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Formulation of PEG-ylated L-asparaginase loaded poly (lactide-co-glycolide) nanoparticles: influence of PEGylation on enzyme loading, activity and *in vitro* release

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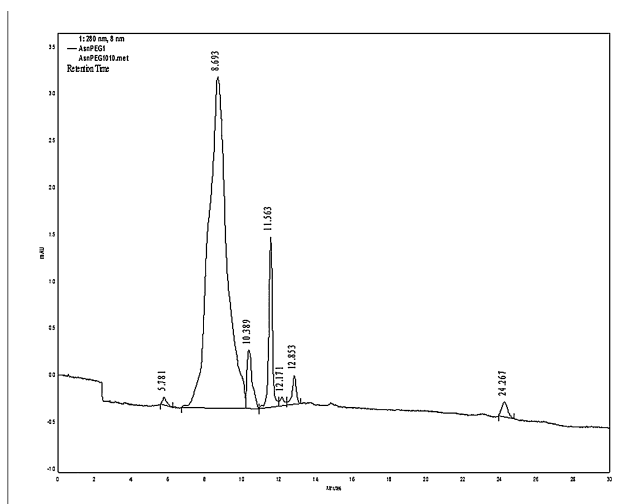
The present paper describes the advantage of PEG-ylation of L-asparaginase before encapsulation over its incorporation in the native form. During encapsulation a considerable amount of native protein undergoes denaturation and forms insoluble aggregates. In an effort to overcome this problem, L-asparaginase was PEG-ylated before subjecting it to the harsh conditions as encountered during double emulsion solvent evaporation technique. L-asparaginase was conjugated with succinimidyl succinate derivative of polyethylene glycol (SS-PEG, MW 5000) followed by characterization of the formed conjugate using size exclusion-HPLC and SDS PAGE. The PEG-ylated L-asparaginase consisted of different isomers from mono to multi PEG-ylated depending upon the number of Lysine residues (14 in case of L-asparaginase) with about 5% as native protein. The specific activity as retained after PEG-ylation was 62.84 ± 8.2% and further about 82.7% of activity was recovered from the particles. Imitated studies with the native protein confirmed the enhanced stability of the conjugated protein when exposed to the organic solvent and sonication and showed comparatively less encapsulation efficiency due to increased hydrophilicity. Release profiles for native as well as conjugated proteins consisted of sustained release of about 66.66% and 44.45% in 28 days, respectively. The decrease in the release can be attributed to the increase in the molecular weight of the conjugated protein. The study finally proved that PEG-ylation protected the enzyme and prevented it from denaturation during encapsulation.

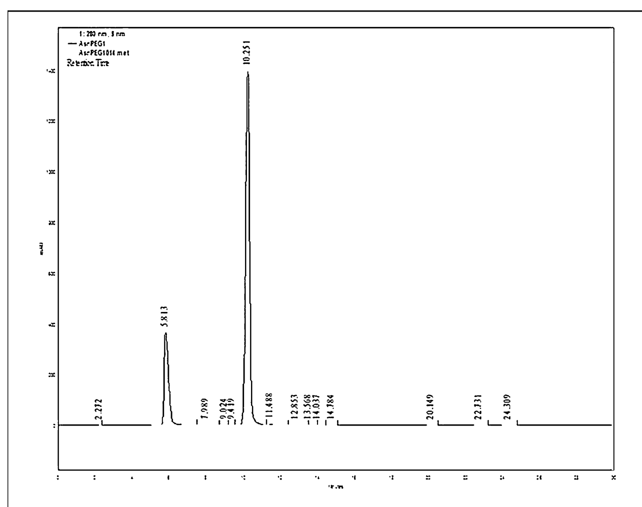
1. Introduction

To develop a suitable delivery system for proteins has always been a challenging task. The issues such as stability during long term storage and developing the ways to control the problems like aggregation and denaturation has always been of major concern to the pharmaceutical industries (Frokjaer and Otzen 2005; Brown 2005). The harsh formulating conditions can cause disruption of their secondary, tertiary and in some cases quaternary structures leading to complete or partial loss of their activity. PEG-ylation is one of the strategies by which aggregation and misfolding of the protein can be avoided. It confers stability by forming a hydrophilic shell around the protein in an organic solvent (Diwan and Park 2001). It also improves the pharmacokinetic properties of the protein (Harris et al. 2001). Decreased immunogenicity is another benefit (Hindsa et al. 2005; Inada et al. 1995).

Bacterial L-asparaginase (L-aspargine amidohydrolase, E.C. 3.5.1.1) is an enzyme of high therapeutic value due to its use in clinical protocols for the treatment of children with acute lymphoblastic leukemia (Qing-Long et al. 2002; Swain et al. 1993). It has been used as a therapeutic agent since Kidd (1953) discovered that it has the ability to hinder the growth of the induced tumors in mice after treatment with hamster serum, which contains high levels of this enzyme (Broome 1963; Oettegen et al. 1967). One of the main drawbacks of L-asparaginase is its short *in vivo* circulating time, which implies the necessity of multiple intravenous injections in order to reach optimum therapeutic levels. Another most important problem is its ability to evoke immunogenic responses, which could be as severe as anaphylactic shocks. To overcome these problems the administration of L-asparaginase in liposomes has previously been suggested (Gaspar et al. 1996; Jorge et al. 1994). Various articles report the difficulties associated with developing effective formulations for such proteins as they are relatively large molecules with secondary, tertiary and quaternary structures (Lu and Park 1995; Wang 1999; Putney and Burke 1998; Weert et al. 2000). Disruption of these structures which occurs readily due to slight change in pH, temperature etc. of the environment can lead to complete or partial loss of activity. This research work mainly deals with preparation and evaluation of PEG-ylated enzyme loaded polymeric particles and to determine if it provides any protection to the enzyme against harsh conditions being encountered during the emulsification process.



(a)



(b)

Fig. 1: a) SE-HPLC chromatogram of the pure protein. Four different peaks may represent the monomeric form of the protein. b) SE-HPLC chromatogram of the PEG-ylated protein. Decrease in the elution time indicating increase in the molecular weight due to PEG attachment

2. Investigations, results and discussion

2.1. Physicochemical characterization of the conjugated protein

Most common sites for PEG-ylation reaction are at lysine residues and the N-terminal group. Lysine is the most prevalent amino acid and can accounts for more than 10% of overall amino acid sequence. With protein alone four peaks corresponding to elution times 8.693, 10.389, 11.563 and 12.853 min were observed. But after PEG modification, surprisingly only a single peak with reduced elution time of 5.813 min was obtained. The reason behind this is not clear but it can be hypothesized that four peaks obtained can be due to four different units of the protein. Crystalline structure studies have shown that asparaginase is a dimmer of the dimmers or in simple terms it is a tetramer and hydrogen and ionic bonds are responsible for its secondary and tertiary structure. Predominantly hydrophobic interactions hold the different units together, which are weak enough to undergo dissociation releasing the four units separately. Coupling of PEG might have caused the units to re-associate and thereby single elution peak at 5.813 min was obtained Fig. 1 (a) and (b), which was less than the four units alone due to increase in the molecular weight.

Further SDS PAGE analysis has shown that the shift from 32 kDa, which corresponds to the molecular weight of each monomer to higher molecular weight. Non specific PEG-ylation has caused an uncontrolled number of PEG moieties to couple with the 24 lysine residues in asparaginase giving a range of protein molecules with different molecular weights. These can be called as ‘PEGmers’. This gives rise to a smear of band Fig. 2 (a) and (b) instead of a single band as obtained with a pure protein. Lysine residues present at or near the active sites of the enzyme possibly affected the specific activity of the protein on PEG modification. The specific activity of the pure enzyme was 1.4 units/mg and after PEG modification, enzyme showed 0.87 units/mg of activity. There was almost $62.84 \pm 8.2\%$ retention of the activity.

2.2. Simulation studies

The steps involved in the double emulsion solvent evaporation that affects the protein stability includes exposure to the organic solvent and stress due to double sonication. These produce detrimental changes in the protein and ultimately lead to the loss in the activity of the protein (Crotts et al. 1997). Both the parameters were studied independently. L-Asparaginase and PEG-ylated L-asparaginase were both studied by size exclusion chromatography with UV-Vis detectors at 280 nm. The first step in the double emulsion solvent evaporation technique is emulsifying the aqueous protein solution with organic solvent solution of the polymer. The protein therefore comes in direct contact with dichloromethane at the interface which can lead to unfolding and subsequent aggregation (Lu and Park 1995). Exposure of both asparaginase and PEG-ylated L-asparaginase to the organic solvent resulted in the formation of insoluble aggregates $14.5 \pm 3.5\%$, $8.5 \pm 1.5\%$, respectively. Significant advantage of PEG-ylated over non PEG-ylated protein could be noticed despite of the presence of 5% of the non PEG-ylated protein.

Upon sonication for 30 s, the percentage of soluble protein found was $95.2 \pm 5.5\%$ for PEG-ylated and $72 \pm 8.3\%$ for non-PEG-ylated, respectively. After 150 s, the same was $80.2 \pm 5.5\%$ and $60 \pm 6.7\%$ for PEG-ylated and non PEG-ylated, respectively and finally after 300 s the loss was considerable with non PEG-ylated, which was about $51 \pm 10.5\%$ as depicted in Fig. 3. The reason for this loss can be attributed to both increased surface area due to sonication, hence more exposure of the protein directly at the dichloromethane and aqueous interface and high shear stress, which facilitate the formation of the aggregated protein.

2.3. Characterization of the nanoparticles

After getting the significant results with PEG-ylation, the PEG-ylated samples were encapsulated in nanoparticles which were further characterized for size and encapsulation efficiencies. The sizes as determined by a dynamic laser scattering method, were both between 150–300 nm. Slight increase in diameter of PEG-ylated L-asparaginase loaded particle relative to L-asparaginase loaded particles, may be attributed to PEG conjugation, which increased the size and molecular weight of the protein. The loading amount and efficiency for free L-asparaginase were $0.932 \pm 0.04\%$ (w/w) and $77.88 \pm 2.01\%$ (w/w), respectively. On the other hand, those of PEG-ylated L-asparaginase particles were $0.802 \pm 0.02\%$ (w/w) and $65.1 \pm 1.47\%$ (w/w). The reduced encapsulation efficiency for PEG-ylated L-asparaginase loaded particles is clearly attributable to the fact that PEG confers hydrophilicity and thus the emulsion formed is comparatively less stable and thus an amount leaches out and is lost

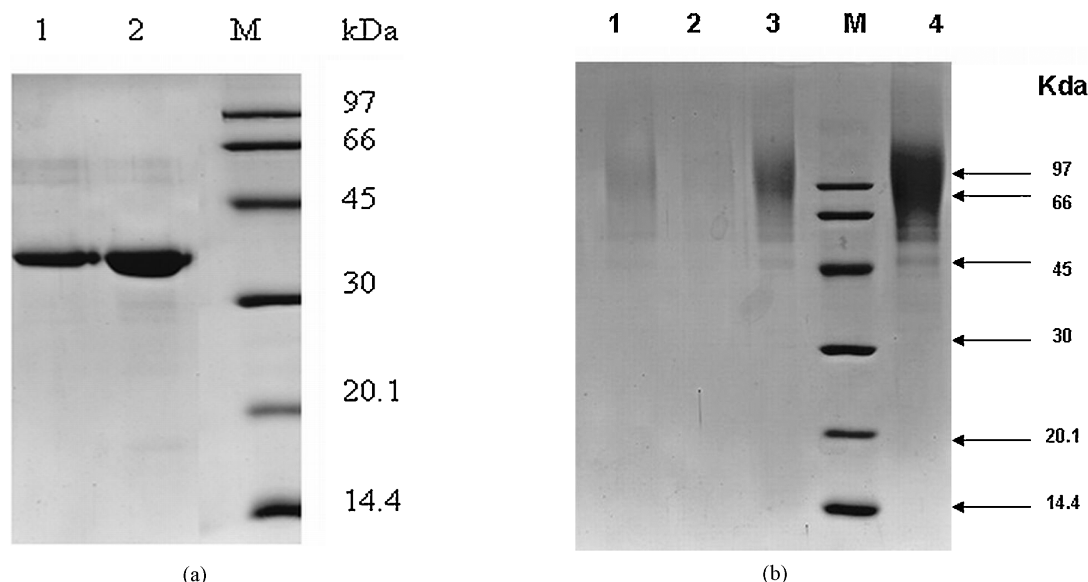


Fig. 2: a) Characterization of pure L-asparaginase under reducing conditions: lane 1 and 2 indicates pure protein and lane 3 indicates the marker protein. The molecular weight could be depicted near about 32 Kda. Characterization of PEG-ylated L-asparaginase under reducing conditions: lane 1, 2, 3 and 4 indicates PEG-ylated protein and lane M indicates the marker protein. Smear of band shows series of proteins of different molecular weight due to PEG-ylation at different positions and varied number of lysine residues

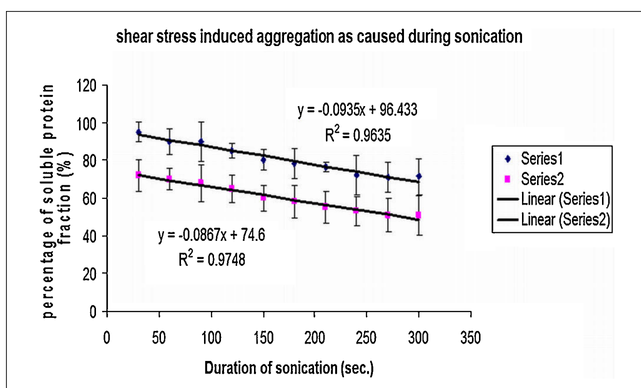


Fig. 3: Shear stress induced denaturation/aggregation as caused during emulsification of aqueous protein solution in dichloromethane. The extent of protein denaturation is expressed as soluble L-asparaginase fraction recovered in PBS after removal of solvent

during washing procedures. For the analysis of the surface, SEM pictures (Fig. 4) were taken which clearly shows that the surface of the particles was smooth, non porous and compact.

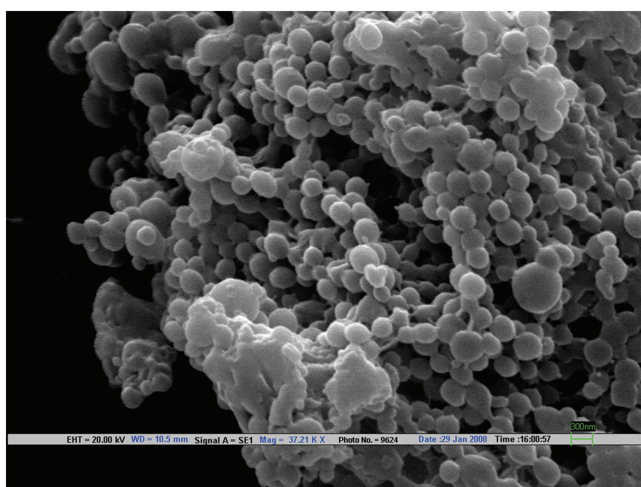


Fig. 4: Scanning electron micrographs of nanaospheres made of PLGA 50:50 (MW 10,000) loaded with L-Asn-mPEG (plz see d order mPEG-Asn)

2.4. *In vitro release studies*

The burst effect of about 38.0% and 28.7% after one day was observed from both the formulations of proteins i.e. L-asparaginase loaded and PEG-ylated L-asparaginase loaded. It has been shown that that the release of protein from particles composed of lactic/glycolic acid polymers occurs by diffusion through aqueous pores that facilitate water-soluble protein release.
From *in-vitro* release experiments of free and PEG modified recombinant asparaginase, it was observed that the burst release after 30 min was very low i.e. about only 7.14% and 5.55%, respectively. This might have occurred due to the higher molecular weight of PEG-ylated asparaginase. But after that there was a (Fig. 5) sustained release and about 66.66% and 44.45% had released in four weeks, respectively. (Fig. 5)

2.5. *Bioactivity evaluation*

Bioactivity of the PEG-ylated protein after release from the particles was almost similar to non encapsulated PEG-Asn i.e. 0.72 ± 0.03 units/mg and 0.87 ± 0.1 units/mg, respectively. Thus, approximately 82.7% of the activity was recovered. PEG formed a protective shell around the protein and prevented it from coming in direct contact with the organic solvent. Its

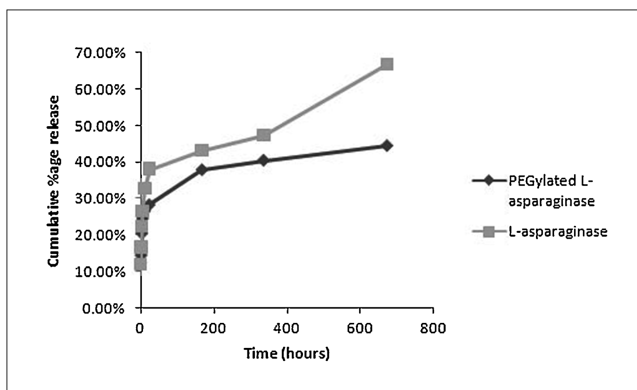


Fig. 5: *In vitro* release kinetics of encapsulated protein from nanospheres carrying native (B) and PEG-ylated L-asparaginase (C)

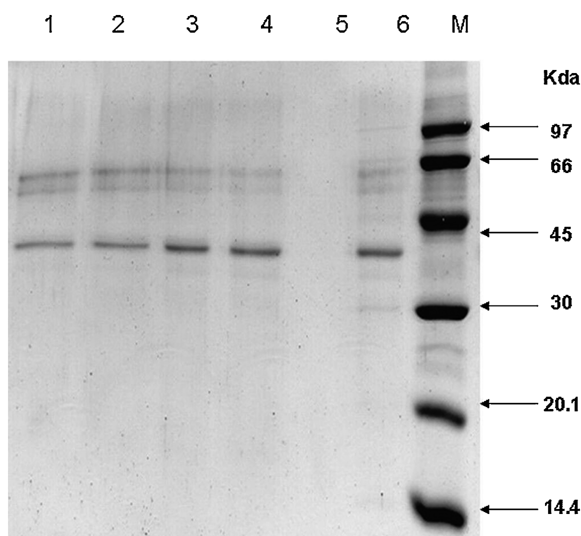


Fig. 6: SDS PAGE profile of the released L-asparaginase from the nanospheres, clearly indicating the aggregation due to two additional bands near 66 Kda

profile on SDS PAGE was also similar to the profile obtained before encapsulation whereas on the other hand for Asn alone the activity before encapsulation was 1.4 ± 0.2 units/mg and after its encapsulation by double emulsion solvent evaporation the specific activity was 0.13 ± 0.01 units/mg thus only $7 \pm 0.007\%$ retention of the activity was obtained. SDS PAGE also showed three bands instead of a single band after release. (Fig. 6) The double bands of a higher molecular weight were obtained, which were due to disruption of the actual protein structure, misfoldings and aggregation, which had led to loss in functionality of the enzyme.

3. Experimental

3.1. Materials

Recombinant asparaginase was received as a gift sample from National Institute of Immunology, succinimidyl succinate monomethoxy polyethylene glycol (5000) was purchased from Sigma (Aldrich, USA) and Poly (D, L- lactide-co-glycolide) copolymer with monomer ratio as 50:50 and molecular mass of 10 kDa from Birmingham Polymer Inc., USA, All other chemicals and reagents were of analytical grade.

3.2. PEG-ylation of L-asparaginase

Non specific covalent modification of lysine residues of asparaginase was carried out using succinimidyl succinate monomethoxy polyethylene glycol (5000) keeping protein to PEG ratio 1:12. Reaction medium used was phosphate buffer prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate, of concentration 50 mM and pH 8.5. Continuous stirring was carried out for 6–7 h at 37 °C. Reaction was terminated using acetic acid. Dialysis by 10 kDa pore size membrane against deionised water was carried out and finally the dialyzed sample was lyophilized without any cryoprotectants and stored at −20 °C.

3.3. Physicochemical characterization of the conjugate

3.3.1. SDS PAGE

The PEG-ylated protein was characterized by SDS PAGE using 4.5% and 12.5% stacking and resolving gel, respectively. The samples were prepared under reducing conditions and were loaded to the gel. Coomassie Brilliant Blue dye as a staining solution.

3.3.2. Size exclusion HPLC

Size exclusion HPLC was carried out using Shimadzu HPLC with high sensitivity UV- Vis photodiode array, fluorescence detectors and Superdex® Gel filtration column (Amersham Biosciences, USA). The column was first equilibrated with phosphate buffer of concentration 50 mM and pH 7.0 followed by the loading of the samples. The elution was carried out by the phosphate buffer saline (PBS, 50 mM, pH 7.0). The flow rate was maintained at 0.6 mL/min. and the eluates were monitored by UV- Vis photodiode arrays at 280 nm.

3.3.3. Specific activity of PEGylated L-asparaginase

The rate of hydrolysis of aspargine was determined by measuring the released ammonia (Mashburn and Wriston 1964). For each different enzyme dilution 0.05 M Tris HCl (pH 8.6) and 0.01 M L-aspargine were added to the test tubes.
Samples were incubated at 37 °C for 5–6 min to achieve temperature equilibration. At zero time and at timed intervals, diluted enzyme samples were added. Clarified by centrifugation and added 0.5 mL of clear supernatant to 7.0 mL reagent grade water. Added 1.0 mL of Nessler's reagent and incubated at room temperature for 10 min. Read at 436 nm. Same procedure was repeated with standard ammonia solution.

3.4. Simulation studies

L-Asparaginase and PEG-ylated L-asparaginase were subjected to the imitated studies where all the conditions, which can be harsh to the protein were studied individually. All the studies were carried out in triplicate.

3.4.1. Studies with organic solvent (dichloromethane)

PEG-ylated and native protein samples were added to the dichloromethane, the solvent most frequently used in double emulsion solvent evaporation technique, in a concentration of 0.1% w/v. The solvent was allowed to evaporate at room temperature and the fraction left was extracted with PBS (50 mM, pH 7.0). The protein concentration was then determined using micro BCA protein assay kit.

3.4.2. Shear stress studies

PEG-ylated and native protein samples were emulsified with dichloromethane (0.1% w/v) using a sonifier (Bandelin Sonifier, duty power 40%) for the duration of 40 to 400 s. The solvent was allowed to evaporate at room temperature and the fraction left was extracted with PBS (50 mM, pH 7.0) followed by determination of the protein concentration using micro BCA protein assay kit.

3.5. Nanosphere preparation

Particles were prepared by the water-in-oil-in-water solvent evaporation technique. Briefly, the method involved preparation of water in oil (w_1/o) primary emulsion by emulsifying 0.1 mL of aqueous protein solution containing 2% sodium bicarbonate and 10% sucrose with 50 mg/mL of PLGA solution in dichloromethane (DCM) using sonication (sonifier, Bandelin duty power 40%, 4 cycles). This was followed by preparation of a secondary emulsion (w_1/o/w_2) between the primary emulsion and water, containing 1% PVA. The secondary emulsion was then stirred for 16–17 h to evaporate DCM. The secondary emulsification step was also carried out using sonication to obtain optimum sized nanoparticles. After the complete evaporation of DCM, the particulate suspension was centrifuged at 30,000 rpm for 20 min. Repeated washing for the complete removal of PVA was carried out. The particles were finally re-suspended in phosphate buffer pH 7.4.

3.6. Characterization of prepared nanospheres

3.6.1. Scanning Electron Microscopy (SEM)

The surface morphology was observed by SEM. Samples were prepared by dropping the particulate suspension on 10 mm X 10 mm aluminum stub and allowing it to dry. The samples were gold coated using a sputter coater (Joel) and viewed through a scanning electron microscope (Jeol JSM 35CF Jeol Instruments, Japan).

3.6.2. Particle size determination

The particle size distribution and polydispersion coefficients were determined by photon correlation spectroscopy (PCS) in a Zetasizer III (Malvern Instruments, Malvern, UK). At least four different batches were analysed to give an average value and standard deviation for the particle diameter.

3.6.3. Protein entrapment

The entrapment efficiency was determined after consecutive extraction of the encapsulated protein from the polymer. For the same, a known amount of particles (10 mg) were dissolved in 1.0 mL of acetonitrile solution to remove the polymer followed by spinning at 20,000 rpm for 10 min. The pelleted protein was collected and the supernatant discarded. The pellet was then re-dispersed in PBS (50 mM, pH 7.4) and the samples were centrifuged again to give washing and to remove the excess polymer. The supernatant

was discarded again and the pellet was re-dissolved in 1.0 mL of 1% SDS. The protein was then estimated using micro BCA assay and took reading at 540 nm.

3.7. *In vitro release profile*

Weighed quantity of nanoparticles were suspended in 1.0 mL of phosphate buffer saline (pH 7.4, 50 mM). They were placed in shaker incubator at 37° C and 200 rpm. At predetermined time intervals, the supernatant was collected and fresh buffer solution was replenished. The supernatant was used to determine the released protein. SDS PAGE and specific activity of the released protein were determined using the same methods as described in the sections 3.3.1 and 3.3.3.

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