Materials Science and Engineering C 47 (2015) 230–236

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Formulations for modulation of protein release from large-size PLGA microparticles for tissue engineering

Roozbeh Qodratnama [a,b,*], Lorenzo Pio Serino [c], Helen C. Cox [a], Omar Qutachi [a], Lisa J. White [a]

[a] Division of Drug Delivery and Tissue Engineering, Wolfson Centre for Stem Cell, Tissue Engineering and Modelling, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, UK
[b] TherapeuTech Ltd., University of Nottingham Institute for Enterprise and Innovation, Sir Colin Campbell Building, Jubilee Campus, Nottingham NG7 2TU, UK
[c] Neotherix Ltd. Research Centre, York Science Park, York YO10 5DF, UK

ARTICLE INFO

Article history:
Received 30 March 2014
Received in revised form 15 October 2014
Accepted 5 November 2014
Available online 7 November 2014

Keywords:
Triblock copolymer
Microparticle
Lysozyme
PLGA–PEG–PLGA
Controlled release
Glass transition temperature

ABSTRACT

In this study we present an approach to pre-program lysozyme release from large size (100–300 μm) poly(DL-lactic acid-co-glycolic acid) (PLGA) microparticles. This approach involved blending in-house synthesized triblock copolymers with a PLGA 85:15. In this work it is demonstrated that the lysozyme release rate and the total release are related to the mass of triblock copolymer present in polymer formulation. Two triblock copolymers (PLGA–PEG1500–PLGA and PLGA–PEG1000–PLGA) were synthesized and used in this study. In a like-for-like comparison, these two triblock copolymers appeared to have similar effects on the release of lysozyme. It was shown that blending resulted in the increase of the total lysozyme release and shortened the release period (70% release within 30 days). These results demonstrated that blending PLGA–PEG–PLGA triblock copolymer with PLGA 85:15 can be used as a method to pre-program protein release from microparticles. These microparticles with modulated protein release properties may be used to create microparticle-based tissue engineering constructs with pre-programmed release properties.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Multifunctional tissue engineering scaffolds produced from microparticles that possess pre-programmed protein release properties can be used for induction of tissue regeneration and for regenerative medicine applications. These scaffolds can potentially create suitable niche for cells to grow and form tissue by possessing growth factor release rates and polymer degradation rate that meet tissue specific needs. The ability to pre-program protein release profiles that mimic the natural release profile of growth factors and hormones accurately is essential to create functional tissue engineering constructs. For example, extracellular matrix (ECM) slowly releases bFGF (beta fibroblast growth factor) and pituitary gland releases hGH (human growth factor) in frequent pulses [1]. The slow degradation rate in some polymers such as PLGA can be utilized to produce multifunctional scaffolds that have both prosthetic and drug delivery properties. PLGA-microparticles can create scaffolds that provide prosthetic support to facilitate tissue regeneration by serving as cell substratum to form tissue *in vitro* or *in vivo*. At the same time, PLGA-microparticles can be loaded with biomolecules and programmed to release the biomolecules in a manner that creates a suitable niche for tissue regeneration.

Microparticles produced from different polymer formulations would possess different release and degradation rates. These microparticles can serve as the building blocks that underlie the multifunctionality of the resultant scaffold. PLGA is one of the widely used polymers in controlled drug delivery. It has a long history of successful use for drug delivery in products such as Zoladex LA [2], Nutropin Depot and Telstar Depot [3]. When PLGA microparticles are used as a controlled delivery system, the weight average molecular weight (Mw) and lactide: glycolide ratio affect the diffusion rate and permeability of the PLGA polymeric matrix which consequently determines the protein release rate and degradation rate of the microparticles. PLGA degrades via hydrolysis of its ester linkages in the presence of an aqueous environment [4,5]. Protein release from PLGA-based microparticles is generally slow and is comprised of three release phases. In the drug delivery context, size of the microparticles affects the release profile and repeatedly in the literature, protein release is achieved from microparticles with a size range less than 50 μm [6]. Large sized microparticles can form highly porous scaffolds that facilitate tissue ingrowth. On the other hand, accelerating protein release in a pre-programmed manner from large sized PLGA microparticles (100–300 μm) has remained a challenge.

The limitations of large-size PLGA-microparticles in terms of drug release may be overcome by using processing methods such as modifying the polymer formulation of the microparticles using more hydrophilic polymers. For example, one of the strategies for improving protein release from PLGA microparticles is blending a hydrophobic foundation polymer such as PLGA or poly(lactic acid) (PLA) with

* Corresponding author at: Division of Drug Delivery and Tissue Engineering, Wolfson Centre for Stem Cell, Tissue Engineering and Modelling, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, UK.
E-mail address: Roozbeh.qodratnama@gmail.com (R. Qodratnama).

http://dx.doi.org/10.1016/j.msec.2014.11.003
0928-4931/© 2014 Elsevier B.V. All rights reserved.

hydrophilic or amphiphilic polymers such as poly(ethylene glycol) (PEG) and chitin [7–10]. Previously, a linear release profile of ovalbumin (OVA) in a 30-day time period was obtained from microparticles (~10 μm) produced from blends of PLGA 50:50 (molecular weight of 35,000 Da) and PEG (molecular weight of 8000 Da) (PLGA:PEG ratio of 1:3 and 1:2) [11].

The application of PLGA–PEG–PLGA (or PLGA–PEO–PLGA) triblock copolymers in controlled drug delivery has been extensively studied mainly as hydrogels [12–19]. These triblock copolymers have acceptable biocompatibility and therefore are suitable for use as biomaterials and medical devices [20–22]. PLGA–PEG–PLGA triblock copolymers hold physicochemical properties that have the potential to overcome the problems associated with protein release from PLGA-based delivery systems. These properties include higher hydrophilicity, accelerated degradation and faster pore formation. Blending PLGA–PEG–PLGA triblock copolymers with PLGA polymer show potential as a tool to accelerate the release of bioactive molecules from delivery systems [23,24].

In this study, two different PLGA–PEG–PLGA triblock copolymers were synthesized and their interaction with water was investigated by studying the sol–gel behavior of the aqueous solution. These two triblock copolymers were used in the fabrication of large size microparticles (100–300 μm). Four different microparticle groups with formulations containing different masses of triblock (10% and 30% w/w) have been compared to a formulation with no triblock i.e. PLGA 85:15. PLGA 85:15 used here was an ester ending polymer with Mw of 118 kDa and a glass transition temperature (Tg) of around 56 °C. Slow degradation rate and slow release profile are generally attributed to high molecular weight and high LA/GA ratio of the polymer used [5,25,26]. The Tg of the above four microparticle groups was measured using rheology. The triblock copolymers were used to decrease the Tg of the polymer formulations and the effect of each triblock copolymer on the Tg was investigated. Morphology and size distribution of microparticles were studied via scanning electron microscopy and laser diffraction. Lysozyme was used as a model protein to study its release kinetics from microspheres produced from PLGA 85:15 blended with PEG-containing triblocks. To study the release behavior of each microparticle group a continuous flow system was used. The effect of each of the PLGA–PEG–PLGA triblock copolymers on the release of lysozyme from microparticles over a 60-day period was investigated separately.

2. Materials and methods

2.1. Materials

All materials are used without further modification and or purification unless otherwise stated. Poly(ethylene glycol) with Mw of 1500 (PEG 1500), poly(ethylene glycol) with Mw of 1000 (PEG 1000), stannous 2-ethylhexanoate (stannous octoate), lysozyme from chicken egg white (EC 3.2.1.17), polyvinyl alcohol (PVA) (Mw: 13–23 kDa, 87–89% hydrolyzed), sodium hydroxide (NaOH), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich, UK. Poly(ethylene glycol) Mw of 6000 (PEG 6000) was obtained from BHD Chemicals and D,L-lactide (LA) from Alfa Aeser, UK. Glycolide (GA) was purchased from PURAC, Gorinchem, Netherlands. Micro bicinchoninic acid (μ-BCA) kit was obtained from ThermoScientific, UK. Poly(DL-lactide-co-glycolide) (PLGA) 85:15 (ester ending, Mw 118 kDa, inherent viscosity 0.6–0.8) was purchased from Lakeshore Biomaterials, Alabama, USA. Dichloromethane (DCM) and dimethylsulfoxide (DMSO) were obtained from Fisher Scientific, UK. Deuterated chloroform ($CDCl_3$) was purchased from Cambridge Isotope Laboratories, MA, USA.

2.2. Synthesis of PLGA–PEG–PLGA triblock copolymers

Two different triblock copolymers were synthesized via ring opening polymerization using PEG1500 and PEG1000 following the method previously described [27]. In brief, the PLGA–PEG1500–PLGA triblock reaction mixture was composed of 5.5 g PEG1500, 9.57 g of LA and 3.08 g of GA (LA:GA molar ratio on feed was 2.5). For synthesis of PLGA–PEG1000–PLGA triblock 5.5 g PEG 1000, 9.97 g LA and 2.68 g GA (LA:GA molar ratio on feed was 3) were used. The PEG component was dehydrated for 3 h at 120 °C and polymerization was continued for 8 h under argon atmosphere at 150 °C.

2.3. Characterization of PLGA–PEG–PLGA triblock copolymers

2.3.1. ^1H NMR characterization

Proton magnetic nuclear resonance (^1H NMR) was used to characterize the triblocks. Spectra were recorded at 400 MHz on a Bruker spectrometer at 25 °C. Triblocks were dissolved (10–30 mg/ml) in deuterated chloroform ($CDCl_3$) containing tetramethylsilane (TMS). The TMS signal was taken as zero chemical shift. Number average molecular weight (Mn) and lactide to glycolide ratio were determined by integration of the peak signals pertaining to each monomer, such as CH_2 of glycolide, CH of lactide, and CH_2–CH_2 of ethylene glycol.

2.3.2. Molecular weight evaluation

Gel permeation chromatography (GPC) was used to determine the weight average molecular weight (Mw) and molecular weight distribution of the triblocks. The analysis was performed using a PL-GPC 50 apparatus at 25 °C. Triblocks were dissolved (10–15 mg/ml) in HPLC grade chloroform ($CHCl_3$). The triblock solutions were filtered using a 0.2 μM Ministar-RC syringe filter unit (Sartorius, Epsom UK) into 2 ml GPC vials. The analysis was performed using chloroform as eluent at a flow rate of 1 ml/min; GPC was calibrated with polystyrene standards. Two PL Gel Mixed-D (5 μm) (7.8 × 300 mm) columns were used for higher resolution. Mw, Mn and polydispersity obtained directly from GPC and reported directly.

2.3.3. Rheological evaluation of aqueous solution of PLGA–PEG–PLGA triblock copolymers

Rheological measurements were performed using a dynamic mechanical analysis rheometer (Anton Paar, Physica MC301). Aqueous solution of PLGA–PEG–PLGA triblock copolymers with different concentrations namely 20, 25, 30 and 35 (%) (w/v) was prepared by addition of the appropriate amount of each triblock copolymer to distilled water (5 ml) and stirred at 4 °C until dissolution. Samples were placed between the 25 mm diameter parallel plates with a gap distance of 0.4–0.5 mm. To study the rheological behavior of the triblock copolymer aqueous solutions, 200 μl of each solution was used. Data were collected under controlled oscillation. Rheology experiments were performed using an environmental chamber exerting air pressure of 5 bar to initiate the apparatus and nitrogen atmosphere 200 In/h throughout the experiment. The temperature changes were controlled using a water bath. A Peltier hood was used to control the temperature inside more accurately and provide a homogenous environment when the parallel plates (25 mm diameter; PP25) were in operation.

2.4. Lysozyme-loaded microparticles

2.4.1. Production of lysozyme-loaded microparticles

To produce lysozyme-loaded microparticles, lysozyme was first micronized via method previously described [28]. Briefly, PEG 6000 (60 mg) was added to glass vial and dissolved in 1 ml distilled water. Chicken egg lysozyme (50 mg) was added to the solution and mixed thoroughly. The PEG/lysozyme solution was frozen using liquid nitrogen and freeze dried for 48 h.

Lysozyme-loaded microparticles were produced using a solid-in-oil–water (S–O–W) method as described by Mortia et al. [29]. This method was optimized to produce a particle size range of 100–300 μm. In total, four different polymer blend formulations were used for preparation of microparticle groups. This was performed by mixing the appropriate amount of PLGA 85:15 and the PLGA–PEG–PLGA of interest. To produce

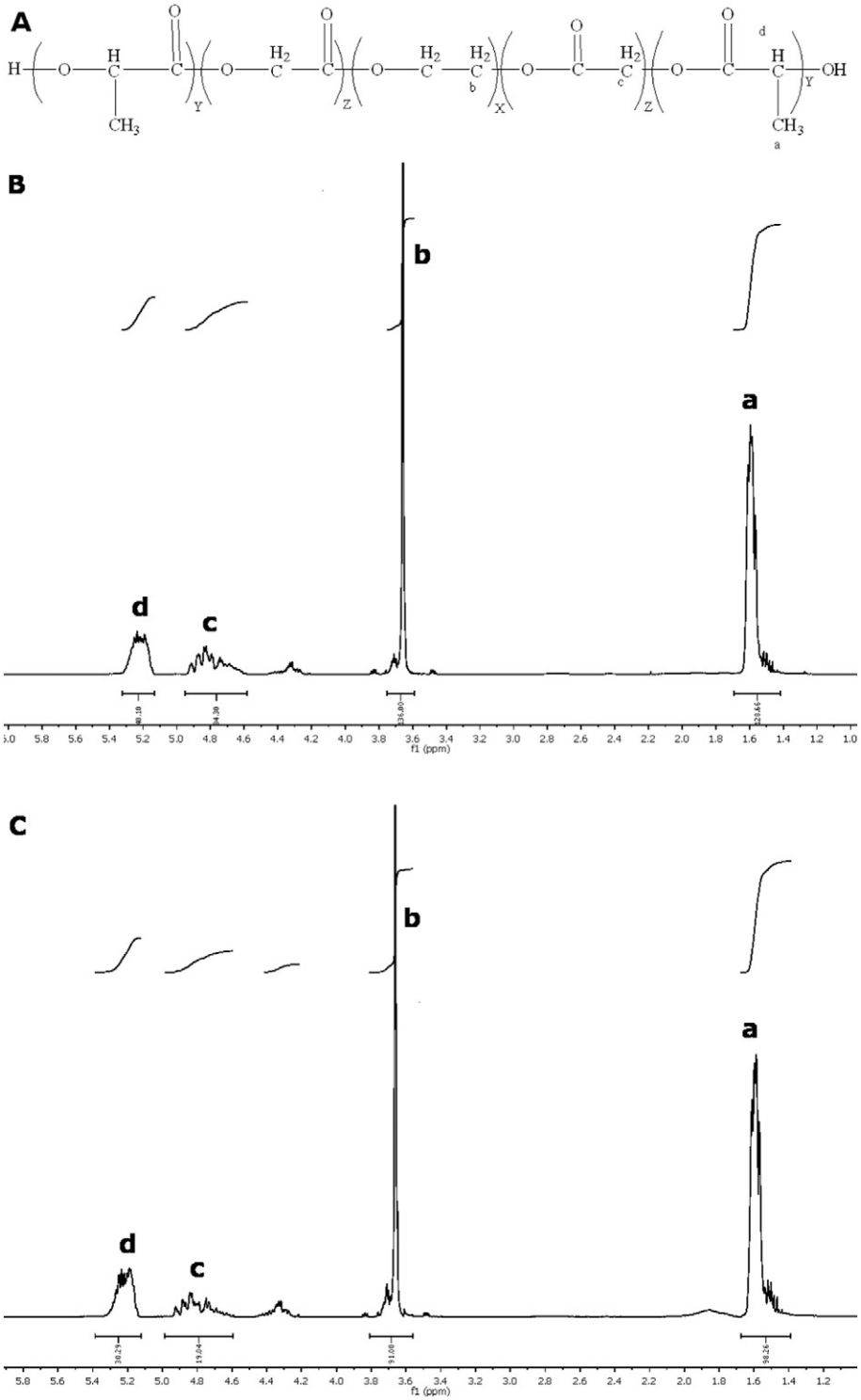

Fig. 1. A) The chemical structure of PLGA–PEG–PLGA triblock copolymer; B) the ^{1}H NMR spectrum of PLGA–PEG1500–PLGA triblock copolymer; C) the ^{1}H NMR spectrum of PLGA–PEG1000–PLGA triblock copolymer. The ^{1}H NMR spectra are plotted as a signal intensity versus chemical shift (ppm: proton precession magnetometer), where the signal peaks are (a) CH_3 of LA, (b) CH_2 of ethylene glycol, (c) CH_2 of GA and (d) CH of LA.

microparticle groups, PLGA/triblock blends (1 g) were dissolved in 3 ml DCM at 25 °C in a glass vial. Micronized lysozyme was also dissolved in DCM (1 ml). The PEG/lysozyme solution was added to PLGA/PLGA–PEG–PLGA solution. To emulsify, 4 ml PVA solution (0.3% (w/v)), was added and mixed using a vortex mixer VM20 mixer (Chiltern Scientific, Bucks, UK) and stabilized for 3–4 h in PVA solution (0.3% (w/v)), after which, the hardened microparticles were washed, sieved and separated by a Retsch AS200 sieve shaker (amplitude 1.40, 40 s interval time for 20 min). Microparticles in the 100–300 μm size range were collected. PLGA 85:15 without triblock was also loaded with micronized lysozyme as a control. Another control group was prepared by fabrication of non-loaded microparticles from PLGA 85:15 with no triblock copolymer.

2.4.2. Measurement of entrapment efficiency

To measure entrapment efficiency (EE), the protein content of microparticles was determined by a method previously described [30]. Briefly,

Table 1
Summary of ^{1}H NMR and GPC results for the PLGA–PEG–PLGA triblock copolymers.

Triblock copolymer	On feed	NMR		GPC		
	LA/GA[a]	Mn[b]	LA/GA[c]	Mn[d]	Mw[e]	PDI
PLGA–PEG1500–PLGA	2.5	2043–1500–2043	3.1	3437	4757	1.38
PLGA–PEG1000–PLGA	3	1443–1000–1443	4.2	1981	2617	1.32

[a] Molar ratio of lactic acid to glycolic acid on feed.
[b] Number average molecular weight calculated from ^{1}H NMR data.
[c] Molar ratio of lactic acid to glycolic acid calculated from ^{1}H NMR data.
[d] Number average molecular weight determined by GPC.
[e] Weight average molecular weight determined by GPC.

10 mg of lysozyme-loaded microspheres was weighed out and dissolved in 750 μl DMSO by shaking for 1 h at RT, followed by addition of 2150 μl of 0.2% NaOH/0.02% SDS solution, and was shaken for 1 h at room temperature (RT). The protein content was then determined by μ-BCA kit according to the instructions of the manufacturer. Briefly, supernatant (150 μl) and μ-BCA kit solution mix (100 μl) were incubated in a 96 well plate for 1 h at 36 °C after which measurement of the absorbance at 562 nm was performed using a TECAN Infinite 200 plate reader. To calculate the protein content each absorbance value was correlated to values obtained from a standard curve prepared from serial dilution of lysozyme concentration (0–200 μg/ml). Only values which were within the linear range of the standard curve were used. Samples which there initial values were above the limit were diluted 2 or 4 times and re-measure accordingly and samples with values lower than this limit were considered immeasurable and not included.

2.4.3. Determination of the glass transition temperature of polymer formulations

To study the change in the Tg of polymer formulations as a function of blending with PLGA–PEG–PLGA triblock copolymers, rheology was performed on melted microparticles. Rheoplus software was used to collect the data. This experiment was performed on all five lysozyme loaded microparticle groups. Microparticles produced from a polymer formulation having PLGA 85:15 were used as control group. Microparticles from each group (300–400 mg) were transferred to the center of the Peltier plate. Microparticles were melted by increasing the temperature to 100–150 °C. Storage and loss moduli and phase angle were collected under controlled oscillation; angular frequency (ω) 1 rad/s; amplitude of 1%, and heating rate of 2 °C/min and 0.1% strain. The temperature ramp was 10 to 80 °C with 35 measuring points. Each sample was run three times.

2.4.4. Scanning electron microscopy (SEM) of microparticles

To study the morphology of microparticles a variable pressure SEM (JEOL 6060LV, Jeol Ltd., UK) was used. Microparticles were sputter-coated on an adhesive stub with gold under argon atmosphere (Balzers SCD 030 Gold Sputter Coater, Liechtenstein) prior to examination.

Table 2
Comparison of sol–gel temperature and gel window for different concentrations of aqueous solutions of triblock copolymers.

Triblock copolymer	Sol–gel temp. (°C)	Gel–sol temp. (°C)	Gel window (°C)
PLGA–PEG1500–PLGA			
20% w/v	33.5	40.8	7.3
25% w/v	33.5	41.0	7.5
30% w/v	33.0	41.0	7.9
35% w/v	33.5	40.3	6.7
PLGA–PEG1000–PLGA			
20% w/v	10.0	16.3	6.3
25% w/v	10.5	16.0	5.5
30% w/v	10.8	15.5	4.7
35% w/v	11.3	15.0	3.7

2.4.5. Determination of the size distribution of microparticles

A laser diffraction method was used to study the size distribution of microparticles. A Coulter LS230 apparatus (Beckman Coulter, UK) was set to use a garnet.rfd optical model. Microparticles (50 mg/ml) were suspended in HPLC grade (HPLC Grade Elga) water and size distribution was recorded after obtaining an obscuration of 8–12% under constant stirring.

2.5. Lysozyme release kinetics

To study the release of entrapped lysozyme from microspheres a set-up previously described by Aubert-Pouessel et al. was employed [31]. Briefly, lysozyme-loaded microparticles (50 mg) were placed into an Omega Column tube (Presearch Ltd., UK). Each end was covered by two 0.5 μm PEEK frits (Presearch Ltd., UK). PEEK material is resistant to protein adhesion [31]. One end was connected to a 20 ml Plastipak syringe using a 1/16″ OD (0.04″ ID) HPLC PEEK tube that was fixed to a Harvard PHD 2000 infusion pump. The other end of the tube was connected to a 15/50 ml centrifuge tube using the same HPLC PEEK tube. The infusion pump was set to provide a continuous infusion rate of 2.0 μl/min. Phosphate buffered saline (PBS) (pH 7.4), was used as eluent buffer. The release study was set up within a temperature-controlled incubator (37 °C). The samples were collected each 24 h in the first week and each 48 to 72 h after that. Supernatant was stored at 4 °C and its protein content was quantified using the μ-BCA kit following the procedure explained in Section 2.4.2.

3. Results and discussion

3.1. ^{1}H NMR characterization and molecular weight evaluation

Bi-functional (α,ω-dihydroxy-terminated) PEGs with molecular weights of 1000 Da and 1500 Da were used to synthesize two compositionally different triblock copolymers via ring opening polymerization from D,L-lactide and glycolide. ^{1}H NMR spectrometry revealed the chemical structure of the synthesized triblocks. The signals pertaining to PLGA–PEG–PLGA are 5.20 ppm for CH of LA, 1.55 ppm for CH_3 of LA, 4.80 ppm for CH_2 of GA, and 3.65 ppm for CH_2–CH_2 of PEG and are shown in Fig. 1. The peaks representing CH of LA, CH_2 of GA, and CH_2–CH_2 of PEG were used for calculation of number average molecular weight (Mn) and LA:GA ratios. The spectra obtained were similar to previously reported spectrum [12]. The structural characteristics calculated from ^{1}H NMR data are summarized in Table 1. Molecular weight and molecular weight distribution of the triblocks were evaluated using GPC. The peaks in the chromatograms represented the triblock copolymers were obtained at the retention time of about 15–16 min. Uni-modal, relatively symmetric and narrow peaks in both chromatograms were obtained that confirm a narrow molecular weight distribution (data not shown). The quantitative data obtained from GPC analysis of the triblock copolymers is summarized in Table 1.

These data (Table 1) demonstrated the difference between the two triblock copolymers synthesized in terms of Mw and LA:GA ratio. The Mw and Mn of the triblock containing PEG 1500 were higher than the one containing PEG 1000 and these were confirmed both by ^{1}H NMR and GPC. These differences resulted in different interactions with water when they are dissolved, as explained in Section 3.2 below.

3.2. Rheological evaluation of aqueous solution of triblock copolymers

Rheological characterization of the aqueous solution of PLGA–PEG–PLGA triblock copolymers synthesized here revealed that both triblock copolymers demonstrated a thermo-reversible sol–gel transition (Table 2). It was shown that aqueous solution of PLGA–PEG1500–PLGA triblock copolymer possesses a gel window of 33–43 °C and the gel window for PLGA–PEG1000–PLGA triblock copolymer was found to be between 10 and 16 °C. The sol–gel transition temperatures for

Table 3
Summary of microparticle size characterization and corresponding entrapment efficiencies (n = 3).

Microparticle polymer formulation	Mean ± STD μm	Entrapment ± STD %
PLGA 85:15; 7E/PLGA–PEG1000–PLGA 90:10	229 ± 76	65 ± 4.2
PLGA 85:15; 7E/PLGA–PEG1000–PLGA 70:30	216 ± 50	68 ± 6.1
PLGA 85:15; 7E/PLGA–PEG1500–PLGA 90:10	222 ± 50	72 ± 4.6
PLGA 85:15; 7E/PLGA–PEG1500–PLGA 70:30	203 ± 71	64 ± 7.6
PLGA 85:15	296 ± 30	85 ± 8.3

PLGA–PEG1500–PLGA and PLGA–PEG1000–PLGA were found to be ~33 °C and ~10 °C, respectively. The higher sol–gel transition temperature of PLGA–PEG1500–PLGA can be attributed to higher hydrophilicity of this polymer. It was shown that lower sol–gel transition temperature is associated with higher hydrophobicity [12]. The gel window of PLGA–PEG1500–PLGA encompassed the physiological and experimental temperature; i.e. 37 °C. On the other hand, the gel window of PLGA–PEG1000–PLGA spanned over a distinctively lower temperature range. The rheological evaluation of the aqueous solution of triblock copolymers showed that these two compositionally different triblock copolymers also interact with water distinctively.

3.3. Size distribution, entrapment efficiency and morphology

Size distribution, entrapment efficiency and morphology are amongst the most important characteristics of microparticles. Size distribution is one of the factors that govern the release behavior of microparticles [32,33]. In this study we have narrowed the size distribution of microparticles by sieving to be able to minimize the effect of microparticle size distribution on release behavior. The mean diameter of the microparticles used for this study was all approximately 200 μm for microparticles with polymeric formulations containing triblocks and around 300 μm for microparticles with no triblocks. There was no statistical difference between the microparticle sizes (Table 3). It has been demonstrated that size-fractionated PLGA microspheres show different release profiles [34]. It was also postulated that there is a correlation between microparticle size and release rate [6].

Measurement of entrapment efficiency (EE) would indicate the mass of protein encapsulated in the microparticles. The method used here [30] is based on dissolution of the polymer matrix in DMSO followed by measurement of the encapsulated protein released into the alkaline environment. The entrapment efficiency of all five microparticle groups is represented in Table 3. The EE of the four microparticles groups containing PLGA–PEG–PLGA in their formulation appeared to be similar and lower than the control group. This can be attributed to the hydrophilicity imposed by the presence of the PLGA–PEG–PLGA triblock copolymer in the four test groups and its absence in the control group; in the sense that during the hardening the hydrophilicity of the test group formulation would attract more water to the polymer matrix and therefore the diffusion of protein moieties to the aqueous environment would be higher. This results in lower EE in this microparticle group.

Scanning electron microscopy (SEM) was used to examine microparticle morphology. SEM images show that all the particles possess smooth surfaces (Fig. 2) with very few pores. For both PLGA–PEG–PLGA formulations, it was observed that blending any of the triblock copolymers with PLGA 85:15 did not affect the morphology of the resulting microparticles with respect to the control group (microparticles produced from pure PLGA 85:15). This can be attributed to the structural similarity of the triblock copolymers with each other and with the PLGA [35].

For tissue engineering purposes, these microparticles could be used in combination or singularly depending on the tissue and the intended indication. Sequential release of multiple growth factors is shown to be a critical factor in neo-tissue formation and being able to pre-program the release would be achievable using this approach.

3.4. Evaluation of the glass transition temperature of polymer formulations

Glass transition temperatures of microparticle melts were measured using a rheometer. The glass transition temperature is the temperature at which the storage modulus declines and becomes lower than the loss modulus as the temperature is increased. The temperature at which the glass transition occurs in the microparticle group produced from PLGA 85:15 without any triblock – that is 56 ± 1.2 °C – is shown in Fig. 3 as a representative. The values for microparticles produced from formulations containing 10% and 30% w/w PLGA–PEG1500–PLGA were 48.6 ± 2.4 °C and 40 ± 1.4 °C, respectively. The glass transition temperature for microparticles with 10% and 30% w/w PLGA–PEG1000–PLGA was 49 ± 1.7 °C and 39.7 ± 2.4 °C, respectively. These data show a correlation between the percentage of PLGA–PEG–PLGA present in the polymer formulation and the Tg (Fig. 3) — in such a way that an increase in the triblock content of the polymer formulation decreased the glass transition temperature of the microparticles. In Fig. 3, there appears to be no difference between PLGA–PEG1500–PLGA and PLGA–PEG1000–PLGA in terms of the effect on the Tg of microparticles in a like-for-like comparison. The high Tg observed in the microparticle group produced from PLGA 85:15 can be attributed to the strong non-covalent

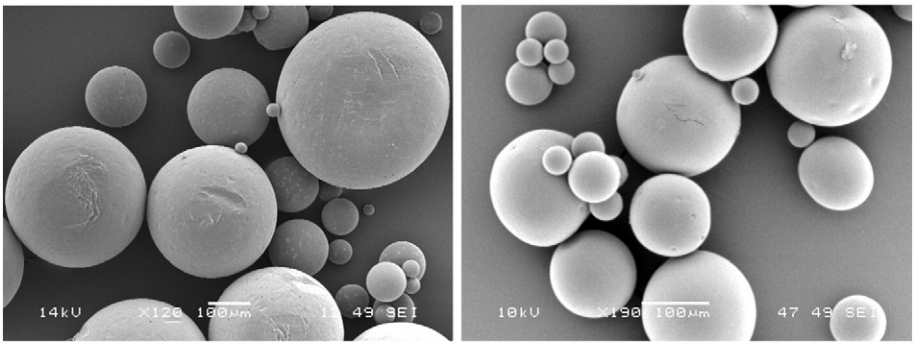

Fig. 2. Representative scanning electron micrograph (SEM) of microparticles fabricated from PLGA85:15 i.e. control group (left) and from PLGA 85:15 containing 30% w/w PLGA–PEG1000–PLGA (right).

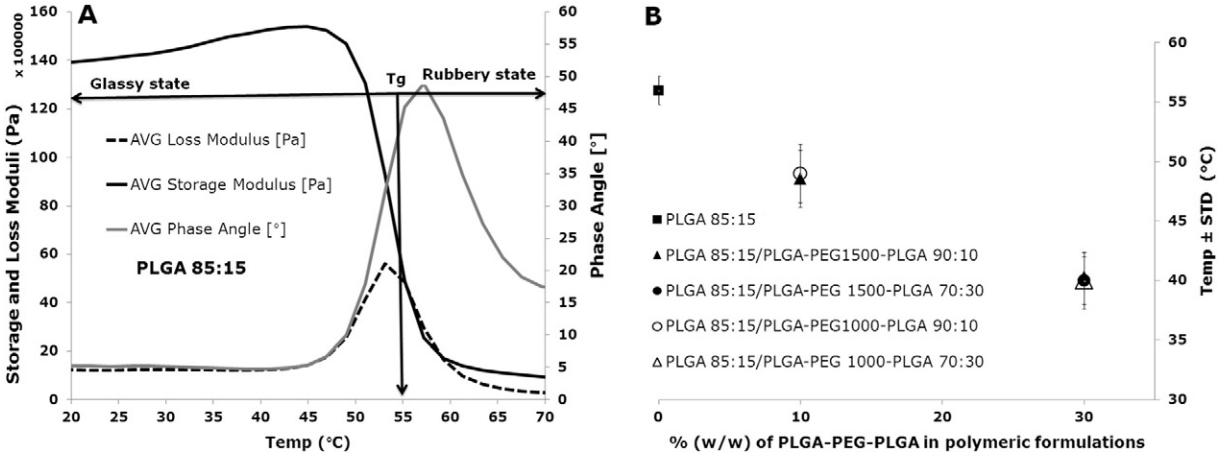

Fig. 3. A) Representative rheology profile of microparticle melts. The graph shows the rheological behavior of PLGA 85:15. The vertical arrow shows the approximate Tg of polymer formulation (56 ± 1.2 °C) (n = 3). B) The effect of blending with triblock copolymers on the Tg of microparticles.

interactions between the polymer cross-linkages that can absorb thermal energy. The decrease in the Tg in other microparticle groups can be attributed to the presence of PLGA–PEG–PLGA triblock that would decrease the strength of the non-covalent interactions between polymer cross-linkages by imposing heterogeneity in the polymeric network. These results show that blending PLGA with PLGA–PEG–PLGA (30% w/w) decreased the Tg to temperatures close to physiological temperature. The proximity of the glass transition temperature to physiological temperature will affect the viscoelastic behavior of the polymer matrix in microparticles and therefore influence the release kinetics of bioactive molecules. Increasing the temperature of the environment was shown to enhance drug diffusion as a function of polymer mobility. Previously, a threefold increase in drug diffusion coefficient had been reported at temperatures near the Tg [36]. It was shown that progesterone release was faster at temperatures above the Tg of PLA-based microparticles and no significant release occurred below the Tg during the period of study; at temperatures above the Tg, drug release rates increase with increase in the temperature [37].

3.5. In vitro release of lysozyme

In this study, the effect of each PLGA–PEG–PLGA triblock copolymer on the release of lysozyme from PLGA 85:15-based microparticles was investigated. In the control group i.e. microparticles with no triblock copolymer a total release of 5.6% (121.4 μg) after 60 days was obtained. Very slow release kinetics is usually expected from PLGA 85:15 [38,39] which is attributed to its predominantly hydrophobic structure and in this case specifically, also attributed to the ester ending and high molecular weight (Fig. 4). The release profile shows that incorporation of PLGA–PEG1000–PLGA has accelerated lysozyme release the same as

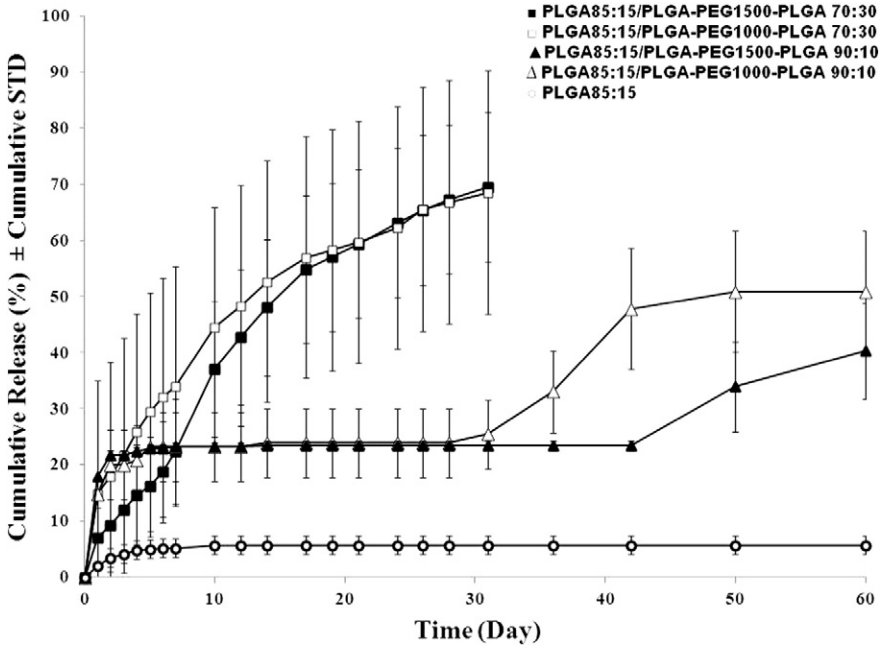

Fig. 4. The cumulative release profiles of microparticles in percent. The profiles obtained from 50 mg microparticles (n = 3). Cumulative STD is calculated based on running some of individual STDs. The profile shows that 70% of entrapped protein is released after 30 days from microparticles with PLGA85:15/PLGA–PEG1500–PLGA 70:30 (■) and PLGA85:15/PLGA–PEG1000–PLGA 70:30 (□) formulations. Nearly 40% of entrapped protein is released from microparticles with PLGA85:15/PLGA–PEG1500–PLGA 90:10 (▲) and PLGA85:15/PLGA–PEG1000–PLGA 90:10 (Δ) formulations. The significant difference ($P < 0.05$) in the release from 30 and 10% triblock containing formulations was observed after day 10. This figure shows the non-significant difference in the release profile of microparticles fabricated from PLGA 85:15 blended with PLGA–PEG1000–PLGA compared to PLGA–PEG1500–PLGA. Data represent the mean (n = 3) and error bars represent cumulative standard deviation (error bars not visible are smaller than the symbol). Statistical analysis was performed using SPSS software (version 16). The paired sample t-test and the ANOVA (general linear model (repeated measures)) were used for the comparison of means. Statistical significance was defined as $p < 0.05$.

the PLGA–PEG1500–PLGA. Non-significant difference ($P < 0.05$) in the release rates was observed from day 1 to day 10 and significant difference in release rate was observed after day 10 ($P < 0.05$). The release is halted after day 30 in microparticle groups containing 30% of either triblocks. After day 30, the release was continued in microparticle groups containing 10% of either triblocks and was increased after day 30 that is only significantly different at one time point between days 30 and 50, however, the total release at the end of the study period was not significantly different.

The lysozyme release was accelerated from microparticles containing 10% w/w PLGA–PEG–PLGA in their formulation. Release profiles obtained from these formulations showed that in total 40.2% (725 μg) and 50.9% (834 μg) lysozyme was released from these microparticles containing PEG 1500 and PEG 1000, respectively, after 60 days. These release profiles represent tri-phasic release profiles and resemble the profile previously reported from microparticles produced from PLGA 50:50 (Mw 7831 Da) with free carboxyl end group in PBS [40]. The release profiles obtained here showed a shift from a bi-phasic release profile in control group to a tri-phasic profile. The fact that the Tg of these microparticle groups (~48 °C) was markedly higher than incubation temperatures (37 °C) eliminates the possibility that the Tg is affecting the release profiles. These release profiles can be attributed to earlier induction of degradation; possibly related to or imposed by presence of PLGA–PEG–PLGA copolymer in the formulation (Fig. 4).

Microparticle groups containing 30% w/w of either triblocks showed gradual and continuous release of lysozyme. In total, 69.45% (1129.3 μg) and 68.5% (1170 μg) of the entrapped protein were released after 30 days from microparticles containing PEG 1500 and PEG 1000, respectively. The release was below detectable levels after day 30. These profiles indicate a continuous release profile. The release profiles obtained from these microparticle groups can be attributed to the proximity of their Tg (~39 °C) to the incubation temperature (37 °C) (Fig. 4). The proximity of environment temperature to the Tg of these formulations, appears to have affected the viscoelastic behavior of these formulations, making them more viscous and therefore the diffusivity was higher in these microparticles. Consequently, higher release rates were obtained. Generally, polymers have more elastic behavior in temperature ranges below their Tg [37]. On the other hand, polymers have more viscous behavior in temperature ranges above or equal to their Tg; and macromolecular mobility is higher. Higher release rates under these conditions can be attributed to drug diffusion through the polymer matrix or combined with diffusion through water-filled pores present in the microparticle [39].

4. Conclusion

Here it is demonstrated that the rate of lysozyme release can be controlled by blending PLGA–PEG–PLGA triblock copolymers with the foundation polymer (PLGA). These data supports the notion that there is a non-significant difference between PLGA–PEG1000–PLGA and PLGA–PEG1500–PLGA in acceleration of lysozyme release from PLGA microparticles; despite differences in the characteristic of these two triblock copolymers. In this work, it was shown that the release rate was correspondent to the mass of triblock blended with the foundation polymer. This release behavior can be attributed to Tg of the polymer formulations. The decrease in the Tg of the polymer formulations and earlier induction of the protein release can be attributed to the blending of the PLGA with PLGA–PEG–PLGA triblock copolymers. It is shown that blending the PLGA 85:15 with PLGA–PEG–PLGA triblock copolymer has decreased the Tg of the microparticles and induced earlier and faster lysozyme release from them. Overall, the release profiles obtained from microparticles containing the same amount of either triblock copolymer appears to be similar. These findings could be used to employ the properties of both the PLGA polymer and the PLGA–PEG–PLGA triblock copolymers to program the release behavior in a way that protein release precedes polymer degradation. This approach can be used to produce multifunctional tissue engineering scaffolds that serve not only as a delivery system for sequential protein release but also as an anchorage for cells to respond to the released biomolecules and provide an appropriate niche for the cells to grow or differentiate. Based on the above findings, tissue engineering constructs can be fabricated that release the encapsulated therapeutic protein prior to polymer degradation; thereby, supporting cellular response to the released biomolecule(s) by providing sufficient anchorage for cells to grow and differentiate.

References

[1] V. Luginbuehl, L. Meinel, H.P. Merkle, B. Gander, Eur. J. Pharm. Biopharm. 58 (2004) 197–208.
[2] B.C. Clark, D.M. Cross, P.R. Gellert, R.S. Kittlety, in: W.I.P. Organization (Ed.), Method for Determining the Release of a Peptide From a Sustained Release Polylactide Formulation, WIPO, UK, 2002.
[3] K.J. Whittlesey, L.D. Shea, Exp. Neurol. 190 (2004) 1–16.
[4] I. Grizzi, H. Garreau, S. Li, M. Vert, Biomaterials 16 (1995) 305–311.
[5] M. Vert, J. Mauduit, S. Li, Biomaterials 15 (1994) 1209–1213.
[6] J. Siepmann, N. Faisant, J. Akiki, J. Richard, J.P. Benoit, J. Control. Release 96 (2004) 123–134.
[7] W. Jiang, S.P. Schwendeman, Pharm. Res. 18 (2001) 878–885.
[8] E.C. Lavelle, M.K. Yeh, A.G.A. Coombes, S.S. Davis, Vaccine 17 (1999) 512–529.
[9] M. Morlock, T. Kissel, Y.X. Li, H. Koll, G. Winter, J. Control. Release 56 (1998) 105–115.
[10] F.-L. Mi, S.-S. Shyu, Y.-M. Lin, Y.-B. Wu, C.-K. Peng, Y.-H. Tsai, Biomaterials 24 (2003) 5023–5036.
[11] M.-K. Yeh, P.G. Jenkins, S.S. Davis, A.G.A. Coombes, J. Control. Release 37 (1995) 1–9.
[12] S.B. Chen, R. Pieper, D.C. Webster, J. Singh, Int. J. Pharm. 288 (2005) 207–218.
[13] B. Jeong, Y.H. Bae, S.W. Kim, Colloids Surf. B: Biointerfaces 16 (1999) 185–193.
[14] D.S. Lee, M.S. Shim, S.W. Kim, H. Lee, I. Park, T.Y. Chang, Macromol. Rapid Commun. 22 (2001) 587–592.
[15] S.M. Li, I. Rashkov, J.L. Espartero, N. Manolova, M. Vert, Macromolecules 29 (1996) 57–62.
[16] I. Rashkov, N. Manolova, S.M. Li, J.L. Espartero, M. Vert, Macromolecules 29 (1996) 50–56.
[17] C. Witt, T. Kissel, Eur. J. Pharm. Biopharm. 51 (2001) 171–181.
[18] L. Youxin, T. Kissel, J. Control. Release 27 (1993) 247–257.
[19] L. Youxin, C. Volland, T. Kissel, J. Control. Release 32 (1994) 121–128.
[20] P. Cerrai, G.D. Guerra, L. Lelli, M. Tricoli, R. Sbarbati Del Guerra, M.G. Cascone, P. Giusti, J. Mater. Sci. Mater. Med. 5 (1994) 33–39.
[21] B. Ronneberger, T. Kissel, J.M. Anderson, Eur. J. Pharm. Biopharm. 43 (1997) 19–28.
[22] T. Kissel, Y. Li, F. Unger, Adv. Drug Deliv. Rev. 54 (2002) 99–134.
[23] R.S. Harland, N.A. Peppas, Eur. J. Pharm. Biopharm. 39 (1993) 229–233.
[24] K.E. Uhrich, S.M. Cannizzaro, R.S. Langer, K.M. Shakesheff, Chem. Rev. 99 (1999) 3181–3198.
[25] T.G. Park, J. Control. Release 30 (1994) 161–173.
[26] T.G. Park, Biomaterials 16 (1995) 1123–1130.
[27] Q.P. Hou, D.Y.S. Chau, C. Pratoomsoot, P.J. Tighe, H.S. Dua, K.M. Shakesheff, F. Rose, J. Pharm. Sci. 97 (2008) 3972–3980.
[28] T. Morita, Y. Horikiri, H. Yamahara, T. Suzuki, H. Yoshino, Pharm. Res. 17 (2000) 1367–1373.
[29] T. Morita, Y. Sakamura, Y. Horikiri, T. Suzuki, H. Yoshino, J. Control. Release 69 (2000) 435–444.
[30] H.K. Sah, J. Pharm. Sci. 86 (1997) 1315–1318.
[31] A. Aubert-Pouessel, D.C. Bibby, M.C. Venier-Julienne, F. Hindre, J.P. Benoit, Pharm. Res. 19 (2002) 1046–1051.
[32] B. Amsden, Pharm. Res. 16 (1999) 1140–1143.
[33] J. Panyam, M.M. Dali, S.K. Sahoo, W. Ma, S.S. Chakravarthi, G.L. Amidon, R.J. Levy, V. Labhasetwar, J. Control. Release 92 (2003) 173–187.
[34] N.S. Berchane, K.H. Carson, A.C. Rice-Ficht, M.J. Andrews, Int. J. Pharm. 337 (2007) 118–126.
[35] S. Chen, J. Singh, Int. J. Pharm. 352 (2008) 58–65.
[36] B.S. Zolnik, P.E. Leary, D.J. Burgess, J. Control. Release 112 (2006) 293–300.
[37] Y. Aso, S. Yoshioka, A. Li Wan Po, T. Terao, J. Control. Release 31 (1994) 33–39.
[38] J.M. Anderson, M.S. Shive, Adv. Drug Deliv. Rev. 28 (1997) 5–24.
[39] N. Faisant, J. Siepmann, J.P. Benoit, Eur. J. Pharm. Sci. 15 (2002) 355–366.
[40] G. Jiang, B.H. Woo, F.R. Kang, J. Singh, P.P. DeLuca, J. Control. Release 79 (2002) 137–145.