PROOF COVER SHEET

Author(s): Hesham M. Tawfeek, Sayed H. Khidr, Eman M. Samy, Sayed M. Ahmed, Elsie E. Gaskell, and Gillian A. Hutcheon

Article title: Evaluation of biodegradable polyester-co-lactone microparticles for protein delivery

Article no: LDDI_A_814060

Enclosures: 1) Query sheet
2) Article proofs

Dear Author,

Please check these proofs carefully. It is the responsibility of the corresponding author to check against the original manuscript and approve or amend these proofs. A second proof is not normally provided. Informa Healthcare cannot be held responsible for uncorrected errors, even if introduced during the composition process. The journal reserves the right to charge for excessive author alterations, or for changes requested after the proofing stage has concluded.

The following queries have arisen during the editing of your manuscript and are marked in the margins of the proofs. Unless advised otherwise, submit all corrections using the CATS online correction form. Once you have added all your corrections, please ensure you press the "Submit All Corrections" button.

Please review the table of contributors below and confirm that the first and last names are structured correctly and that the authors are listed in the correct order of contribution.

Contrib. No.	Prefix	Given name(s)	Surname	Suffix
1		Hesham M.	Tawfeek	
2		Sayed H.	Khidr	
3		Eman M.	Samy	
4		Sayed M.	Ahmed	
5		Elsie E.	Gaskell	
6		Gillian A.	Hutcheon	

AUTHOR QUERIES

Q1: Please provide complete details for reference 57. Please note reference 56 is also missing

Q2: Please provide figure caption for "Figure 2".

Q3: Please provide better quality artwork for figure 3.

Drug Development and Industrial Pharmacy

http://informahealthcare.com/ddi
ISSN: 0363-9045 (print), 1520-5762 (electronic)

Drug Dev Ind Pharm, Early Online: 1–10
 DOI: 10.3109/03639045.2013.814060

informa healthcare

Evaluation of biodegradable polyester-co-lactone microparticles for protein delivery

Hesham M. Tawfeek[1,2], Sayed H. Khidr[2], Eman M. Samy[2], Sayed M. Ahmed[2], Elsie E. Gaskell[1], and Gillian A. Hutcheon[1]

[1]*School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, UK and* [2]*Department of Industrial Pharmacy, Faculty of Pharmacy, Assiut University, Assiut, Egypt*

Abstract

Poly(glycerol adipate-co-ω-pentadecalactone) (PGA-co-PDL) was previously evaluated for the colloidal delivery of α-chymotrypsin. In this article, the effect of varying polymer molecular weight (M_W) and chemistry on particle size and morphology; encapsulation efficiency; *in vitro* release; and the biological activity of α-chymotrypsin (α-CH) and lysozyme (LS) were investigated. Microparticles were prepared using emulsion solvent evaporation and evaluated by various methods. Altering the M_W or monomer ratio of PGA-co-PDL did not significantly affect the encapsulation efficiency and overall poly(1,3-propanediol adipate-co-ω-pentadecalactone) (PPA-co-PDL) demonstrated the highest encapsulation efficiency. *In vitro* release varied between polymers, and the burst release for α-CH-loaded microparticles was lower when a higher M_W PGA-co-PDL or more hydrophobic PPA-co-PDL was used. The results suggest that, although these co-polyesters could be useful for protein delivery, little difference observed between the different PGA-co-PDL polymers and PPA-co-PDL generally provided a higher encapsulation and slower release of enzyme than the other polymers tested.

Keywords

α-chymotrypsin, biodegradable polyesters, lysozyme, microparticles, PGA-co-PDL, protein delivery

History

Received 16 October 2012
Revised 18 April 2013
Accepted 4 June 2013
Published online ■ ■ ■

Introduction

Numerous protein and peptide pharmaceuticals such as recombinant human growth hormone, gaserelin acetate, leuprolide acetate and recombinant bovine somatropin have already received approval from regulating authorities worldwide[1]. However, there are many difficulties associated with delivering biopharmaceutical drugs. The oral route of administration of proteins results in substantial degradation and poor bioavailability[2], therefore, parenteral delivery is usually preferred. However, proteins often exhibit short half-lives in serum, thus requiring frequent administration to maintain their plasma level[3]. To prolong the therapeutic level of proteins, controlled release is required and this can be achieved using biodegradable polymers[4]. A range of formulation methods have been utilized to encapsulate proteins in polymeric micro- and nanoparticles, but water-in-oil-in-water (w/o/w) emulsion solvent evaporation is the most frequently used method. Difficulties in the encapsulation of proteins are related to their high molecular weight ($M_{W)}$, high water solubility and instability upon exposure to formulation conditions[5]. An initial burst release followed by slow, incomplete release of the native protein as a result of protein instability and aggregation has also been recognized as a major problem[6]. Interactions between the protein and the polymer also influence the release profile. These interactions are dependent on protein M_W; isoelectric point; amino acid composition; and hydrophobicity, as well as polymer M_W and chemistry[1]. Polymer properties such as M_W, copolymer composition and crystallinity can also be tailored to alter polymer degradation and subsequent drug release profiles[7,8]. For example, an increase in the M_W of Poly(lactic-co-glycolic acid) (PLGA) resulted in longer degradation times and slower release of bovine serum albumin and tetanus toxid[9,10]. Bovine serum albumin (BSA) and lysozyme (LS) were encapsulated using two different M_Ws of PLGA by (w/o/w) solvent extraction and oil-in-oil (o/o) solvent evaporation systems[11]. BSA was efficiently encapsulated independently of PLGA M_W, whereas the encapsulation of LS was favored with low M_W PLGA.

Although the choice of polymer is critical, few new polymers have been developed for specific drug delivery applications, and mono- and copolymers of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) are commonly adopted due to their widespread availability and approval for human use. One alternative is to develop new polymeric delivery systems to release the protein and retain bioactivity over the required target period[12].

A family of biodegradable polyesters with backbone functionality, synthesized via the enzyme catalyzed transesterification of a combination of activated diacids, glycerol and lactone monomers has been designed to overcome the lack of chemical functionality of the commonly used polyesters[13,14]. The free hydroxyl group from the glycerol monomer allows for the attachment of chemical moieties such as pharmaceutically active drugs, hence introducing the potential for the controlled incorporation and release of desired molecules (drugs, proteins and peptides). In addition, the physical characteristics (hydrophilicity and hydrophobicity) of these polymers can easily be manipulated by varying the

Address for correspondence: Dr Gillian A. Hutcheon, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK. Tel: 0151 2312130. E-mail: G.A.Hutcheon@ljmu.ac.uk

backbone chemistry[15]. Previously, poly(glycerol adipate) (PGA) and poly(glycerol adipate-co-ω-pentadecalactone) (PGA-co-PDL) have been investigated for the delivery of dexamethasone phosphate[16] and ibuprofen[17]. More recently, PGA-co-PDL has shown promise as a sustained release carrier for pulmonary delivery using the model drug, sodium flourescein[18]. PGA-co-PDL (1:1:1, M_W 30.0 KDa) has also previously been used to prepare α-chymotrypsin (α-CH)-loaded microparticles via the double (w/o/w) emulsion solvent evaporation method[19,20]. In the initial w/o emulsification step, a lipophilic surfactant is incorporated to aid the emulsification of the aqueous drug solution and the organic phase containing the polymer. Gaskell et al. found that on average 22.1 μg α-CH per 1 mg PGA-co-PDL was encapsulated, and there was a loss of enzyme bioactivity during encapsulation followed by a further gradual loss upon release[19]. The low amount of α-CH encapsulated is typical of these systems due to the diffusion of the protein from the inner to outer aqueous phases during particle formation and upon solvent evaporation. These different previous studies have all utilized a 1;1;1 ratio of monomers, and the M_W of the particular polymers used varied depending upon the M_W achieved during synthesis. Which, given the nature of these reactions, can be difficult to precisely control. It is therefore not known whether the copolymer composition or M_W may influence the characteristics of the particles formed.

Polymer properties such as molecular weight Mw, copolymer composition and crystallinity can be tailored to alter polymer degradation and the consequent drug release profiles as well as the microparticles characteristics. The nature of the protein encapsulated can also affect the particle formation, loading, release and bioactivity profiles[21].

Therefore this study is an extension of the work presented by Gaskell et al., examining the effect of small changes in polymer Mw and copolymer composition on the encapsulation efficiency, loading, particle size, morphology, *in vitro* release and bioactivity of two different proteins, α-CH (25 kDa) and LS (14 kDa). These enzymes differ in size (LS, 14 KDa, α-CH, 25 KDa), isoelectric point (LS, 11.2, α-CH, 9.1) and stability (LS is more stable than α-CH).

Materials and methods

Materials

Novozyme 435 (a lipase from *Candida antarctica* immobilized on a microporous acrylic resin) was purchased from Bio Catalytics (USA) and stored over P_2O_5 at 5°C prior to use. Glycerol, 1.3-propandiol, ω-pentadecalactone, α-chymotrypsin (type II from bovine pancreas), lysozyme (from chicken egg white), aerosol OT (dioctyl sodium sulphosuccinate), poly(vinyl alcohol) (PVA, M_W 9–10 KDa, 80% hydrolyzed), azocasein, 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside, citric acid, trichloroacetic acid (TCA) and sodium citrate were all obtained from Sigma-Aldrich Chemicals (UK). Dichloromethane and N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES) were purchased from BDH (UK). Tetrahydrofuran (THF) was purchased from Fisher Scientific. Phosphate buffered saline tablets at pH 7.4 were obtained from Oxoid (UK). Divinyl adipate (DVA) was obtained from Fluorochem (UK). A polystyrene standards kit was purchased from Supelco (USA).

Polymer synthesis

The copolymers PGA-co-PDL and PPA-co-PDL were synthesized, processed and characterized using methods adapted from Thompson et al.[22] and further described by Gaskell et al.[19] Polymer M_W was varied by controlling the reaction time. Reaction times of 6, 18 and 24 h were used to prepare PGA-co-PDL (1:1:1) with a M_W of 11.4, 26.0 and 39.2 KDa, respectively. The ratio of divinyl adipate (DVA) and glycerol (1:1) to ω-pentadecalactone was varied to produce polymers theoretically containing 1:1:0.5 and 1:1:1.5 of DVA, glycerol and ω-pentadecalactone, respectively. Using the same reaction conditions, PPA-co-PDL with a Mw of 22.0 KDa was synthesized from a 1:1:1 molar ratio of DVA: 1.3-propanediol: ω-pentadecalactone over 24 h.

The polymers were characterized by gel permeation chromatography, GPC (Viscotek TDA Model 300 ran by OmniSEC3 operating software precalibrated with polystyrene standards) and ^{1}H-NMR spectroscopy (Bruker AVANCE 300 operated via XWIN-NMR v3.5). ^{1}H-NMR (δ_H $CDCl_3$, 300 MHz) PGA-co-PDL (1:1:0.5): 1.34 (s, 11H, H-g), 1.65 (m, 8H, H-e, e', h), 2.32 (m, 6H, H-d, d', i), 4.05 (q)-4.18 (m) (6H, H-a, b, c, f), 5.2 (s, H, H-j), PGA-co-PDL (1:1:1): 1.34 (s, 22H, H-g), 1.65 (m, 8H, H-e, e', h), 2.32 (m, 6H, H-d, d', i), 4.05 (q)-4.18 (m) (6H, H-a, b, c, f), 5.2 (s, H, H-j) and PGA-co-PDL (1:1:1.45) 1.30 (s, 32H, H-g), 1.68 (m, 9H, H-e, e', h), 2.32 (m, 6H, H-d, d', i), 4.05 (q)-4.18 (m) (6H, H-a, b, c, f), 5.2 (s, H, H-j). Protons a to j are illustrated in Figure 1.

Particle preparation

The multiple emulsion-solvent evaporation (w/o/w) technique was employed for the encapsulation of α-CH and LS as reported previously[19]. Briefly, a 1% (v/v) solution of protein (100 mg mL^{-1}) in phosphate buffered saline (PBS) pH 7.4 was added dropwise to a homogenizing solution of polymer (30 mgmL^{-1}) and aerosol AOT (2 mM) in dichloromethane (15 ml) and emulsified using a IKA yellowline DI 25 basic at 8000 rpm for 30–40 s. This first emulsion was then gradually added to a mixing 1% (w/v) PVA solution (135 ml). This w/o/w emulsion was left to mix with a Silverson L4 RT mixer at 1000 rpm for 3 h to allow for dichloromethane evaporation at 25 °C. The particles obtained were collected by centrifugation (EBA 20, Hettich) at 6000 g for 6 min at room temperature. The supernatant was labeled ''wash 1'' and retained for further analysis. The microparticles were re-suspended in 120 ml PBS buffer to remove the residual PVA present on the surface of the particles and centrifuged as before. The collected supernatants were labeled ''wash 2''. The microparticles were then filtered, vacuum-dried overnight and stored in the fridge. Three batches of each type of particle were prepared.

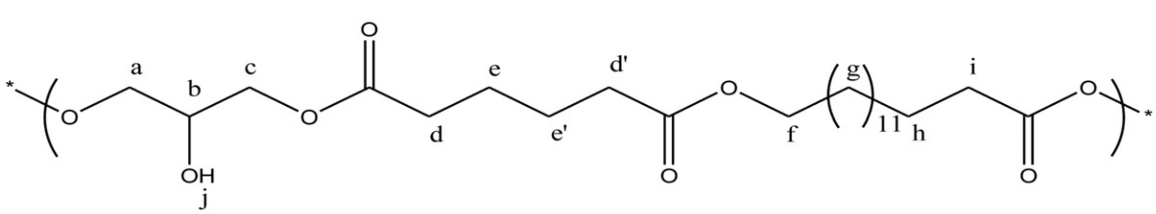


Figure 1. Chemical structure of PGA-co-PDL (1:1:1).

Particle characterization

The particles were visualized by scanning electron microscopy (FEI – Inspect S Low VAC Scanning Electron Microscope). A suspension of particles in water was deposited on 13 mm aluminum stubs layered with a sticky conductive carbon tab and air dried. An atomic layer of gold was deposited onto the particle containing stubs using an EmiTech K 550X Gold Sputter Coater, 25 mA for 3 min.

Particle size and size distribution were determined by a laser scattering device (Beckman Coulter LS 13 320, with aqueous liquid module) according to the method described by Pamujula et al.[23] The Frauenhofer method was used to calculate the size distribution of particles in water. The results obtained from measurements of at least three batches of microparticles were described by the volumetric mean diameter of the microparticles (VMD) in micrometers. Equation (1) gives the formula for the span of the volume distribution, which measures the width of the size distribution relative to the median diameter (d[v,50]). A more heterogeneous size distribution gives a large span value[24].

$$Span = \frac{d[v, 90] - d[v, 10]}{d[v, 50]} \tag{1}$$

Powder X-ray diffraction (PXRD) patterns were collected by using a Rigaku Miniflex X-ray diffractometer. Samples were finely ground and packed into an aluminum sample holder. Patterns were collected between 5° and 50° 2θ, at increments of 0.02° 2θ, scanning speed 2°min-1, voltage 30 KV, current 15 mA using CuKα (1.54 Å) radiation.

Drug loading and encapsulation efficiency

The theoretical encapsulation efficiencies and enzyme loading from three different batches of microparticles were calculated from the measurement of the non-encapsulated protein fraction present in the wash samples (Equation 2) and with the assumption that no protein was lost during the preparation and processing of the particles[19]. The enzyme loading (μg/mg) was determined using (Equation 3).

$$\text{Encapsulation Efficiency } (\%) = \frac{\text{Protection not washed out (mg)}}{\text{Amount of protetin initially added (mg)}} \times 100 \tag{2}$$

$$\text{EnzymeLoading} = \frac{\text{Total amount of encapsulated enzyme } (\mu g)}{\text{Total amount of polymer (mg)}} \tag{3}$$

In vitro *release of enzyme from microparticles*

Sacrificial sampling was used to observe the release of the enzyme from the particles. In a clean dry 1.5 ml microtube, 10 mg of vacuum-dried particles and 1 ml of phosphate buffer saline pH 7.4 at 37 °C were placed under sink conditions. The microtubes were then incubated at 37 °C in an orbital shaker (IKA KS 130) at 250 rpm. Samples were removed at increasing time points over 24 h and centrifuged (5 min at 13 500 rpm (17 000 *g*), accuSpin Micro 17) to collect the particles. The supernatants were retained for analysis by the protein assays described below.

The bioactivity of both enzymes was presented as the bioactive fraction of the released enzyme. This was calculated from the ratio of enzyme concentration determined from enzyme activity and the total enzyme concentration as determined by UV spectroscopy using the methods described below[25].

Methods for assessing protein content and activity

The encapsulation washes (wash 1 and 2) and supernatants from the release studies were analyzed for protein content[19] and activity using the following methods.

UV spectrophotometry

To determine the total protein content in a sample, the absorbance was measured at 282 nm for both α-CH and LS, (UV/VIS spectrophotometer Lambda 40, Perkin Elmer, run via the UV WinLab version 2.80.03 software).

Azocasein assay

The proteolytic activity of α-CH following release from particles was determined using a chromogenic-based technique as modified by Gaskell et al.[19] Briefly, 50 μL sample, standard or blank and 200 μL of azocasein (10 mg/ml), prepared in 25 mM HEPES buffer were incubated for 3 h at 37°C. The reaction was terminated by addition of 750 μL of 0.3 M trichloroacetic acid to precipitate the undigested protein–chromogenic conjugate and the samples were centrifuged for 5 min at 13 500 rpm (17 000 g) (accuSpin Micro 17) to remove the precipitate. Blank samples were prepared using deionized water to determine the amount of azo-dye released nonenzymatically from the substrate. Absorbance of the samples was recorded at 415 nm compared to blank reagent samples using UV/VIS spectrophotometer Lambda 40, Perkin Elmer, using the UV WinLab version 2.80.03 software. Three replicates of each sample were obtained and processed.

Muramidase assay

The muramidase activity of LS was determined using the method described by Telkov et al.[26] Supernatant (760 μL) was incubated with 8 μM 4-Methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside in 50 mM citrate buffer, pH 6.0, in the presence of 5 mM $MgSO_4$ for 3 h at 37 °C. The fluorescence intensity was measured using a fluorescence spectrophotometer (Varian Cary Eclipse, operated via the Cary Eclipse Advanced Reads Application version 1.1 (132) software) at an excitation wavelength of 350 nm and an emission wavelength of 450 nm.

Statistical analysis

Statistical analysis was performed using student t-paired test. The F-test was used to test the significance of variance. The statistical significance level was set at $p \leq 0.05$.

Results and discussion

The aim of this research was to investigate if changes to the M_W and chemistry of PGA-co-PDL would alter the encapsulation, release and bioactivity of α-CH and LS loaded into microparticles fabricated by a w/o/w double emulsion solvent evaporation technique.

Polymer synthesis and characterization

The lipase catalyzed ring opening polymerization of an equimolar quantity of DVA, glycerol and ω-pentadecalactone produced PGA-co-PDL (1:1:1) of different M_Ws (11.2, 26.0 and 39.2 KDa) by altering the time in contact with the lipase (6, 18 and 24 h, respectively) (Figure 1). A maximum M_W for this type of polymer is usually obtained around 24 h synthesis followed by a subsequent decrease in M_W as hydrolytic reactions dominate[27]. This means that the range and difference in M_Ws achievable is small and can be difficult to control. The incorporation of 1,3-propandiol in place of the glycerol produced PPA-co-PDL (1:1:1,

M_W 22.0 KDa) which is more hydrophobic than PGA-co-PDL as it does not have pendant hydroxyl groups.

A different set of polymers with a constant 1:1 ratio of DVA and glycerol, but with either 0.5 or 1.5 equivalents of ω-pentadecalactone, was also prepared (1:1:0.5, M_W 23.0 KDa and 1:1:1.45, M_W 34.0 KDa). These polymers should be more (1:1:1.45) and less (1:1:0.5) hydrophobic than PGA-co-PDL (1:1:1) depending on the relative number of hydroxyl groups. It is difficult to control the M_W of these polymers as an increase in the amount of ω-pentadecalactone increases the polymer M_W obtained. This means it can be difficult to directly compare the effect of monomer ratio on polymer and particle properties as there is also a difference in M_W. ^{1}H-NMR integration patterns were used to confirm that the monomeric content in the polymers were as expected and comparable to that reported in previous work[22]. The difference in the number of protons at δ1.34 is indicative of the different proportions of pentadecalactone within the polymer backbone (1:1:0.5 (11H), 1:1:1 (22H) and 1:1:1.45 (32H)).

Particle characterization

Protein-containing and blank particles containing no protein were prepared from each of the different polymers. The mean median of particle diameters (d_{50}) of three separate batches of α-CH- or LS-loaded microparticles and the span values are presented in Table 1.

The particle sizes obtained ranged between 9 and 18 µm. The particles prepared from PGA-co-PDL (1:1:0.5) were aggregated so no size data was obtained for this polymer. There was no significant difference observed between the sizes of most of the α-CH- or LS-loaded particles for the different polymers used except with PGA-co-PDL (1:1:1, 39.2 KDa) where significantly larger LS-loaded particles were obtained ($p < 0.05$). Previously, it was reported that the higher the M_W or concentration of polymer in the emulsion, the larger the diameter of the produced particles[28]. It was not anticipated that any great differences in particle size would be observed because the polymer M_W range studied was small, and the stirring speed, solution concentrations and the organic phase volume were fixed which are the main contributing factors affecting particle size[20]. Additionally, analysis of the span values (see Table 1) indicates that all microparticles produced had a large size distribution which made it difficult to draw any real trends from the data obtained.

The morphology of microparticles is very important as it influences particle degradation and hence can affect the protein release[29]. Moreover, particle morphology is dependent on the nature, composition and M_W of the polymer[30,31] as well as the particle formulation conditions[20].

The SEM images of the external structure of α-CH loaded PGA-co-PDL microparticles prepared from PGA-co-PDL (1:1:1) of different M_W are presented in Figure 2(A–C). Almost spherical microparticles with a slightly irregular shape and a rough ridged surface were observed. A high variability in microparticle size was noted during the SEM analysis which supports the span value data shown in Table 1. A similar morphology was also observed with LS-loaded microparticles fabricated from the same polymers (Figure 2G–I). Hence, changing either the polymer M_W or the type of protein encapsulated did not alter the particle morphology.

Altering the chemistry did, however, have an effect on particle morphology. PGA-co-PDL (1:1:0.5) produced small, aggregated, non-uniform particles (Figure 2D), and increasing the lactone content within the polymer changed the particle morphology slightly. With both α-CH- and LS-loaded PGA-co-PDL (1:1:1.45) particles, some of the particles appeared irregular in shape with rough surfaces, while the others were spherical with a slightly smoother surface than those prepared from PGA-co-PDL (1:1:1) (Figure 2E and J). These smooth particles were more similar to those obtained from PPA-co-PDL (Figure 2F and K). A similar morphology to α-CH-loaded microparticles was observed with the LS-loaded microparticles (Figure 2H–K). Thompson et al. reported similar morphological characteristics for particles prepared from PGA-co-PDL and PPA-co-PDL[22]. Drug-free and ibuprofen-loaded microspheres[17] produced using PGA-co-PDL were rough with a ridged morphology, whereas the equivalent PPA-co-PDL microspheres were smooth.

Drug loading and encapsulation efficiency

Polymer M_W, degree of hydrophilicity, polymer chemistry, volume of organic phase and enzyme and polymer concentration play an important role in determining the amount of enzyme encapsulated. It was reported that increasing the M_W of poly (ε-caprolactone), PLA and PLGA increased the encapsulation efficiency and the mean particle size due to the increased viscosity of the organic phase, which reduces protein diffusion into the external aqueous phase before polymer hardening[8,32]. Partitioning of the drug from the internal to the external aqueous phase limits the encapsulation efficiency and drug loading in particles prepared via the emulsion solvent evaporation technique. During particle formation, solvent removal and polymer precipitation can alter the amount of the protein that partitions into the external aqueous phase[33]. It was previously determined that 3 h was the optimum time for PGA-co-PDL protein-containing particle formation as this provided enough time for the solvent to evaporate yet minimized enzyme diffusion to the aqueous phase[19].

The encapsulation efficiencies and enzyme loading from three different batches of microparticles prepared using different polymers are presented in Table 2.

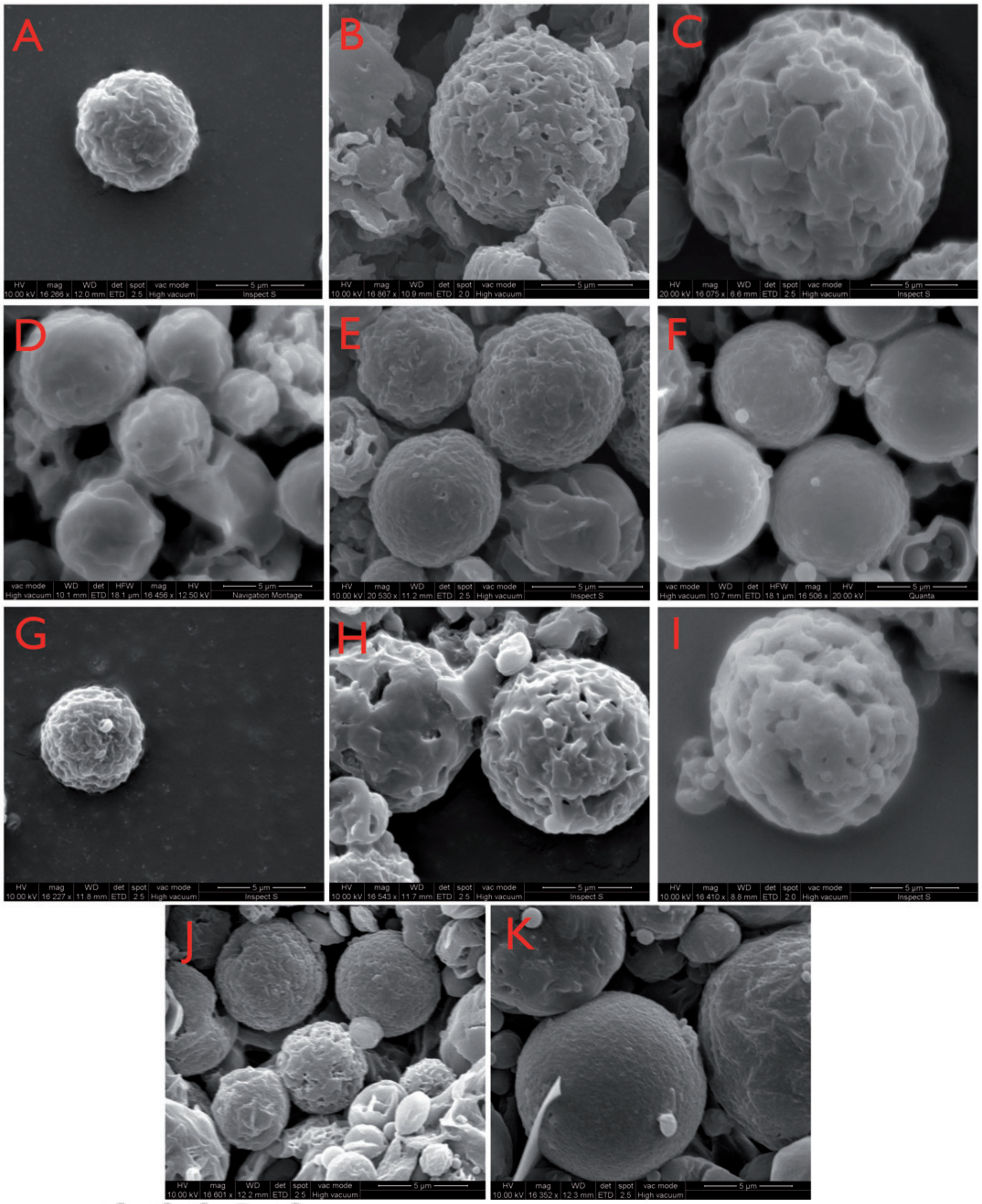
Increasing the M_W of PGA-co-PDL had no significant effect on either the encapsulation efficiency or α-CH loading ($p > 0.05$). However, a shift in PGA-co-PDL M_W from 11.4 to 39.2 KDa might not be large enough to induce a significant increase in the viscosity of the organic phase, leading to a change in enzyme loading. The degree of crystallinity of the polymer is another important factor affecting drug encapsulation as drugs will tend to be encapsulated in the amorphous region of the polymer[34].

Table 1. The mean median of particle size and the span values for α-CH- and LS-loaded microparticles prepared via the w/o/w double emulsion solvent evaporation technique. The results are the mean of three different prepared batches ± S.D.

	Mean median of particle size (µm)		Span values	
Polymer type	CH	LS	CH	LS
PGA-co-PDL (1:1:1, M_W 11.4 KDa)	13.6 ± 1.4	9.3 ± 1.4	2.2 ± 0.2	2.1 ± 0.2
PGA-co-PDL (1:1:1, M_W 26.0 KDa)	14.4 ± 2.9	12.2 ± 0.9	1.9 ± 0.2	2.3 ± 0.3
PGA-co-PDL (1:1:1, M_W 39.2 KDa)	13.8 ± 2.9	17.5 ± 0.6	2.8 ± 1.2	3.3 ± 0.6
PGA-co-PDL (1:1:1.45, M_W 34.0 KDa)	9.6 ± 0.81	15.1 ± 1.0	1.6 ± 0.3	2.1 ± 0.2
PPA-co-PDL (1:1:1, M_W 22.0 KDa)	10.0 ± 1.2	14.4 ± 1.5	2.2 ± 0.5	2.6 ± 0.4

DOI: 10.3109/03639045.2013.814060

COLOR Online / B&W in Print



Q2

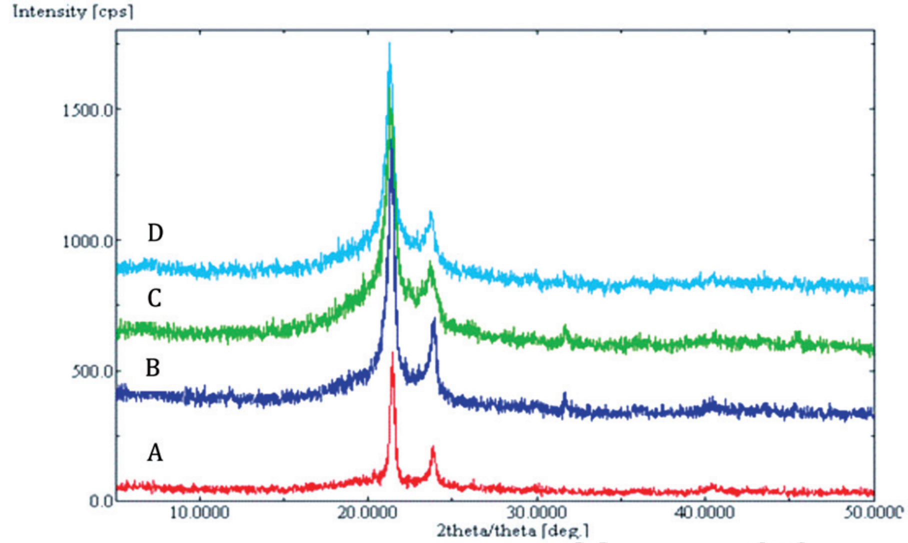
Figure 2. ■■■.

Table 2. Encapsulation efficiencies (%) and enzyme loading (μg/mg particle) of α-Chymotrypsin (α-CH) and Lysozyme (LS) within polymeric particles formulated over 3 h via the multiple emulsion solvent evaporation technique. The amount of α-CH or LS added into the aqueous phase was 150 mg. The results are the mean of three different prepared batches ± S.D.

	CH		LS	
Polymer type	EE (%)	Loading (μg/mg particle)	EE (%)	Loading (μg/mg particle)
PGA-co-PDL (1:1:1, M_W 11.4 KDa)	14.83 ± 1.5	49.39 ± 4.9	32.05 ± 3.3	108.65 ± 11.5
PGA-co-PDL (1:1:1, M_W 26.0 KDa)	12.52 ± 4.4	41.70 ± 0.01	32.62 ± 0.5	107.50 ± 2.1
PGA-co-PDL (1:1:1, M_W 39.2 KDa)	19.40 ± 2.5	64.60 ± 8.4	30.11 ± 4.0	103.60 ± 10.8
PGA-co-PDL (1:1:0.5, M_W 23.0 KDa)	20.41 ± 2.5	68.03 ± 6.4	25.84 ± 2.6	86.17 ± 10.6
PGA-co-PDL (1:1:1.45, M_W 34.0 KDa)	23.94 ± 3.1	79.80 ± 7.6	**33.93 ± 1.1	**113.10 ± 5.8
PPA-co-PDL (1:1:1, M_W 22.0 KDa)	*38.58 ± 6.4	*128.50 ± 12.7	36.40 ± 2.84	121.33 ± 11.6

**Significant difference PGA-co-PDL (1:1:1.45, Mw 34.0 KDa) versus PGA-co-PDL (1:1:0.5, Mw 23.0 KDa), *significant difference PPA-co-PDL (22.0 KDa) versus PGA-co-PDL (26.0 KDa) at $p < 0.05$.

Figure 3. X-ray diffraction pattern of α-Chymotrypsin-loaded particles formulated from A, PPA-co-PDL (22.0 KDa); B, PGA-co-PDL (11.4 KDa); C, PGA-co-PDL (26.0 KDa); and D, PGA-co-PDL (1:1:1.5, Mw 34.0 KDa).



The PXRD patterns illustrated in Figure 3 indicate that both PGA-co-PDL and PPA-co-PDL are semicrystalline copolymers. Both PGA-co-PDL and PPA-co-PDL showed characteristic peaks at 21.5° and 24° 2θ. PGA-co-PDL of different M_{Ws} have the same XRD patterns, indicating they have the same level of crystallinity, and this may explain the similar encapsulation efficiencies observed. However, the PXRD pattern for PPA-co-PDL has a flatter baseline between 0° and 20° 2θ, indicating that it is a more crystalline material. This difference in degree of crystallinity between PGA-co-PDL and PPA-co-PDL may have influenced microparticle formation but does not explain the increased encapsulation efficiency observed with PPA-co-PDL.

Furthermore, changing the polymer composition by altering the pentadecalactone monomeric ratio from 0.5 to 1.5 molar ratio significantly ($p < 0.05$) increased the encapsulation efficiency of LS-loaded microparticles. An increase was also observed with α-CH-loaded particles, but this was not significant ($p > 0.05$). Compared to PGA-co-PDL, utilizing the more hydrophobic polymer (PPA-co-PDL) a significant ($p < 0.05$) increase in encapsulation efficiency and α-CH loading (from 12.52 ± 4.42 to 38.58 ± 6.48% and 41.70 ± 0.01 to 128.50 ± 12.70, respectively) was observed. The highest α-CH and LS encapsulation efficiency and loading were obtained from the most hydrophobic polymer, PPA-co-PDL. These results suggest that the more hydrophobic polymers demonstrate better encapsulation efficiency and drug loading of both enzymes compared to the less hydrophobic variants.

Similarly, McGee et al. showed that ovalbumin-loaded microparticles prepared with PLGA with higher lactide to glycolide content (85:15) gave higher protein loading compared to the more hydrophilic one with 50:50 lactide to glycolide ratio[35]. Also, higher amounts of bovine albumin were encapsulated using PLGA (75:25) compared to the more hydrophilic PEGylated PLGA co-polymer[36].

Comparing the encapsulation efficiencies and enzyme loading for both enzymes, it was found that LS showed a higher encapsulation and loading compared to α-CH with all the PGA-co-PDL variants assessed (Table 2). LS is a smaller, positively charged enzyme that has the ability to be adsorbed onto the surface of polymers and this adsorption will affect its encapsulation and release kinetics[37]. Furthermore, as previously reported[37,38], the temperature rises during the emulsification steps and the adjustment of the pH to 7.4 can lead to favorable conditions for LS adsorbing onto polymers. This could result in increased amounts of LS being encapsulated within PGA-co-PDL. Also, we cannot neglect that using 1% PVA as an emulsifier imparts a negative charge to the surface of PGA-co-PDL and PPA-co-PDL which would support enzyme binding. It was reported that PVA, which is physically entrapped within the surface layer of the polymer, imparts a negative surface charge on the microparticles produced[39,40]. However, comparable amounts of 128.5 ± 12.7 and 121.33 ± 11.6 µg/mg particle of α-CH and LS were encapsulated, using PPA-co-PDL. This represents a significant increase over PGA-co-PDL for α-CH- but not LS-loaded particles.

In vitro release

It was anticipated that polymer M_W and polymer backbone chemistry would be important factors affecting the drug release[21]. Varying the M_W, varies the degradation rate of the polymer and release kinetics of the drug can be controlled accordingly[41]. Additionally, the hydrophobicity of the polymer can affect the drug release by reducing the rate of water penetration into the microspheres and drug egress to some extent compared to the less hydrophobic polymers[42]. Furthermore, different particle morphologies may affect the protein release profile through its effect on the microspheres porosity and the distribution of the drug within the matrix[29,43].

The release profiles of either α-CH or LS under sink conditions from different batches are shown in Figures 4 and 5. Figure 4 shows the release of α-CH from microparticles prepared using different polymers over 24 h into PBS buffered saline. Most of the α-CH-loaded microparticle formulations showed a biphasic release pattern with an initial high burst release phase followed by a continuous release phase for the first 5 h which became constant till the end of the release study. The extent of the burst release varied between different microparticle formulations, depending on the polymer used, and a notable difference was observed between PGA-co-PDL (1:1:1 26.0 KDa or 11 KDa) and the other polymers.

Other research groups[11,33] have observed that increasing polymer M_W led to a decrease in the total amount of enzyme released. In this study, there was no general trend observed between increasing M_W and decreasing enzyme release, which may be because the differences in M_W were small, but there was significantly less release after 24 h with PGA-co-PDL (39 KDa) particles compared to PGA-co-PDL (26 KDa or 11 KDa) particles. Varying the proportion of PDL within the polymer from 0.5 to 1.5 mole equivalents did not have any consistent effect on the

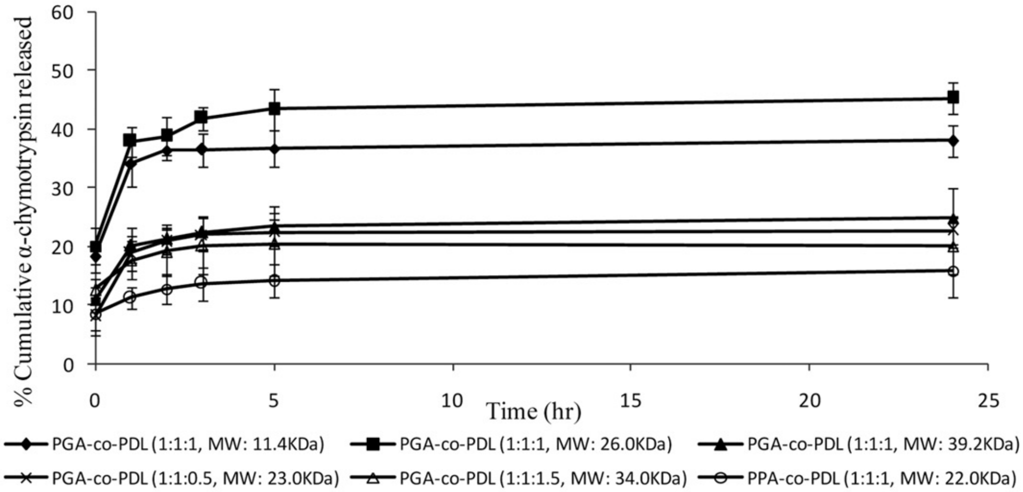


Figure 4. Release profiles of α-Chymotrypsin from polymeric microparticles prepared via the multiple emulsion solvent evaporation technique. The results are the mean of three different prepared batches at each time point ± S.D.

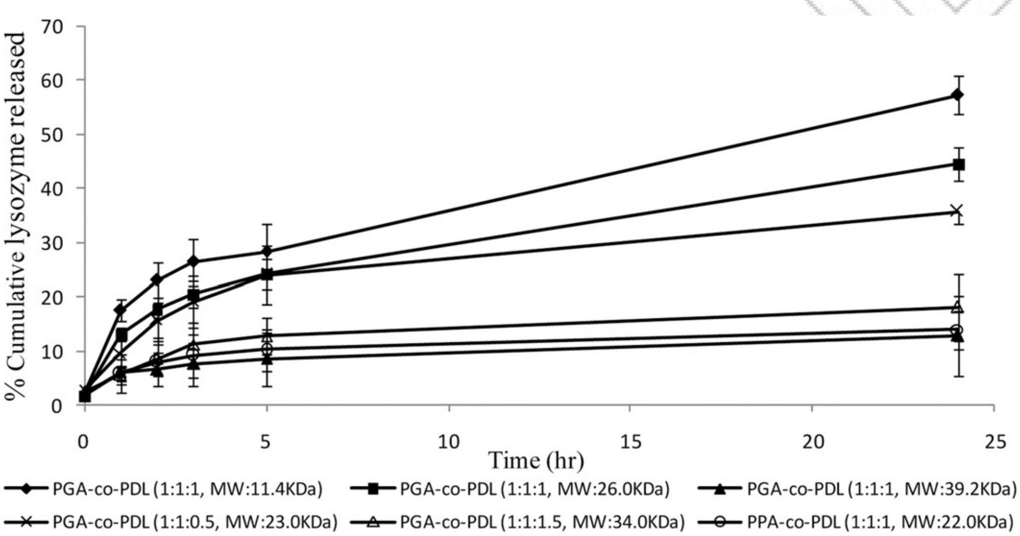


Figure 5. Release profiles of lysozyme from polymeric microparticles prepared via the multiple emulsion solvent evaporation technique. The results are the mean of three different prepared batches at each time point ± S.D.

α-CH release from microparticles. The biggest difference in release was found when comparing PGA-co-PDL (26 KDa) with the more hydrophobic polymer of a comparable M_W, PPA-co-PDL (22 KDa). Compared to PPA-co-PDL, PGA-co-PDL showed a significantly ($p < 0.05$) higher burst release of α-CH (20.13 ± 3.0% compared to 8.54 ± 2.7%) and a greater amount of release after 24 h in PBS buffer (45.28 ± 2.7% compared to 15.84 ± 4.5%). Furthermore, PPA-co-PDL demonstrated the lowest burst and total release of α-CH of all the prepared microparticles.

The initial burst release phase of α-CH from these microparticles could be due to the rapid release of protein near to the surface of microparticles which accumulates at the water/oil interface during the solvent evaporation process. The release of the protein entrapped within the polymeric matrix causes a continuous release of α-CH during the first 5 h. Furthermore, the constant release phase could be attributed to the protein aggregation and degradation that occurs during the release process. Despite the higher encapsulation efficiency gained from PPA-co-PDL, these particles demonstrated a slower burst and continuous release rate compared with PGA-co-PDL with comparable Mw. This might be due to the higher hydrophobicity and slower rate of degradation of this polymer (unpublished data). The lower surface area available for contact with the dissolution medium and the large particle size could be other contributing factors toward this slow release as denser microparticles with smooth surfaces will usually produce a lower rate of initial release compared with rough, porous microparticles. This is in agreement with Thompson et al. who observed a similar effect for ibuprofen release from PGA-co-PDL and PPA-co-PDL microparticles[17].

The release profiles of LS from the different polymeric microparticles are shown in Figure 5. In this case, the LS-loaded microparticle formulations showed a very small initial burst phase followed by continuous release until the end of the release study at 24 h. With LS there was a general trend of increasing PGA-co-PDL (1:1:1) M_W and decreasing enzyme release. The release of LS from the 39 KDa polymer was significantly lower, and there was less difference observed between the 26 KDa and 11 KDa variants. Although, as with α-CH, there was a difference in the release of LS from PPA-co-PDL (22 KDa) and PGA-co-PDL (26 KDa) of a comparable $M_{W,}$ with LS the release profile of the PPA-co-PDL particles was virtually the same as that of PGA-co-PDL (39 KDa).

It was observed that the pattern of LS release was different from that obtained with α-CH. α-CH release was characterized by an initial burst followed by a slow continuous release phase for the first 5 h then a plateau was reached. On the other hand, LS showed a lower burst release followed by a higher continuous release phase. This was especially evident with the lower M_W PGA-co-PDL. The lower burst release could be attributed to the more efficient encapsulation of LS inside the microparticles with minimum amounts remaining adsorbed on the surface. Stronger binding of LS to these polymers could be another reason for this as LS is cationic and these particles have a slightly anionic surface from incomplete removal of PVA.

With all the microparticles studied, an incomplete release of enzyme from these was observed even after 3 weeks. This has been observed by many researchers, and it might be due to degradation of the protein during the manufacturing of the microparticles[44]. Formation of intermolecular linkages, hydrolysis of the protein molecule and the nonspecific adsorption between polymer and protein either physically or chemically can lead to protein degradation[45].

Enzyme bioactivity

Retaining biological activity is crucial for the delivery of enzymes and peptides, and preservation of the tertiary structure is required to maintain activity. Enzyme activity before and after encapsulation and upon release can be monitored to investigate the effect of these processes on biological activity. Many researchers have estimated the bioactivity of LS by measuring the rate of degradation of *Micrococcus luteus* cells[25,46]. However, this method is not always reproducible because of the dependence on the ionic strength of the medium[47,48]. Different methods using small synthetic substrates have been developed, investigated and recommended for accurate determination of LS[49–51].

Observation of the bioactive fraction of α-CH released from microparticles prepared using PGA-co-PDL and PPA-co-PDL (Figure 6) indicates that the maximum bioactivity was observed at zero hours and ranged between 27% and 60%. This was followed by a sharp decrease in activity during release into PBS buffer (pH 7.4). It was noticed that α-CH released from PGA-co-PDL exhibited a maximum activity of between 40% and 60%, and PPA-co-PDL showed the lowest activity of ~27% at zero hour. Furthermore, a gradual loss in bioactivity was recorded for all the α-CH-loaded microparticles investigated. The reduction in activity of α-CH could be attributed to conformational changes in the α-CH active site during emulsification. The homogenization and use of organic solvents are considered important steps in causing protein deactivation and aggregation resulting in a low bioactive fraction at zero hour[52–54]. The gradual loss in activity during *in vitro* release was most likely due to autolysis and protein fragmentation[53]. This finding is similar to what was already reported by Gaskell et al. where they found that α-CH released from PGA-co-PDL-loaded microparticles lost its bioactivity gradually with an onset of loss due to proteolysis upon 2 h release[19].

At zero hour of release, LS retained almost 100% of its initial bioactivity within all the particles investigated. Then, with time it began to gradually lose its bioactivity (Figure 7). The higher M_W polymer, PGA-co-PDL (1:1:1, 39.0 KDa), and the more hydrophobic polymers, PGA-co-PDL (1:1:1.45) and PPA-co-PDL, showed a significantly ($p < 0.05$) higher bioactive fraction, after 5 and 24 h release, compared to the other co-polymers. The maximum LS bioactive fraction was found using PGA-co-PDL (1:1:1.45, M_W 34 KDa) and PGA-co-PDL (1:1:1, M_W 39.2 KDa) 0.78 ± 0.08 and 0.42 ± 0.02, respectively, after incubation in PBS for 24 h.

LS is a relatively stable enzyme[55] which can better withstand the harsh condition of the emulsification process and this was confirmed by the retention of its bioactivity at zero time of release (bioactive fraction ranged from 0.9 to 1.03 for all the investigated polymers, Figure 7). Similarly, it was reported by Giteau and coworkers that the LS released from PLGA microspheres was still biologically active compared to α-CH, peroxidase and β-galactosidase-loaded PLGA microspheres[57]. However, during *in vitro* release there was a gradual decrease in the bioactive fraction which could be attributed to the effect of PBS buffer on the released LS. So, the nature of the release medium on the enzyme activity is very important, as many proteins are not stable in buffer media at 37 °C. However, for most studies the choice of release medium is dictated by the *in vivo* target for delivery of the enzyme. Jiang et al. investigated protein stability and protein–polymer interactions in different release media and their effect on protein release profiles from PLGA microspheres using LS as a model protein[37]. They found that LS showed a higher stability at pH 4.0 acetate buffer and pH 2.5 glycine buffer, whereas at pH 7.4 PBS, the stability was low and significant protein adsorption was evident. Furthermore, the higher bioactive fraction of LS in PGA-co-PDL (1:1:1, Mw, 39.2 KDa) and PGA-co-PDL (1:1:1.45) could

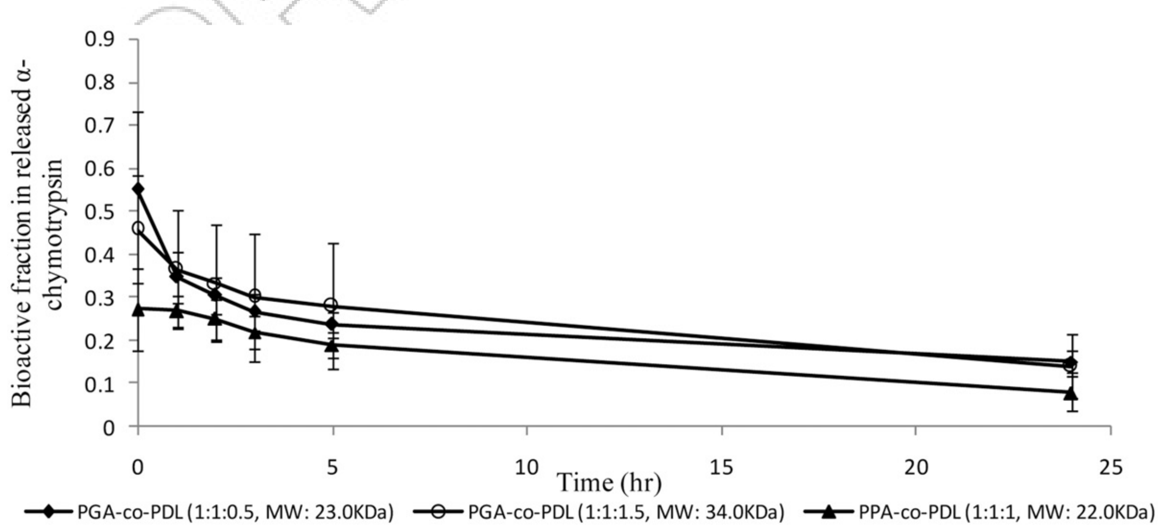


Figure 6. Bioactive fraction of released α-Chymotrypsin from (A) PGA-co-PDL (1:1:1, MW 11.4, 26.0 and 39.2 KDa) and (B) PGA-co-PDL (1:1:0.5, MW 23.0 KDa, 1:1:1.5, MW 34.0 KDa) and PPA-co-PDL (1:1:1, MW 22.0 KDa) in PBS buffer, pH 7.4. Triplicate samples were used from two different prepared batches at each time point ± S.D.

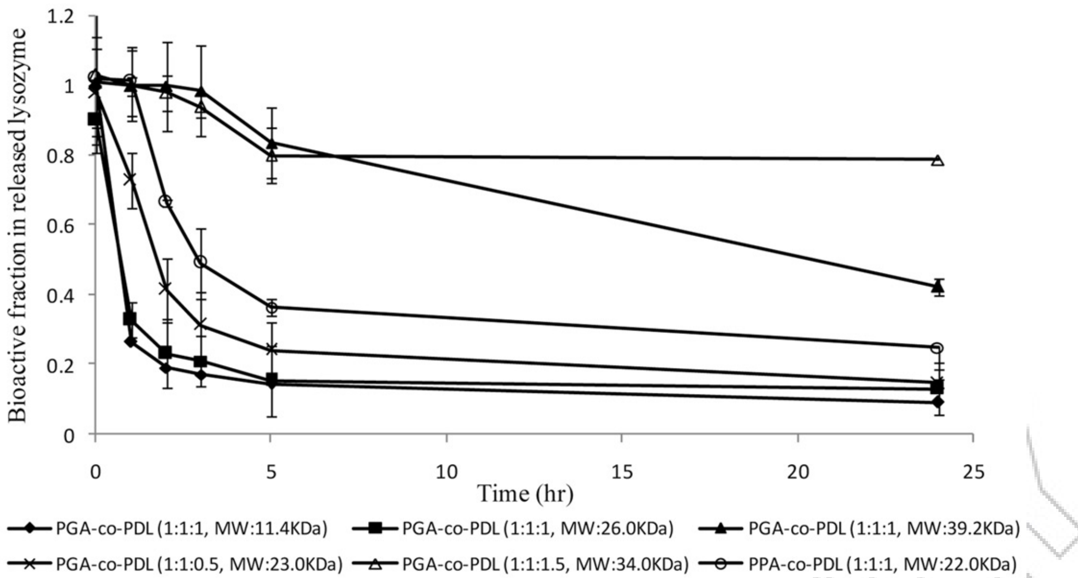


Figure 7. Bioactive fraction of released lysozyme from the different investigated polymers in PBS buffer, pH 7.4. Triplicate samples were used from two different prepared batches at each time ± S.D.

possibly be attributed to the higher solubility of these polymers in DCM compared with PPA-co-PDL and the lower M_W PGA-co-PDL polymers. Additionally, the longer the contact time of the enzyme in the organic phase, the more enzyme activity would be lost. Thus, a higher solidification rate would be beneficial in retaining the LS biological activity. Similar results were reported by Ghaderi and Carlfors regarding stability of LS during emulsification process within PLGA[48]. Future work will focus on enhancing macromolecule encapsulation efficiency as well as maintaining stability during the manufacturing process. For example, the use of additives to protect the protein structure or the application of alternative formulation methods such as spray drying or s/o/w emulsions may substantially reduce the loss in bioactivity during encapsulation.

Conclusion

This research has shown that altering the M_W of PGA-co-PDL from 11.2 to 39.2 KDa had little impact on particle morphology, size, encapsulation efficiency or bioactivity of α-CH- and LS-loaded microparticles. Altering the polymer chemistry had a greater effect, as a higher encapsulation efficiency and drug loading of both α-CH and LS were obtained with PPA-co-PDL compared to PGA-co-PDL particles. A biphasic release pattern was obtained with all microparticles studied, and the release profiles varied according to the polymer used. A lower burst and continuous release was obtained for both enzymes with the more hydrophobic polymers, PPA-co-PDL and PGA-co-PDL (1:1:1.45) and with the higher M_W PGA-co-PDL (39.2 KDa). Furthermore, a very low burst release was recorded with LS compared to α-CH with all the investigated polymers.

One benefit of the low impact of small changes in M_W or PDL content on encapsulation and release is that batch-to-batch variations in the polymers should not have a demonstrable effect on either the properties of particles formed or the encapsulation and release data obtained. These findings suggest that more substantial changes to polymer properties are required to significantly influence the encapsulation and release of proteins. The nature of this type of polymerization reaction means that it is difficult to achieve higher M_W materials and extend the range of M_Ws studied. Small changes to the polymer chemistry has been shown to have a greater effect, hence future studies will focus on further modifying the polymer chemistry either by incorporating different monomers into the backbone or via modification of the pendant hydroxyl groups.

Declaration of interest

The authors report no conflicts of interest.

Hesham Tawfeek thanks the Ministry of Higher Education, Egyptian government, for funding the research presented within this paper.

References

1. Sinha VR, Trehan A. Biodegradable microspheres for protein delivery. J Control Rel 2003;90:261–80.
2. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm 1999;185:129–88.
3. Pérez C, Castellanos IJ, Costantino HR, et al. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. J Pharm Pharmcol 2002;54:301–13.
4. Chen S, Pieper R, Webster DC, Singh J. Triblock copolymers: synthesis, characterization, and delivery of a model protein. Int J Pharm 2005;288:207–18.
5. Pisal DS, Kosloski MP, Baluiyer SV. Delivery of therapeutic proteins. J Pharm Sci 2010;99:2557–75.
6. Castellanos IJ, Cruz G, Crespo R, Griebenow K. Encapsulation-induced aggregation and loss in activity of γ-chymotrypsin and their prevention. J Control Rel 2002;81:307–19.
7. Hutchinson FG, Furr BJA. Biodegradable polymer systems for the sustained release of polypeptides. J Control Rel 1990;13:279–94.
8. Jalil R, Nixon JR. Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties. J Microencapsulation 1990;7:297–325.
9. Panyam J, Dali MM, Sahoo SK, et al. Polymer degradation and *in vitro* release of a model protein from ($_{D,L}$-lactide-co-glycolide) nano-and microparticles. J Control Rel 2003;92:173–87.
10. Alonso MJ, Gupta RK, Min C, et al. Biodegradable microspheres as controlled-release tetanus toxid delivery systems. Vaccine 1994;12:299–306.
11. Blanco D, Alonso MJ. 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 1998;45:285–94.
12. Shantha Kumar TR, Soppimath K, Nachaegari SK. Novel delivery technologies for protein and peptide therapeutics. Curr Pharm Biotech 2006;7:261–76.

13. Kline BJ, Beckman EJ, Russell AJ. One-step biocatalytic synthesis of linear polyesters with pendant hydroxyl groups. J American Chem Soc 1998;120:9475–80.
14. Namekawa S, Uyama H, Kobayashi S. Enzymatic synthesis of polyesters from lactone, dicarboxylic acid divinyl esters, and glycols through combination of ring–openening polymerization and polycondensation. Biomacromolecules 2000;1:335–8.
15. Kallinteri P, Higgins S, Hutcheon G, et al. Novel functionalized biodegradable polymers for nanoparticle drug delivery systems. Biomacromolecules 2005;6:1885–94.
16. Puri S, Kallinteri P, Higgins S, et al. Drug incorporation and release of water soluble drugs from novel functionalized poly(glycerol adipate) nanoparticles. J Control Rel 2008;125:59–67.
17. Thompson CJ, Hansford D, Higgins S, et al. Evaluation of ibuprofen-loaded microspheres prepared from novel copolyesters. Int J Pharm 2007;329:53–61.
18. Tawfeek H, Khidr S, Samy E, et al. Poly(Glycerol Adipate-co-ω-Pentadecalactone) spray-dried microparticles as sustained release carriers for pulmonary delivery. Pharm Res 2011;28:2086–97.
19. Gaskell EE, Hobbs G, Rostron C, Hutcheon GA. Encapsulation and release of alpha-chymotrypsin from poly(glycerol adipate-co-omega-pentadecalactone) microparticles. J Microencapsulation 2008;25:187–95.
20. O'Donnell PB, McGinity JW. Preparation of microspheres by solvent evaporation technique. Adv Drug Deliv Rev 1997;28:25–42.
21. Morales JO, Joks GM, Lamprecht A, et al. A design of experiments to optimize a new manufacturing process for high activity protein-containing submicron particles. Drug Dev Ind Pharm 2013; Epub ahead of print.
22. Thompson CJ, Hansford D, Higgins S, et al. Enzymatic synthesis and evaluation of new novel ω-pentadecalactone polymers for the production of biodegradable microspheres. J Microencapsulation 2006;23:213–26.
23. Pamujula S, Graves RA, Kishore V, Mandal TK. Preparation and *in vitro* characterization of amifostine biodegradable microcapsule. Eur J Pharm Biopharm 2004;57:213–18.
24. Rabbani NR, Seville PC. The influence of formulation components on the aerosolisation properties of spray dried-powders. J Control Rel 2005;110:130–40.
25. Srinivasan C, Katare YK, Muthukumaran T, Panda AK. Effect of additives on encapsulation efficiency, stability and bioactivity of entrapped lysozyme from biodegradable polymer particles. J Microencapsulation 2005;22:127–38.
26. Telkov MV, Deminal GR, Voloshin SA, et al. Proteins of the Rpf (Resuscitation Promoting Factor) family are peptidoglycan hydrolases. Biochemistry 2006;71:414–22.
27. Kline BJ, Beckman EJ, Russell AJ. One-step synthesis of linear polyesters with pendant hydroxyl groups. J Am Chem Soc 1998;120:9475–80.
28. Watts PJ, Davies MC, Melia CD. Microencapsulation using emulsification/solvent evaporation: an overview of techniques and applications. Crit Rev Ther Drug Carr Sys 1990;7:235–59.
29. Tan H, Ye J. Surface morphology and *in vitro* release performance of double-walled PLLA/PLGA microspheres entrapping a highly water-soluble drug. Applied Surf Sci 2008;255:353–6.
30. Cai C, Mao S, Germershaus O, et al. Influence of morphology and drug distribution on the release process of FITC-dextran-loaded microspheres prepared with different types of PLGA. J Microencapsulation 2009;l26:334–45.
31. Tuncay M, Cali S, Ka HS, et al. Diclofenac sodium incorporated PLGA (50:50) microspheres: formulation considerations and *in vitro*/*in vivo* evaluation. Int J Pharm 2000;195:179–88.
32. Youan BBC, Benoit MA, Baras B, Gillard J. Protein-loaded poly(ε-caprolactone) microparticles. I. Optimization of the preparation by (water-in-oil)-in water emulsion solvent evaporation. J Microencapsulation 1999;16:587–99.
33. Mittal G, Sahana DK, Bhardwaj V, Ravi Kumar MNV. Estradiol loaded PLGA nanoparticles for oral administration: effect of polymer molecular weight and copolymer composition on release behavior *in vitro* and *in vivo*. J Control Rel 2007;119:77–85.
34. Kim BK, Hwang SJ, Park JB, Park HJ. Characteristics of felodipine-located poly(ε-caprolactone) microspheres. J Microencapsulation 2005;22:193–203.
35. McGee PJ, Davis SS, O'Hagan DT. Zero order release of protein from poly(-lactide-co-glycolide) microparticles prepared using a modified phase separation technique. J Control Rel 1995;34:77–86.
36. Li Y, Pei Y, Zhang X, et al. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. J Control Rel 2001;71:203–11.
37. Jiang G, Woo BH, Kang F, et al. Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres. J Control Rel 2002;79:137–45.
38. Tsai T. Adsorption of peptides to poly(D,L-lactide-co-glycolide): 2. Effect of solution properties on the adsorption. Int J Pharm 1996;127:43–52.
39. Singh M, Briones M, Ott G, O′Hagan D. Cationic microparticles: a potent delivery system for DNA vaccine. Proc Natl Acad Sci USA 2000;97:811–16.
40. Bala I, Bhardwaj V, Hariharan S, et al. Design of biodegradable nanoparticles: a novel approach to encapsulating poorly soluble phytochemical ellagic acid. Nanotechnology 2005;16:2819–22.
41. Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials 2000;21:2475–90.
42. Edlund U, Abertsson AC. Degradable polymer microspheres for controlled drug release. Adv Polymer Sci 2002;157:67–112.
43. Luan X, Skupin M, Siepmann J, Bodmeier R. Key parameters affecting the initial release (burst) and encapsulation efficiency of peptide-containing poly(lactide-co-glycolide) microparticles. Int J Pharm 2006;324:168–75.
44. Crotts G, Sah G, Park TG. Adsorption determines *in vitro* protein release rate from biodegradable microspheres: quantitative analysis of surface area during degradation. J Control Rel 1997;47:101–11.
45. Viswanathan NB, Patil SS, Pandit JK, et al. Morphological changes in degrading PLGA and P(DL)LA microspheres: implications for the design of controlled release systems. J Microencapsulation 2001;18:783–800.
46. Ghaderi R, Carlfors J. Biological activity of lysozyme after entrapment in poly($_{D,L}$-lactide-co-glycolide)-microspheres. Pharm Res 1997;14:1556–64.
47. Davies RC, Neuberger A, Wilson BM. The dependence of lysozyme activity on pH and ionic strength. Biochim Biophys Acta 1969;178:294–305.
48. Chang KY, Carr CW. Studies on the structure and function of lysozyme. I. The effect of pH and cation concentration of lysozyme activity. Biochim Biophys Acta 1971;229:496–503.
49. Osawa T. Lysozyme substrates. Synthesis of p-nitrophenyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-(1→6)isomer. Carbohydr Res 1966;1:435–43.
50. Osawa T, Nakazawa Y. Lysozyme substrates. Chemical synthesis of p-nitrophenyl O-(2-acetamido-2-deoxy-β-Image-glucopyranosyl)-(1→4)-O-(2-acetamido-2-deoxy-β-Image-glucopyranosyl)-(1→ 4)-2-acetamido-2-deoxy-β-Image-glucopyranoside and its reaction with lysozyme. Biochim Biophys Acta 1966;130:56–63.
51. Ballardie FW, Capon B, Cuthbert MW, Dearie WM. Some studies on catalysis by lysozyme. Bioorg Chem 1977;6:483–509.
52. Van de Weert M, Hennink WE, Jiskoot W. Protein instability in poly(lactide-co-glycolic acid) microparticles. Pharm Res 2000;10:1159–67.
53. Pérez-Rodriguez C, Montano N, Gonzalez K, Griebnow K. Stabilization of α-chymotrypsin at the CH_2CL_2/water interface and upon water-in-oil-in-water encapsulation in PLGA microspheres. J Control Rel 2003;89:71–85.
54. Sah H. Protein behavior at the water/methylene chloride interface. J Pharm Sci 1999;88:1320–5.
55. Giteau A, Venier-Julienne MC, Marchel S, et al. Reversible protein precipitation to ensure stability during encapsulation within PLGA microspheres. Eur J Pharm Biopharm 2008;70:127–36.