Encapsulation and release of α-chymotrypsin from poly(glycerol adipate-co-ω-pentadecalactone) microparticles

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Abstract

Polymer based microparticles are increasingly becoming of interest for a variety of applications including drug delivery. Recently poly(glycerol adipate) (PGA) and poly(glycol adipate-co-ω-pentadecalactone) have shown promise for delivery of dexamethasone phosphate and ibuprofen. In this paper the copolyester poly(glycol adipate-co-ω-pentadecalactone) was evaluated as a colloidal delivery system for encapsulated therapeutic proteins. Enzyme containing microparticles were prepared via the double water-in-oil-in-water (w/o/w) emulsion-solvent evaporation methodology. α-chymotrypsin was used as a model proteolytic enzyme and its transfer was monitored during the emulsification process, in addition to *in vitro* release from formed particles. On average 22.1μg protein per 1mg polymer was encapsulated, although gradual loss of activity of the protein, once released, was recorded. The work presented shows the potential of this polyester as a delivery system for enzymes via microparticles, with improvements to the system achievable via polymer and process optimisation. The pendant hydroxyl groups on the polymer backbone provide future capacity for tailored alteration of the physical and chemical properties of the polymer, in addition to covalent attachment of various compounds.

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Introduction

For the controlled delivery of various synthetic drugs and biomacromolecules a range of natural and artificial vehicles have been used. Liposomes (Sharma and Straubinger, 1994), micelles (Onyuksel *et al.*, 1994), polysaccharides (Sharma *et al.*, 1995) and virus vectors (Schreier, 1994) all provide media for administration of drugs. Additionally, micro/nanoparticles prepared from synthetic biodegradable polymers have also been employed (Crotts and Park, 1995; Davis *et al.*, 1996; Sinha *et al.*, 2004). Amongst the commonly used polymers for colloidal drug delivery approved for human use are poly(lactic acid) (Guiziou *et al.*, 1996), poly(lactic-co-glycolic acid) (Aguiar *et al.*, 2004; Bilati *et al.*, 2005) and poly(ε-caprolactone) (Kim *et al.*, 2005; Le Ray *et al.*, 2003). There is also a growing list of novel synthetic biodegradable polymers that are being investigated for their potential in drug delivery systems, including polycarbonates, polyanhydrides, polymalic acid, polyphosphazenes, polyaminoacids (Davis *et al.*, 1996), polyesters (Breitenbach *et al.*, 2000; De Jesús *et al.*, 2002) and poly-N-isopropylacrylamide and other responsive polymers (Alexander and Shakesheff, 2006).

Microparticles are of particular interest in drug administration as they provide a useful means of controlling drug delivery and release, in addition to being cheaper, easier to produce, more stable and highly manipulative in their physical characteristics, compared to the other formulations available. The incorporation of drugs into these systems can be achieved via encapsulation during particle formation.

There is an array of particle preparation methodologies available using preformed polymers offering the choice of colloidal systems with different physical and chemical properties. The most commonly used particle preparation procedure enabling drug encapsulation is emulsion-solvent evaporation (Obeidat and Price, 2003; Ogawa *et al.*, 1988; Watts *et al.*, 1990). This methodology has been applied to a diverse range of lipophilic drugs (Juni *et al.*, 1985; Kim *et al.*, 2005; Ruan and Feng, 2003), however as such is not suitable for the encapsulation of hydrophilic drugs and biomolecules (Jalil and Nixon, 1990). Modifications to the emulsion-solvent evaporation technique have enabled successful entrapment and subsequent release of proteins and other hydrophilic compounds (Aguiar *et al.*, 2004; Crotts and Park, 1995; Gaspar *et al.*, 1998; Song *et al.*, 1997; Zhang and Zhu, 2004). The multiple emulsion-solvent evaporation methodology (Ogawa *et al.*, 1988) involves preparing an internal

water-in-oil (w_1/o) emulsion, where the inner aqueous phase contains the chosen hydrophilic active and the oil phase the selected polymer and appropriate surfactant. This emulsion is further emulsified with a continuous water phase containing an appropriate stabiliser. The thus formed water-in-oil-in-water (w_1/o/w_2) emulsion is mixed until the solvent evaporates and solid particles are formed. Such a system should allow for encapsulation of both hydrophilic and lipophilic molecules within the one colloidal system (Perez *et al.*, 2000). Adaptations to this methodology have allowed for a diverse range of hydrophilic compounds to be encapsulated.

The multiple emulsion system for particle preparation is reliant upon a number of factors: amount and chemical/physical nature of the polymer, characteristics of the stabilisers, ratio and volumes of the phases, time and mixing speed of the different emulsifications, solvent choice, etc. With the solvent-evaporation particle preparation procedure the purpose of the surfactant is to stabilise the formed emulsion for a short time, while the solvent evaporates, thus preventing coalescence and aggregation of the droplets within the emulsion. The choice of stabiliser very much depends on the type of emulsion to be stabilised. The effects of surfactants are not limited to the preparation procedure of the particles, but also have an influence on the characteristics of the particles and hence release of active (Graves *et al.*, 2005).

The purpose of entrapment of the hydrophilic drugs or biomacromolecules is to obtain sustained release of the active over a period of time. The release mechanism of the entrapped active molecules depends on their location within the particles. Desorption from the particle surface, diffusion through the polymer matrix, erosion of the polymer matrix or combinations of these are possible. The release profile of a given compound from a microparticle is governed by many parameters, including the nature of the polymer used, conditions of particle preparation, physical properties of the particles and the release environment. The particle surface morphology has also been shown to affect the release patterns (Le Ray *et al.*, 2003). In principle, it should be possible to manipulate the release profiles of any colloidal drug delivery system to match the needs of the application.

The main properties that make colloidal systems good for drug delivery are the biocompatible (non-toxic) nature of the polymer based system, adequate biodegradation rates to enable the controlled release of the accompanying active molecule and suitability for the targeted therapeutic levels, in addition to drug incorporation amounts. Poly(glycol adipate) offers backbone functionality via

pendant hydroxyl groups, which provide future potential for alteration of its physical and chemical properties, in addition to covalent attachment of various compounds. The possibility of tailoring the polymer chemistry expands its potential application in controlled delivery of a variety of molecules, compared to the commonly used polymers. Previous work on these and similar materials has focused on optimisation of nanoparticulate delivery systems for hydrophilic dextramethasone phosphate (Kallinteri *et al.*, 2005) and hydrophobic ibuprofen (Thompson *et al.*, 2006; Thompson *et al.*, 2007). This work explores the potential of poly(glycol adipate-co-ω-pentadecalactone) in colloidal delivery systems affording sustainable release of an encapsulated therapeutic protein.

Materials and methods

Materials

Glycerol, ω-pentadecalactone, Novozyme 435 (a lipase from *Candida antartica* immobilised on a macroporous acrylic resin), α-chymotrypsin (type II from bovine pancreas), Aerosol OT (dioctyl sodium sulfosuccinate), poly(vinyl alcohol) (PVA, 9-10kMw, 80%, Sigma), azocasein, 4-methylumbelliferyl-p(N,N,N-trimethylammonium) cinnamate (MUTMAC) and sodium orthoborate were all obtained from Sigma-Aldrich Chemicals (UK). Dichloromethane, trichloroacetic acid (TCA), sodium hydroxide, N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) and tetrahydrofuran (THF) were purchased from BDH (UK) whereas, phosphate buffered saline tablets at pH7.4 were obtained from Oxoid (UK). Divinyl adipate was obtained for Fluorochem (UK).

Polymer synthesis

The copolymer poly(glycol adipate-co-ω-pentadecalactone) was synthesised via an enzyme catalysed procedure adapted from Thompson *et al.* (2006). Briefly, 0.05mol of glycerol, divinyl adipate and ω-pentadecalactone were added to a 250mL two-necked round bottom flask followed by 15mL of tetrahydrofuran. This was allowed to equilibrate to $50^{\circ}C$ in a water bath, followed by the addition of 2.5% (w/v) of Novozyme 435 washed down with a further 5mL THF. An open top condenser was fitted to the flask. Stirring commenced at 2000rpm (setting 6 on the Heidolph RZR1

stirrer) using a Teflon shaft and paddle and the reaction was allowed to proceed for 24h. The resulting waxy liquid was further processed and analysed as outlined previously by Thompson *et al.* (2006) obtaining a white powder stored at room temperature. The polymer was characterised by GPC and NMR. The GPC system (Viscotek TDA Model 300 ran by OmniSEC3 operating software) was pre-calibrated with polystyrene standards (EasiCal A and B, Polymer Laboratories).

Particle preparation

For effective encapsulation of the model enzyme α-chymotrypsin a multiple emulsion-solvent evaporation technique was employed (Ogawa *et al.*, 1988). The copolymer poly(glycerol adipate-co-ω-pentadecalactone) was dissolved in dichloromethane. The surfactant, chosen to stabilise the first water-in-oil (w/o) emulsion, was an ion-pairing anionic surfactant, Aerosol OT. It was dissolved in the oil phase with the polymer at a sub-critical micellial concentration of 2mM (Huibers *et al.*, 1997).

The multiple emulsions were prepared as follows. A 1% (v/v) α-chymotrypsin stock solution (100mg mL^{-1} made up in phosphate buffered saline at pH7.4) was added drop-wise to a homogenising solution of polymer (30mg mL^{-1}) and Aerosol OT (2mM) in dichloromethane (15mL). The Polytron probe homogeniser (PT2100, 11000rpm for 1min) was used to emulsify this water-in-oil emulsion. This configured the 'first emulsion', a water-in-oil system. This was then gradually added to a mixing 1% (w/v) PVA solution (135mL in a 200mL glass beaker) to form the water-in-oil-in-water (w/o/w) multiple emulsion. The emulsion was left to mix with the Silverson L4 RT mixer at 1000rpm for the required time to allow for dichloromethane evaporation.

Single emulsion particles were prepared as controls. Here the oil phase (15mL) containing the polymer (30mg mL^{-1}) and Aerosol OT (2mM) was emulsified with the 1% (w/v) PVA aqueous solution containing 150mg α-chymotrypsin, using the Silverson L4 RT mixer at 1000rpm for the required time period.

The particles obtained were collected by ultracentrifugation (rotor Ti70, Beckman L80 ultracentrifuge) at 30,000g for 15min. The supernatants were labelled as 'wash 1' samples and retained for further analysis. Each pellet was re-suspended in 20mL deionised water to further remove residual PVA, and centrifuged as before, at 30,000g for 15min. These supernatants were collected as 'wash 2' samples. The particle-

containing pellets were re-suspended in 1.5mL deionised water, deep-frozen at -80°C prior to being freeze dried (Edwards Freeze Drier Super Modulyo).

The particles were visualised by scanning electron microscopy (JSM Jeol 840 Scanning Electron Microscope). The 13mm aluminium stubs were layered with a carbon tab and 10-20μL of particle suspension in water was deposited on the surface and air dried. An atomic layer of gold was deposited onto the particle containing stubs using the Polaron E 5000 Gold Sputter Coater. Images were taken using the Rontech Image Capture System.

Partitioning of chymotrypsin from the emulsion

Single and multiple emulsions were prepared as described above. Samples (1mL) of the emulsion formed were taken at timed intervals and the phases separated by centrifugation (2min at 13500rpm, MiniSpin Eppendorf). Initially there were two liquid phases (aqueous and polymer-containing oil) and the clear upper aqueous phase was retained at 4°C for further analysis. As the dichloromethane evaporated, upon 2.5h, solid polymer pellets were obtained and the aqueous supernatants collected and stored at 4°C. All the aqueous phases were subsequently assessed for protein content by measuring UV absorbance at 282nm, see below.

Release of chymotrypsin from optimised particles

Three batches of particles were made via the double emulsion solvent evaporation technique as described above. The particles were collected after 3h allowing sufficient time for the solvent to evaporate. As controls, particles were prepared via the single emulsion solvent evaporation procedure, as described previously. They were collected after 3h mixing, processed and freeze dried as for the double emulsion particles.

Into clean dry 2mL microtubes, 10mg of freeze-dried particles was deposited. To each one of these, 1mL of phosphate buffered saline pH7.4 at 37°C was added. The microtubes were then incubated at 37°C in the orbital shaker set at 100rpm (Innova$^{\text{TM}}$ 4340, New Brunswick Scientific). To observe the release of enzyme from the particles sacrificial sampling was employed. The samples were removed at increasing time points and centrifuged (5min at 13500 rpm, MiniSpin, Eppendorf) to collect particles. The supernatants obtained were collected and stored at 4°C for further

analyses. The protein contents of the collected supernatants were determined using the assays described below.

Methods for assessing protein content and activity

The washes collected (wash 1 and 2) and supernatants obtained throughout the release studies were analysed for protein content and activity using the following methods:

Azocasein assay. The proteolytic activity of chymotrypsin following incorporation and subsequent release from particles was determined using a chromogenic based technique – the azocasein assay (Charney and Tomarelli, 1947).

The procedure was modified from the literature (Brock *et al.*, 1982). To 200μL of 10mg mL^{-1} azocasein, made in 25mM HEPES buffer (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), 50μL sample, standard or blank were incubated overnight (16h) at 37°C. The reaction was stopped by addition of 750μL of 0.3M trichloroacetic acid to precipitate the undigested protein-chromophore conjugate. The samples were centrifuged (5min at 13,500 rpm, MiniSpin, Eppendorf) to remove the precipitate. To 200μL of the supernatant formed, 100μL of 0.5M sodium hydroxide was added to intensify the orange colour formed. Blank samples were made using deionised water to determine the amount of released azo-dye from the substrate non-enzymatically. Absorbance was read at 415nm wavelength and compared to reagent blank samples using a multiwell spectrophotometer (Benchmark Microplate Reader, Biorad). Three replicas of each sample were obtained and processed.

UV absorbance. To determine the total protein content present in the samples, their absorbance was measured at the optimum wavelength determined for chymotrypsin, 282nm (UV/VIS spectrometer Lambda 40, Perkin Elmer, run via the UV WinLab version 2.80.03 software). The Plastibrand UV cuvettes (Fisher) were used.

MUTMAC assay. The chymotrypsin active site titration method described by (Gabel, 1974) was used to assess residual active chymotrypsin in the samples. A 0.2mM solution of the fluorogenic compound 4-methylumbelliferyl-p(N,N,N-trimethylammonium) cinnamate (MUTMAC) was prepared as the enzyme substrate.

The reactions were set up in black 96 fluorescent plates (SLS) as follows. To 200μL sodium orthoborate buffer (0.1M at pH7.5), 50μL MUTMAC solution was added together with 50μL sample/standard/blank. These were mixed thoroughly prior to excitation at 360nm and measuring emission at 450nm. Measurements were taken on the fluorescence spectrophotometer, Varian Cary Eclipse, operated via the Cary Eclipse Advanced Reads Application version 1.1 (132) software.

Results and discussion

The focus of the work presented here was to evaluate the potential of the described polyester as a colloidal vehicle for protein delivery. Throughout the development of the enzyme-containing colloidal systems, α-chymotrypsin was chosen as the model enzyme, as it is commercially readily available with high units of activity. Much work has been done on the chymotrypsins (Bender and Killheffer, 1973), thus a literature-based background knowledge of this enzyme and the economic advantages made it a feasible candidate for development of the delivery system. Additionally, investigations of the encapsulation of chymotrypsin into poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres have previously been reported (Perez-Rodriguez *et al.*, 2003), however most of the literature focuses on other proteins including lysozyme (Jiang *et al.*, 2002), bovine serum albumin (Panyam *et al.*, 2003), asparaginase (Gaspar *et al.*, 1998), etc. Here the encapsulation and activity of α-chymotrypsin were monitored during the emulsification process and upon *in vitro* release from the poly(glycol adipate-co-ω-pentadecalactone) based delivery system.

Polymer synthesis

The enzyme catalysed poly(glycol adipate-co-ω-pentadecalactone) had a molecular weight of 30kDa compared to polystyrene standards. Integration patterns of the ^{1}H NMR demonstrated a 1:1:1 ratio of the three monomers used and the random nature of the polymer was confirmed by ^{13}C NMR (Thompson *et al.*, 2006).

Partitioning of chymotrypsin from double emulsion

To encapsulate chymotrypsin within the polymer colloidal system, a water-in-oil-in-water multiple emulsion system was employed. Here the enzyme was dissolved in the

inner water phase and this was emulsified, initially within the polymer-containing oil phase, followed by a second outer aqueous phase. Diffusion of the chymotrypsin from the internal water section through the oil phase into the outer continuous water phase affects the encapsulation efficiency. To assess this 'movement' of chymotrypsin during the particle formation process, its concentration in the outer aqueous phase was monitored over a period of time. Initially the chymotrypsin diffused through the liquid oil phase containing the polymer. Upon solvent evaporation the enzyme continued to diffuse through the solid polymer particles into the surrounding aqueous phase. Whether this partitioning is due to micellar transport through the oil phase supported by the surfactants present, formation of hydrophilic channels or any other route, remains unclear.

Once the multiple emulsion was formed, samples were taken at regular time intervals and the oil and aqueous phases separated by centrifugation. The protein content in the aqueous phase was measured using the UV assay (figure 1). A steady increase in chymotrypsin concentration was observed over time, changing from $0.893(\pm0.060)$mg mL^{-1} at 0min to $1.152(\pm0.037)$mg mL^{-1} at 7h. Once the solvent evaporated, upon 2.5h mixing, the enzyme continued to be released from the particles formed into the outer aqueous environment at a similar rate.

In the single emulsion control systems the protein concentration showed no significant change in concentration over the time period monitored, see figure 1.

[Insert FIGURE 1 about here]

From these observations it was concluded that the most convenient length of time for particle formation, under the given conditions, was 3h. This time allowed for evaporation of the solvent leading to solid polymer colloidal particles, yet minimised the subsequent loss of chymotrypsin.

Release of α-chymotrypsin from optimised particles

Once the particle preparation time was optimised at 3h, three separate batches of multiple emulsion particles were prepared containing α-chymotrypsin and one via the single emulsion procedure. Figure 2 shows SEM images of particles prepared via this multiple emulsion-solvent evaporation procedure. They ranged in size from 2μm to 12μm and had a furrowed surface morphology. These particles were used to observe the release of the enzyme. The total protein present in the wash samples, collected

during particle preparation, is shown in table 1. High levels of PVA in the samples were considered to cause overestimation protein measurements using the UV and azocasein assays (unpublished observations).

[Insert FIGURE 2 about here]

From the results obtained it was observed that on average 10mg of chymotrypsin was encapsulated within the particles from the 3 batches, see table 1. The percentage encapsulation efficiencies for the 3 batches (D1 to D3) were calculated as the percentage of total protein not washed out during particle preparation, equation (1). The enzyme load was defined as the amount of enzyme per unit polymer weight and the equation used is given below, equation (2).

$$Encap.Efficiency(\%) = \frac{Protein\_not\_washed\_out(mg)}{Protein\_amount\_added(mg)} x100 \qquad (1)$$

$$Enzyme\_Load(\%) = \frac{Total\_enzyme\_amount(mg)}{Total\_polymer\_amount(mg)} x100 \qquad (2)$$

[Insert TABLE 1 about here]

Therefore the encapsulation efficiency calculated was, on average, 6.63($\pm$3.55)% for the three batches made. Whereas, the enzyme load was estimated to have been, on average, 2.21($\pm$1.18)%, based on the amount of enzyme washed out of the polymer delivery formulation. Expressed as a percentage these values appear low due to the high amount of total protein added to the system (150mg). However, they are comparable to similar examples within the current literature. The average amount of protein encapsulated is 22.1 μg per 1mg poly(glycol adipate-co-ω-pentadecalactone), which is comparable to that reported by Perez-Rodriguez *et al.* (2003): 16.7μg chymotrypsin encapsulated per 1mg PLGA polymer. There are considerable differences in the encapsulation efficiencies of the 3 separate batches prepared possibly reflecting the variation in the encapsulation procedure and errors inferred during measurement of washed-out protein in the presence of higher PVA concentrations. Nonetheless, they are exemplary of the results possible from such systems and are represented here as a 'proof-of-principle' concept.

The temporal chymotrypsin release samples collected were analysed for total protein content via the UV absorbance assay, see figure 3. From the release data it was observed that maximal release was achieved upon 2h incubation.

[Insert FIGURE 3 about here]

The average maximal release concentrations for all batches of particles from 2h onwards were further used to calculate the total amount of protein releasable from the particles, see table 2. The calculated amounts of enzyme encapsulated for the 3 batches, table 1, were supported by the estimated amount of enzyme subsequently released from the particles, shown in table 2. These were further expressed as encapsulation yields, using equation (3) and enzyme load, using equation (2) above.

$$Encap.Yield(\%) = \frac{Protein\_released(mg)}{Protein\_amount\_added(mg)} x100 \tag{3}$$

[Insert TABLE 2 about here]

The encapsulation efficiencies in table 1 were calculated by measurement of the non-encapsulated protein present in the wash samples and are based on the assumption that there is no protein loss during the preparation and processing of particles. Also, due to the influence higher concentrations of PVA can have on the UV protein concentration measurements, an overestimation of the non-encapsulated enzyme was predicted. Thus, an underestimation of the encapsulation efficiencies may have occurred.

The encapsulation efficiency and yield are based on different presumptions; the former centring on how much is not present in the samples and the latter how much is. Nonetheless, only with the D2 particles (multiple emulsion particles-batch 2) did they differ significantly at p=0.05 confidence level (encapsulation efficiency 10.31% and encapsulation yield 6.48%). This lead to the presumption that most of the chymotrypsin encapsulated was released. Loss of protein due to non-specific adsorption to the surface may be accountable for the minimal discrepancies in the values obtained and were not quantified in this study. The possibility of a second phase of enzyme release that would coincide with particle/copolymer degradation should not be neglected. Monitoring the release of protein over longer periods of time may reveal more information on this. No protein release was observed from the control single emulsion particles, thus it was concluded that all the surface-adsorbed protein was removed during the wash step. The absorbance readings taken at 282nm for these release samples were of a negligible negative value (ranging from -0.0650 to –0.0887 A.U.), thus confirmed no hindrance of protein UV absorbance measurement from other components used in the preparation of the particles.

The physiological activity of the released chymotrypsin was estimated via the active site titration (MUTMAC) assay. This fluorescent based assay revealed a

decrease in amount of active sites over time. Maximal activity was observed upon 1h. Assessing the proteolytic activity of the released chymotrypsin via the colorimetric azocasein assay revealed a similar activity profile, with an onset of loss of proteolysis upon 2h release (figure 4). This confirms the above active site titration results.

[Insert FIGURE 4 about here]

These two assays were used to measure the amount of active enzyme released from particles prepared from multiple emulsions. The fluorescent MUTMAC assay is based on the irreversible binding of an α-chymotrypsin substrate, (N,N,N-trimethylammonium)cinnamate, and subsequent release of the fluorophore 4-methylumbelliferone. This results in a direct correlation between active sites present and fluorescent intensity. The azocasein assay is based on the release of sulfanilamide covalently linked to casein upon proteolytic digestion of this generic substrate. The released sulfanilamide is measured spectrophotometrically and reflects the proteolytic activity of the sample. Both techniques revealed a reduction in activity, implying that some form of conformational change to the active site of chymotrypsin had occurred.

Control experiments were set where 1mg mL^{-1} chymotrypsin in PBS was incubated at 37°C and 100rpm for 5.5h. No detrimental effect on chymotrypsin activity was observed. Hence it was concluded that the enzyme released from particles did not loose its activity due to the incubation conditions but during the emulsification process. Perez-Rodriguez *et al.* (2003) reported the detrimental effect the dichloromethane/water interface has on chymotrypsin inactivation and aggregation during the process of encapsulation into PLGA particles, suggesting a combination of suitable excipients may reduce the observed protein unfolding. Furthermore, Castellanos *et al.* (2002) address the effects of various physical and chemical parameters on the integrity and activity of encapsulated γ-chymotrypsin. They observed an increased level of aggregation and loss of activity upon release of the enzyme when encapsulated via the solid-in-oil-in-water (s/o/w) technique. However, they do demonstrate the beneficial effects of co-lyophilising the enzyme with poly(ethylene-glycol) (PEG) prior to encapsulation suggesting this as a potential preventative measure. Similarly, a decrease in enzyme activity was also observed by Gaspar *et al* (1998) when they encapsulated L-asparaginase in PLGA nanoparticles. They monitored the release and activity of L-asparaginase and noted a decline in

activity upon 2 or 7 days, depending on the polymer molecular weight. Contrary to above literature, their investigation into whether the particle preparation process damages the enzyme resulted in negative results and the authors concluded no aggregation or cleavage of the protein occurs during the emulsification steps of particle preparation. Nonetheless, upon longer release studies (3 weeks), some physical alteration of the released L-asparaginase was observed with SDS-PAGE, leading to the assumption that the enzyme may undergo denaturation once released from the colloidal systems. The decline in activity in the current work occurred much sooner than that reported in the literature, with a gradual decrease in activity observed after 2h release; refer to figure 4.

The influence of polymer molecular weight on protein encapsulation has been previously addressed with higher molecular weights polymers producing particles with slower release rates than lower molecular weight polymers (Gaspar *et al.*, 1998; Song *et al.*, 1997). Thus, simply altering the molecular weight of poly(glycerol adipate-co-ω-pentadecalactone) would afford adjustments in size range and performance of the colloidal delivery system. Research into the effect different physical properties of the functional copolymers may have on protein encapsulation could reveal a more efficient system. Additionally, further aspects of optimisation of such a delivery system may address the issue of the enzyme activity. Lyoprotectants have been used to decrease the adverse effects freeze-drying has on proteins (Gupta and Roy, 2004). Various other additives to the system have been shown to improve the encapsulation and subsequent release of hydrophilic compounds. Addition of salt to the outer aqueous phase decreases the proportion of partitioned protein by depressing its aqueous solubility (Dinarvand *et al.*, 2005). Alternatively, spray drying has been effectively used to encapsulate insulin in PLGA microparticles with reported minimal denaturation of the protein upon release (Quiglia *et al.*, 2003).

Future aspects of the optimisation of the poly(glycerol adipate-co-ω-pentadecalactone)/protein delivery system may also include assessment of various excipients to improve delivery and activity of the therapeutic enzyme in addition to evaluating the colloidal system capacity for different protein targets. Degradation profiles of the polymer formulation and its effects on the delivery of drugs should be further investigated.

Conclusions

These initial investigations of utilising functional enzyme-synthesised polyesters for delivery of proteins via microparticles indicate the potential for future development of such systems. Within the poly(glycol adipate-co-ω-pentadecalactone) colloidal system 22.1 μg protein was encapsulated per 1mg polymer. Release of this protein from the particles was observed over 7h and a continuous loss of enzyme activity was recorded during this time period. Altering the chemistry of the polymer backbone, attaching functional moieties, or drugs and/or protein, to the polymer would offer a variety of physical characteristics required to improve the delivery vehicle and enable combined delivery of both hydrophobic drug and hydrophilic enzyme. Modifications to the emulsion-solvent evaporation procedure may afford ameliorated enzyme activity upon release, providing a more suitable system for delivery of pharmaceutical proteins.

Previous studies have been performed on biocompatibility of the commonly used polyesters for various applications (Vaquette *et al*., 2006). Kallinteri *et al*. (2005) investigated the cytotoxicity of microspheres of poly(glycol adipate) and acylated derivatives of this polymer designed for parenteral delivery. They concluded low toxicity of their delivery systems. Given the nature of the copolymers used in this study it is anticipated that they will exhibit similar biocompatibility to the individual PGA and polylactones and this is currently under investigation.

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Table 1.   Encapsulation efficiencies of α-chymotrypsin within the particles formulated over 3h via the multiple emulsion solvent evaporation technique. Separate batches of enzyme containing particles were prepared: D1, D2 and D3. The amount of α-chymotrypsin added into the emulsion was 150mg (1.5mL of 100mg mL^{-1}). The α-chymotrypsin (CT) concentration in the washes was determined via the UV absorbance assay. The amount of α-chymotrypsin encapsulated was determined as the amount added minus the amount washed out.

Batch	Sample	Amount of CT washed out (mg)	Total CT amount washed out (mg)	Encap. amount of CT (mg)	Encap. efficiency (%)	Enzyme load (%)*
D1	wash 1 wash 2	133.67 6.47	140.14	9.86	6.58	2.19
D2	wash 1 wash 2	126.59 8.11	134.69	15.31	10.21	3.40
D3	wash 1 wash 2	136.66 8.69	145.35	4.65	3.10	1.03

* per 450mg polymer

Table 2. Encapsulation yield of α-chymotrypsin within double emulsion particles calculated as the total amount of protein releasable from the system under the given conditions. The maximal release concentration of α-chymotrypsin (CT) was determined from the release profile, figure 2, as the average concentration of both sets of results from 2h to 5.5h incubation for each batch of particles. The amount of α-chymotrypsin added into the particle preparation emulsions was 150mg (1.5mL of 100mg mL^{-1}).

Batch	Average max. release of CT (±st.deviation) (mg mL^{-1})	Amount of CT released* (mg)	Encapsulation yield (%)	Enzyme load (%)*
D1	0.207 (±0.024)	9.32	6.21	2.07
D2	0.216 (±0.026)	9.73	6.48	2.16
D3	0.145 (±0.028)	6.53	4.36	1.45

* per 450mg polymer

Figure legends

Figure 1. Diffusion of α-chymotrypsin from the internal aqueous phase of a multiple emulsion. The concentration of protein in the outer aqueous phase was measured using the UV absorbance assay. The dichloromethane evaporated from the double emulsion system within 2.5h, indicated by the arrow. Four replicate systems of the double emulsions were prepared (Double 1-4). The three single emulsion systems were monitored as controls (Single 1-3). The results are represented as means of 3 separate readings of the samples, and standard deviations are shown.

Figure 2. Scanning electron microscope images of particles formed by the multiple emulsion solvent evaporation technique. Image A) was taken at x1000 magnification and the scale bar represents 30μm, whereas image B) x3000 rpm and the scale bar represents 10μm.

Figure 3. Release profiles of α-chymotrypsin from particles formed over 3h by the multiple emulsion solvent evaporation methodology. Three batches were assessed: D1, D2 and D3. Duplicate samples were obtained for each time point from each batch. The results are expressed as means of 3 separate measurements of the samples, with the average standard deviation being 0.02861±0.0008 and represented only on the D3 run 2 5h point for clarity of graph.

Figure 4. Activity of released enzyme compared to total protein. Proteolytic activity of the samples was determined via the azocasein assay and compared to a α-chymotrypsin standard curve. Total protein content was assessed using UV absorbance at 282nm assay and comparing with a α-chymotrypsin standard curve. Release profile repeated in triplicate with 3 measurements made for each sample. Results represented as mean±standard deviation.
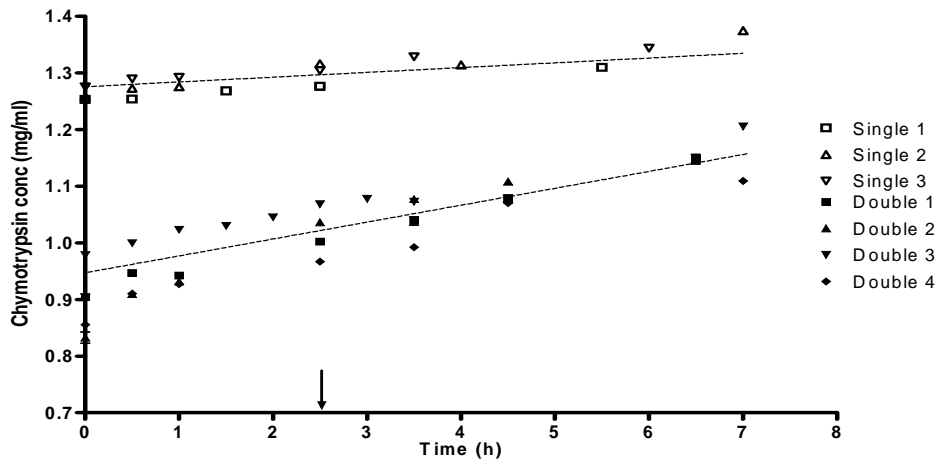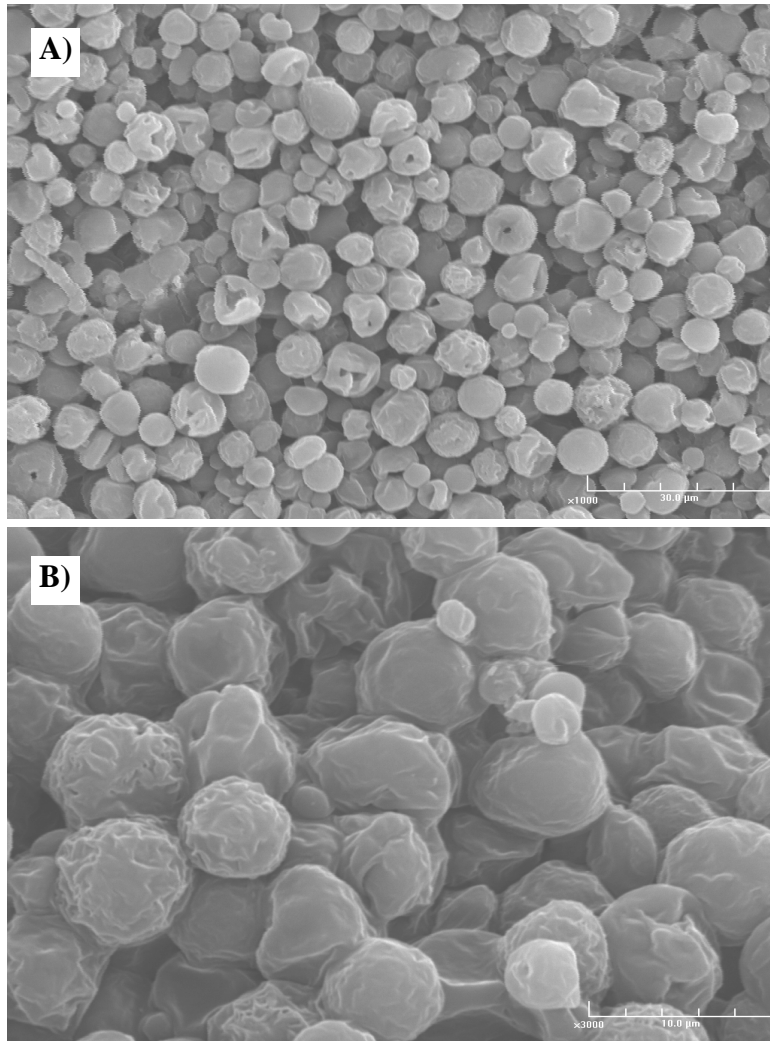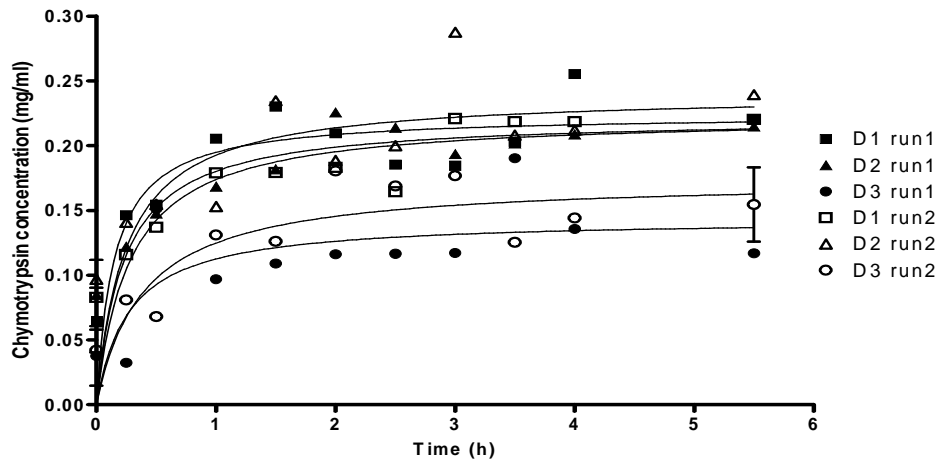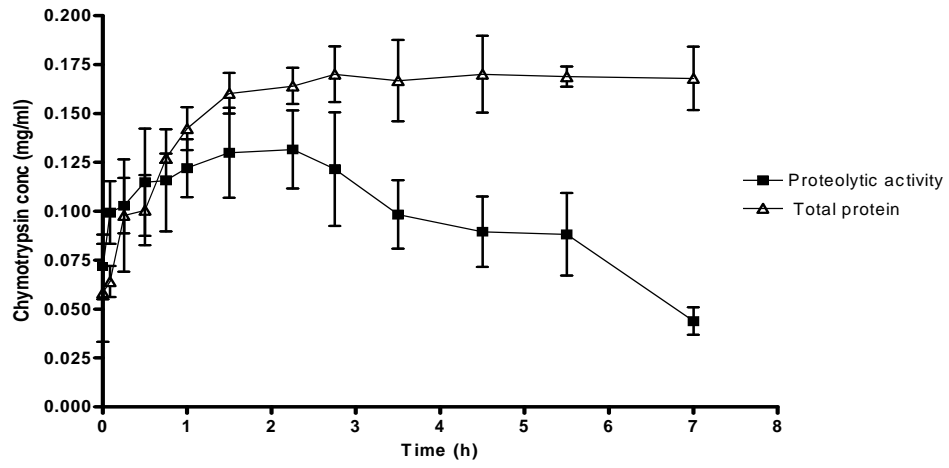
Figure 1.

Figure 2.

Figure 3.

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