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Synthesis and characterisation of a new pH-sensitive amphotericin B—poly(ethylene glycol)-*b*-poly(L-lysine) conjugate

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Dedicated to Professor Vojeslav Štěrba on the occasion of his 85th birthday

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Abstract—This paper reports on the synthesis, characterisation, and efficiency of a new intravenous conjugate of amphotericin B (AMB). Twelve molecules of AMB were attached to block copolymer poly(ethylene glycol)-*b*-poly(L-lysine) via pH-sensitive imine linkages. In vitro drug release studies demonstrated the conjugate ($M_w = 26{,}700$) to be relatively stable in human plasma and in phosphate buffer (pH 7.4, 37 °C). Controlled release of AMB was observed in acidic phosphate buffer (pH 5.5, 37 °C) with the half-life of 2 min. The LD_{50} value determined in vivo (mouse) is 45 mg/kg.

The polyene macrocyclic antifungal antibiotic amphotericin B (AMB) belongs among life-saving drugs[1,2] in treatment of systemic fungal diseases due to failure of patient's immunity caused by severe illnesses.[1] Amphotericin B was isolated by Trejo and Bennett[3] half a century ago along the Orinoco River in Tembladora (Venezuela) from the *Streptomyces nodosus*. However, clinical application of AMB is limited by its poor solubility and also potential serious organ toxicities, particularly nephrotoxicity, which mainly appear to be dose-dependent.[4] A number of conjugates have been used in order to increase the therapeutic index of AMB: they enable solubilisation in aqueous media and also continuous dosage, which usually lowers the toxicity, too.[5] The conjugates clinically applied so far are non-covalent bio-degradable phospholipid complexes of the type amphotericin B lipid complex (ABLC).[6]

Such lipid formulations of amphotericin B often significantly increase the antifungal activity in vitro as compared with the amphotericin B itself.[7] In our previous paper, we described the preparation of conjugate of AMB with poly(ethylene glycol) (PEG), where AMB is bound partly by covalent bond and partly by non-covalent interactions.[8] In another type of conjugate, AMB was attached to PEG by means of labile carbamate link,[9] which decomposes in blood plasma by 1,6-benzyl elimination mechanism[10] to give free AMB ($t_{1/2} = 1.5$–3 h, blood rat plasma). However, this principle is not generally suitable and applicable to any arbitrary group of medical drugs. For instance, in the case of PEG-AMB conjugates this system of 1,6-benzyl elimination leads to a relatively fast release of free AMB directly into blood circulation, which rapidly increases its concentration in kidneys, and this is undesirable especially because of its nephrotoxicity. The AMB set free in the blood undergoes subsequent distribution[11] among the present lipoproteins [high-density-lipoprotein (HDL) and low-density-lipoprotein (LDL)], and it is presumed[12] that the increased level of the complexes of AMB with LDL results in the nephrotoxic effect of AMB. Another disadvantage of the above-described AMB conjugates lies in low capacity of the poly(ethylene glycol) chain,[13,14] the relatively large carrier molecule carrying only two AMB molecules. This present paper deals with synthesis and characterisation of a new type of conjugate of AMB with block copolymer poly(ethylene glycol)-*b*-poly(L-lysine) (PEG-*b*-PLL), which is considered

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Keywords: Drug delivery; Amphotericin B; pH-sensitive conjugate.

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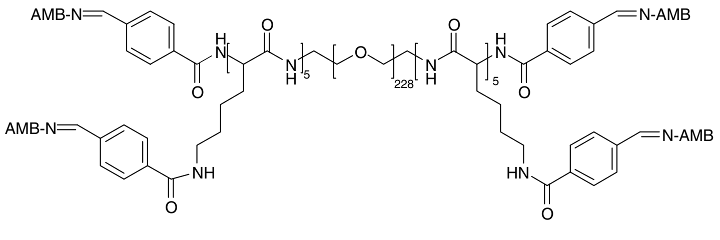
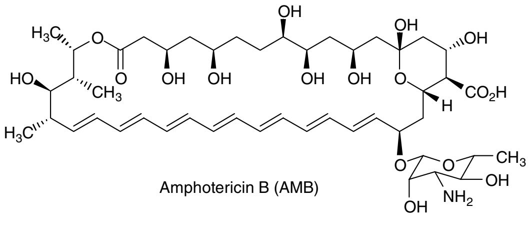
to be fully biocompatible, non-toxic and has been often used for drug or gene delivery systems[15] or for biomimetic applications.[16] However, in comparison with the poly(ethylene glycol) itself[14], it enables attachment of a larger number of AMB molecules, whereby it approaches the supramolecular arrangement similar to the attachment of four amphotericin B molecules to one molecule of calix[4]arene.[17] The bond type between the carrier PEG-*b*-PLL and AMB was chosen in such a way as to prevent release of free AMB in blood circulation during intravenous application: the targeted release of AMB only takes place at the location of fungal pathogens. One of the methods that enable targeted antimycotic action is based on the fact that a number of pathogens—inter alia fungal agents—start such biochemical processes in the attacked tissue[18] that lead to local decrease of pH value down to pH $\approx$5. This finding has been recently used in construction of highly effective pH-sensitive lipid formulations (liposomes) of nystatin[19] and the covalent conjugate poly(ethylene glycol)-AMB.[13] We have chosen imine group as pH-sensitive link between amino group of mycosamine AMB and polymeric carrier. This link should be sufficiently stable at the pH values usual in blood and tissues (pH 7.4–7.6), e.g., in blood plasma and/or blood serum. On the other hand, it should be very easily hydrolysable at lower pH value (Fig. 1).

First, we used well-known NCA method[15]: ring-opening polymerization of α-(N^{ε} -benzyloxycarbonyl)-L-lysine[15,20] initiated by α,ω-diaminopoly(ethylene glycol)[14] (M_w = 10,000) and followed by hydrogenolytic deblocking of benzyloxycarbonyl group to obtain the block polymer poly(ethylene glycol)-*b*-poly(L-lysine) (PEG-*b*-PLL) (M_w = 13,200).[21] This block copolymer was characterised by means of GPC[22], ^{1}H NMR[22], IR spectroscopy[22] and elemental analysis.[22] The results of GPC, elemental analysis and integral intensities of ^{1}H NMR signals of individual hydrogen atoms indicate[21] that at either end of the poly(ethylene glycol) chain there are (statistically) five L-lysine units. A comparison of integral intensities of hydrogen signals of ε-CH_2 group in ^{1}H NMR spectrum[21] at 2.74 ppm and at 3.01 ppm indicated that approximately 40% of poly(L-lysine) chain is present in the α-helix arrangement (deuteriochloroform solution), while the remaining approximately 60% is in the form of random coil.[23] In the second step, we prepared poly(ethylene glycol)-*b*-poly(*N*-(4-formylphenylcarboxamino)-L-lysine)[24] (PEG-*b*-PFLL) by reaction of PEG-*b*-PLL with 4-formylbenzoic acid,[19,25] which was activated with diisopropylcarbodimide (DIC). The IR spectrum of PEG-*b*-PFLL contains the typical band of aldehyde group (1701 cm^{-1}), and the results of ^{1}H NMR measurements indicate[23,24] that all the terminal amino groups of PEG-*b*-PLL have been quantitatively substituted with formylbenzoic acid, which means that at either end of the chain there are six aldehyde functional groups attached to aromatic ring. Moreover we have found from ^{1}H NMR[24] that due to this substitution the proportion of the chain in α-helix arrangement decreased to 20% in accordance with Ref. 23 The synthesis of the conjugate of PEG-*b*-PFLL with AMB involves reaction of terminal aldehyde functional groups in the PEG-*b*-PFLL chain with the primary amine group of amphotericin B in the presence of activated molecular sieves 4 Å and trimethyl orthoformate to form the imine bond[25] (Schiff's base) (Scheme 1).

The final conjugate, poly(ethylene glycol)-[*b*-poly(L-lysine)$_5$]$_2$-(AMB)$_{12}$ (PEG-*b*-PFLL-AMB), was characterised[26] by means of infrared spectroscopy: the IR spectra exhibit band at 1638 cm^{-1} which is typical for $CH{=}N$ bond. The ^{1}H NMR spectrum was unclear, and the individual signals could not be assigned. The GPC method was used for determination of M_w, and the HPLC technique for determination of purity of the conjugate (the content of free AMB was always less than 1 mol %). Furthermore, by means of UV/Vis spectroscopy (typical maxima corresponding to polyene system of AMB[26]) it was proved that the content of AMB in the conjugate corresponds to the molar ratio of 1:12 (PEG-*b*-PLL/AMB). The prepared conjugate is very well soluble in water and forms finely opalescent solutions. The next part of our work deals with investigation of behaviour of the conjugate prepared in various

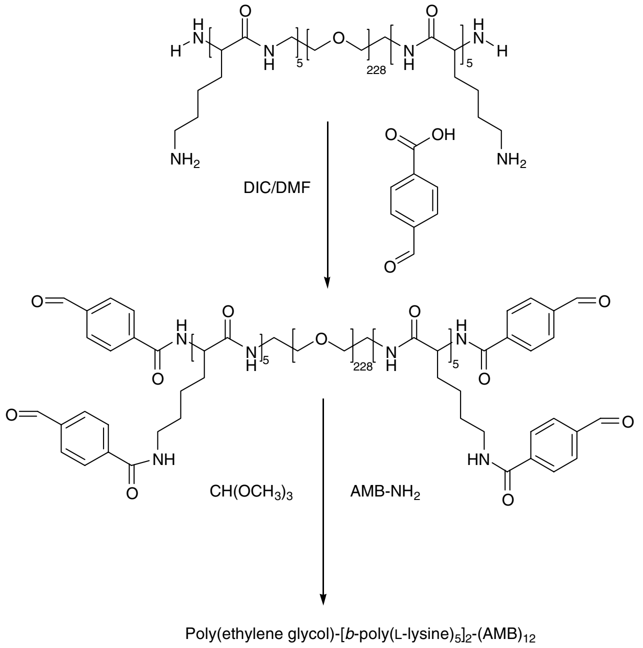


Poly(ethylene glycol)-[*b*-poly((L-lysine)$_5$]$_2$(AMB)$_{12}$



Amphotericin B (AMB)

Figure 1. Structure of poly(ethylene glycol)-[*b*-poly(L-lysine)$_5$]$_2$-(AMB)$_{12}$.



Scheme 1. Synthesis of poly(ethylene glycol)-[*b*-poly(L-lysine)$_5$]$_2$-(AMB)$_{12}$.

media. First, we studied the stability of the conjugate in phosphate buffers and then in human blood plasma. The time changes of the conjugate concentration in human blood plasma during 24 h at 37 °C were negligible and their values were comparable with error of estimation.[27] From the time changes in the UV/Vis spectra we have found that in solutions of phosphate buffer with pH 7.4, (7×10^{-2} M, 37 °C) the conjugate is relatively stable, being hydrolysed only very slowly at the imine bond, so that less than 5 mol % AMB is released from the conjugate during 24 h. The conjugate has been designed in order to achieve targeted release of AMB only in the places with pathologically lowered pH value. The solution of phosphate buffer[28] (pH 5.5; 7×10^{-2} M) was adopted as a model of medium imitating affected tissue with lowered pH value. The acid-catalysed[29] hydrolysis of the conjugate was accompanied by decrease of absorbance at 346 nm and an increase of absorbance at 386 and 409 nm (Fig. 2).

The absorption band at 409 nm is specific[30] for monomeric AMB, and its increase with time corresponds to the rate of free AMB release. From the absorbance–time dependence at 409 nm we calculated[28] the pseudo-first-order rate constant for AMB release from conjugate which is $k_{\mathrm{obs}} = (5.6 \pm 0.1) \times 10^{-3}\ \mathrm{s}^{-1}$, i.e., $t_{1/2} = 2$ min. Acute toxicity was tested[31] on mice with the conjugate PEG-*b*-PFLL-AMB in comparison with AMB itself. Most deaths occurred 5 min to 24 h after iv administration of the tested compounds. When compared to control, PEG-*b*-PFLL-AMB increased LD$_{50}$ 12 times for conjugate itself or five times related to content of AMB in polymer (see Table 1).

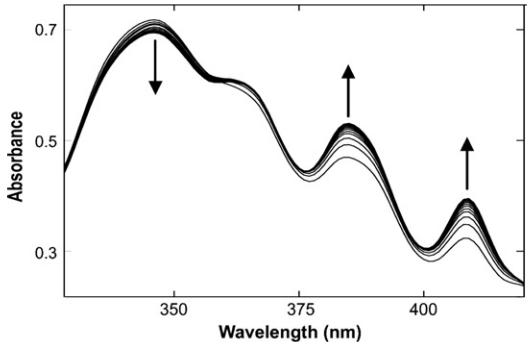


Figure 2. Time changes of UV/Vis spectrum of conjugate in phosphate buffer (pH 5.5; 7×10^{-2} M).

Table 1. Acute toxicity of conjugate: poly(ethylene glycol)-[*b*-poly(L-lysine)$_5$]$_2$-(AMB)$_{12}$ (PEG-*b*-PFLL-AMB)

Compound	Control (AMB)	Conjugate
LD$_{50}$ mouse iv (mg/kg)	3.7	45.1
95% confidence intervals (mg/kg)	3.2–4.2	42.1–48.1

Both the toxicity of the conjugate found in vivo and the results of hydrolysis study in vitro are in accordance with the presumption that our suggested system of pH-sensitive release of AMB minimizes the level of free AMB and of its complexes with LDH in blood circulation and thereby lowers the risk of toxic injury to patient[12]. In contrast to the earlier[8,9,13] intravenous conjugates of AMB with poly(ethylene glycol)s releasing free AMB in blood circulation, we have suggested and prepared new conjugate allowing selective release of AMB only through acid-catalysed hydrolysis of imine bond, i.e., only at the places of anticipated activities of fungal pathogen. Our new conjugate contains 12 molecules of amphotericin B attached to one molecule of biocompatible carrier, namely poly(ethylene glycol)-*b*-poly(L-lysine). In conclusion it can be stated that the conjugate prepared by us represents another promising candidate for new intravenous forms of AMB, suitable for further tests on animal model.

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21. Poly(ethylene glycol)-*b*-poly(L-lysine) (PEG-*b*-PLL) (M_w = 13 200): yield 98%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.38 (m; 30 H; $(CH_2)_3$), 2.74 (br s; 4H, ε-CH_2, (α-helix)), 3.01 (br s; 6H, ε-CH_2, (random coil)), 3.69 (m, 114H, $(CH_2)_2O$), 5.38 (br s, 5H, CH); IR (KBr) *ν*: 3339, 3114, 2942, 2866, 2337, 1852,1814, 1783, 1657, 1630, 1537, 1455, 1258, 1139, 923, 748, 696,963, 531 cm^{-1}; for $C_{516}H_{1036}N_{22}O_{238}$ (%): C, 54.58; H, 9.13; N, 2.71; found: C, 54.81; H, 9.32; N, 2.98; M_w/M_n = 1.16.
22. Gel permeation chromatography (GPC) was used for estimation M_w of polymers. The measurements were performed with HEMA-BIO columns (hydrophilic modified HEMA-Gel, particle size 10 μm porosity 40/100/300/1000) at room temperature using 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 PEGs with varying molecular weights (PSS, Polymer Standard Service GmbH, Mainz, Germany); the ^{1}H NMR spectra were measured in deuteriochloroform ($CDCl_3$), on a Bruker AMX 360 apparatus and a Bruker 500 Avance apparatus, respectively; The IR spectra were measured using Nicolet Impact 400 spectrometer. The samples (0.2 mg) were measured in KBr pellets (200 mg).
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26. Poly(ethylene glycol)-[*b*-poly((L-lysine)$_5$]$_2$-(AMB)$_{12}$ (PEG-*b*-PFLL- AMB) (M_w = 26 700), yield: 86%; IR (KBr) *ν*: 3341, 3116, 2945, 2886, 2741,2337, 1852, 1814, 1719,1693, 1637, 1630, 1537, 1467, 1256, 1201,1139, 923, 748,696, 963, 531 cm^{-1}; UV–vis (λ_{max}: 346, 367, 386, 409 nm); for $C_{1176}H_{1948}N_{34}O_{466}$ (%) calculated: C, 58.82; H, 8.13; N, 1.98; found: C, 58.99; H, 8.36; N, 2.20; M_w/M_n = 1.11.
27. A micro-test-tube was charged (from a pipette) with 500 μl sample of plasma or serum (from healthy donors of plasma at transfusion station) and 500 μl aqueous solution of conjugate PEG-*b*-PFLL-AMB to obtain the final concentration of the conjugate equal to 1×10^{-1} M. The samples were incubated at 37 °C. After the incubation, at definite intervals: 0 (immediately), 1, 2 –10 h and after 24 h they were stabilised with 100 μl of 4% $ZnSO_4{\cdot}7H_2O$. The mixture was shaken for 1 min and then centrifuged for 3 min at 14000 rpm. The supernatant (20 μl) was introduced to a column LiChroCART® 125 × 4 mm packed with LiChrospher® 100 RP-18e 5 μm (MERCK); mobile phase: acetonitrile, 2×10^{-2} M chelaton II.
28. The kinetic measurements were performed on an HP VU/VIS 8453 Diode Array apparatus using 1 cm closable quartz cells in thermostat block at 37 °C. The cell was charged with 2 ml phosphate buffer (pH 7.4, 7×10^{-2} M or pH 5.5, 7×10^{-2} M), and after attaining the mentioned temperature, 15 μl methanolic solution of conjugate PEG-*b*-PFLL-AMB was injected into the cell to obtain the final concentration of substrate equal to approximately 5×10^{-5} M. The measured absorbance-time (*A*–*t*) dependences were used to calculate the observed rate constants of pseudo-first order (k_{obs} (s^{-1})) from the equation: $k_{obs}t = \ln \Delta A + \text{const.}$, where $\Delta A = (A_\infty - A_t)$ or $(A_t - A_\infty)$.
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31. Acute toxicity was tested for amphotericin B (Bristol-Myers Squibb) as a control and for PEG-*b*-PFLL-AMB. For both the compounds tested, the mice were divided into six groups of 10 males, and the compound was administered intravenously via the tail vein at doses carefully determined after a series of preliminary trials. Toxic signs and mortality were monitored for 14 days after administration. LD_{50} values and the corresponding confidence limits were determined by non-linear regression using GraphPad Prism (version 4; GraphPad software Inc., San Diego, CA, USA).