Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 342 (2007) 72–77
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

# Influence of end groups on *in vitro* release and biological activity of lysozyme from a phase-sensitive smart polymer-based *in situ* gel forming controlled release drug delivery system

Sumit Chhabra, Vishal Sachdeva, Somnath Singh *

*Department of Pharmacy Sciences, School of Pharmacy and Health Professions, Creighton University Medical Center, Omaha, NE 68178, USA*

Received 12 January 2007; received in revised form 13 April 2007; accepted 30 April 2007
Available online 6 May 2007

**Abstract**

Phase-sensitive *in situ* gel forming controlled release formulations of lysozyme were prepared using poly lactic acid (PLA) and/or poly glycolic acid (PGA) based polymers differing in end groups in addition to composition, and a solvent system consisting of various ratios of benzyl benzoate (BB) and benzyl alcohol (BA). The amount of lysozyme in the released samples was determined by measuring absorbance at 280 nm using suitable controls to nullify the effect of absorption of formulation degradation products. Biological activity of lysozyme was studied by an enzyme activity assay using *Micrococcus lysodeikticus* as substrate. Polymers bearing carboxylic acid end group were not soluble in 100% BB but polymers having ester end groups were soluble up to 27% (w/v) except polymer 4. A biphasic release profile consisting of slower first phase followed by faster second phase was observed. Formulations prepared from polymer with carboxylic acid groups showed significantly ($p < 0.05$) lower burst release (4%) than those containing ester end groups (20–30%). However, formulations consisting of polymer with carboxylic acid end groups showed significantly ($p < 0.05$) faster release rate of incorporated lysozyme, although the total amount released was less in comparison to the total amount released from formulations prepared using polymers containing ester end groups. The mean percentage specific enzyme activity (MPSEA) data were supported by the release profiles. In conclusion, polymer end groups may influence the release profiles of a protein from an *in situ* gel depot forming controlled release formulations.


*Keywords:* Phase sensitive; Smart polymer; End groups; Lysozyme

## 1. Introduction

Proteins possess unique physical and chemical properties which create difficulties in formulation and delivery. With the tremendous growth of biotechnology and sequencing of the human genome, a large number of therapeutically active proteins are being developed (Lee, 2002). Proteins are susceptible to degradation by gastric enzymes and acidic environment, first pass metabolism, and pulmonary proteases activity; therefore, they are mainly administered by parenteral route. Since most of protein drugs are cleared rapidly from systemic circulation, they must be given frequently and in high dose which may cause toxicity and meet poor patient compliance (Robinson and Talmadge, 2002).

As a consequence, increasing attention has been focused on methods for administering these biologically active agents continuously at a sustained rate for an extended period of time. The primary method of accomplishing controlled release has been through incorporating the biologically active agents within polymers.

*In situ* gel forming, injectable phase-sensitive, smart polymer systems have been found as promising controlled drug delivery systems (Al-Tahami et al., 2006; Singh and Singh, 2004; Hatefi and Amsden, 2002). The injectable gel systems which utilize a polymeric matrix and a solvent/solvents, have been investigated which takes advantage of solvents differing in their relative hydrophilicity/hydrophobicity (Okumu et al., 2001). The hydrophilic solvents used by various researchers are benzyl alcohol, *N*-methyl pyrrolidone, and ethanol, and the hydrophobic solvents used are benzyl benzoate, miglyol, and triacetin (Cleland et al., 2001). The hydrophilic solvent leaves the gel quickly upon injection while the hydropho-

* Corresponding author. Tel.: +1 402 280 3548; fax: +1 402 280 3320.
*E-mail address:* ssingh@creighton.edu (S. Singh).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.04.034

bic solvent leaves the gel very slowly in comparison to a hydrophilic solvent. Consequently, a shell that is spongy and porous forms rapidly around the exterior of the gel with a hydrophilic solvent; while a shell also forms with the hydrophobic solvent, but it is smoother and has much smaller pores which slows water penetration to the gel thereby decreasing the rate of polymer degradation by hydrolysis and controlling the release of the incorporated protein (Chandrashekhar et al., 2001; Cleland et al., 2001; Duenas et al., 2001; Eliaz and Kost, 2000; Ravivarapu et al., 2000a,b). The polymers used must be biodegradable and biocompatible, e.g. polylactic acid, polyglycholic acid, poly lactic-co-glycholic acid, poly ε-caprolactone, etc.

The functional groups residing at the end of these polymers may influence the release profiles of the incorporated proteins. However, there is a little information available regarding the influences of these end groups on the various parameters controlling the release and stability of the incorporated/entrapped proteins. Polymer end groups influence surface properties of polymers which could be critical for rate of release of drugs from nanoparticles and microspheres (Jalbert et al., 1997). Polymer end groups could affect the sensitivity of chemical amplification resist systems based on acid catalysis in a way altogether different from the conventional resist systems (Ito et al., 1992). A patented surface modifying end groups (SMEs) technology can generate desired surface chemistry without even using surface modifying additives which sometimes have limitations in generating the desired surface properties (Ward and White, 1996).

End group modification in addition to, drug loading, polymer molecular weight, and copolymer composition, is reported to be a critical factor affecting the *in vitro*/*in vivo* release of a drug from nanoparticles. The intravenous administration of monomethoxy (polyethylene glycol)-poly (lactide-co-glycolide)-monomethoxy (polyethyleneglycol) [mPEG-PLGA-mPEG] nanoparticles of mitoxantrone in mice resulted in prolonged mitoxantrone residence in systemic blood circulation compared to the intravenous administration of PLGA nanoparticles without the presence of methoxy end groups (Duan et al., 2006). End group modification is also reported to influence the release of the drug from a multilayered PLGA stent matrix. The release of sirolimus from a bi-layer polymeric film used in stent was found to decrease when the drug-eluting layer was made from PLGA bearing a lauryl ester end group instead of acid group (Wang et al., 2006).

In an *in situ* forming PLGA based microparticle formulation of leuprolide, PLGA with free carboxylic acid end groups led to a lower drug release compared to PLGA with esterified end groups (Luan and Bodmeier, 2006). End group is also found to influence encapsulation efficiency of a drug in a microsphere formulation. In a PLA and PLGA based microspheres higher encapsulation of huperzine was found when the polymer was containing free carboxylic acid group at the end of polymer chain (Gao et al., 2006).

Therefore, in this study we have investigated the influence of carboxylic acid and ester groups at the end of PLA/PLGA based polymer on the rate of release and biological activity of a model protein lysozyme from *in situ* gel forming controlled release formulations.

## 2. Material and methods

### 2.1. Materials

All the polymers ranging in IV from 0.19–0.32 dl/g [(Resomer® R 202 S, 202 H, and 203 S, and Resomer® RG 502 S and 502 H] were purchased from Boehringer Ingelheim (Petersburg, VA, USA) and used as obtained without any purification. Lysozyme (L6876) and *Micrococcus lysodeikticus* ATCC# 4698 (M3770) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). BB and BA were purchased from Acros Organics, New Jersey, USA. All other chemical used were of analytical reagent quality.

### 2.2. Methods

#### 2.2.1. Preparation of injectable polymer solution

Different concentrations (5–27%, w/v) of each polymer (Polymers 1–5) were added to different solvent mixtures in a glass vial and kept in a shaking water bath (37 °C, 35 rpm) for 24 h. Solubility was determined by visual inspection. Injectability of the polymer solution was determined by passing through a 22-gauge needle. The solvent mixtures were prepared by mixing two solvents (BB and BA) in different ratios (100:0–60:40, respectively). Those polymer solutions were chosen for the study, which were easily injectable at 37 °C through a 22-gauge needle.

#### 2.2.2. Preparation of polymer–drug solution

Injectable polymer solutions were mixed with a fixed concentration (5%, w/v) of protein (lysozyme) and homogenized (5000 rpm, 1 min). Eight formulations differing in polymer composition, end groups, intrinsic viscosities, and solvent composition were prepared by following the steps shown in Fig. 1.

#### 2.2.3. In vitro release

Five hundred microliters of the formulation were injected into 10 ml of isotonic phosphate buffer (pH 7.4) kept in a glass vial. The formulation immediately changed into a gel depot. The vials containing *in situ* formed gel depot were kept in a reciprocal shaking water bath (Precision Scientific, Winchester, VA) at 37 °C and 35 rpm for the entire period of study. One milliliter of aliquot was withdrawn at specified time points which were used for determining quantity, biological activity, and conformational stability of lysozyme. The volume withdrawn was replaced with fresh releasing media.

#### 2.2.4. Quantification by UV–vis spectrophotometer

One milliliter of the suitably diluted sample/standard was used for measuring the absorbance in UV–vis spectrophotometer (UV 1700, Shimanzu, Kyoto, Japan) at 280 nm. Samples from formulation without lysozyme were used as blank control for absorbance. Amount of lysozyme released in the samples

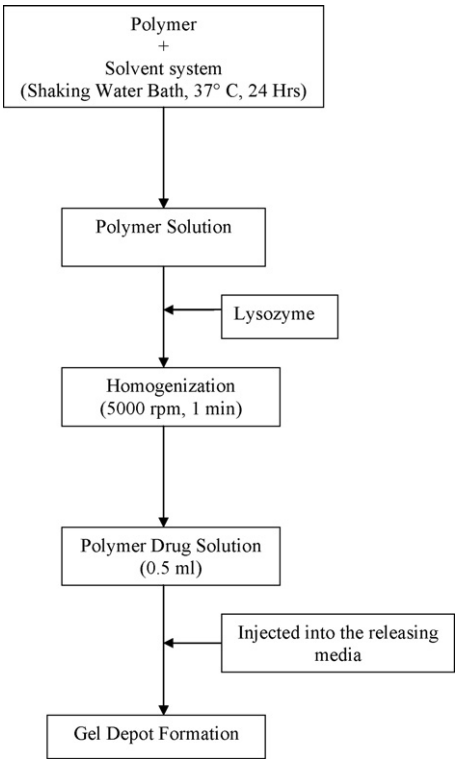


Fig. 1. Preparation of polymer–drug solution.

was obtained from the standard curve and corrected for sample removal (Hayton and Chen, 1982).

*2.2.5. Biological activity of lysozyme by enzyme activity assay*

Suitable amount of *Micrococcus lysodeikticus* cell suspension (0.015%, w/v) was prepared by using mixing *Micrococcus lysodeikticus* (Sigma Chemicals, St. Louis, MO) in potassium phosphate buffer (pH 6.24 at 25 °C). Two and a half milliliters of this cell suspension were taken into a spectrophotometeric cell, and hundred microliters of an appropriated diluted lysozyme sample (200–400 lysozyme units/ml) was added to it. The rate of decrease in absorbance at 450 nm was monitored by a UV spectrophotometer (UV 1700, Shimanzu, Kyoto, Japan) during the total period of 3 min. Number of biologically active lysozyme units were determined by using following formula (Shugar, 1952):

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

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.

*2.2.6. 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

### *3.1. Polymer solubility in various solvent systems*

Table 1 lists the characteristics and solubility of the polymers used in this study. Solubility of polymers was determined in a solvent system consisted of different ratios of BB and BA. A mixture of BB and BA provides an easy tool to manipulate to obtain a solvent system of optimal hydrophilicity or hydrophobicity for a particular polymer. Polymers 2 and 3 were found insoluble in BB which might be due to their relative hydrophilicity provided by the presence of acidic end groups because all other polymers were found in BB.

Polymers 1, 4 and 5 were relatively hydrophobic in nature due to the presence of ester end groups and therefore were soluble in BB. The maximum solubility of polymers 1 and 5 observed was 27% (w/v) but for polymer 4, it was 20% (w/v) in BB which might be due to polymer composition. Polymer 4 is consisted of PLA and GA (50:50) but polymers 1 and 5 are entirely consisted of PLA only (Table 1 and Fig. 2a and b). Consequently, polymer 4 could be expected to be less hydrophobic than polymer 1 or 5. Therefore, polymer 4 gets solubilized to a lesser extent (20%) in comparison to polymer 1 or 5 (27%) in BB, a more hydrophobic solvent than BA (Merck Index, 2001),

Polymer 2 demonstrated greater solubility in the solvent system containing mixture of BB and BA (70:30), while polymer 3 favored the two solvents in a ratio of 30:70 which too can be explained on the basis of polymer composition. Polymer 3 consists of PGA in addition to the PLA but polymer 2 is com-

Table 1
Characteristics of polymers used for preparing sustained release formulations

| Polymer | Composition | Intrinsic viscosity (dl/g) | Hydrophilicity[a] | Degradation[a] | End groups | Solvent system (BB:BA) | Concentration[b] (w/v%) |
|---|---|---|---|---|---|---|---|
| Polymer 1 | PLA* | 0.22 | Low | Slow | Ester | 100:00; 70:30 | 27 |
| Polymer 2 | PLA* | 0.20 | High | Fast | Acid | 70:30 | 27 |
| Polymer 3 | PLGA** | 0.19 | High | Fast | Acid | 30:70 | 20 |
| Polymer 4 | PLGA** | 0.19 | Low | Slow | Ester | 100:00; 70:30 | 20 |
| Polymer 5 | PLA* | 0.32 | Low | Slow | Ester | 100:00; 70:30 | 27 |

* PLA = Poly (DL-lactide).
** PLGA = Poly (DL-lactide-co-glycolide).
[a] Comparative values expected on the basis of polymer structures.
[b] Maximum concentration obtained at 37 °C.

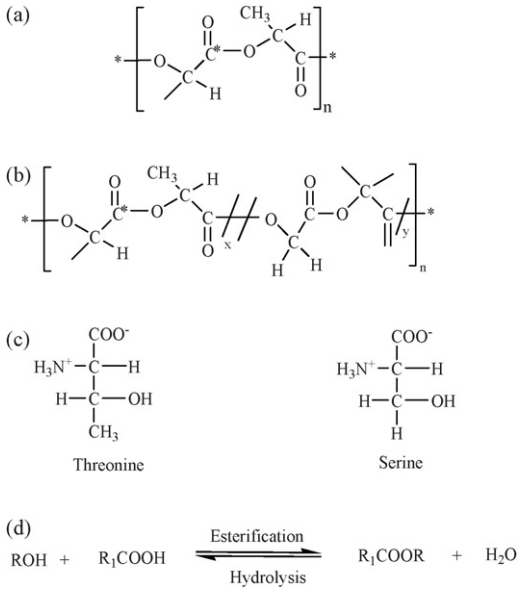


Fig. 2. Structure of polymers used, amino acid residues of lysozyme possessing OH group, and their interaction with polymer end groups. (a) Structure of polymer 1 (*$OCH_2CH_3$), 2 (*OH), and 5 (*$OCH_2CH_3$); (b) structure of polymer 3 (*OH) and 4 (*$OCH_2CH_3$) where * and *n* represent the end group and polymer chain length, respectively; (c) structure of threonine and serine amino acid residues having alcoholic hydroxyl groups; (d) possible interaction between hydroxyl groups of amino acid residues of lysozyme and free carboxylic groups of polymers.

posed of PLA only (Table 1 and Fig. 2a and b). Since, GA is relatively hydrophilic than PLA; the polymer 3 prefers a solvent system containing greater fraction of less hydrophobic solvent component BA to get dissolved.

### 3.2. *In vitro* release profile

Fig. 3 shows the percentage cumulative release of lysozyme from formulations 1–8 at different time points through 20 weeks. The general trend of release pattern is biphasic characterized by slower initial phase followed by faster second phase. The second phase appeared at different time points (ranging from 2 to 12 weeks) for different formulations, which could be explained on the basis of difference in polymer composition, polymer end groups and/or composition of solvent system.

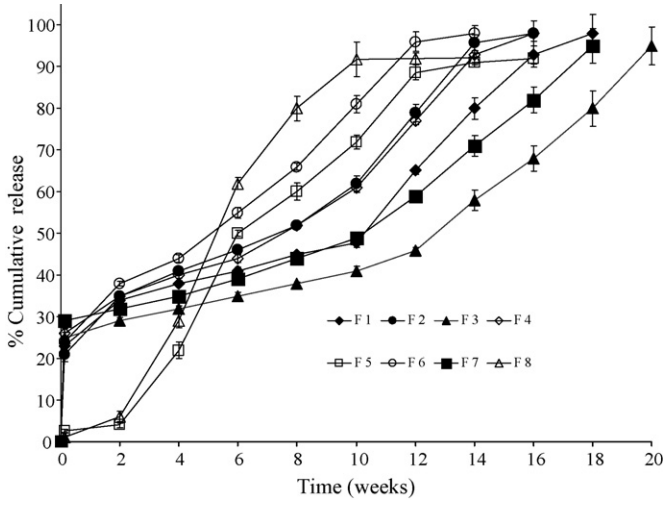


Fig. 3. *In vitro* percentage cummulative release of lysozyme from formulations 1–8 (F1–F8).

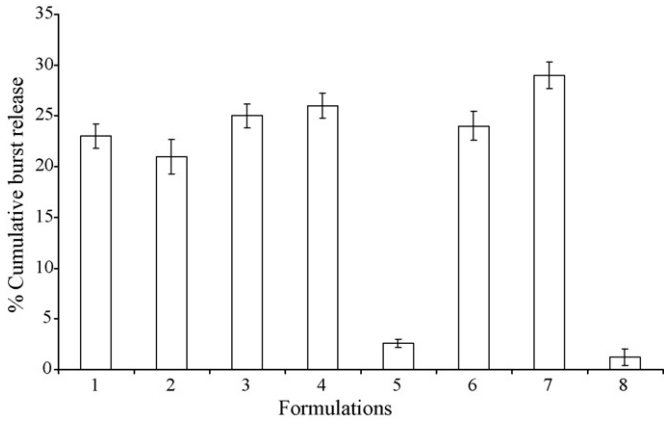


Fig. 4. *In vitro* percentage burst release of lysozyme from formulations 1–8 (F1–F8).

Formulations 5 and 8 were made with polymers 2 and 3, respectively, which had carboxylic acid as end groups, while other formulations made with polymers having ester as end groups (Table 1 and Fig. 2a and b). Carboxylic acid end groups might be thought to form some kind of chemical linkage (Fig. 2d) with hydroxyl group bearing amino acid residues (Fig. 2c) of lysozyme, thereby, making the first phase of the biphasic release pattern short and slow in comparison to those of rest of the formulations. Therefore, the burst release determined for formulations 5 and 8 were up to 4% while the rest of the formulations showed burst release in the range of 20–30% of the incorporated lysozyme (Fig. 4).

The burst release is reported to be controlled by the rate of gelation. The higher the rate of gelation, greater would be the burst release and more hydrophobic (or less hydrophilic) a formulation is, the sooner it would form the gel depot *in situ* (Brodbeck et al., 1999a,b; Chen and Singh, 2005a,b; Singh and Singh, 2004; Wang et al., 2003). Formulation 5 and 8 are relatively less hydrophobic than other formulation; therefore, they showed lower burst release which could be attributed to slower rate of gelation expected with these formulations. The release in first 24 h is treated as burst release.

After this initial burst release, the rest of the release is predominantly controlled by rate of degradation of polymer by hydrolysis. The more hydrophobic a formulation is, the greater it would resist the entry of water and therefore, the slower rate of degradation. All of the formulations were prepared from polymers of comparable molecular weight (IV 0.19–.22 dl/g) except polymer 5 which is of greater molecular (IV 0.32 dl/g) and has ester end group. Therefore, formulations 3 and 7 showed significantly ($p < 0.05$) greater control on the release of incorporated lysozyme than rest of the formulation because they were prepared using polymer 5. Formulations 5 and 8 showed significantly ($p < 0.05$) lower control over the release of incorporated

Table 2
The sustained release formulations of lysozyme

| Formulation | Polymer | Polymer concentration (w/v%) | Lysozyme concentration (w/v%) | Solvent %BB | System %BA |
|---|---|---|---|---|---|
| Formulation 1 | Polymer 1 | 15 | 5 | 100 | 0 |
| Formulation 2 | Polymer 4 | 15 | 5 | 100 | 0 |
| Formulation 3 | Polymer 5 | 15 | 5 | 100 | 0 |
| Formulation 4 | Polymer 1 | 15 | 5 | 70 | 30 |
| Formulation 5 | Polymer 2 | 15 | 5 | 70 | 30 |
| Formulation 6 | Polymer 4 | 15 | 5 | 70 | 30 |
| Formulation 7 | Polymer 5 | 15 | 5 | 70 | 30 |
| Formulation 8 | Polymer 3 | 15 | 5 | 30 | 70 |

lysozyme than other formulations because they were prepared from polymers 2 and 3 containing carboxylic acid end groups, respectively.

Moreover, lysozyme was never released from formulations 5 and 8 completely (maximum 92%) contrary to rest of the formulations where lysozyme got released up to 98% which might be due to some kind of chemical linkages between carboxylic acid groups (available in formulations 5 and 8) and hydroxyl groups of lysozyme (Fig. 2d). Some of these linkages might not have been hydrolyzed even after 10 or 12 weeks which is supported by no increase in lysozyme in the released sample obtained after 10 weeks from formulation 5 and 8. Possible interaction of a peptide (leuprolide) with esterified end group of a polymer (PLGA) were implicated in explaining the release profile of leuprolide from a microsphere formulation (Blanco and Alonso, 1998). In another study of chitosan based thermoset of indomethacin, its release at a controlled rate is reported to be due to the interaction of its aliphatic carbonyl group with $NH_2$ group of the chitosan backbone (Gong et al., 2006).

Although formulations 3 and 7 are prepared from same polymer 5, the rate of release of lysozyme from 7 was significantly ($p < 0.05$) greater than that from 3 which might be due to the difference in solvent system used in their preparation (Table 2). Formulation 7 used a mixture of BB and BA (70:30) whereas only BB was used for formulation 3. BA being more hydrophilic than BB, comes out sooner during the gelation process resulting in relatively increased porosity of the gel depot formed. Porosity facilitates access of water to the polymer back-bone which causes polymer degradation via the hydrolysis process (Graham et al., 1999; Eliaz and Kost, 2005).

### 3.3. Biological activity of the released lysozyme

Table 3 shows the MPSEA of lysozyme released from formulations 1–8. MPSEA was calculated for all formulations at the time of exhaustion when no increase in cumulative percentage release was found with time. The MPSEA values were found to be in the range of 30–52% which could be due to the differences in exhaustion time. A formulation exhausting the release of lysozyme sooner, would be in contact with the releasing media for lesser amount of time; therefore, lesser decrease in its enzyme activity in comparison to formulation exhausting in longer period of time. Therefore, formulation 8 showed significantly greater ($p < 0.05$) MPSEA than other formulations as it got exhausted sooner (around 10 weeks) than other formulations.

In 24-h samples lowest MPSEA was determined in samples from formulations 5 and 8 which might be due to the lower amount of lysozyme released by these formulations. Formulation 5 and 8 are containing carboxylic acid end groups which have resulted in lower burst release from them.

Furthermore, a higher MPSEA observed in samples from formulation 5 and 8 indicates that the putative chemical linkage did not have any permanent destructive effect on the enzyme activity of the lysozyme. This might be due to the regeneration of hydroxyl groups involved in the possible covalent bond formation with the carboxylic group via the mechanism of hydrolysis. In all the formulations, MPSEA was found to be greater in comparison to MPSEA observed in lysozyme solution kept under similar conditions (37 °C at 35 rpm) at all the time points except day 1. The decrease in MPSEA in formulation on day 1 might be due to influence of solvent on surrounding media on lysozyme. However, a better MPSEA in formulations at all other time points

Table 3
Percentage specific enzyme activity (%SEA)[a] (mean ± S.D; $n = 3$) of lysozyme released from various formulations

| Weeks | Lysozyme | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 100 ± 0.0 | ND | ND | ND | ND | ND | ND | ND | ND |
| 0.143 | 97 ± 1.5 | 94 ± 0.6 | 96 ± 0.6 | 95 ± 0.6 | 96 ± 0.9 | 85 ± 2.5 | 97 ± 0.4 | 96 ± 1.1 | 87 ± 2.7 |
| 4 | 64 ± 2.1 | 70 ± 0.8 | 85 ± 0.8 | 65 ± 1.2 | 76 ± 0.8 | 65 ± 0.7 | 87 ± 0.8 | 71 ± 0.9 | 67 ± 1.8 |
| 8 | 49 ± 1.8 | 59 ± 0.5 | 64 ± 1.1 | 52 ± 0.5 | 61 ± 1.4 | 55 ± 1.9 | 66 ± 0.6 | 56 ± 0.9 | 56 ± 1.5 |
| 12 | 26 ± 0.8 | 45 ± 1.8 | 51 ± 0.5 | 38 ± 1.7 | 48 ± 1.7 | 49 ± 1.8 | 50 ± 0.9 | 41 ± 0.6 | 52 ± 1.1 |
| 16 | 10 ± 0.7 | 32 ± 0.7 | 35 ± 2.1 | 35 ± 2.1 | 30 ± 2.1 | 35 ± 1.2 | ND | ND | ND |
| 20 | ND | ND | ND | ND | 22 ± 1.9 | ND | ND | ND | ND |

F, formulation; ND, not detected.
[a] $\text{SEA}(\%) = \frac{\text{SEA in sample}}{\text{SEA in freshly prepared lysozyme}} \times 100$.

support the protective effect of these formulations on the enzyme activity of lysozyme.

## 4. Conclusion

Polymer end groups influenced release profiles of lysozyme from phase-sensitive smart polymer formulations as indicated by lower burst release which should be substantiated by using various model proteins differing in number of amino acid residues bearing hydroxyl groups. The influence of end groups on the rate of gelation and the gel morphology would be included in future studies. The nature of interaction between polymer end groups and protein and its influence on their stability would provide us tool to reduce burst release of the incorporated protein.

## Acknowledgement

This study was financially supported by Health Future Foundation, Creighton University.

## References

Al-Tahami, K., Meyer, A., Singh, J., 2006. Poly lactic acid based injectable delivery systems for controlled release of a model protein, lysozyme. Pharm. Dev. Technol. 11, 79–86.

Brodbeck, K.J., DesNoyer, J.R., McHugh, A.J., 1999a. Phase inversion dynamics of PLGA solutions related to drug delivery. Part II. The role of solution thermodynamics and bath-side mass transfer. J. Control. Rel. 62, 333–344.

Blanco, D., Alonso, M.J., 1998. Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants. Eur. J. Pharm. Biopharm. 45, 285–294.

Brodbeck, K.J., Pushpala, S., McHugh, A.J., 1999b. Sustained release of human growth hormone from PLGA solution depots. Pharm. Res. 16, 1825–1829.

Chandrashekhar, B.L., Madril, D., Tow, K., Balliu, C., Bawa, R.S., 2001. Sustained release of leuprolide acetate from an in-situ forming biodegradable polymeric implant as the delivery vehicle. Proceed. Int. Symp. Control. Rel. Bioact. Mater. 28, 6406.

Chen, S., Singh, J., 2005a. Controlled delivery of testosterone from smart polymer solution based systems: in vitro evaluation. Int. J. Pharm. 295, 183–190.

Chen, S., Singh, J., 2005b. In vitro release of levonorgestrel from phase sensitive and thermosensitive smart polymer delivery systems. Pharm. Dev. Technol. 10, 319–325.

Cleland, J.L., Daugherty, A., Mrsny, R., 2001. Emerging protein delivery methods. Curr. Opin. Biotechnol. 12, 212–219.

Duan, Y., Sun, X., Gong, T., Wang, Q., Zhang, Z., 2006. Preparation of DHAQ-loaded mPEG-PLGA-mPEG nanoparticles and evaluation of drug release behaviors in vitro/in vivo. J. Mater. Sci. Mater. Med. 17, 509–516.

Duenas, E., Okumu, F., Daugherty, A., Cleland, J.L., 2001. Sustained delivery of rhVEGF from a novel injectable liquid, PLAD. Proceed. Int. Symp. Control. Rel. Bioact. Mater. 28, 6216.

Eliaz, R.E., Kost, J., 2000. Characterization of a polymeric PLGA-injectable implant delivery system for the controlled release of proteins. J. Biomed. Mater. Res. 50, 388–396.

Eliaz, R.E., Kost, J., 2005. Applications of injectable polymeric implants for protein and DNA delivery. Israel J. Chem. 45, 437–444.

Gao, P., Ding, P., Xu, H., Yuan, Z., Chen, D., Wei, J., Chen, D., 2006. In vitro and in vivo characterization of huperzine a loaded microspheres made from end-group uncapped poly(D,L-lactide acid) and poly(D,L-lactide-co-glycolide acid). Chem. Pharm. Bull. 54, 89–93.

Gong, K., Darr, J.A., Rehman, I.U., 2006. Supercritical fluid assisted impregnation of indomethacin into chitosan thermosets for controlled release applications. Int. J. Pharm. 315, 93–98.

Graham, P.D., Brodbeck, K.J., McHugh, A.J., 1999. Phase inversion dynamics of PLGA solutions related to drug delivery. J. Control. Rel. 58, 233–245.

Hatefi, A., Amsden, B., 2002. Biodegradable injectable in situ forming drug delivery systems. J. Control. Rel. 80, 9–28.

Hayton, W.L., Chen, T., 1982. Correction of perfusate concentration for sample removal. J. Pharm. Sci. 71, 820–821.

Ito, H., England, W.P., Lundmark, S.B., 1992. Effects of polymer end groups on chemical amplification. Proc. SPIE 1672, 2–14.

Jalbert, C., Koberstein, J.T., Hariharan, A., Kumar, S.K., 1997. End group effects on surface properties of polymers: semiempirical calculations and comparison to experimental surface tensions for α, ω-functional poly(dimethylsiloxanes). Macromolecules 30, 4481–4490.

Lee, H.J., 2002. Protein drug oral delivery: the recent progress. Arch. Pharm. Res. 25, 572–584.

Luan, X., Bodmeier, R., 2006. Influence of the poly(lactide-co-glycolide) type on the leuprolide release from in situ forming microparticle systems. J. Control. Rel. 10, 266–272.

Merck Index, 2001. 13th ed., Merck & Co., Inc., Whitehouse, NJ, p. 1132.

Okumu, F.W., Daugherty, A., Dao, L., Filder, P.J., Brooks, D., Sane, S., Cleland, J.L., 2001. Sustained delivery of growth hormone from a novel injectable liquid, PLAD. Proceed. Int. Symp. Control. Rel. Bioact. Mater. 28, 6210.

Ravivarapu, H.B., Moyer, K.L., Dunn, R.L., 2000a. Parameters affecting the efficacy of a sustained release polymeric implant of leuprolide. Int. J. Pharm. 194, 181–191.

Ravivarapu, H.B., Moyer, K.L. Dunn, R.L., 2000b. Sustained activity and release of leuprolide acetate from an in situ forming polymeric implant. AAPS Pharm. Sci. Techol. 1 (article-1).

Robinson, S.N., Talmadge, J.E., 2002. Sustained release of growth factors. In Vivo 16, 535–540.

Shugar, D., 1952. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. Biochim. Biophys. Acta 8, 302–309.

Singh, S., Singh, J., 2004. Controlled release of a model protein lysozyme from phase sensitive smart polymer systems. Int. J. Pharm. 271, 189–196.

Wang, L., Kleiner, L., Venkatraman, S., 2003. Structure formation in injectable poly (lactide-co-glycolide) depots. J. Control. Rel. 90, 345–354.

Wang, X., Venkatraman, S.S., Boey, F.Y., Loo, J.S., Tan, L.P., 2006. Controlled release of sirolimus from a multilayered PLGA stent matrix. Biomaterials 27, 5588–5595.

Ward, R.S., White, K.A., 1996. Surface-Modifying End Groups for Biomedical Polymers. US Patent 5,589,563.