Research Paper

Synthesis, characterization and in-vitro antitubercular activity of isoniazid-gelatin conjugate

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Abstract

Objectives A novel and simple method to synthesize antitubercular-protein conjugate by solid phase synthesis was developed employing a carboxypolystyrene resin. The aim was to covalently bind a drug with antitubercular activity, isoniazid, to a biomacromolecule, gelatin, widely used in the pharmaceutical, cosmetic and food industry.

Methods Calorimetric and ^{1}H NMR analyses were performed to verify the bond formation between the antitubercular drug and gelatin. After absorption isoniazid delivers toxic metabolites and so an oxidation test with *tert*-butyl hydroperoxide was performed to assess the amount of toxic metabolites released from the prodrug (gelatin linked to isoniazid), compared with isoniazid itself.

Key findings Spectrophotometric analysis revealed that the protein derivative was an excellent isoniazid prodrug since there was a 40% reduction in release of toxic metabolites (isonicotinic acid) by the prodrug. The results clearly showed that antitubercular moieties, covalently linked to a natural polymer, allowed the introduction of peculiar features for specific pharmaceutical applications into the macromolecule. In addition, antitubercular activity of the new polymer was determined by Middlebrook 7H11 medium against *Mycobacterium tuberculosis* complex.

Conclusions The new isoniazid-gelatin conjugate showed significant antitubercular activity and for this reason should be useful as an efficacious tool in the treatment of tuberculosis.

Introduction

Gelatin is the thermally and hydrolytically denatured product of collagen, the most abundant protein in animals, and is used extensively for industrial, pharmaceutical, and medical applications.[1] Moreover, it represents one of the most used natural materials, widely employed due to its biocompatibility, biodegradation, nontoxicity and nonimmunogenicity.[2,3] Due to the various potential uses of gelatin, it is useful to investigate its modification to develop new materials with improved properties.[4]

Tuberculosis is a major burden in many developing countries and its treatment involves continuous and frequent multiple drug dosing.[5–7] This problem could be solved with the introduction of long-duration drug formulations releasing the antimicrobial agents in a slow and sustained manner, which would allow reduction in frequency and dosing numbers.[8] Colloidal carriers such as liposomes, microspheres and

nanoparticles are a well-known example of this strategy.[9–12] Today, versatility of particulate technology enables tailoring of nanoparticle-based drug delivery systems with consideration of the target, desired pharmacokinetic profile, and route of administration. The obvious advantages of an inhaled therapy include direct drug delivery to the diseased organ, targeting to alveolar macrophages harbouring the mycobacteria, reduced risk of systemic toxicity and improved patient compliance. Moreover, in contrast to oral administration, inhaled drugs are not subjected to first-pass metabolism. Beginning with the respiratory delivery of a single antitubercular drug, it is now possible to transport multiple drugs simultaneously with a greater therapeutic efficacy. In this context, gelatin was chosen due to the presence of many functional groups on this protein that makes it an ideal candidate for conjugation with isoniazid, the first-line antituberculosis medication in
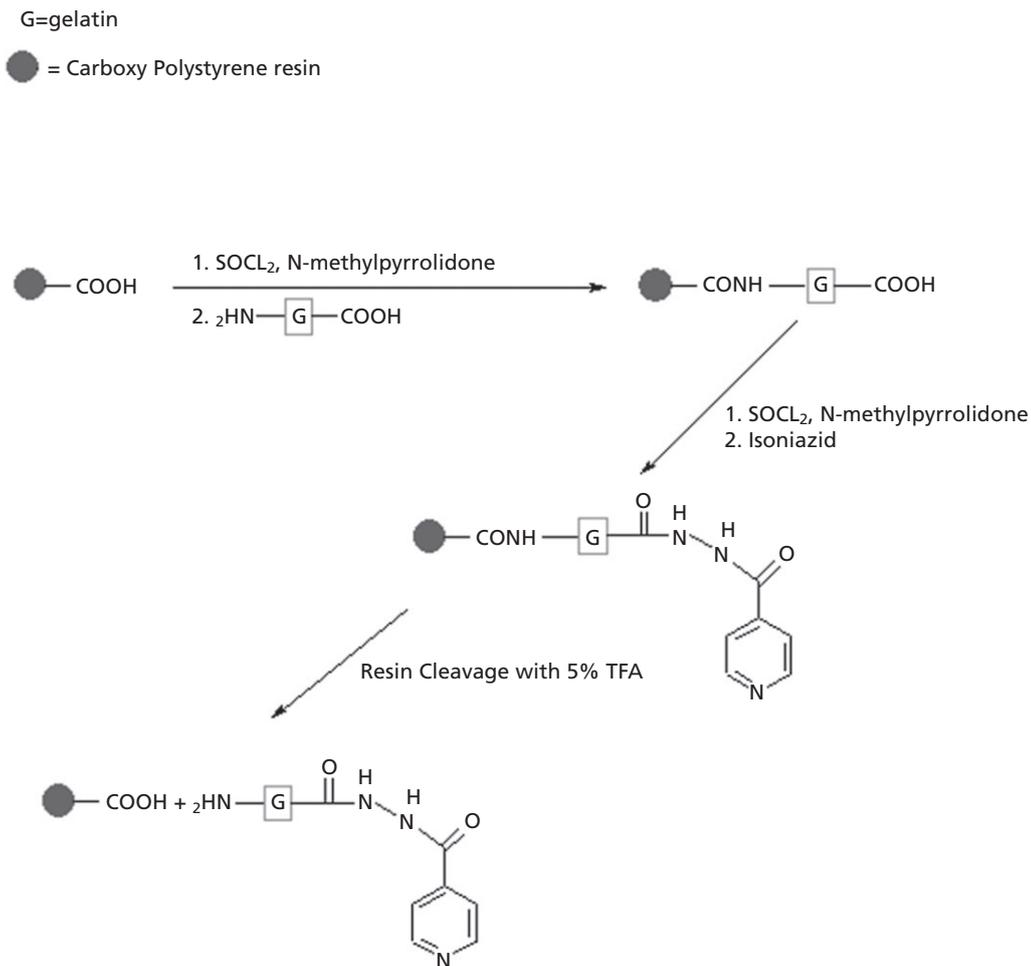
G=gelatin

⬤ = Carboxy Polystyrene resin

Figure 1 Synthetic route to isoniazid-gelatin derivative. TFA, trifluoroacetic acid.

tuberculosis prevention and treatment. In recent years, biodegradable polymeric nanoparticles have attracted considerable attention as potential drug delivery systems, especially for their ability to target particular organs/tissues.[13] Nanaoparticles show different promising properties, useful to reach these objectives, such as versatility in formulation, sustained release properties, sub-cellular size, and biocompatibility with tissue and cells. Moreover, they show high stability, high carrier capacity, and feasibility of the incorporation of both hydrophilic and hydrophobic substances. Furthermore, they can be administered by several routes, including oral and pulmonary application.[14] In particular, nanoparticle-based drug delivery systems have a considerable potential for tuberculosis treatment. The aim of this work was to obtain a novel antitubercular-gelatin conjugate for the potential development of nanoparticle carriers useful for pulmonary delivery of isoniazid and other loaded drugs such as rifampicin, as an efficacious tool in the treatment of tuberculosis. For this purpose isoniazid was covalently linked to the biodegrad-

able polymer gelatin. The synthetic strategy proposed in this study allows a protein matrix covalently linked to an antitubercular drug to be obtained, useful for the realization of nanoparticulate systems, by a spray-drying technique, carrying in the surface and in the inner part, two drugs with synergic actions. Gelatin derivative was prepared through solid phase synthesis using a carboxypolystyrene resin and characterized by ^{1}H NMR spectroscopy. Its antibacterial activity against *Mycobacterium tuberculosis complex* was evaluated also. Moreover, the gelatin derivative was subjected to an oxidation test to verify if the obtained pro-drug was able to protect isoniazid from the production of hydrazine, one of its toxic metabolites. The aim of the test was of preliminarily verifying the in-vitro stability of the isoniazid-gelatin conjugate to the oxidation induced by an oxidating agent, known as *tert*-butyl hydroperoxide (*tert*-BOOH). It was not predictive of the potential improved safety for the conjugate in the clinical setting but only of an increased stability of the drug covalently linked to gelatin.[15]
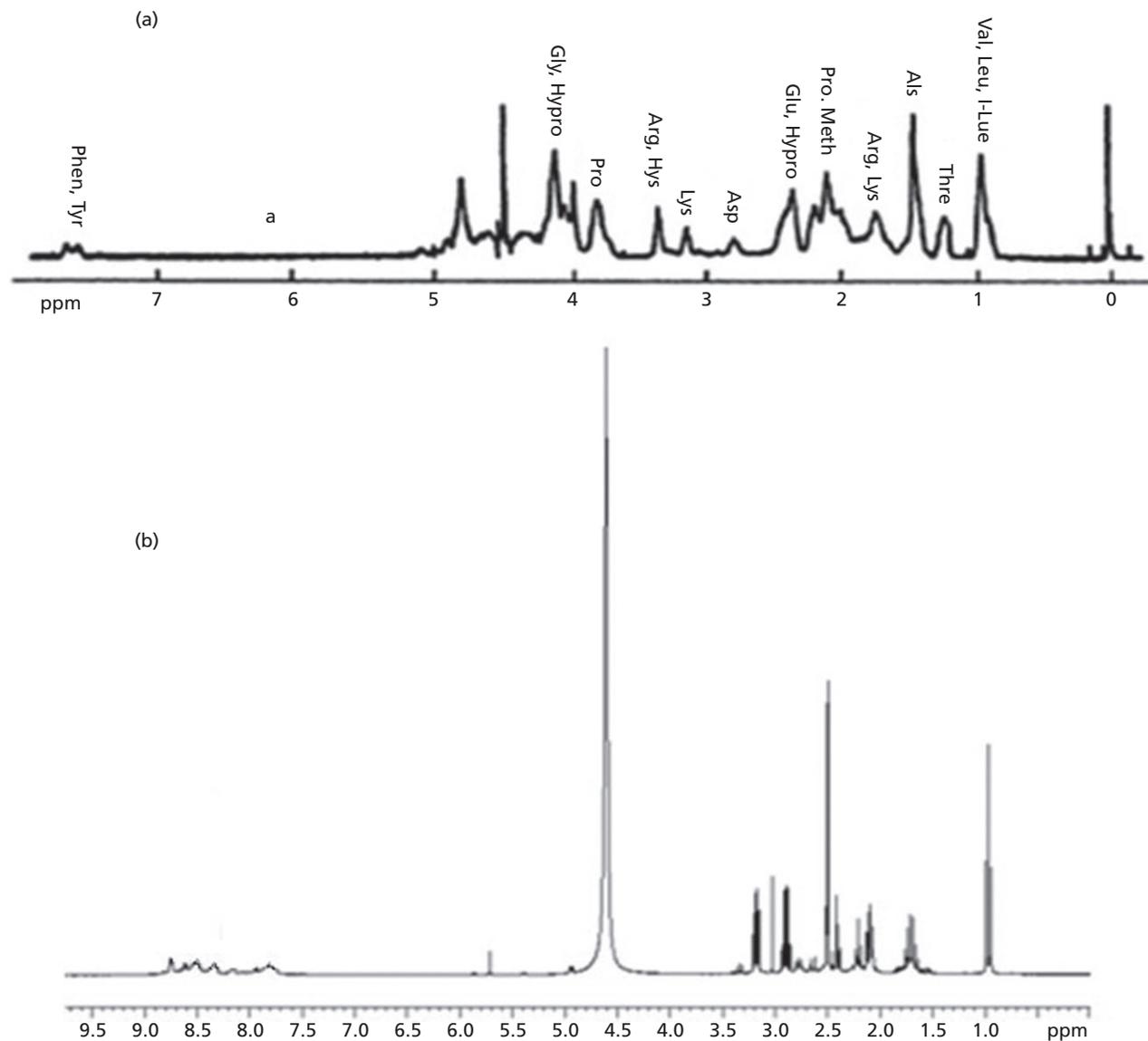
Figure 2 ^{1}H NMR spectra of commercially available gelatin (a) and of isoniazid-gelatin conjugate (b).

Materials and Methods

Materials

All solvents of analytical grade were purchased from Carlo Erba Reagents (Milan, Italy). Dichloromethane, diethylether, N-methyl pyrrolidone, thionyl chloride, gelatin (mixture type A and B with 160 Bloom grams and unknown molecular weight), isoniazid and trifluoroacetic acid were purchased from Sigma-Aldrich (Sigma Chemical Co, St Louis, MO, USA). Resin (carboxypolystyrene HL, 100–200 mesh, 1% DVB) was purchased from Merck AG. Middlebrook 7H11 medium was purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

Measurements

^{1}H NMR spectra were processed using a spectrometer Burker VM30; chemical shifts are expressed in δ and referred to the solvent. UV-vis spectra were realized through a UV-530 JASCO spectrophotometer. The samples were lyophilized utilizing a freeze-drying micro moduly apparatus Edwards Weisselberg (Kinnelon, NJ, USA). The calorimetric analyses (differential scanning calorimetry (DSC)) were performed using a Netzsch DSC200 PC.

Antitubercular activity was evaluated with a Becton Dickinson Detection Instrument (Becton Dickinson, USA).
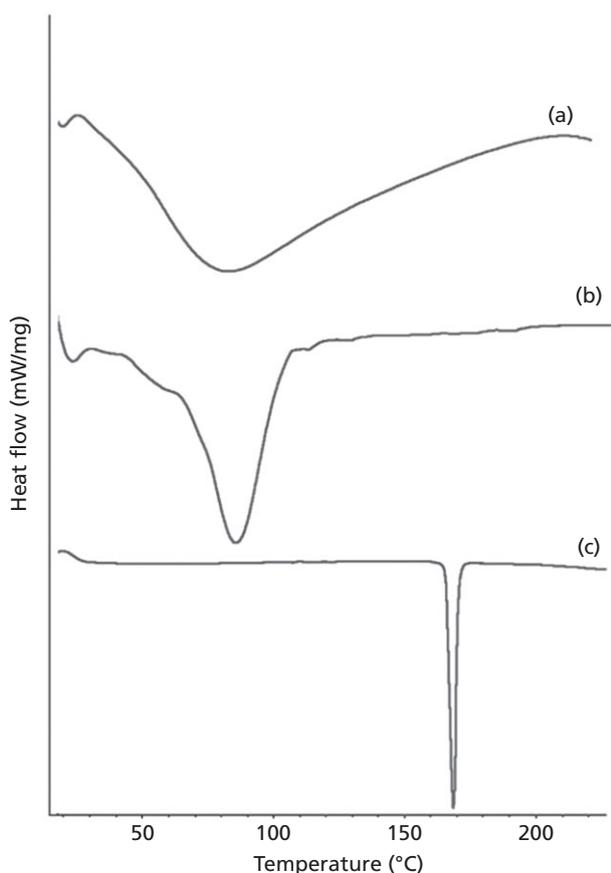
Figure 3 Calorimetric analysis of (a) gelatin (b) isoniazid-gelatin, and (c) isoniazid.

Synthesis of the antitubercular-gelatin conjugate

The reaction was carried out according to the procedure reported in literature.[16, 17] In a Disa vial equipped with magnetic stirrer, carefully flamed and maintained in an inert atmosphere, containing 0.4 g carboxypolystyrene resin suspended in 20 ml N-methyl pyrrolidone, 0.0435 ml thionyl chloride (6.45×10^{-4} mol) and 0.32 ml pyridine (3.96×10^{-3}) were added. The reaction mixture was continuously stirred magnetically at 25°C and after three hours, gelatin 0.017 g was added. The reaction mixture was left under reflux at 100°C, maintained in continuous magnetic stirring, and after one hour 0.082 g isoniazid (6.45×10^{-3} mol) and 0.32 ml pyridine (3.96×10^{-3} mol) were added. The reaction was left under reflux for approximately 72 h at 25°C. At the end of the reaction, initially 20 ml 5% trifluoroacetic acid in dichloromethane, at 60°C for 60 min, was added to the mixture to promote the cleavage of the resin, which was then totally recovered by filtration. Finally, diethyl ether was added to allow the precipitation of the gelatin derivative. Under our

experimental conditions, trifluoroacetic acid in a final concentration of 5% did not hydrolyse isoniazid. This hydrolysis occurred in 48 h.[18] The precipitate was filtered, washed with ethanol, dried and finally dissolved in distilled water, frozen and lyophilized to remove the aqueous solvent. The reaction product was analysed by ^{1}H NMR (D_2O). Yield was 1.34 g (0.082 g isoniazid).

Calorimetric analysis of the antitubercular-gelatin conjugate

Calorimetric analyses of the samples were carried out using DSC. In a standard procedure approximately 6.0 mg dried sample was placed inside a hermetic aluminum pan and sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25 to 300°C under a dry nitrogen atmosphere with a flow rate of 25 ml/min and heating rate of 5°C/min.

Oxidation test

The gelatin derivative and the commercially available isoniazid were, separately, solubilized in a solution consisting of 6 ml phosphate buffer (PBS) pH = 7.4 and 500 μl *tert*-BOOH (0.25×10^{-3} mol). The solutions obtained were left for 2 h in the dark at 37°C in a shaking bath.[19,20] Subsequently, the formation of one of the major toxic metabolites, isonicotinic acid, was monitored by measuring the absorbance at a wavelength of 261.5 nm ($\varepsilon = 3520$ l/mmol/cm).

Antitubercular activity

The antitubercular activity of the isoniazid-gelatin conjugate was tested in Middlebrook 7H11 medium using a double dilution technique.[21] Briefly, the polymer was diluted to obtain a solution 2 μg/ml and was then submitted to a double dilution. Each of these concentrations (100 μl) was inoculated in a Mycobacteria Growth Indicator Tube (MGIT) that was incubated in a MGIT 960 Becton Dickinson Detection Instrument (Becton Dickinson, USA) for the antimicrobial activity measurement.

Results and Discussion

Synthesis of isoniazid-gelatin conjugate

Gelatin is a natural and nontoxic polymer, commonly used for pharmaceutical and biomedical applications due to its biodegradability and biocompatibility in physiological environments. It was chosen as the polymer backbone to be functionalized with isoniazid to obtain a biomacromolecule with raised antitubercular properties. Gelatin is obtained by controlled hydrolysis of an insoluble protein, collagen, which is found abundantly in the connective tissues. The specific release of drugs in the lungs can be affected through
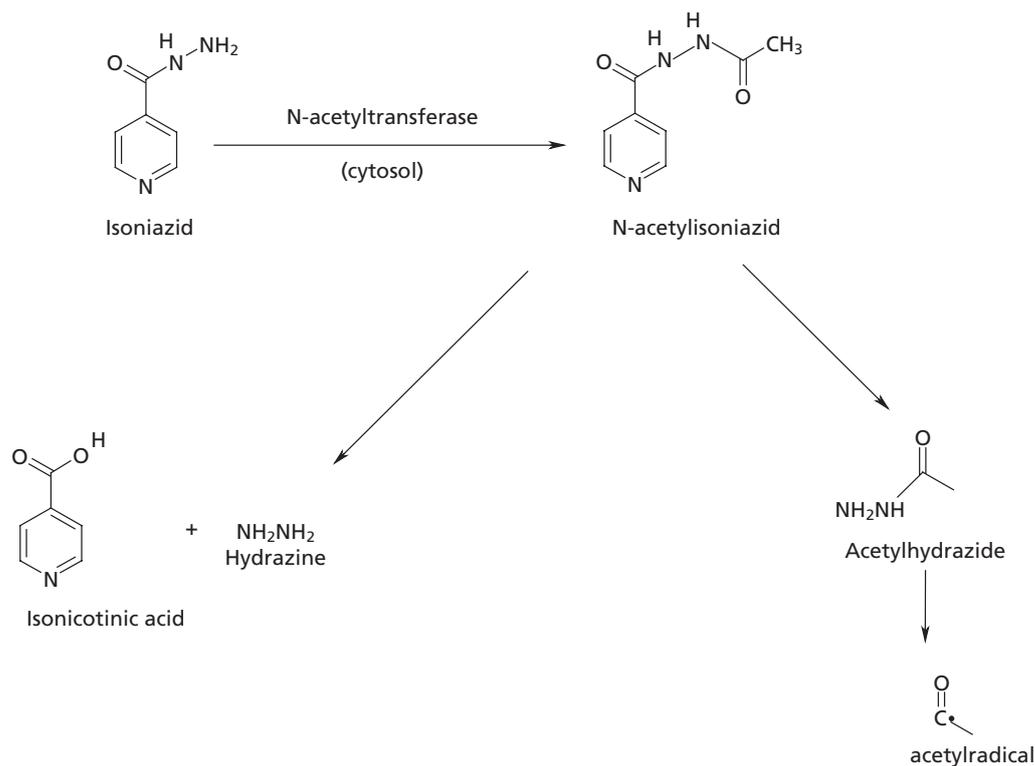
Figure 4 Conversion of isoniazid in to its toxic metabolites.

the action of two enzymes: the esterases and the amidases. Derivatization of the gelatin with isoniazid was obtained by a heterogeneous reaction of amidation. This biopolymer could be used to obtain nanocapsules, useful to entrap other antitubercular drugs (rifampicin, pyrazinamide etc.) to be administered by inhalation. The purpose of this reaction, in the heterogeneous phase, was the derivatization of gelatin with isoniazid. The reaction occurred in N-methyl pyrrolidone and initially promoted the chlorination of C-terminal groups of the resin by using thionyl chloride (SOCl₂) in excess. Pyridine (Py) (nucleophilic catalyst) and gelatin were added to form an amide bond with the resin. The Py buffers the acid environment. Thionyl chloride then reacted with the gelatin C-terminal group. By adding isoniazid, we allowed the formation of an amide bond between the terminal acyl chloride group of gelatin and isoniazid hydrazinium group. To achieve the cleavage of the resin, 5% trifluoroacetic acid in dichloromethane (Figure 1) was used. The solution was filtered and the resin was recovered and dried under vacuum. The solution was then mixed with diethyl ether to promote the precipitation of the gelatin derivative. The biopolymer was collected by filtration, dissolved in distilled water and frozen. After that the product was collected by lyophilization.

Characterization of isoniazid-gelatin conjugate using ¹H NMR

The spectrum of gelatin showed the presence of numerous peaks characteristic of amino acids forming the peptide. The spectrum of the gelatin derivative displayed the presence of isoniazid benzene rings in the range of aromatic groups (7.5–9 ppm), thus confirming the existence of the covalent bond between gelatin and isoniazid (Figure 2).

Characterization of isoniazid-gelatin conjugate by differential scanning calorimetry

The calorimetric analysis revealed the presence of a commercial gelatin broad endothermic peak centred at 85°C. On the other hand, as confirmation of derivatization the DSC spectrum of the gelatin derivative revealed the absence of the pure isoniazid transition peak (170°C). Thermal characterization of the prepared conjugates was performed by recording DSC thermograms of dried antitubercular-gelatin, blank gelatin, and pure isoniazid (Figure 3). As far as DSC of gelatin was concerned, a broad endothermic peak located around 60–180°C was assigned to the glass transition of α-amino
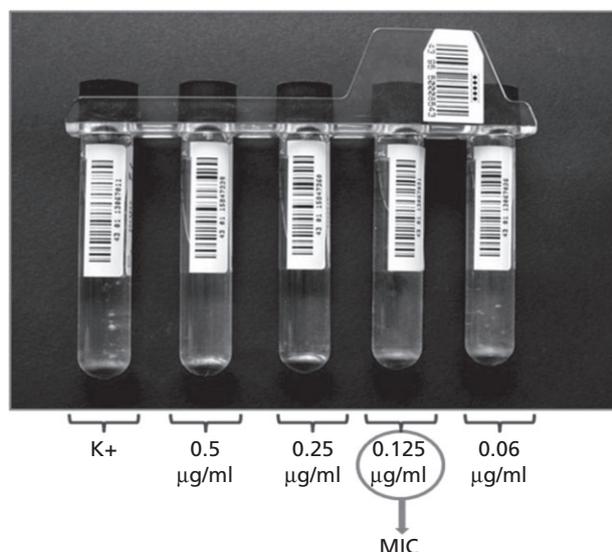
Figure 5 Minimum inhibitory concentration (MIC) value of isoniazid-gelatin conjugate against *Mycobacterium tuberculosis complex*. K⁺ is the positive growth control.

acid blocks in the peptide chains; ΔH_t associated to this transition was -242.1 J/g of protein derivative.

Oxidation test

The oxidation test was performed to assess the amount of toxic metabolites released from the prodrug (gelatin linked to isoniazid) compared with isoniazid. Initially a solution consisting of PBS and a pro-oxidant agent (*tert*-BOOH) was prepared, and then the prodrug and the drug were introduced separately in to it. Spectrophotometric analysis allowed us to assess the amount of metabolites released by drug and prodrug, respectively, after insulting them with *tert*-BOOH. There was a 40% reduction in metabolites, such as hydrazine, being released from the prodrug compared with the drug. The test revealed that our derivative was an excellent prodrug that could reduce the intrinsic toxicity of isoniazid (see Figure 4).

Antitubercular activity evaluation

The isoniazid-gelatin conjugate was tested for its antimyco-bacterial activity *in vitro* against *Mycobacterium tuberculosis complex* in Middlebrook 7H11 medium by the double dilution technique. The polymer has shown significant antimicrobial activity expressed as the minimum inhibitory concentration (MIC). MIC is the minimum concentration of a compound required to give 90% inhibition of bacterial growth. As shown in Figure 5, the conjugate obtained exhibited excellent antimycobacterial activity equal to 0,125 μg/ml and was analogous to that of isoniazid *in vitro* (0.1 μg/ml).

Conclusions

Gelatin was suitably derivatized with isoniazid to prepare a new biopolymer to be used as an isoniazid prodrug and as material to encapsulate rifampicin or other drugs for the treatment of tuberculosis. The synthesis on solid phase allowed the initial formation of an amide bond between the N-terminal group of gelatin and the C-terminal groups of the resin. The C-terminal group of gelatin was then activated with thionyl chloride. When the derivative obtained was treated with isoniazid it led to the desired product. The presence of this derivatization was confirmed by ¹H NMR and DSC analyses. An in-vitro test was used to assess the antibacterial activity of the conjugate. This test showed a positive behaviour of polymer in the inhibition of bacteria proliferation. The results suggested that the biomaterial possessed an excellent antitubercular activity comparable with that of free isoniazid *in vitro*. Binding of the antitubercular drug to a highly biocompatible and economic polymer such as gelatin, obtained a derivative that could be used for the preparation of drug delivery systems to be administered by inhalation. Oxidation studies showed that the drug, covalently linked to gelatin, released a smaller amount of toxic metabolites compared with the free drug. Using special techniques such as spray drying or the Wurster process, antitubercular gelatin could be used to encapsulate other drugs such as rifampicin or pyrazinamide.[22,23] The nanocapsules resulting from this process would thus have an important synergistic effect on tuberculosis.[24] The amidases present both in lungs and in plasma could split the link so they could deliver the isoniazid first and then, gradually, the other antitubercular encapsulated drug.[25] However, since the entity of metabolic actions in the lungs is not well known, it could be possible for isoniazid linked to gelatin to display its antitubercular action staying attached to the protein without being hydrolysed from it, as its active moiety was not involved in this bond.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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