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International Journal of Pharmaceutics 271 (2004) 189–196
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Controlled release of a model protein lysozyme from phase sensitive smart polymer systems

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Received 13 May 2003; received in revised form 10 November 2003; accepted 10 November 2003

Abstract

The purpose of this study was to investigate the suitability of phase sensitive smart polymer-based protein formulations in order to deliver a model protein, lysozyme, in a conformationally stable and biologically active form at a controlled rate over extended period of time. Four different formulations, using D,L-poly(lactide) (D,L-PLA) and a solvent mixture of different ratios of benzyl benzoate (BB) and benzyl alcohol (BA), were prepared. Conformational stability and biological activity of lysozyme were studied by differential scanning calorimeter and enzyme activity assay, respectively. We found a significant ($P < 0.05$) increase in burst and rate of release of incorporated lysozyme from formulations containing greater proportion of BA. In order to increase the conformational stability and biological activity of lysozyme, we incorporated mannitol as stabilizer into formulations. Mannitol increased the conformational and biological activity of lysozyme in comparison to the control formulation prepared without mannitol. In conclusion, phase sensitive smart polymer-based delivery systems were able to deliver a model protein, lysozyme, in a conformationally stable and biologically active form at a controlled rate over extended period of time.

Keywords: Controlled release; Differential scanning calorimeter; Smart polymer; Lysozyme; Conformational stability; Biological activity

1. Introduction

There have been enormous advances in recent years in the field of protein and peptide engineering. With the tremendous growth of biotechnology and recent sequencing of the human genome, it is possible to produce significant numbers of therapeutically active proteins (Lee, 2002). Currently, a wide variety of delivery systems exist for drug substances, but the traditional drug development of proteins has relied on parenteral injections of liquid formulations. The delivery method for proteins should be critically evaluated by considering the effect of these methods on the biological activity and conformational stability of the protein in the formulation as well as the toxicity and ease of manufacturing of the protein formulation. The implant delivery systems are able to overcome most of the above obstacles except their complex and costly manufacturing (Jeong et al., 1999; Benoit et al., 2000; Fowler et al., 2000; Stevenson and Tan, 2000). Pulmonary delivery generally leads to low protein bioavailability in comparison to injections (Shen et al., 1999). Although, transdermal and oral delivery systems have met significant advancement in recent years, logarithmic increase in bioavailability is required to make them commer-

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doi:10.1016/j.ijpharm.2003.11.010

cially viable delivery routes (Naik et al., 2000; Stoll et al., 2000). Therefore, an efficient delivery system for proteins is the key to commercial success of current and future biotechnology products (Cleland et al., 2001).

"Smart polymers" that display a physiochemical response in nonlinear fashion to external stimuli (i.e. temperature, pH, solvent, magnetic field, electric field, ultrasound, etc.) are widely explored as potential drug-delivery systems (Ishishara et al., 1984; Langer, 1990; Thomas et al., 1995; Hoffman, 1995; Klein, 2000; Kost and Lapidot, 2000). Several approaches have been reported for the delivery of bioactive molecules in controlled and pulsatile manner using polymeric carrier (Kikuchi and Okano, 2002).

In situ gel forming, injectable, phase sensitive smart polymer systems (smart polymer mixed with organic solvents) have been found promising to control the drug delivery of proteins (Brodbeck et al., 1999; Chandrashekhar et al., 2001). These injectable formulations allow ease of manufacture, single injections of high doses with small volumes and small needle, and enhanced protein stability (Cleland et al., 2001). A solution of PLA in the mixture of non-toxic hydrophilic and hydrophobic organic solvents has been used for the preparation of phase sensitive smart polymer formulations (Okumu et al., 2001). An instantaneous gel is formed in situ after injecting these formulations. The hydrophilic solvent leaves the gel causing the formation of a shell around the exterior of the depot, while the hydrophobic solvent remains behind slowing water penetration to decrease the rate of polymer hydrolysis and potentially increase protein stability (Eliaz and Kost, 2003; Ravivarapu et al., 2000a; Duenas et al., 2001).

The exact way that the injected solution responds to its physiologic surroundings determines the final depot morphology and, consequently, its eventual release characteristics. Fundamental parameters of the gel formation kinetics include the water influx rate and the gelation rate (Ravivarapu et al., 2000b). The water influx rate refers to the diffusion of water from the physiologic surroundings and subsequent accumulation within the injected polymer solution. As many drugs of interest are water-soluble, the water influx rate is critical because it determines the rate at which the drugs dissolve and therefore how readily they are able to diffuse through the gel implant (Ravivarapu et al., 2000c). The gelation rate is the rate at which the solution is transformed into a soft gelled implant. It will determine the properties of the diffusional path that the drug molecules must take as they leave the gel implant (Graham et al., 1999).

Proteins possess unique physical and chemical properties, which shall create difficulties in formulation and delivery. Unlike conventional small molecular weight compounds, proteins being polypeptides consist of a regularly repeating backbone with distinctive side chains that interact with each other to contribute to the three dimensional conformational structure (Dibiase and Kottke, 2000). In environments other than their physiological ones, therapeutic proteins may rapidly denature and easily lose their conformational stability and biological activity (Jen and Merkle, 2001).

In this study, we investigated the in vitro release, conformational stability and biological activity of a model protein, lysozyme, from phase sensitive smart polymer-based drug delivery systems.

2. Materials and methods

2.1. Materials

D,L-Polylactide (PLA, RESOMER® R 202 inherent viscosity 0.19 dl/g) was purchased from Boehringer Ingelheim, Petersburg, VA. Lysozyme (EC 3.2.1.17) from chicken egg white and Micrococcus Lysodeikticus (*Micrococcus luteus*) were purchased from Sigma Chemical Company, St. Louis, MO. Micro BCA protein assay reagent kit was purchased from Pierce Biotechnology, Inc., Rockford, IL. All other chemicals used were of analytical grade.

2.2. Preparation of phase sensitive in situ gel depot forming system

Polymer solutions were prepared by placing the mixture of polymer (PLA) and solvents [benzylbenzoate (BB) and benzyl alcohol (BA)] in shaker water bath at 37 °C for 24 h. Lysozyme (with/without mannitol) was homogenized (8000 rpm, 2 min) with polymer solution. Four formulations were prepared differing in amount of BB and BA (Table 1). Injectability of formulations was observed through 25-gauge needle.

Table 1
Formulation compositions of phase-sensitive smart polymer-based lysozyme delivery systems

Formulations	D,L-PLA (%, v/v)	BB (%, v/v)	BA (%, v/v)	Lysozyme (%, w/v)
1	5	100	–	5
2	5	95	5	5
3	5	90	10	5
4	5	85	15	5

D,L-PLA: D,L-poly(lactide), BB: benzyl benzoate, and BA: benzyl alcohol.

2.3. In vitro release of lysozyme

Five hundred microliters of the formulation containing 25 mg of lysozyme was injected into 15 ml of isotonic phosphate buffer (pH 7.4) contained in a glass vial. The formulation immediately changed into gel depot, which is due to the change in environment from organic to aqueous. The vials containing in situ formed gel depot were kept in reciprocal shaking water bath (Precision Scientific, Winchester, VA) at 37 °C and 35 rpm for the entire period of study. Five milliliters of aliquots were withdrawn from releasing media without disturbing or breaking the gel structure. The volume withdrawn was replaced with fresh releasing media. The amount of lysozyme in the released samples was determined by MicroBCA protein assay method (Smith et al., 1985). Briefly, samples were centrifuged at $4229 \times g$ for 30 min. One milliliter of suitably diluted supernatant/standard was mixed with 1 ml of working reagent solution made by mixing Micro BCA Reagent A, B, and C in the ratio of 50, 48, and 2, respectively. This mixture was incubated at 60 °C for 60 min, cooled to room temperature, and used for measurement of absorbance at 562 nm by UV-Vis spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Samples from formulation without lysozyme was used as blank control for absorbance. Amount of lysozyme in the released samples was obtained from the standards curve and corrected for sample removal (Hayton and Chen, 1982).

2.4. Conformational stability of lysozyme by differential scanning calorimeter (DSC)

Conformational stability of lysozyme in 2 weeks-released samples was evaluated by using an ultra sensitive DSC (VP-DSC, MicroCal, Northampton, MA). All samples were centrifuged and supernatants were filtered through 0.1 μm filter. These samples and buffer (releasing media) were degassed by stirring under vacuum before loading into the sample and reference cells, respectively. The heat flow required to keep the sample cell and reference cell at same temperature was recorded at temperature range of 25–95 °C at scan rate of 1.5 °C/min. To ensure that the heat transition during protein conformational alterations is the only source of thermal difference between sample cell and reference cell, a baseline thermogram was obtained by loading the releasing buffer in both—sample cell and reference cell. This baseline was subtracted from sample thermogram during data analysis. The transition curve was fitted by non-2-state model (MN2state) which used the Levenberg/Marquardt non-linear least-square method. The calorimetric enthalpy (ΔH) was taken as the area under the transition curve. Number of peak was selected one. All data manipulations were performed by using Origin® software (MicroCal Software, Inc., Northampton, MA) provided with the instrument. Mid point transition temperature (T_m) and ΔH were used as conformational stability indicating thermodynamic parameters. Experiments were carried out in triplicate and data were reported as mean ± S.D. Increase in ΔH and T_m for all of the samples from different formulations in comparison to lysozyme solution maintained at identical conditions, was interpreted as an indication of stabilizing effect provided by different components of the formulations.

2.5. Biological activity of lysozyme by enzyme activity assay

For enzyme activity assay, a portion of vigorously shaken *M. luteus* stock suspension (0.01%, w/v) into phosphate buffer (0.1 M, pH 7.0) was diluted so that it had an A450 between 0.2 and 0.6. Three milliliters of this diluted *M. luteus* solution was taken into a spectrophotometer cell and 0.1 ml of an appropriately diluted lysozyme sample was added to it. The rate of decrease of absorbance at A450 was monitored by UV spectrophotometer during a total incubation period of 2 min at 25 °C. Slope of the linear portion of the plot (between absorbance and time) in absorbance units per min gave the amount of lysozyme in enzyme unit (EU)

(Shugar, 1952). Unit of biologically active lysozyme was determined by using the following formula:

$$\text{Units of lysozyme/ml sample} = \frac{(\Delta A_{450\,\text{nm}}/\text{min Test} - \Delta A_{450\,\text{nm}}/\text{min Blank})(\text{df})}{(0.001)(0.1)}$$

where, df: dilution factor; 0.001 = change in absorbance at A as per the unit definition; 0.1 = volume (in ml) of sample/standard used.

2.6. Data analysis

Statistical comparisons were made using Student's t-test and analysis of variance (ANOVA). The level of significance was used as $P < 0.05$.

3. Results and discussion

Fig. 1 shows the in vitro release profiles of lysozyme from gel depot formulations. The amount of lysozyme in day 1 samples was treated as burst release. We found 9.59 ± 1.43%, 14.08 ± 0.34%, 23.45 ± 0.30%, and 33.12 ± 1.45% burst release of lysozyme from formulations 1, 2, 3, and 4, respectively. Higher release rate was found with formulations containing greater proportion of BA (hydrophilic fraction).

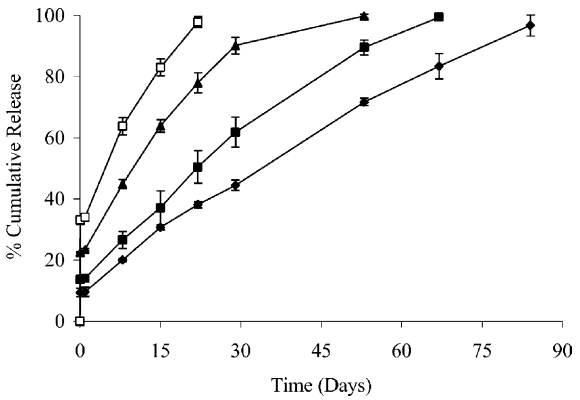

Fig. 1. Percentage cumulative release of lysozyme from in situ formed gel depot of different phase-sensitive polymer formulations. Keys: (◆) benzyl benzoate (100%, v/v), (■) benzyl benzoate (95%, v/v) + benzyl alcohol (5%, v/v), (▲) benzyl benzoate (90%, v/v) + benzyl alcohol (10%, v/v), (□) benzyl benzoate (85%, v/v) + benzyl alcohol (15%, v/v).

In this study, we have investigated a mixture of hydrophobic (BB) and hydrophilic (BA) solvents, which are used in injectable formulations (Ash, 1996). We found greater burst and rate of release from formulations containing greater proportions of BA in solvent mixtures, which are due to faster gelation and/or polymer degradation (Graham et al., 1999; Eliaz and Kost, 2003). The range of burst release obtained is generally lower than microsphere formulations where proteins might get adsorbed at polymer surface (Perez et al., 2002).

We have used D,L-PLA (inherent viscosity 0.19 dl/g) which has optimal viscosity for easier injectability through 25-gauge needle. We obtained release rate in the range of 270–800 μg/day from different formulations. The release rate can be manipulated by varying solvent composition and polymer concentration to meet the need of patients in case of therapeutic proteins.

We observed increases in the area under the transition peak of DSC thermograms from formulations 1 through 4 (Fig. 2). All of the formulations showed significant ($P < 0.05$) increase in the area under the transition curve in comparison to the control. Formulation 4 showed greater area under the transition curve than other formulations. Table 2 shows increase in conformational stability as indicated by significant increase ($P < 0.05$) in ΔH with increase in hydrophilic fraction of the solvent mixtures of the formulations 1–4 in comparison to the control. However, ΔH values of

Table 2
Mid point transition temperature and calorimetric enthalpy of lysozyme released from in situ formed gel depot determined by DSC

Formulations	Transition temperature T_m (°C) (mean ± S.D., $n = 3$)	Calorimetric enthalpy ΔH (cal/mol) × 10^4 (mean ± S.D., $n = 3$)
Lysozyme (freshly prepared)	73.3 ± 0.5	9.6 ± 0.5
Control	73.1 ± 0.1	2.8 ± 0.6
1 (BB 100%)	73.3 ± 0.2	3.5 ± 0.4
2 (BB 95% + BA 5%)	73.3 ± 0.3	4.6 ± 0.2
3 (BB 90% + BA 10%)	73.5 ± 0.6	6.2 ± 0.6
4 (BB 85% + BA 15%)	73.6 ± 0.2	7.2 ± 0.7

Control: lysozyme (5%, w/v in releasing buffer) kept at 37 °C in oscillating water bath for 2 weeks, BB: benzyl benzoate, and BA: benzyl alcohol.

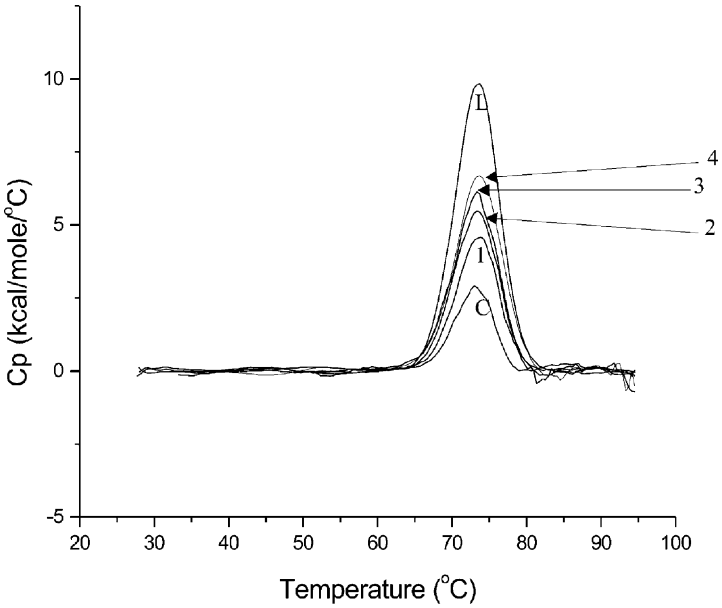

Fig. 2. DSC thermograms of 2 weeks released lysozyme from different phase-sensitive polymer gel formulations. Experiments were performed in triplicate. Keys: C: control (lysozyme in releasing buffer kept at 37 °C in oscillating water bath for 2 weeks), 1: formulation 1, 2: formulation 2, 3: formulation 3, 4: formulation 4, L: freshly prepared lysozyme.

lysozyme in the in vitro released samples are lower than freshly prepared solution, which may be due to prolonged contact of lysozyme with releasing buffer and various formulation components.

DSC is ideally suited to the study of protein thermal denaturation in solution since it measures directly the forces stabilizing the conformational structure. The characterization of protein unfolding, using several biophysical methods, has led to the notion that a loss in compact structure resulting in non-native conformational change has a dramatic effect on aggregation (Azuaga et al., 2002; Photchanachai et al., 2002), deamidation (Barone et al., 1997; Pikal and Rigsbee, 1997), and oxidation (Freeman et al., 1997; Komsa et al., 2000). Biophysical studies have provided information about the relationship between protein unfolding and degree of stability (Sebastien et al., 1999). T_m and ΔH have been attributed to a level of stability provided by additives under screening studies (Chang et al., 1993; Izutsu et al., 1990; Remmele et al., 1998). We found greater ΔH values for formulations containing greater proportions of BA, which might suggest higher conformational stability of the released lysozyme from such formulations. However, this needs further investigation and confirmation using other techniques such as FTIR spectroscopy, size-exclusion chromatography, gel electrophoresis, and circular dichroism (Perez et al., 2002; Van de Weert et al., 2000).

Table 3
Specific enzyme activity of lysozyme in the 2 weeks released sample from in situ formed gel depot polymeric formulations

Formulations	Specific enzyme activity (EU/mg) $\times 10^3$ (mean ± S.D., $n = 3$)
Lysozyme (freshly prepared)	45.0 ± 2.1
Control	13.0 ± 1.0
1 (BB 100%)	16.0 ± 0.1
2 (BB 95% + BA 5%)	21.0 ± 1.1
3 (BB 90% + BA 10%)	29.0 ± 1.3
4 (BB 85% + BA 15%)	44.0 ± 0.1

Control: lysozyme (5%, w/v in releasing buffer) kept at 37 °C in oscillating water bath for 2 weeks, BB: benzyl benzoate, and BA: benzyl alcohol.

Table 3 provides data on the specific enzyme activity of lysozyme in the released samples. We found significantly greater ($P < 0.05$) enzyme activity for lysozyme in all of the released samples in compari-

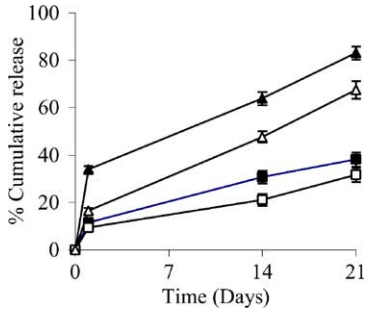

Fig. 3. Comparison of percentage cumulative release of lysozyme from polymer formulations with and without mannitol. Keys: (■) formulation 1 without mannitol, (□) formulation 1 with mannitol, (▲) formulation 4 without mannitol, (Δ) formulation 4 with mannitol.

son to the control sample. The specific enzyme activity data supported the conformational stability results as higher specific enzyme activity was observed for lysozyme from formulations containing greater proportions of BA.

Fig. 3 compares the release profile of lysozyme from formulations 1 to 4 with and without mannitol. We found significant decrease ($P < 0.05$) in burst release as well as rate of release of lysozyme from both the formulations containing mannitol in comparison to identical formulations without mannitol. Fig. 4 shows the DSC thermograms of released samples from formulations 1 to 4 with and without mannitol. We found significant ($P < 0.05$) increase in the area under the transition peak for both formulations containing mannitol in comparison to identical formulation without mannitol. All the formulations showed significant ($P < 0.05$) increase in the area under the transition peak as well as transition temperature in comparison to the control sample. Table 4 compares the conformational stability and specific enzyme activity of lysozyme in the released samples from formulations 1 to 4 with and without mannitol. We found significant increase ($P < 0.05$) in the conformational stability and specific enzyme activity of lysozyme from formulations containing mannitol than without mannitol.

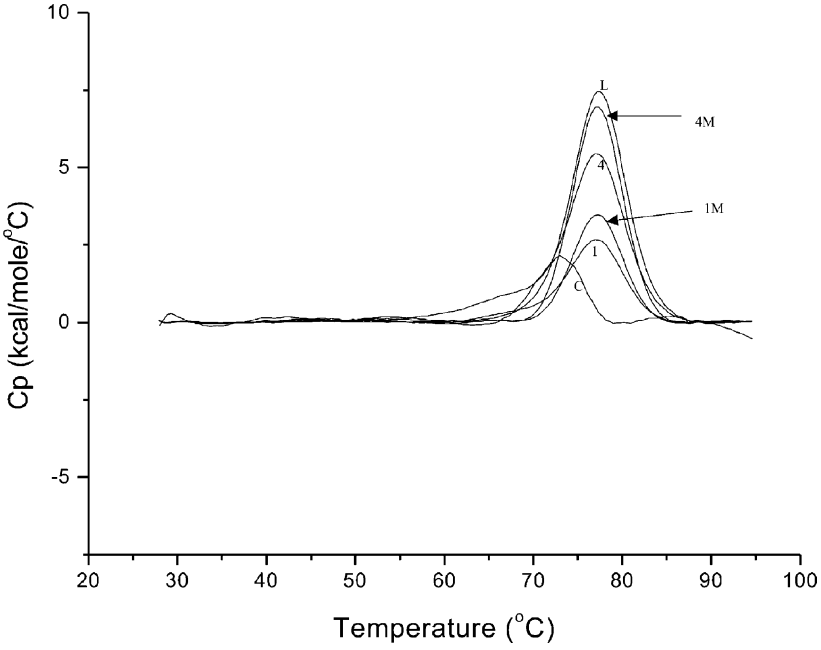

Fig. 4. DSC thermograms of 2 weeks released lysozyme from different phase-sensitive polymer gel formulations with or without mannitol. Keys: C: control (lysozyme in releasing buffer kept at 37 °C in oscillating water bath for the entire period of study), 1: formulation 1 without mannitol, 1M: formulation 1 with mannitol, 4: formulation 4 without mannitol, 4M: formulation 4 with mannitol, L: freshly prepared lysozyme.

Table 4
Comparison of conformational stability and specific enzyme activity of lysozyme from polymer formulations with and without mannitol

	Lysozyme[a]	Formulation 1		Formulation 4	
		Without mannitol	With mannitol	Without mannitol	With mannitol
T_m (°C)	67.2 ± 0.3	73.1 ± 0.1	76.9 ± 0.4	73.6 ± 0.1	77.3 ± 0.1
$\Delta H \times 10^4$ (cal/mol)	1.3 ± 0.1	2.8 ± 0.6	4.5 ± 0.1	6.2 ± 0.6	8.5 ± 0.2
SEA $\times 10^3$ (EU/mg)	11.0 ± 0.1	16.0 ± 0.4	20.0 ± 0.1	29.0 ± 0.1	34.0 ± 1.1

T_m: mid point transition temperature, ΔH: calorimetric enthalpy, SEA: specific enzyme activity. Data are presented as (mean ± S.D., $n = 3$).
[a] Lysozyme solution (5%, w/v) kept at 37 °C for 2 weeks.

Polyols are reported to stabilize proteins in aqueous solution against various destabilizing factors by a preferential hydration mechanism and/or hydrogen bonding (Lee and Timasheff, 1981; Allison et al., 1999). Therefore, the released lysozyme from formulations 1 to 4 prepared with mannitol exhibited better stability and biological activity. The lower burst and slower release rate are potentially due to the formation of hydrogen bonds between mannitol and polymer/lysozyme resulting in decrease in diameter of interconnected diffusional pathways inside the polymer gel via which the incorporated protein is diffusing (Bawa et al., 1985) and/or due to reduced solubility of lysozyme in the releasing media in the presence of polyol in the formulations.

The conformational stability and specific enzyme activity from all of the formulations were significantly ($P < 0.05$) greater than that from lysozyme solution (5%, w/v), which indicates stabilizing effect of formulation components. ΔH and T_m obtained for samples from all of the formulations were lower in comparison to that from freshly prepared lysozyme solution. However, this is expected to improve significantly with in vivo situation, as the released protein will be assimilated immediately. We used phosphate buffered saline (pH 7.4) as releasing media. However, pH at the interface of the polymer and buffer will be different from the bulk pH, which can affect the model protein.

In conclusion, phase sensitive smart polymer-based formulations may be used to deliver proteins in a conformationally stable and biologically active form at a controlled rate over extended period of time. However, further studies employing wide range of therapeutic proteins differing in their size and conformational structure, are warranted to make it a generally acceptable delivery method for proteins.

Acknowledgements

We acknowledge the financial support from NIH grant #HD 41372, and assistance of Babu Medi in the preparation of the manuscript.

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