Potential Tuberculostatic Agents: Micelle-Forming Copolymer Poly(ethylene glycol)-Poly(aspartic acid) Prodrug with Isoniazid

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Summary

With the objective of obtaining slow-acting isoniazid derivatives, of potential use as chemoprophylactics or chemotherapeutics in tuberculosis, the micelle-forming copolymer of poly(ethylene glycol)-poly(aspartic acid) prodrug with isoniazid was synthesized. The derivative obtained was found to be active in *Mycobacterium tuberculosis* culture, with a minimal inhibitory concentration (MIC) 5.6 times lower than that of the tuberculostatic drug.

Introduction

Tuberculosis is presently a major world-wide health problem. About one third of the world population is infected with the bacilli, two million people are infected each year, and eight thousand die of the disease every day[1]. In Brazil, 160 thousand people are infected with the bacilli yearly, and the future outlook is alarming. It is a paradox that a disease from the 19th century still exists on such a scale in the 21st century!

Changes in the tuberculosis epidemiology pattern are due to several factors, e.g., the emergence of multi-drug resistant bacilli, infections by complex *Mycobacterium avium*, difficulties in the absorption of chemotherapeutic agents in patients co-infected with HIV, and lack of patient compliance. These factors contribute to the difficulties in the selection of effective drugs, and lengthen the duration of treatment [2]. Therefore, more effective antimycobacterial agents are in constant demand.

Despite the resistance it elicits, isoniazid has been one of the drugs of choice in standard tuberculosis treatment [3,4]. In pulmonary and extra-pulmonary tuberculosis, the drug is used for a long period of time – at least six to twelve months – in association mainly with rifampin and pyrazinamide. For chemoprophylactic purposes, specially in co-infections with AIDS [5], isoniazid has been used alone for ca. six months [3–5]. The non-compliance of the patient with this long treatment period has been considered one of the main causes for the increase in the multi-drug resistant tuberculosis. DOTS – Directly Observed Treatment Short-course – has been used in an attempt to decrease the discontinuation of the treatment, and hence the occurence of drug-resistant tuberculosis [6].

One of the main goals of the WHO tuberculosis group[7] is to design slow-acting drugs that could be prescribed in treatment schedules with a higher probability of being followed, although long enough to assure that the disease is cured. This is also true for chemoprophylactic schedules, since they are considered for people who are infected, but have not yet developed the disease.

One of the approaches employed to obtain slow-acting drugs is the so called "prodrug design" [8–10]. The drug is transformed into an inactive transport form, which in vivo, through enzymatic or chemical processes, delivers the active compound, which interacts with its receptor. In this approach, macromolecules have been extensively used as carriers [11–18].

The conjugation of a polymer with a drug is effective when it results in overcoming either pharmaceutical or pharmacokinetic problems associated with some drugs. Natural, synthetic, biodegradable, and non-biodegradable macromolecules have been tested with the objective of decreasing the toxicity and prolonging the action of drugs [18]. Many polymers have been used as drug carriers of diverse therapeutic classes [8,14–18,24].

The products of these drug-polymer conjugations show different pharmacokinetic features which depend, inter alia, on: polymer structure, charge and molecular weight distribution, body fluid stability, the nature of drug-polymer association, and hydrosolubility of the complex formed [19]. Micelle-forming polymers have been introduced in order to solve the problem of low hydrosolubility of polymer-drug conjugates.[25,26] This approach has increased interest in studying these systems because the micelles formed enhance the drug stability, as well as its transport through the biological membrane [26,27]. One of the main factors that affects drug transport through biological barriers is the micelle size; this can be controlled by varying the polymer chain length. According to Yokoyama et al. [25], aggregates whose diameter is similar to that of virus circulate in the blood without provoking capillary embolus, escape renal excretion and unspecific uptake by the RES system, and easily penetrate into the target cells through blood vessels.

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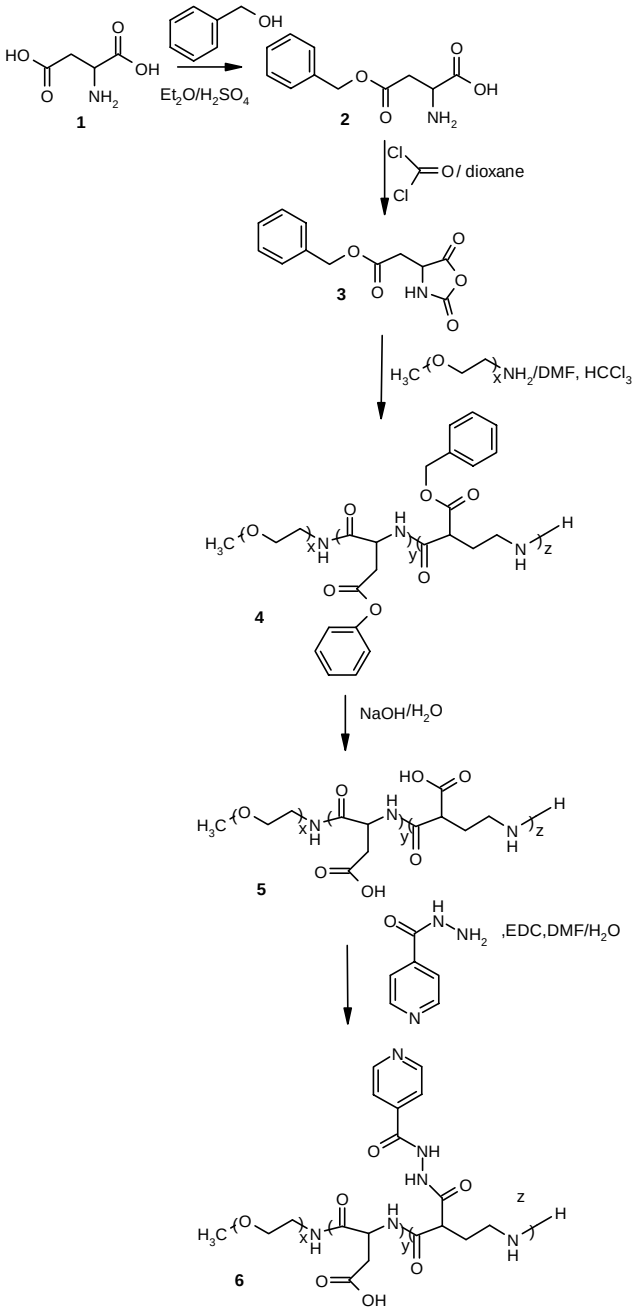

Figure 1. Synthetic pathway of isoniazid conjugate-copolymer poly(ethylene glycol)-poly(aspartic acid).

Being enclosed within the micellar domain the drug is prevented from precipitating and protected from the enzyme attack in the blood. Additionally, the micelles stabilise the macromolecular structure through inter and intra-molecular hydrophobic interactions [25]. The micellar hydrophilic domain is responsible for the drug's interaction with biocomponents, e.g. proteins, cells, leading to the pharmacokinetic drug profile, whereas the hydrophobic portion is involved in the pharmacodynamic phase, that means drug-receptor interactions [19].

The objective of our work was to obtain micelle-forming polymer prodrugs derived from isoniazid with controlled delivery of the tuberculostatic, that can be used either as chemoprophylactics or as chemotherapeutics in tuberculosis.

Isoniazid may undergo a significant first-pass effect: it is biotransformed by *N*-acetyl transferase into *N*-acetyl-isoniazid in the liver. In adult fast acetylators its half-life is 0.5 to 1.6 h and in slow acetylators it is 2 to 5 h; in children 1.5 to 15 years old, the drug half-life is 2.3 to 4.9 h. Liver and kidney damage and peripheral neuritis are the main untoward effects of the drug [4]. As already mentioned, designing slow-acting tuberculostatics is one of the approaches that fits WHO recommendations[7] in order to prevent the discontinuation of treatment, decreasing resistant strain emergence. It is worth noting that the prolongation of action through prodrug design leads to a parallel decrease of toxicity [8,9].

The present paper reports the first step of the design of a slow-acting isoniazid prodrug, i.e the synthesis and in vitro culture tests with *M. tuberculosis*.

The synthesis was carried out by Cammas and Kataoka's [11] method, and is shown in Figure 1. L-Aspartic acid (**1**) was protected with the benzyl group to give β-benzyl-L-aspartate (BLA) (**2**). This was converted into β-benzyl-L-aspartate *N*-carboxyanhydride (NCA-BLA) (**3**) by condensation with phosgene. Reaction with α-methyl-g-aminepolyoxyethylene, MW 5,000, gave the copolymer poly(ethylene glycol)-poly(aspartic) acid (PEG-BLA) (**4**), from which (**5**) was obtained by alkaline hydrolysis of the protecting group. The isoniazid conjugate-copolymer poly(ethylene glycol)-poly(aspartic acid) (**6**) was synthesised by condensation of the later with isoniazid using EDC.

Results and Discussion

The synthesis of BLA was carried out by the method of Benoiton [28]. We have found that the yield was higher, ca. 50%, when the temperature was maintained between 13 and 17 °C. Lower yields, ca. 40% were obtained at temperatures above 17 °C. Pyridine must be slowly added, under continuous, efficient stirring, in order to prevent the formation of a pasty product. Stirring must be maintained until the solution is milky. The solvent evaporation allows the product to precipitate.

NCA-BLA synthesis was first based on method B, reported by Karlson [29]. Modifications of the original conditions lead to the general method described herein. NCA-BLA formation was evidenced by solubilization of the BLA suspension, and had occurred much faster after phosgene introduction, i.e. in 3 minutes as compared with ca. 1 hour according to the original procedure. Differences in crystallinity of BLA, and/or in phosgene flow rate may account for the much faster reaction in our case. Unlike the oily reaction product reported by Karlson [29], we obtained a yellow solid whose isolation was easier than that originally described.

Compared to the IR spectrum BLA, that of NCA-BLA shows bands at 1862 cm^{-1} 1781–1800 cm^{-1}, 1722–1723 cm^{-1} and 1584–1464 cm^{-1}. These are characteristic of $\nu_{C=O}$ of *N*-carboxyanhydride five-membered rings, $\nu_{C=O}$ of ester carbonyl groups, $\nu_{C=C}$ of aromatic rings, respectively [30]. The ^{13}C NMR δ C=O chemical shift at ca. 151–152 ppm for the

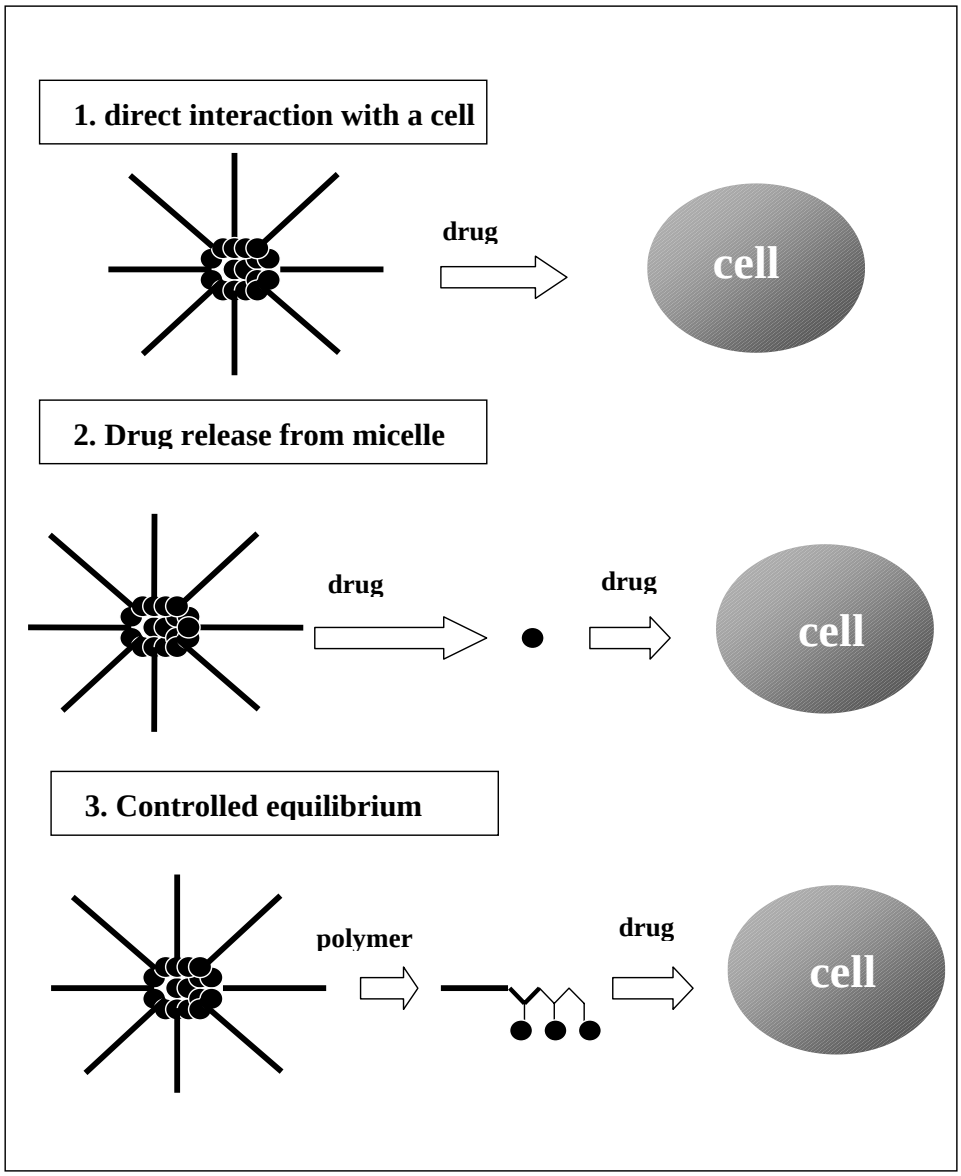

Figure 2. Schematic representation of the mechanism of action of micelle-forming drug polymers (adapted from ref. [25]).

less electrophilic carbonyl is indicative that the cyclization has occurred.

PEG-PBLA synthesis occurs via CH_3-PEG-NH_2 as a polymerization initiator. Its terminal amine group promotes the nucleophilic addition to the more eletrophilic carboxyl group of NCA-BLA. This synthesis followed the method published Yokoyama et al. [25,30] and the ^{1}H-NMR and ^{13}C-NMR agree with the expected structure. The chemical shift at ca. 165–167 ppm confirmed the formation of the amide linkage between PEG derivative and PBLA. Identification of the copolymer is made difficult by the presence of PEG homopolymer impurity. Therefore, Yokoyama *et al.*[25] used alcohols (methanol to *n*-butanol) in order to precipitate PEG-PBLA from its chloroform solution, whereas Cammas and Kataoka[11] preferred isopropyl alcohol. Although we have used the latter solvent, complete elimination of contaminants (i.e., PEG homopolymers) has not been achieved, as showed by the peaks at 23 and 30 ppm.

PEG-PASP synthesis was carried out by removing the protecting benzyl group through alkaline hydrolysis [25,26], a step whose completeness has been confirmed by ^{1}H and ^{13}C NMR spectroscopy. Thus the characteristic proton peaks of C_6H_5-C***H*****$_2$** (5.0–5.2 ppm) and C_6***H*****$_5$**-CH_2 (7.7–7.4 ppm) and the ^{13}C peaks 51–53 ppm [31] are all absent in the spectra of PEG-PASP.

The conjugation of INH with PEG-PASP followed the methodology described by Yokoyama *et al.* [25,30] ^{1}H NMR spectrum shows chemical shifts due to the aromatic protons of INH at 7.1–8.5 ppm. Although the amide protons have not been detected probably because of line broadening by the

attached nitrogen, the corresponding carbonyl group was identified at 170–172 ppm in the ^{13}C NMR spectrum. Free carboxyl groups in PEG-PASP and PEG-PASP-INH were determined by acid-base titration and the content in INH was determined through comparison, which showed that the drug is conjugated to 64.87% of the polymer carboxyl groups.

We have characterised the drug-polymer conjugate formed by dye (Sudan III) solubilization, and quasi-elastic light scattering. The cmc was found to be 5×10^{-5} mol/L, and the mean R_h was 266 ± 27 nm, much higher than the optimum (50 nm) considered by Yokoyama et al. [25]. The uncertainty in R_h is due to the weak scattering intensity of the sample. We plan to investigate the effects of reaction conditions (temperature and stirring speed) on particle size.

The drug-polymer conjugate was submitted to biological in vitro assay in *M. tuberculosis* culture. Isoniazid, PEG-BLA, and PEG-PASP were included as control. MIC was 0.8 μg/mL for PEG-PASP-INH and 0.2 μg/mL for INH alone. No activity was observed neither for PEG-BLA nor for PEG-PASP. The drug-polymer conjugate contains 0.036 μg/mL of INH (64.87% substitution), i.e., a 5.6 times improvement in tuberculostatic action has been achieved with the polymeric drug.

At least three hypotheses (Figure 2) have been advanced by Yokoyama *et al.*[25] to explain the mechanism of action of these micelle-forming polymers. In the first, the micelles interact directly with the target, in the second, the drug is released prior to interacting with the receptor centre, whereas in the third, the activity is due to the drug-polymer conjugate itself.

The most probable mechanism in our case is the third, in which INH is released after the micelle internalisation and enzyme cleavage. The possible increase in permeability due to surface activity of the polymers can be discarded since the carrier polymers, PEG-BLA and PEG-PASP, did not show activity. Besides, INH has a rigid structure-activity relationship that limits its molecular modification, requiring the drug to be released prior interacting with the receptor [3]. Notwithstanding, further studies are needed to explain the mechanism involved.

Those preliminary results in biological assays are indicative that the prodrug has permitted a higher level of drug penetration in the micro-organism. This is quite promising and further work is being done in order to decrease the micelle size, allowing better penetration into pulmonary capillaries and alveolus.

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Experimental

Isoniazid was obtained from FURP (Fundação do Remédio Popular, Brasil). α-methyl-ω-aminepolyoxyethylene (MW 5,000) was purchased from Shearwater, Inc., USA, and EDC, from Aldrich. Solvents and other common reagents are from different sources.

The polymers were submitted to lyophilization in Edwards equipment model L4KR and MLW-LGAO5.

Free carboxyl groups were determined in PEG-PASP and PEG-PASP-INH aqueous solutions by titration with 0.1 M NaOH using phenolphthalein indicator.

Elemental analyses were performed at the Institute of Chemistry, University of São Paulo with a Perkin-Elmer 24013 Elemental Analyzer. Melting points were determined with a Büchi apparatus and were not corrected. IR analyses were carried out with a Shimadzu model IR-470 spectrophotometer, using KBr pellets. ^{13}C-NMR and ^{1}H-NMR were carried out with a Bruker Advance DPX300 spectrometer (operating at 300.13 MHz for ^{1}H), and samples were dissolved in DMSO-d_6 or D_2O.

Critical micelle concentration (cmc) of PEG-PASP-INH aqueous solution (concentrations from 5×10^{-8} to 5×10^{-3} μg/mL) was determined with a Beckman DU 70 Uv-vis spectrophotometer, by measuring the absorbance, *A*, at 519 nm of solubilized Sudan III dye. A plot of *A* versus log [surfactant] gave two straight lines intersecting at the cmc.

Mean micelle hydrodynamic radius, R_h, was determined by quasi-elastic light scattering with a Malvern 4700 MW apparatus, equipped with a 60 mW He/Ne laser source, from the equation [32]:

$$R_h = \frac{kT}{6\pi\eta D_0}$$

where k, T, η, D_0 refer to the Boltzmann constant, the absolute temperature, the solvent viscosity (cP), and the micellar translational diffusion coefficient, respectively.

Synthesis

β-*Benzyl-L-aspartate (BLA)*[28]

Sulfuric acid (40 mL) was slowly added to a cold mixture of anhydrous ethyl ether (400 mL) and benzyl alcohol (400 mL). After removal of the ether under reduced pressure, aspartic acid (53.2 g; 0.4 mol) was added in small portions, under constant magnetic stirring, the mixture was maintained overnight at 13 to 17 °C. To the vigorously stirred mixture were added absolute ethyl alcohol (800 mL), followed by anhydrous pyridine (200 mL), and stirring was continued until an opalescent solution was obtained. This was maintained at –30 °C overnight and the precipitate was filtered off. The product was lyophilized and stored under reduced pressure. Yield: 50%. Mp: 217–220 °C; Anal. $C_{11}H_{10}NO_4$.

β-*Benzyl-L-aspartate N-Carboxyanhydride (NCA-BLA)*[29]

Phosgene was bubbled into a stirred suspension of BLA (20 g; 0.089 mol) in dioxane (400 mL), at 50 °C, until complete dissolution. The solvent and excess of phosgene were removed under nitrogen at 40 °C. An ethyl acetate:petroleum ether mixture (1:1) was then added and the product filtered off, then suspended in a 1:1 mixture of chloroform:2-butanol, and the suspension maintained overnight at –30 °C. The product was filtered off and dried under reduced pressure. Yield: 44%. Mp: 126–129 °C; Anal. $C_{11}H_{12}NO_5$. IR (KBr, cm^{-1}): ν = 3310 (NH), 2940 (CH), 1862 (CO), 1801 (CO), 1723 (CO), 1506–1463 (C=C), 757–699 (CH). ^{1}H-NMR (DMSO-d_6,) δ = 8.29(sl, NH, 1H), 7.45–7.36(m, ArH, 5H), 5.2–5.1 (s, $ArCH_2$, 2H), 2.7–2.6, CH_2NH, 2H). ^{13}C-NMR (DMSO-d_6,) δ = 171.08 (C1), 169.38 (C11), 152.25 (C12), 135.57 (C3), 128.55 (C8), 128.31 (C4, C5), 128.19 (C6, C7), 66.40 (C2), 53.68 (C10), 34.70 (C9).

*Poly(ethylene glycol)-poly(*β*-benzyl-L-aspartate) (PEG-PBLA)*[25,30]

A solution of α-methyl-ω-aminepolyoxyethylene (MW 5,000, 3.00 g; 0.0006 mol) in 13 mL of bidistilled chloroform was added to a stirred solution of NCA-BLA (7.25 g; 0.29 mol) in 11 mL of DMF and 100 mL of bidistilled chloroform. The reaction was maintained under nitrogen for 70 hours, with stirring. The product was then mixed with ethyl ether and the precipitate filtered off, dried under reduced pressure, re-dissolved in chloroform and added to ethyl ether. The precipitate obtained was dried under reduced pressure. ^{13}C-NMR(D_2O): δ = 170.56 (C7), 169.95 (C4), 136.74 (C9), 129.84 (C12), 127.75 (C10, C13), 127.75 (C11,C14), 72.67 (C2), 67.03 (C8), 37.04 (C3).

Copolymer Poly(ethylene glycol)-Poly(aspartic acid) (PEG-PASP) [25,30]

A solution of 34.5 mL of NaOH 0.43 M in water-methanol-isopropyl alcohol (1:2:2) was added to a solution of PEG-PBLA (2.7 g) in acetic acid (27 mL) and the resultant was added to ethyl ether (40 mL). The precipitate was dissolved in distilled water and dialysed against distilled water in a dialysis membrane (cut off 1,000) for 4 hours. The solution was then lyophilised. ^{1}H-NMR (D_2O): δ = 4.37(s, CH_a- and $_b$-amide), 3.43 (m, CH_2PEG), 3.19 (s, CH_3), 2.77 (sl, CH_2 a-amide). ^{13}C-NMR (D_2O): δ = 172.26 (C7,C11), 163.32 (C4), 69.98 (C2), 51.13 (C10), 35.06 (C3). Free COOH: 0.595 mEq/g polymer.

Isoniazid Conjugate-Copolymer Poly(ethylene glycol)-Poly(aspartic acid) (PEG-PASP-INH) [25,30]

Isoniazid (0.18 g; 0.0013 mol) was dissolved in DMF (0.8 mL), then added to a solution of EDC (0.52 g; 0.0033 mol) and PEG-PASP (1.51 g) in distilled water (48.3 mL), the mixture was stirred for 4 hours at 0 °C. EDC (0.52 g; 0.0033 mol) was then added and the stirred mixture maintained for 24 hours at room temperature. The solution obtained was dialysed against acetate buffer pH 5.0 (dialysis tubing cut off 1,000), for 4 hours, against water, dialysed for an additional 4 hours (dialysis tubing cut off 12,000–14,000), then lyophilised. ^{1}H-NMR (D_2O, d): 8.13 (sl, N***H*** polymer), 7.8–7.3 (dl, CH_{INH}), 4.84 (s, NH-NH), 4.62 (sl, CHα- and β-amide), 3.65 (m. CH_2PEG), 3.36(s, OCH_3), 2.11 (sl, CH_2 α-amide). ^{13}C-NMR (D_2O, ppm): 176.42 (C7), 172.26 (C12), 165.27 (C4,C8), 150.09(C16,C17), 122.36 (C14,C15), 68.98 (C2), 58.79(C1), 39.51(C3), 34.89(C6). Free COOH: 0.209 mEq/g polymer.

In Vitro Biological Assay

MIC determination was carried out in autoclaved (121 °C/15 min) Middlebrook $7H_9$ culture medium (4.7 g) mixed with sterile water (900 mL) and glycerol (2 mL). After cooling, 20 mL of OADC (oleic acid, albumin, dextrose and catalase) was added for each 180 mL of culture medium. Isoniazid and a negative control were compared to the synthesised polymers, PEG-BLA AND PEG-PASP, in 8 twofold-concentrations each, from 3.2 to 0.025 mg/mL, using 8 mL of culture medium. Plates with 96 wells and 200 mL of culture medium/well were used for μMIC determination.

M. tuberculosis H37Rv strain was used in growth logarithm phase.

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