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Article

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PEGylation of Albumin Nanoparticles Immobilized with the Anti-Tuberculosis Drug "Isoniazid"

Polyethylene glycol (PEG) is widely used in nanomedicine to extend the circulation time of a drug in the blood and increase drug efficacy. Conjugation by attaching polyethylene glycol to an albumin macromolecule and nanoparticles is a well-established technique known as PEGylation. The aim of this research was to prepare and evaluate serum stable long circulating PEG-albumin-isoniazid nanoparticles for the treatment of Mycobacterium tuberculosis which can improve its therapeutic effect by increasing its permeability, solubility and accumulation in aveolar macrophages. For the first time, PEGylated BSA nanoparticles loaded with isoniazid were synthesized by desolvation using urea and cysteine as denaturing and stabilizing agents. Nanoparticles with an average size of up to 300 nm were obtained by varying the PEG concentration. The polydispersity index of all particle charges was less than 0.1, indicating monodisperse size. The ζ potential values indicate sufficient physical stability of the nanoparticles. SEM images showed that the particles were spherical in shape. The TGA and DSC results obtained confirm that drug loading does not affect the structure of the polymer. Based on FT-IR studies, the absence of chemical interactions between PEGylated BSA nanoparticles and isoniazid was established. In *in vitro* release studies, the nanoparticles were demonstrated to have a prologue release.

Keywords: nanoparticles, bovine serum albumin, polyethylene glycol, isoniazid, desolvation, hydrophilic drugs, anti-tuberculosis drugs.

Introduction

Recently, albumin-based nanoparticles have been actively used as pharmaceutical and functional carriers due to their low toxicity and biodegradability, as evidenced by the numerous studies conducted on the subject [1-3]. Albumin nanoparticles have several advantages as a drug delivery system, such as biodegradability, stability, particle surface modification, ease of particle size control, and they also have fewer toxicity issues such as immunogenicity [4, 5].

The process of modifying albumin molecules with various polymeric compounds has recently become a relevant area of research. There is worldwide experience in the production of drug nanoparticles by binding — linking inert macromolecules of polyethylene glycol (PEG). Immobilized by drugs PEG nanoparticles not only ensure good tolerability but also have an improved pharmacokinetic profile, promote deep penetration of molecules and provide additional protection against proteolytic enzymes [6, 7].

Polyethylene glycol is one of the medicinal synthetic polymer injectables that can be used for the organism and is approved by the Food and Drug Administration. There are numerous benefits of PEGylated nanosystems, such as those in Figure 1 [8, 9].

The desolvation method is most commonly used to synthesize albumin-based nanoparticles [10–12]. The desolvation method allows the synthesis of nanoparticles by a simple process of adding desolvating agents, such as ethanol or acetone, to albumin solutions containing drugs. Desolvating agents change the structure of albumin and reduce its solubility, leading to the formation of precipitates in the form of protein nanoparticles [2]. Once the nanoparticles are formed, they are bonded with bridging agents such as glutaraldehyde.

In previous studies, glutaraldehyde was mainly used as a crosslinking agent for the preparation of PEGylated albumin-based nanoparticles: the authors of [13] obtained PEGylated nanoparticles prepared from human serum albumin (HSA); Thadakapally, et al. [14] conducted research to obtain serum-stable long-

circulating polymeric curcumin nanoparticles and synthesized nanoparticles using serum albumin and polyethylene glycol; the authors of [15] work synthesized serum-stable long-circulating PEGylated paclitaxel-BSA nanoparticles for breast cancer treatment.

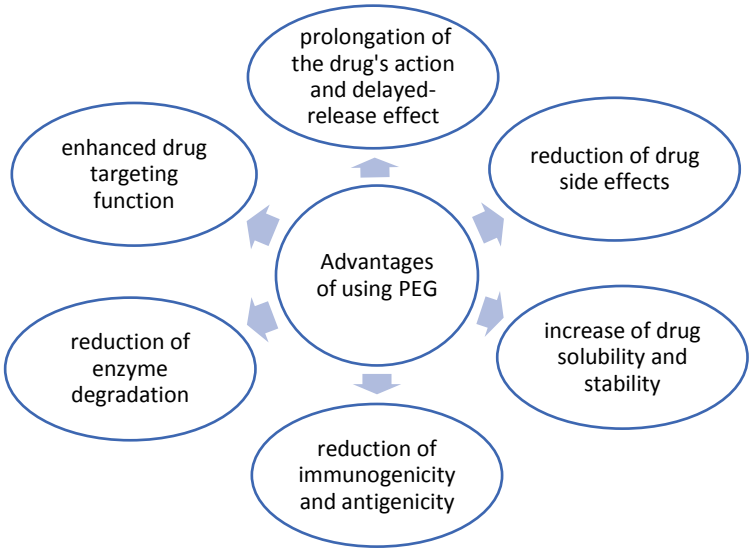


Figure 1. Advantages of PEGylated nanoparticles

In our study, we suggest replacing the synthetic stabilizer with natural materials such as urea and cysteine [16–18]. We first proposed a method for the preparation of PEG-BSA NPs using the natural components urea and L-cysteine. In this case, urea plays the role of a chaotropic agent that unfolds albumin chains and increases the availability for interaction with L-cysteine. As a result, the thiol-disulfide exchange reactions between the PEG-BSA NPs macrochains are stabilized [17].

In this research we aimed to improve the properties of the anti-tuberculosis drug isoniazid by PEGylation of bovine albumin nanoparticles. Thus, the main aim of this research was to prepare and evaluate serum stable long-term circulating PEG-albumin-isoniazid nanoparticles (PEG-BSA-INH NPs) for the treatment of Mycobacterium tuberculosis, capable of improving its therapeutic effect by increasing its permeability, solubility and accumulation in aveolar macrophages.

Experimental

Materials

Isoniazid (INH) with an in-medical purity of over 99 %, bovine serum albumin (lyophilized powder, 98 %) (BSA), L-cysteine (98.5 %) and polyethylene with MW 4000 and 6000 were supplied by Sigma Aldrich (Germany). Ethanol was purchased from DosFarm (Almaty, Kazakhstan). Urea (99.5 %) was purchased from ChemPribor SPb (St. Petersburg, Russia). Sodium hydrophosphate and potassium dihydrophosphate were used to prepare a phosphate-buffered saline solution.

Preparation of PEG-BSA-INH NPs

PEG-BSA-INH nanoparticles were prepared by desolvation with some additions as in [16–18]. According to this technique, a given amount of bovine serum albumin (concentration 20 mg/mL) and PEG with MW 4000 or 6000 (10–100 mg/mL) were dissolved in distilled water while stirring at 200 rpm. Then an aqueous solution of urea was added (its concentration was 7 mol/L) and treated with an ultrasonic bath; the ultrasonic period was 3 min. Pre-prepared isoniazid at a concentration of 4 mg/mL was added to the resulting suspension. Ethanol was then added to each albumin solution at a rate of 1 mL/min to form a colloidal dispersion of albumin nanoparticles with PEG. A given amount of L-cysteine aqueous solution (concentration 2.5 mg/mL) was then added. The reaction mixture thus obtained was stirred continuously for 2 h. The produced nanoparticles were separated by centrifugation (MiniSpin, Eppendorf, Hamburg, Germany) at 14,000 rpm for 15 min, then the suspension of nanoparticles was washed three times with distilled water.

Particle size, polydispersity index, ζ-potential and morphology of the PEG-BSA-INH NPs

The polydispersity (PDI) and particle size were determined on a laser particle size detector (Malvern Zetasizer Nano S90, Malvern Instruments Ltd., Malvern, UK) using dynamic light scattering (DLS). Each batch of nanoparticles was appropriately diluted with distilled water immediately after production. ζ-potential was determined with a Zeta potential analyzer using Phase Analysis Light Scattering (NanoBrook ZetaPALS, Brookhaven Instruments Corporation, Nova Instruments, USA). The surface morphology of PEG-BSA-INH NPs was investigated by scanning electron microscopy (MIRA 3LM TESCAN, Brno, Czech Republic, EU).

Encapsulation efficiency, loading capacity and PEG-BSA-INH NPs' yield

The amount of isoniazid loaded into PEG-BSA nanoparticles was determined by measuring the amount of unentrapped drug in the supernatant. Isoniazid was analyzed by high performance liquid chromatography (HPLC) (Shimadzu LC-20 Prominence). The encapsulation efficiency, loading capacity and yield of nanoparticles were calculated as follows:

$$\text{Encapsulation Efficiency}(\%) = \frac{\text{Mass of INH in NPs}}{\text{Mass of total INH}} \times 100\ \%;$$

$$\text{Loading Capacity}(\%) = \frac{\text{Mass of INH in NPs}}{\text{Total mass of NPs}} \times 100\ \%;$$

$$\text{Nanoparticles Yield}(\%) = \frac{\text{Total mass of NPs}}{\text{Total mass of INH} + \text{Total mass of polymer}} \times 100\ \%.$$

In vitro study of drug release from polymer NPs

To investigate the *in vitro* release of isoniazid from NPs: A dialysis bag (MWCO: 1 kDa) containing 3 mL of phosphate-buffered saline (PBS) with PEG-BSA-INH nanoparticles was placed in a beaker with 250 ml of PBS. The beaker was placed in a water bath, at 37 °C. Dialysates were sampled periodically (3 mL at a time). To study the degree of INH release from the polymer nanoparticles, the amount of drug released was recorded by HPLC and calculated by formula:

$$\text{Drug Release}(\%) = \frac{\text{Mass of released drug}}{\text{Mass of the total drug in nanoparticles}} \times 100\ \%.$$

Thermogravimetric analysis and differential scanning calorimetry

Polymer and nanoparticle behaviour of PEG-BSA-INH during thermal degradation was studied with a thermogravimetric analyzer and differential scanning calorimetry (LabSYS evo TGA/DTA/DSC, Setaram, France); the instrument was operated from 30 °C to 600 °C under nitrogen at a flow rate of 30 ml/min and a heating rate of 10 °C/min.

Study of prepared nanoparticles by Infrared spectroscopy

IR spectroscopy (FSM 1202, Infraspek Ltd., Russia) was used to identify the samples. FT-IR spectra were determined using the KBr method. 3 mg of the sample was mixed with 100 mg of KBr and a pellet was prepared. The IR range investigated was 4000 to 400 cm^{-1} and resolution of FT-IR spectra was 8 cm^{-1}.

Statistical analysis

The data are expressed as mean ± standard deviation. The analysis was carried out statistically by Minitab 19 Statistical Software. The data were analyzed by one-way analysis of variance (ANOVA).

Results and Discussion

PEGylation of albumin involves a chemical reaction between albumin reactive groups (amino acid side chains, N-terminal amino group or C-terminal carboxylic acid) and PEG [19]. Recently, disulfide bonds have been considered as targets for PEGylation; disulfide bridges are known to be present in small amounts in albumin macromolecules, which makes PEGylation at these sites attractive because of the possibility of producing homogeneous nanoparticles [8]. In terms of drug delivery system development, the most attractive aspect of cysteine is that it provides an accessible chemoselective site for surface modification without alter-

ing the tertiary structure of albumin [20-22]. An example of the production of albumin-specific binding sites via cysteine is the PEGylated product "CIMZIA®" (UCB Pharma S.A., Belgium) [19].

In previous work [18], we synthesized bovine albumin nanoparticles with the anti-tuberculosis drug "Isoniazid". Cysteine, which is capable of cleaving intramolecular disulfide bonds in proteins, was used to form the nanoparticles. This leads to a more complete unfolding of the polypeptide chain and facilitates cysteine attachment to intramolecular S-S bridges within the BSA globule [16–17, 22]. We suggest that the addition of urea and L-cysteine unfolds the albumin globule and opens the disulfide bridges, where PEG molecule attachment occurs more easily. Using the optimized parameters of BSA-INH NPs, we added different concentrations of PEG in the synthesis of PEGylated NPs. Thus, PEG-BSA-INH nanoparticles were obtained by desolvation (Fig. 2). The specifications of the produced NPs are shown in Table.

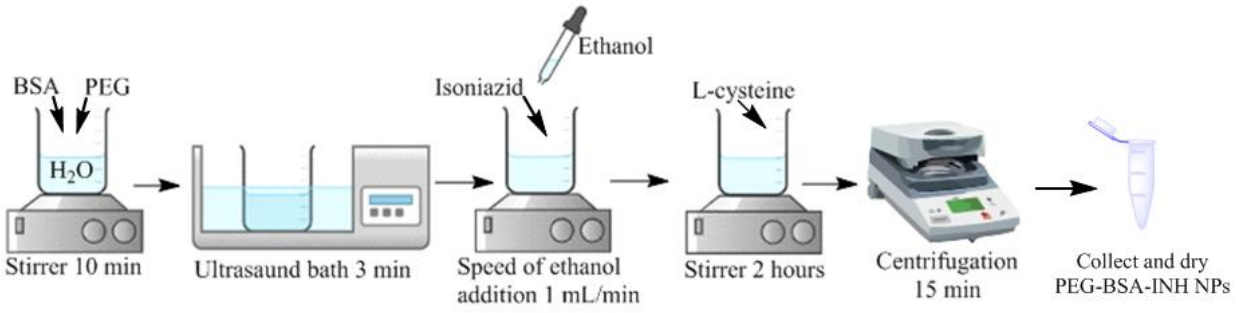


Figure 2. Scheme for producing PEG-BSA-INH nanoparticles

T a b l e

Characteristics of the PEG-BSA-INH nanoparticles

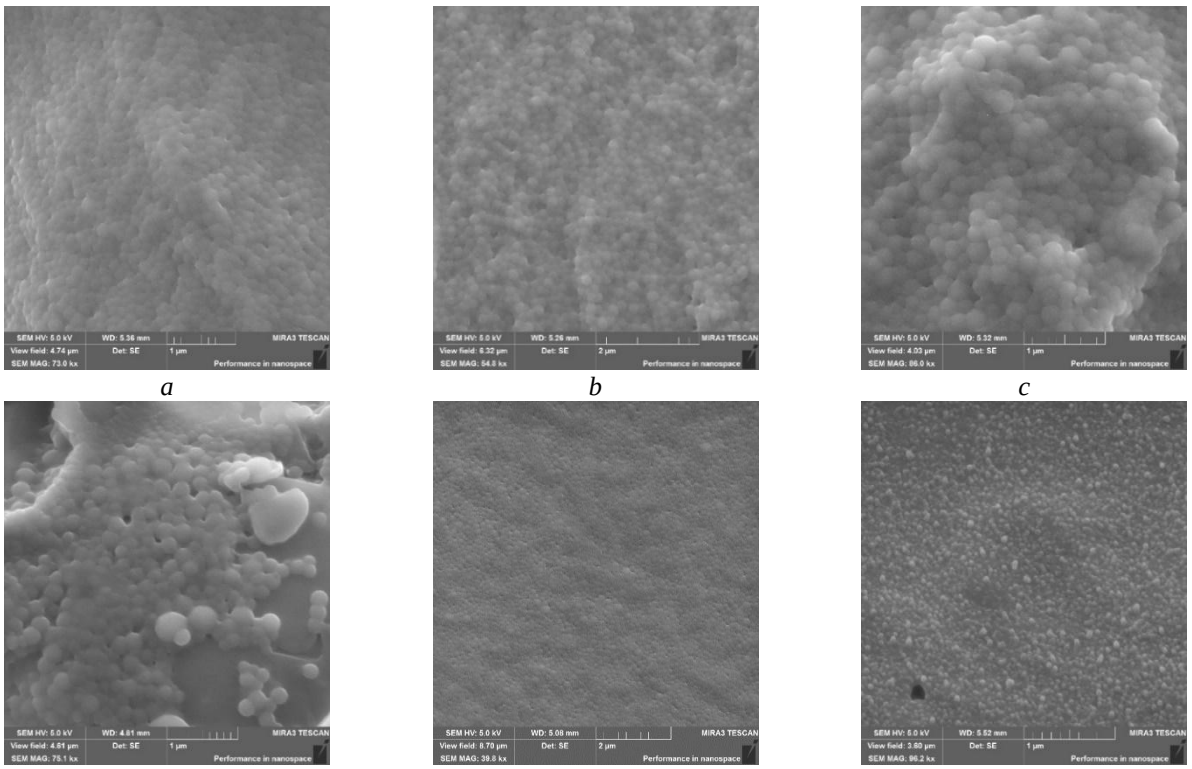
Formulation	PEG type	[PEG], mg/mL	Size, nm	PDI	ζ potential, mV	Encapsulation efficiency, %	Loading capacity, %	NPs' yield
B10	–	–	197.6±2.5	0.068±0.01	–28±6	50±3	38±4	26±3
BP410	4000	10	237.5±2.7	0.037±0.01	–34±4	68±3	18±2	47±3
BP420	4000	20	212.8±5.2	0.069±0.03	–28±1	75±1	22±2	28±6
BP440	4000	40	246.7±9.3	0.027±0.01	–41±3	74±7	19±1	22±4
BP480	4000	80	226.1±2.5	0.060±0.01	–21±7	80±5	24±5	14±2
BP4100	4000	100	225.7±5.8	0.059±0.02	–29±3	79±2	24±3	11±1
BP610	6000	10	249.7±8.1	0.040±0.02	–37±2	80±8	20±2	49±4
BP620	6000	20	245.1±5.9	0.069±0.02	–36±1	78±2	22±3	34±4
BP640	6000	40	239.8±1.6	0.039±0.03	–49±3	78±3	20±3	23±4
BP680	6000	80	271.2±9.7	0.078±0.04	–40±3	75±3	25±4	14±2
BP6100	6000	100	287.9±5.4	0.155±0.02	–44±9	77±4	27±5	11±2

PEG is known to coordinate about 3 water molecules per monomer unit, which gives PEG a large hydrodynamic volume [23]. Therefore, PEGylation of nanoparticles should lead to an increase in the hydrodynamic particle diameter as measured by DLS, which is an indirect reference to the degree of modification [24]. PEGylated and non-PEGylated BSA nanoparticles were compared with respect to particle size and polydispersity using DLS. Without PEGylation the particle diameter was 197.6±3 nm (B10 formulation). Particle size was found to be influenced by the molecular weight of the polyethylene glycol, while PEG concentrations were practically not affected. Therefore, with PEG 6000 the particles had a larger diameter than with PEG 4000. The highest added concentration of PEG 6000 (100 mg/mL) resulted in particles with a diameter 50 nm larger than those without added PEG. The polydispersity index of all particle charges was less than 0.1, indicating monodisperse size.

To confirm the stability of PEG-coated nanoparticle suspensions, the ζ-potential was measured and compared with non-PEG-coated BSA nanoparticles. Table shows the change in ζ-potential as a function of increasing concentration of added PEG. In contrast to the particle size, the ζ-potential changed significantly compared to the non-PEG-added BSA nanoparticles. In all cases, stable nanoparticle systems were produced,

with PEG-6000 obtaining nanoparticles with a ζ-potential potential of about -40 mV or more. In addition, parameters such as the encapsulation efficiency, the loading capacity and the yield of nanoparticles are also important factors in the production of nanoparticles. With increasing concentration of PEG, a decrease in the yield of nanoparticles and an increase in the loading capacity were observed as for PEG-4000 and PEG-6000 (Table).

Particle size and surface morphology of PEGylated albumin nanoparticles were performed by scanning electron microscopy (SEM). The images were analyzed using Image J software and the average particle size of BP410, BP420 and BP480 nanoparticles was 161.0±8, 201.5±2 and 226.2±3 nm, respectively. And for BP610, BP620 and BP680 nanoparticles, the average size was 231.4±5, 107.8±7, 117.7±4 nm, respectively. The morphology of the particles was smooth and spherical (Fig. 2).

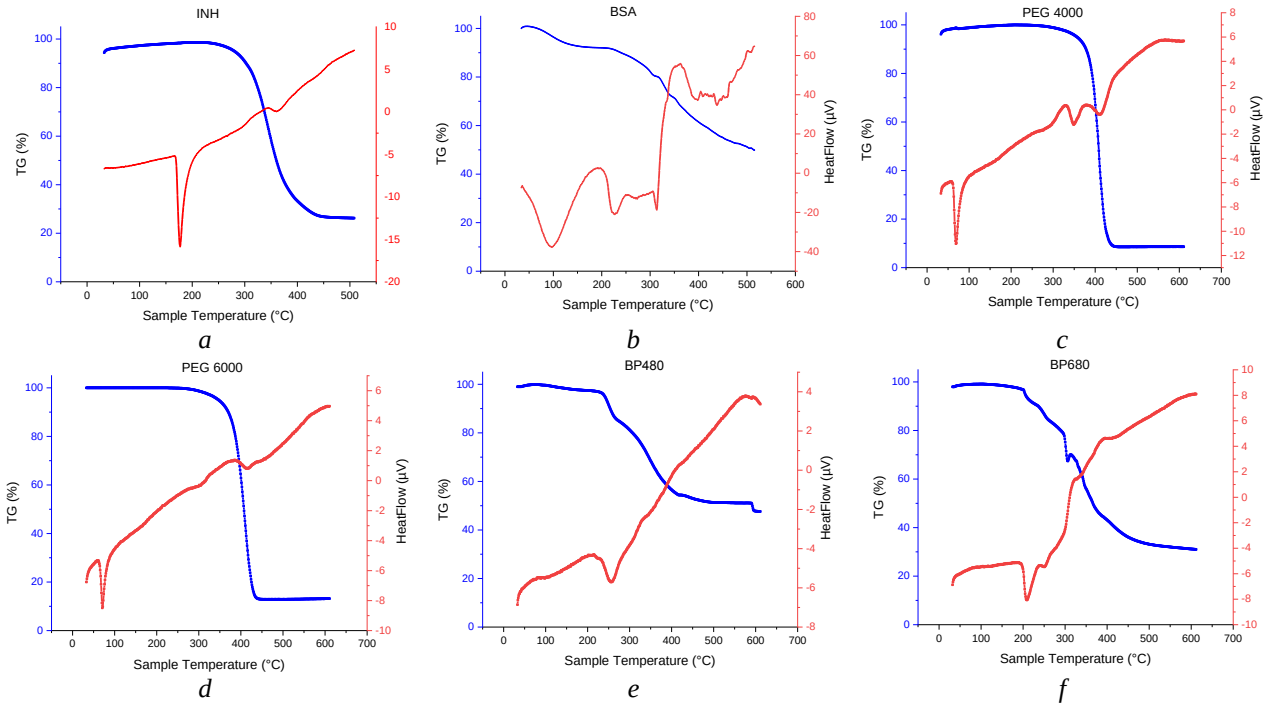


a — BP410; *b* — BP420; *c* — BP480; *d* — BP610; *e* — BP620; *f* — BP680

Figure 2. SEM images of isoniazid loaded PEG-BSA nanoparticles

Further, it was necessary to establish whether the drug substance affects the structure of the polymer. On this basis, we selected thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) as instruments (Fig. 3).

The thermal stability of isoniazid loaded nanoparticles (BP480 and BP680) as well as isoniazid, albumin, PEG4000 and PEG6000 was studied by TGA and DSC (Fig. 3). The DSC curve of isoniazid showed an endothermic peak at 177.2 °C, the associated melting, followed by another endothermic event at 345.6 °C due to the material decomposition (Fig. 3*a*). The mass loss occurred between 250 and 430 °C and was 72 % [24, 25]. The DSC data for BSA shows a three-step thermal degradation, so endothermic event at 98.9 °C related to moisture evaporation, 227.3 °C and 313 °C related to the degradation of the BSA amide bonds [26, 27]. The DSC curves for PEG4000 and PEG6000 showed an endothermic melting event at 70 °C, without mass loss (Fig. 3*c* and *d*). Thermogravimetric analysis for PEG4000 and PEG6000 showed a mass loss of more than 90 % in the temperature range of 300–440 °C. For BP480 there was an endothermic peak at 260 °C, between 220–410 °C there was a mass loss of about 40 % (Fig. 3*e*). For BP680, an endothermic peak was observed at 210 °C, with a mass loss of up to 60 % in the range of 200–440 °C. (Fig. 3*f*). However, compared to the curves of pure BSA and PEG, a significant movement towards higher temperatures was detected. This is probably due to the action of isoniazid. Data obtained by TGA and DSC analysis suggest that drug loading has no effect on the polymer structure.



a — INH; *b* — BSA; *c* — PEG 4000; *d* — PEG 6000; *e* — BP480; *f* — BP680

Figure 3. Thermal behavior of the components of the system and the produced nanoparticles

FT-IR analysis of samples was performed to investigate the interaction between the drug and the polymer. The FT-IR spectra of BSA, isoniazid, PEG and INH loaded PEGylated albumin nanoparticles are shown in Figure 4.

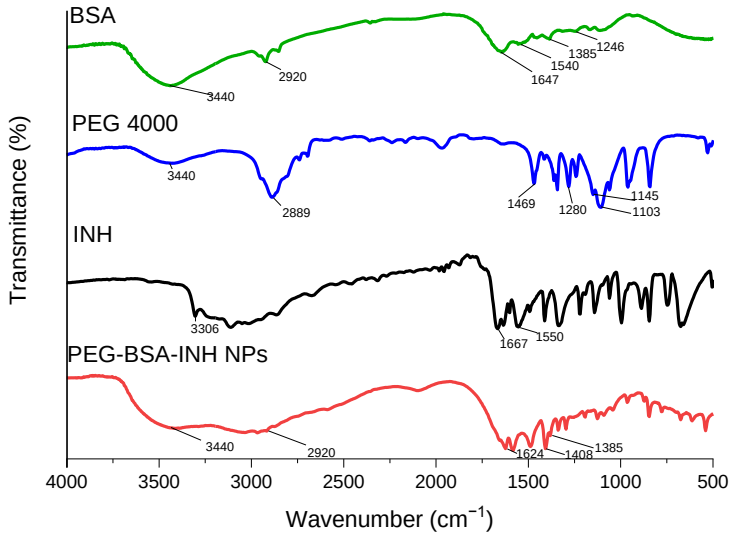


Figure 4. FT-IR spectra for isoniazid loaded PEGylated BSA nanoparticles

In FT-IR spectra of BSA, there were detected bands at 3440 cm^{-1} and 2920 cm^{-1} which show A-amide bound to N-H and B-amide bound to the free ion, respectively. The bands at 1647 cm^{-1}, 1540 cm^{-1} and 1246 cm^{-1} demonstrate C=O stretching vibrations of amide-I, N-H bending vibrations of amide-II, C-N stretching vibrations of amide-III, respectively, confirming the helical structure of BSA [17, 18]. For pure isoniazid broad strong characteristic peaks at 3306 cm^{-1} correspond to N-H stretching, C-H stretching vibrations of the heteroaromatic structure occur at 3100 cm^{-1} for asymmetric stretching, troughs at 1412 cm^{-1} are attributed to C–C symmetric ring vibrations, C-N stretch absorption of aliphatic amines is weak and occurs at 1060 cm^{-1}. The bands at 671 cm^{-1} and 1556 cm^{-1} are attributed to the C-C=O and H-N-N bend, respective-

ly [28]. There are no differences in the IR spectra of PEG due to their different molecular weights. The characteristic absorption bands for polyethylene glycol at 3440 cm^{-1} are due to the O-H stretching band, 2889 cm^{-1} is due to aliphatic C-H stretching, the bands at 1280 cm^{-1} and 1242 cm^{-1} are due to asymmetric C-O and C-O-C stretching vibrations, respectively [29, 30]. The spectrum of PEG-BSA-INH-NPs demonstrates representative peaks for the structure of albumin and isoniazid, indicating the absence of chemical interaction between PEGylated BSA nanoparticles and isoniazid.

The kinetics of drug release from nanoparticles in the creation of isoniazid loaded polymer complexes should be studied to confirm the prolonged action of the prepared NPs. The release kinetics of INH from PEG-BSA nanoparticles were investigated in phosphate buffer at pH 7.4 by dialysis. The degree of INH release from the polymer was calculated from the concentration of the released drug, which was determined by HPLC, the results of which are presented in Figure 5.

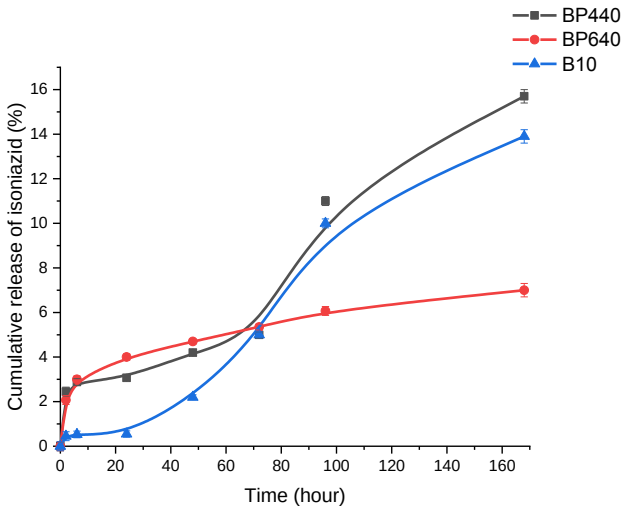


Figure 5. Investigation of isoniazid release from the polymer matrix by dialysis in phosphate-buffered saline

The data presented in Figure 5 shows that all samples demonstrate sustained release throughout the study period (7 days/ 168 hours). Thus, our study clearly demonstrates the potential for sustained release of isoniazid from PEGylated albumin nanoparticles. This makes it possible to prevent drug spikes in the blood and maintain therapeutic concentrations for a long time, indicating the potential of these particles as drug carriers for isoniazid delivery [31–34]. A study of release kinetics demonstrates a small Berst effect and prolonged release with a gradual release of isoniazid. Thus, these results provide a basis for the development of low-toxicity isoniazid prolonged-release chemotherapeutic forms.

Conclusions

PEG-BSA-INH nanoparticles were successfully synthesised by desolvation. The resulting particles were nanosized, as confirmed by DLS and SEM images. SEM images also showed that the particles were spherical in shape. Thus, the scanning microscopy results confirmed the formation of PEG-BSA-INH nanoparticles and also showed a decrease in particle size, which improved the solubility and permeability of the preparation. Based on FT-IR studies, it is clear that PEG was incorporated into the nanoparticles. Thus, the composition can be regarded as PEGylated albumin nanoparticles with isoniazid. The ζ-potential had been determined after the nanoparticles were obtained and the values indicated sufficient physical stability of the nanoparticles. In *in vitro* release studies, it was demonstrated that the nanoparticles had a prologue release. This suggests the possibility of sustained release of the drug *in vivo* as well.

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