suspension of five colonies 1 mm diameter in saline solution for 24 h. Suspension density adjustment was performed spectrophotometrically, so that the absorbance of each suspension to correspond to that produced by McFarland standard 0.5 at 530 nm. The final density of the suspensions varied from 1×10^6 to 2.5×10^3. These suspensions were diluted 1:100 with sterile saline solution and 1:20 in RPMI 1640-MOPS, so that the final cell density to vary from 0.5×10^3 to 2.5×10^3. The witness suspension was obtain by adding 900 μL yeast inocula to 100 μL RPMI 1640-MOPS DMSO 1:10 dilution. Growth factor was determined by comparing

turbidity in sample tubes with witness tube and CMI was calculated as the minimal antifungal agent concentration that caused growth total inhibition.

Results and discussion

Phase solubility studies

The solubility of Ny in water is very low 4×10^{-4} M at 37 °C, as described in literature [2]. In Fig. 1 it is shown the solubility curve obtained for Ny in presence of PEO (Fig. 1) in distilled water. As one can see the concentration of Ny in water increases linearly up to 38.4×10^{-4} M, the resulting linear curves can be classified, in general, as type AL (linear positive isotherm), as described in literature [9]. Tenfold increase of the Ny water solubility indicates that Ny–PEO conjugate is a proper candidate for Ny complexation, due to its enriched capacity of increasing Ny low water solubility.

Differential scanning calorimetry

DSC is the most appropriate method to unveil useful information on solid-state interactions between Ny and PEO (Fig. 2). As seen from Fig. 2 DSC melting thermogram of Ny is typical for a crystalline anhydrous substance, with a sharp fusion endotherm ($T_{peak} = 160$ °C). PEO exhibits a melting endothermic peak at 35 °C observable both in pure and PEO–Ny physical mixture (in the same ratio as in the conjugate). This peak disappears completely in case of the Ny–PEO due probably to its coverage by the Ny macrocycle. The interaction between the two components seems to affect in a much less degree Ny which peak is just slightly shifted to 158 °C comparing with the pure

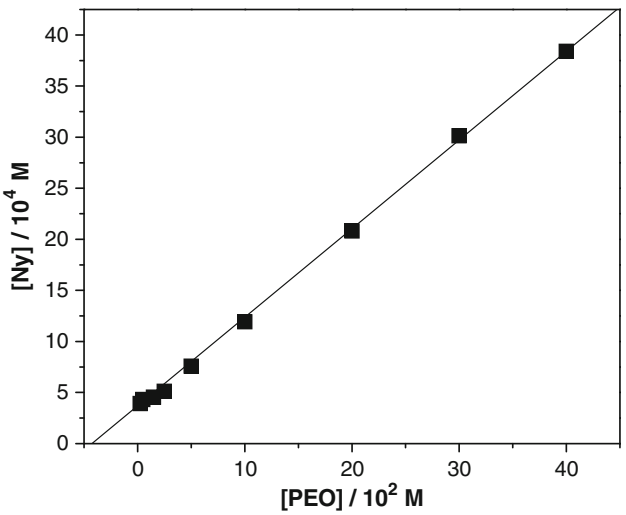

Fig. 1 Ny–PEO phase solubility behavior

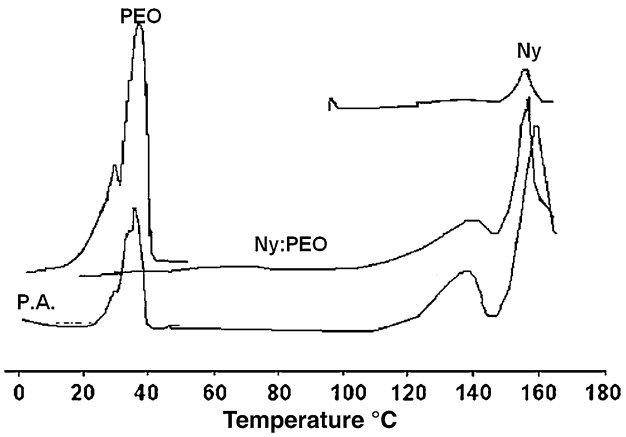

Fig. 2 DSC thermograms for Ny, PEO, their physical mixture (P.A.) and Ny–PEO conjugate

Ny and physical mixture. That might be due to the entrance of the polymeric chain inside the cavity of the Ny macrocycle, preventing PEO from melting up to temperatures that usually characterize Ny molecules. The inclusion ratio of PEO in Ny was equal to 60%.

X-ray diffraction pattern studies

X ray diffraction patterns of pure Ny and PEO, and their corresponding solid are shown in Fig. 3. As observable from Fig. 3 both X-ray diffractograms for Ny and PEO exibit sharp diffraction peaks are present, indicating their crystalline state. More intriguing is the behavior of the Ny–PEO conjugate that seems to indicate a higher crystallinity than the initial components probably due to the formation of a new structure with higher internal order degree, comparing with the physical mixture of Ny and PEO that exhibit the peaks characteristic for the pure components (Fig. 3).

The interplanar distances for all above investigated samples are presented in Table 1. As can be seen from Fig. 3 and Table 1, Ny–PEO complex exhibit a different (individual) structure. We could recognize only three common diffusion reflexes (d = 10.76 and 8.85 Å from Ny, 4.2 from PEO) in the conjugate structure due to internal reorganization caused by Ny and PEO interaction and probable hydrogen bonding formation.

Nuclear magnetic resonance studies

^{1}H-NMR studies reveal useful information about the interaction between the two components in the Ny–PEO conjugates. The spectra of pure Ny and PEO and the corresponding Ny–PEO conjugate, obtained in DMSO, are shown in Fig. 4. As seen from Fig. 4c the spectra of the Ny–PEO conjugate and pure Ny (Fig. 4a) look quite

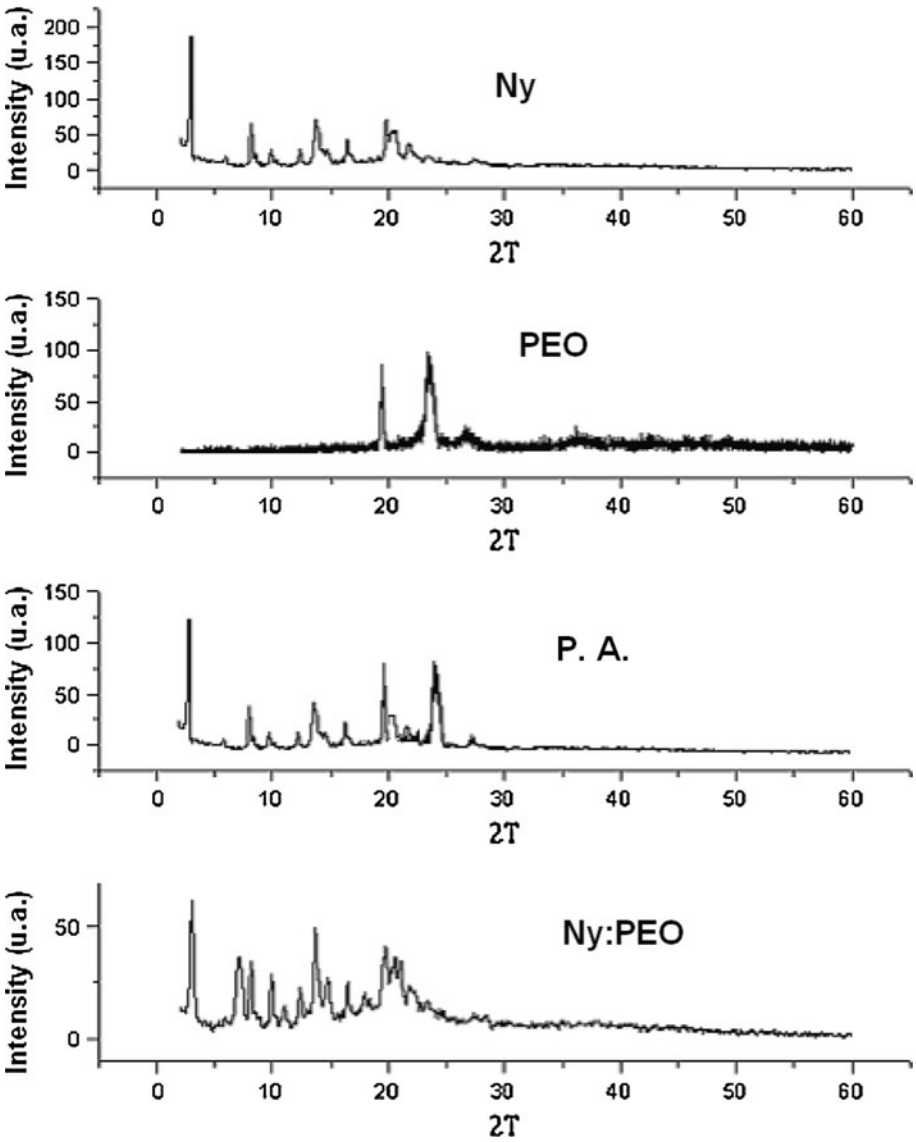

Fig. 3 XRDP of the starting Ny, PEO, their physical mixture (P.A.) and the Ny–PEO conjugate sample

Table 1 Interplanar distances calculated from XDRP

Sample	d (Å)
Ny	30.02, 10.78, 14.74, 6.42, 5.39, 4.47, 4.05
PEO	4.56, 3.79
Physical mixture	30.02, 10.78, 14.74, 6.42, 5.39, 4.56, 4.47, 3.79
Ny–PEO	29.13, 12.4, 10.78, 8.86, 7.14, 6.47, 5.37, 4.94, 4.48, 4.31, 4.04

similar except the changes involving the shift of both methyl (H-36) and OH groups (H-3 and H-10) due to some small conformational changes and hydrogen bonding effects imposed by the hydroxyl group at C-10 [17]. Quite different from Ny behavior PEO ^{1}H-NMR indicates the shifting of both $-CH_2$ (1′) and OH protons (1″), from 3.75 ppm, respectively 2 ppm to 3.42, respectively 1.87 ppm, indicating the formation of hydrogen bonding with the Ny macrocycle as suggested also by X ray diffraction patterns studies above described [18]. Using the ratio between the integrals determined for OH protons from PEO structure and H-10 that are situated quite separated in the conjugate spectra was possible to calculate the ratio between Ny and PEO in the conjugate and was determined as 6.1 Ny cycles per PEO chain (confirming the results obtained by UV measurements described in section Methods 3). No chemical interaction and no degradation of Ny was observed.

Release kinetics from Ny–PEO conjugates

Ny releasing from the Ny–PEO conjugates (Figs. 5, 6) presents an interesting kinetics. As depicted in Fig. 5a the absorbance of the Ny dissolved in water at 306 nm increases in time, and by reference to the calibration curve

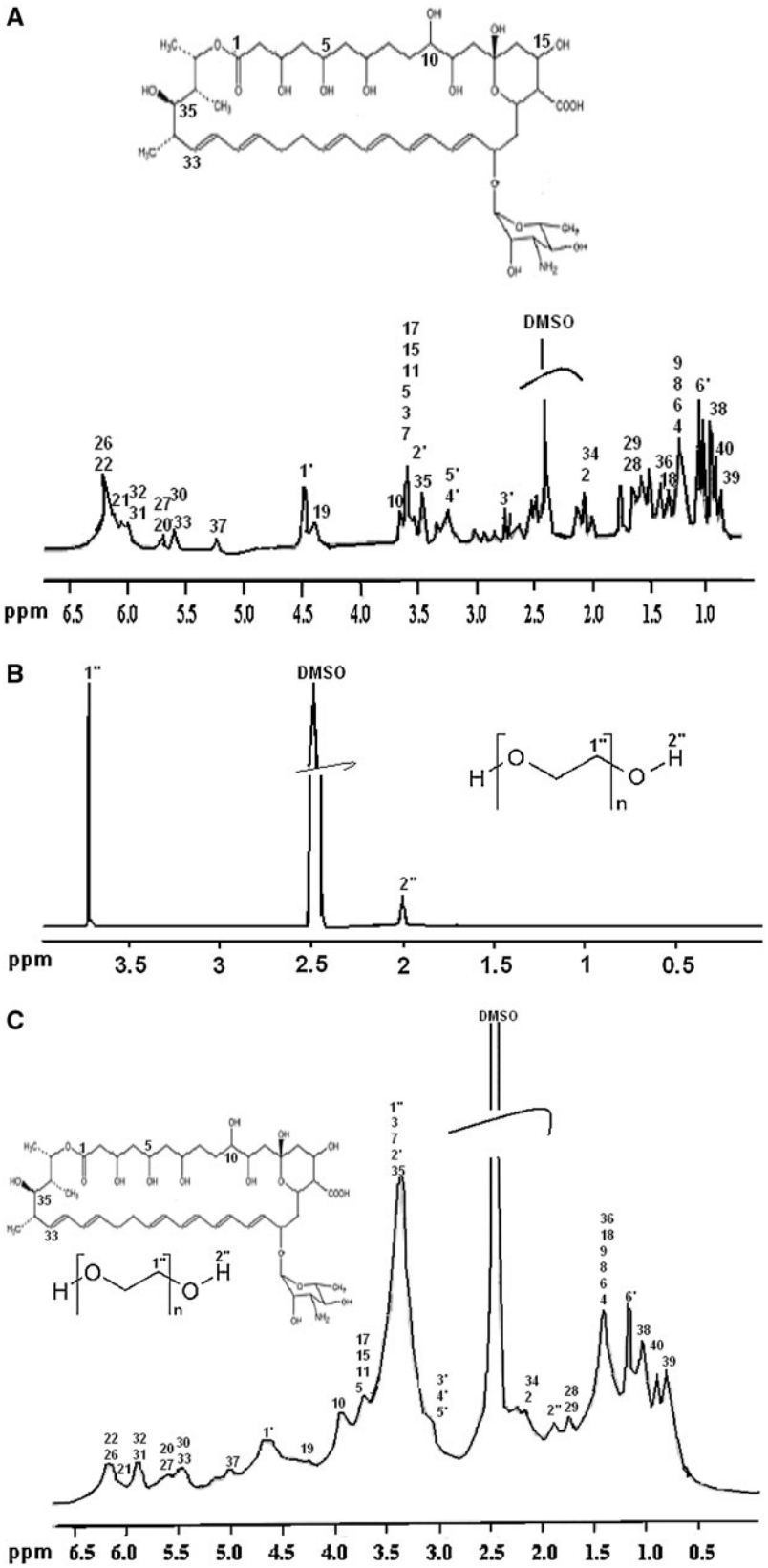

Fig. 4 ^{1}H-NMR spectra of Ny (**a**), PEO (**b**), and Ny–PEO conjugate (**c**)

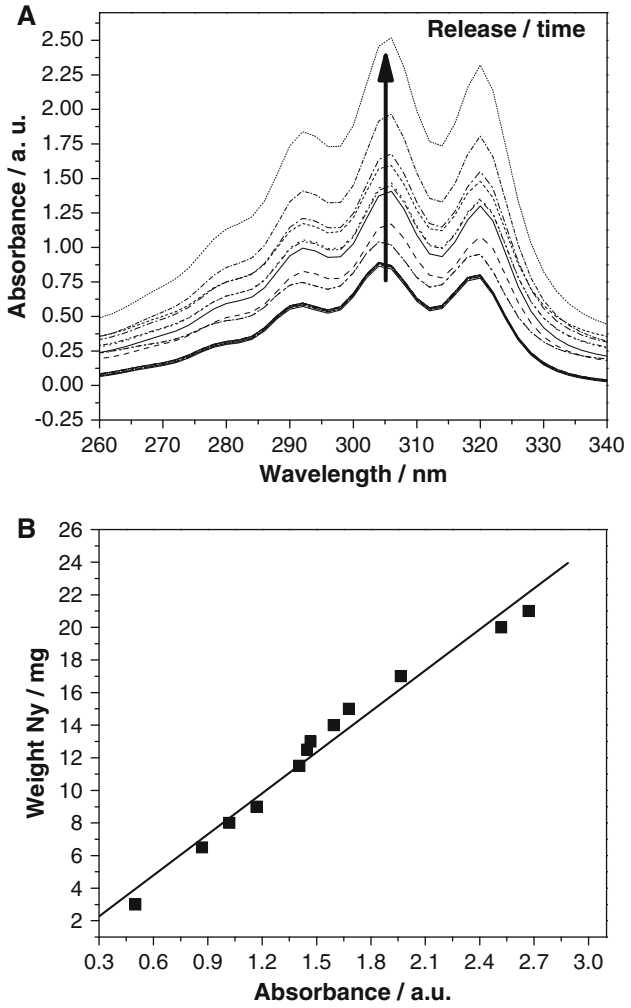

Fig. 5 The dependence of the absorption at 306 nm of the release time (**a**); and the calibration curve of Ny (**b**)

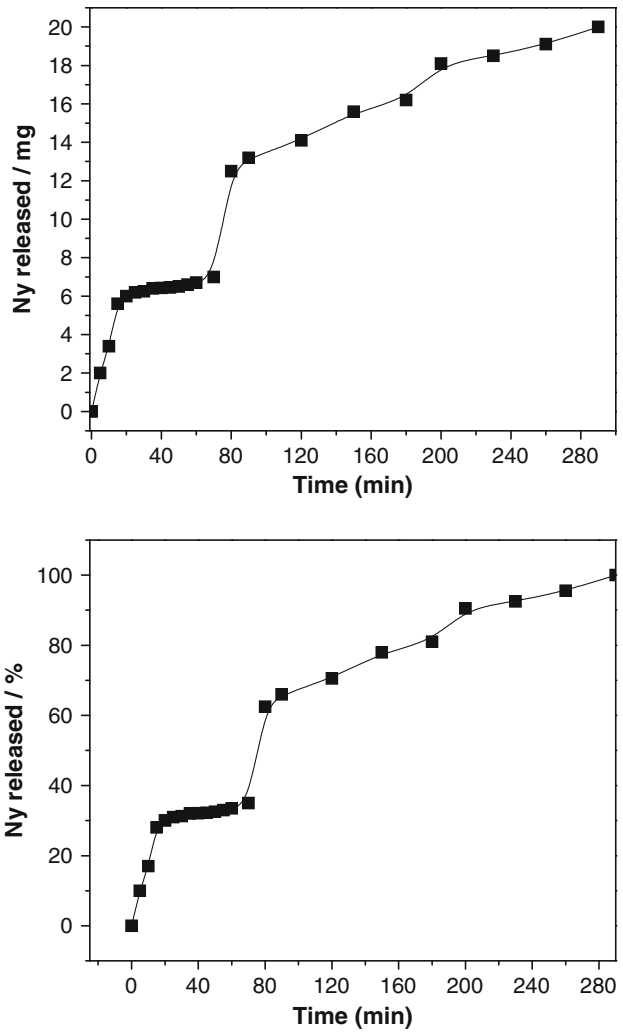

Fig. 6 Ny releasing behavior from Ny–PEO conjugate

of the Ny in water determined in the range of Ny quantities (mg) appropriated to those obtained during the time release (Fig. 5b) we obtained the Ny release curve presented in Fig. 6 As one can observe from Fig. 6 in the first 15 min of solubilization the total released quantity of Ny increases, when 1/3 from the total drug quantity had been dissolved. Starting from this point the quantity of Ny dissolved remains almost constant until 70 min and it grows rapidly up to 120 min when is released almost 14 mg from the 20 initially dissolved. The release curve presents an ascendant trend up to the ending at 300 min, in 180 min only 6 mg was dissolved the same quantity as initial dissolved in the first 15 min. We can conclude that first the drug is released fast at the beginning due to the fast interaction with the water solvent and is reducing in time due to modulation of drug diffusion induced by PEO relaxation. Almost 40% of Ny total content is released in 1 h less than lactose-Ny layer and almost as the double polymer layer Ny (6 h) described in the literature [19].

In vitro antifungal activity

As reported in the literature CMI is equal to 0.96 mg/L for pure Ny [10] and was obtained as 0.83 mg/L for Ny–PEO conjugates (Ny content). The efficacy of the conjugated drug increased, as expected, still the enhancement was not as high as initially expected correlated probably with the kinetics of releasing from the new conjugates with PEO higher than 1.25 mg/L reported by [13].

Conclusions

We synthesized a new conjugate in the solid state based on Ny and PEO and we characterized it by DSC, XRDP and ^{1}H-NMR (that indicated a ratio of complexation of 6.1 Ny units per PEO cycle and the stabilization of the structure by hydrogen bonding). No structural changes of Ny in the conjugate and no chemical interaction between the

components were detected. Phase solubility studies indicated that the solubility of pure Ny significantly increased when compared to that of the pure Ny (more than ten times) indicating the solubilization behavior induced by PEO presence. Ny is released from these conjugates in a quite interesting manner, fast at the beginning up to 15 min when 1/3 from the entire quantity is dissolved, and almost constant up to 60 min, and again increasing up to 300 min indicating a sustained release with quite interesting potential applications. CMI for Ny–PEO conjugates on *Candida albicans* ATCC 10231 is equal to 0.83 mg/mL, proving the increase of Ny antifungal activity after complexation with PEO.

Acknowledgments This research was financially supported by European Social Fund –„Cristofor I. Simionescu" Postdoctoral Fellowship Programme (IDPOSDRU/89/1.5/S/55216), Human Resources Development 2007–2013. The authors will like to express their gratitude to Dr. Valeria Harabagiu, Dr. Xenia Patras and Dr. Aurica Farcas for their advice and guidance in understanding the complexity of the described phenomena.

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