Effect of poly(ethylene glycol) content and formulation parameters on particulate properties and intraperitoneal delivery of insulin from PLGA nanoparticles prepared using the double-emulsion evaporation procedure

Yusuf A. Haggag[1, 2], Ahmed M. Faheem[3], Murtaza Tambuwala[1], Mohamed A. Osman[2], Sanaa A. El-Gizawy[2], Barry O'Hagan[4], Nigel Irwin[1] and Paul A. McCarron[1*]

[1]*School of Pharmacy and Pharmaceutical Sciences, Saad Centre for Pharmacy and Diabetes, Ulster University, Cromore Road, Coleraine, Co. Londonderry, BT52 1SA, UK*

[2]*Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Tanta, Tanta, Egypt.*

[3]*University of Sunderland, Department of Pharmacy, Health and Well-being, Sunderland, SR1 3SD, UK*

[4]*School of Biomedical Sciences, Ulster University, Cromore Road, Coleraine, Co. Londonderry, BT52 1SA, UK*

***Corresponding author**

School of Pharmacy and Pharmaceutical Sciences,

Saad Centre for Pharmacy and Diabetes,

Ulster University,

Cromore Road, Coleraine, Co. Londonderry, BT52 1SA, UK

Tel: +44 (0) 28 701 23285

p.mccarron@ulster.ac.uk

(Faheem and McCarron made equal contributions to the work)

Abstract

Context Size, encapsulation efficiency and stability affect the sustained release from nanoparticles containing protein-type drugs.

Objectives Insulin was used to evaluate effects of formulation parameters on minimising diameter, maximising encapsulation efficiency and preserving blood glucose control following intraperitoneal (IP) administration.

Methods Homogenisation or sonication was used to incorporate insulin into poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles with increasing PEG content. Effects of polymer type, insulin/polymer loading ratio and stabiliser in the internal aqueous phase on physicochemical characteristics of NP, *in vitro* release and stability of encapsulated insulin were investigated. Entrapment efficiency and release were assessed by radioimmunoassay and bicinconnic acid protein assay, and stability was evaluated using SDS-PAGE. Bioactivity of insulin was assessed in streptozotocin-induced, insulin-deficient Type I diabetic mice.

Results Increasing polymeric PEG increased encapsulation efficiency, whilst absence of internal stabiliser improved encapsulation and minimised burst release kinetics. Homogenisation was shown to be superior to sonication, with NP fabricated from 10% PEG-PLGA having higher insulin encapsulation, lower burst release and better stability. Insulin-loaded NP maintained normoglycaemia for 24 hours in diabetic mice following a single bolus, with no evidence of hypoglycaemia.

Conclusions Insulin-loaded NP prepared from 10% PEG-PLGA possessed therapeutically useful encapsulation and release kinetics when delivered by the IP route.

Key words insulin, nanoparticles, diblock copolymers, encapsulation efficiency, intraperitoneal bioactivity

1. Introduction

Effective insulin administration underpins clinical management of Type 1 diabetes (1). Daily routines of insulin injection are a familiar feature, the discomfort of which contributes in part to poor patient adherence to prescribed therapy (2). Although insulin is the most common treatment option for the Type 1 diabetic patient, its use is commonplace in therapy of the Type 2 patient, who suffers uncontrolled blood glucose levels unresponsive to diet, exercise, weight control and oral hypoglycaemic medication (3).

Advances in formulation design that incorporate insulin are wide-ranging and form the basis of generic protein-based therapeutics (4). Polymeric nanoparticles (NP) feature often and are used to incorporate an array of therapeutic peptides and proteins (5). Their development is driven, in part, by excellent safety profiles relevant to human use (6). Biodegradable poly(esters), such as poly(D,L-lactic-co-glycolic acid) (PLGA), are popular matrix materials and often modified by co-polymerisation with poly(ethylene glycol) (PEG). This alters hydrophobicity, enhances the drug loading, controls the burst effect, prolongs *in vivo* circulation time by avoiding phagocytosis and, consequently, improves overall bioavailability (7). These polymers have been used to encapsulate *inter alia* lysozyme, recombinant human epidermal growth factor and luteinising hormone-releasing hormone agonist, leading to improvement of pharmacokinetic profiles and minimising frequency of administration (8, 9). They are generally fabricated using emulsion solvent evaporation or solvent-displacement techniques, which rely on primary and secondary emulsion phases of either organic or aqueous character (10).

Studies describing encapsulation of insulin into biodegradable polymeric devices highlight certain problems. A poorly controlled initial release phase is commonplace and

leads to hypoglycaemic shock (11). However, the primary concern is poor stability of insulin after exposure to formulation conditions present during the emulsification and solvent removal-based processes (12). The primary emulsification phases are of particular concern (13). Emulsification of the primary aqueous protein solution with an immiscible solvent, such as dichloromethane, facilitates protein aggregation at the aqueous–organic interfacial boundary (14). The end result is incomplete release (15) or low encapsulation efficiencies (16).

There is a need to improve the sustained release kinetics of insulin and biologically active peptides from biodegradable carriers, whilst preserving activity during fabrication. Formulation studies using potent peptides is often hampered given that available quantities of peptide are often low. Using model drugs, such as insulin, is a common strategy which gathers preliminary data. Formulation of insulin-loaded NP by the double-emulsion technique is commonly done by shearing the system with either sonication or homogenisation (17). Encapsulating insulin using sonication gives high insulin burst release and low entrapment efficiency as troublesome consequences (18). Emulsification using homogenising maintains a linear release profile and higher encapsulation efficiency of hydrophilic drugs that cannot be achieved in particles fabricated by sonication (17), but details of stability are less well documented. Therefore, in this study, sustained release NP for parenteral insulin delivery were evaluated for stability and biological activity during both fabrication steps and release. The formulation strategy used was a modified double-emulsion solvent evaporation technique adopting either homogenisation or sonication to optimise the entrapment efficiency and the initial release of insulin from PLGA and its diblock copolymers containing 5% and 10% PEG. The effect of polymer type,

insulin/polymer loading ratio and concentration of poly(vinyl alcohol) in the internal aqueous phase, on the physicochemical characteristics of insulin-loaded NP, together with *in vitro* release profiles and stability were investigated. Furthermore, we evaluated *in vivo* insulin activity using the IP route, as administration is relatively straightforward in the murine model. It is also relevant to the development of continuous intraperitoneal insulin infusion. IP insulin has been shown to provide adequate glycaemic control, which appears superior to that seen following treatment with conventional SC insulin (19). However, the approach is not without its clinical difficulties and more data is needed to assess long-term safety, which will include evaluation of novel delivery strategies, such as nanoparticulate platforms (19).

2. Materials and Methods

2.1 Materials

PLGA (Resomer®RG 503H) with an average molecular weight of 34 kDa and a lactide-to-glycolide ratio of 50:50 was purchased from Sigma Chemical Co. (St. Louis, USA). Two PEG-PLGA diblock copolymers (Resomer® RGP d 5055 (5% PEG) and Resomer® RGP d 50105 (10% PEG) with PEG molecular weight of 5 kDa, were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Bovine insulin (51 amino acids, MW5.734 kDa), poly(vinyl alcohol) (PVA) 87-89% hydrolysed (MW 31.000-50.000) and phosphate buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, USA). A Micro BCA® Kit was obtained from Pierce Ltd. (Rockford, IL). Dichloromethane was of HPLC grade and all other reagents were of analytical grade or higher purity. Milli-Q-water was used throughout the study.

2.2 Preparation of insulin-loaded NP

A modified, double-emulsion, solvent evaporation technique was used in this work, as illustrated schematically in Fig. 1 (20). Insulin was dissolved in 0.2 ml of internal aqueous phase (0.1 M HCl), which was then emulsified at 6,000 rpm (Silverson L5T, Silverson Machines Ltd., Buckinghamshire, UK) for 2 minutes into 2.0 ml of dichloromethane (DCM) containing 10% w/v of the polymer type under investigation. The primary emulsion (w/o) was injected directly into 50 ml of PVA solution (external aqueous phase) under agitation. Emulsification continued at 10,000 rpm for 6 minutes to produce the secondary emulsion using the same homogeniser.

147 For insulin-loaded NP prepared by sonication, insulin was dissolved in 0.2 ml of
148 0.1 M HCl and then mixed with 2.0 ml of DCM containing 10% w/v of 10% PEG-PLGA.
149 The primary and secondary emulsification steps were performed using an ultrasonic
150 processor equipped with a XL-2020 3.2 mm probe (Misonix Incorporated, NY, USA) in
151 an ice bath for 2 minutes. The emulsion was stirred overnight under vacuum to evaporate
152 the DCM and prevent pore formation on the surface of the NP. After formation, NP were
153 collected by centrifugation at 10,000 x *g* for 30 minutes at 4 °C (Sigma Laborzentrifugen
154 GmbH., Germany), washed three times with ultrapure water and 2% w/v sucrose solution
155 and lyophilised using freeze drying (Labconco., Missouri, USA). The freeze-dried NP
156 were stored in a desiccator at ambient temperature. The formulation variables and
157 identifier codes are listed in Table 1.
158

159 **2.3 NP characterisation**

160 Freeze-dried NP samples (5.0 mg) were mixed with ultrapure water to a suitable
161 concentration and suspended using vortex mixing for 3 minutes. Particle size and its
162 distribution (polydispersity index, PDI) were measured using dynamic light scattering
163 (Zetasizer 5000, Malvern Instruments Ltd., Malvern, UK). NP zeta potential was
164 quantified using laser Doppler anemometry (Zetasizer 5000 (Malvern Instruments Ltd.,
165 Malvern, UK), following dispersal and adjustment of conductivity with 0.001 M KCl. All
166 measurements were performed in triplicate.

167 The NP surface morphology was observed by scanning electron microscopy (SEM)
168 (Quanta 400 FEG, FEI Ltd., Oregon USA). An aliquot of NP was mounted on carbon tape
169 and sputter-coated with gold under vacuum in an argon atmosphere before observation.

2.4 Determination of insulin loading and entrapment efficiency.

Insulin loading was determined using a direct colorimetric method (20). A weighed sample of NP was dissolved in 0.5 ml of 1.0 M NaOH and incubated overnight at 37 °C. The solution was neutralised with 0.5 ml of 1.0 M HCl, centrifuged for 5 minutes at 10,000 x *g* and the supernatant analysed for insulin content using a bicinchoninic acid assay (Micro BCA®) (21). Percentage entrapment efficiency (%EE) was expressed as a ratio of the determined insulin loading to the maximum theoretical loading. An indirect radioimmunoassay method was used to determine insulin content in the supernatant phase during drug release analysis (22). A standard curve of bovine insulin prepared over a concentration range of 3.9×10^{-3} to 2.0×10^{1} ng ml^{-1} was used.

2.5 *In vitro* release studies

Lyophilised insulin-loaded NP (5.0 mg) were suspended in a release medium of 1.0 ml PBS (pH 7.4) and incubated at 37 °C using a reciprocal shaking water bath at a fixed speed of 100 rpm. Samples (100 µl) were taken at predetermined time intervals of 1, 12, 24, 48, 96, 120, 144 and 168 hours and replaced with fresh PBS at the same temperature. The collected samples were centrifuged for 5 minutes at 10,000 x *g* and the insulin content in the supernatant determined using the indirect radioimmunoassay, as described in section 2.4. Each experiment was performed in triplicate.

2.6 Determination of residual PVA

The residual mass of PVA attached to the NP surface after washing was determined by a colorimetric method based on the reaction with iodine. A sample of freeze-dried NP (2.0 mg) was incubated in 0.5 M NaOH for 15 minutes at 60 °C, neutralised by addition of 1.0 M HCl and the final volume adjusted to 5.0 ml with water. To each NP sample, 3.0 ml of iodine reagent (0.65 M solution of boric acid, 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M)) and 1.5 ml of water were added. The absorbance of each sample was measured using visible spectroscopy (Agilent 8453, Agilent Technologies, Palo Alto, CA, USA) at 690 nm after 15 minutes incubation and compared to a standard plot of known PVA concentration (23). Residual PVA was expressed as a percentage mass fraction of the total NP mass.

2.7 *In vitro* stability and protein integrity studies

SDS-PAGE analysis was performed using a BioRad Mini Protean II gel apparatus (Hercules, CA, USA). The final supernatant obtained after 7 days of the *in vitro* release experiment was used as a sample for this study. The sample was prepared under non-reducing conditions for application on a NuPAGE® gel consisting of 4% and 12% stacking and resolving gels, respectively. A fixative solution of Coomassie Brilliant Blue was employed to stain and reveal the protein bands. Insulin dispersed in PBS (pH 7.4) was used as control to simulate release conditions. Electrophoresis was run in constant current mode (50 mA) and fixed voltage modes (60 V and 120 V) during stacking and running stages, respectively (24). The GelDoc-It™ image system was used to record the position of protein bands.

2.8. *In vivo* studies

Experimental animals

Young (8-week-old) male National Institutes of Health Swiss mice (18–20 g) (Harlan, UK) were age matched, divided into four groups and housed individually in an air-conditioned room at 22±2 °C with a 12:12 hour light-dark cycle (08:00–20:00) and free access to a standard diet and water *ad libitum*. To induce Type I diabetes, weight-matched mice received a single intraperitoneal injection of streptozotocin (STZ, 150 mg kg^{-1}) dissolved in citrate buffer (pH 4.5). Mice with fasting blood glucose level >8 mmol l^{-1} 72 hours after STZ administration were considered diabetic and included in the study. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 and Ulster University's Animal Ethics Committee guidelines.

In vivo bioactivity of insulin formulation

Four groups (n=6) of insulin-deficient STZ-induced diabetic mice were used for this study. Insulin-loaded NP and free insulin were suspended in sterile PBS and injected intraperitoneally at a dose of 25 U kg^{-1} body weight. The two control groups comprised mice treated with saline or blank NP. Blood samples were withdrawn by tail vein puncture prior to injection and at predetermined time points thereafter. Blood glucose and insulin were measured. Blood glucose concentrations were determined instantaneously by the glucose oxidase method using a handheld Ascencia Counter meter (Bayer Healthcare, UK) (25), while plasma insulin was measured by radioimmunoassay (22), as described previously.

2.9 Statistical analysis

Results are presented as mean ± SD and mean ± SEM for *in vitro* and *in vivo* studies, respectively. Comparative analyses between groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Area-under-the-curve (AUC) analysis was performed using the trapezoidal rule with baseline correction. A value of $p<0.05$ was considered statistically significant.

3. Results and discussion

3.1 Effect of polymer type on 9% insulin-loaded NP (F1, F4 and F7)

The physicochemical characterisation of insulin-loaded NP fabricated with three different polymer types is shown in Table 1. For an insulin loading of 9%, PEGylated NP (F4 and F7) prepared from 5% and 10% PEG-PLGA diblock copolymers, respectively, were significantly lower in size ($p<0.05$) than NP prepared from PLGA (F1). This can be explained by the short chain length of PEG-PLGA compared to PLGA, as well as the presence of covalently linked hydrophilic PEG that decreased polymer association during NP formation, which eventually resulted in the formation of smaller particles (26). All insulin-loaded NP formulations showed low PDI values ranging from 0.21 to 0.43. Increasing PEG content in the polymer backbone caused a further decrease in the NP size, leading to the smaller size of F7 compared to F4. These results are in good agreement with previous reports (20).

PLGA NP exhibited higher negative zeta potential values when compared to PEGylated NP (F4 and F7), but no significant difference between the two latter types was observed. PEG chains on the NP surface are expected to mask surface charge arising from PLGA (7). This reduction of surface charge is a drawback for the PEGylated types, given that NP are notoriously difficult to stabilise when dispersed or suspended in aqueous media. Attractive forces cause NP to agglomerate or aggregate, and surface charges play an important role in preventing this effect. However, during this work, no discernible effects relating to aggregation were observed. To obtain long-term stable NP samples, the dispersions were freeze-dried and NP were re-dispersed in a suitable medium prior to the *in vitro* or *in vivo* experiments.

PEGylation resulted in a significant increase ($p<0.05$) in insulin loading and encapsulation efficiency. PEG-PLGA is an amphiphilic polymer and creates a benign environment for encapsulating hydrophilic molecules, like insulin, through its hydrophilic PEG chains. This slows peptide diffusion to the external aqueous phase and results in high loading efficacy (27). The higher encapsulation efficiency of F7 compared to F1 and F4 is due to the lower solubility of 10% PEG-PLGA in DCM, which resulted in faster polymer precipitation that prevented insulin escape to the outer phase and consequently increased its entrapment efficiency (28).

In vitro release profiles of F1, F4 and F7 are shown in Fig. 2. Insulin-loaded NP prepared with PEGylated polymers displayed an initial burst release, followed by a steady release profile. The burst release from insulin-loaded PLGA NP was smaller in comparison to PEGylated polymers, followed by a more sustained phase. The burst release of insulin is associated with its peripheral loading, which enables it to diffuse quickly towards the release medium (29) and is more prevalent in the PEGylated types. The burst release was faster and significantly higher from F4 and F7 when compared to F1 ($p<0.05$), with greater cumulative release after 24 hours. Results showed sustained release of insulin from F1, whereas release from F4 and F7 approached complete release over a 7-day period. The release of insulin from PEGylated NP is governed by its diffusion through a swollen inner structure formed after contact with the aqueous release medium due to the presence of hydrophilic PEG moieties in the polymer matrix (20).

3.2 Effect of insulin/polymer loading ratio

The influence of insulin loading (9%, 6% and 3%) is captured in comparisons of formulations of PLGA (F1, F2 and F3), 5% PEG-PLGA (F4, F5 and F6) and 10% PEG-PLGA (F7, F8 and F9). Results from Lamprecht *et al*. (30) show that there is no effect of protein loading on NP size. However, the results in this work showed that increasing insulin loading from 3% to 9% resulted in a significant increase ($p<0.05$) in the size of PLGA and PEGylated NP. In addition, blank NP were significantly lower in size when compared to insulin-loaded types. This is likely due to insulin in the primary emulsion droplets driving up their size, and insulin present on the NP surface, which together increases the mean NP diameter.

Increasing the ratio of insulin to polymer had a significant effect on PDI values and encapsulation efficiency ($p<0.05$), but no effect on surface charge. Importantly, the increase in insulin loading resulted in a significant decrease ($p<0.05$) in %EE. Although this may at first seem counter-intuitive, it can be explained in terms of concentration gradients driving drug out of the primary droplets. Increasing insulin concentration gradients in this way may deplete droplets of payload more effectively and dump free insulin into the continuous water phase (30).

The *in vitro* release profiles and the initial bursts were directly related to the degree of theoretical insulin loading, as shown in Fig. 3, 4 and 5. There was a positive correlation between insulin loading and the extent of the initial burst phase. High insulin burst and rapid release rate could be attributed to the drug concentration gradient between the NP and the release media. Moreover, the diffusion of surface-associated insulin facilitates the

311 formation of water-filled channels that allow subsequent elution of the core-residing insulin
312 (31, 32).
313
314 **3.3 Effect of internal aqueous phase stabiliser concentration**
315 PVA was used as a stabilising agent in this study and set at three different concentrations,
316 namely 0.00%, 1.25% and 2.50% w/v. The resulting physicochemical properties of
317 different NP formulations of PLGA (F3, F10 and F11), 5% PEG-PLGA (F6, F12 and F13)
318 and 10% PEG-PLGA (F9, F14 and F15) are shown in Table 1. The NP size was
319 significantly decreased ($p<0.01$) by PVA addition to the primary emulsion. Increasing
320 PVA concentration caused further decreases in the mean NP size. There are differing views
321 in the literature on whether the primary or secondary emulsion phases have the greater
322 influence on particle size. It is proposed that PVA stabilises the inner water droplets against
323 coalescence, which produces smaller primary emulsion droplets and resultantly smaller NP
324 sizes (31). However, this is in contrast to findings of Bilati *et al*. (33) who concludes that
325 the secondary emulsification step effectively determines the final particle size in double-
326 emulsion techniques. In this current study, particle size reduction was more significant in
327 PEGylated NP when compared to PLGA NP ($p<0.05$). Since PLGA is more hydrophobic
328 than a diblock copolymer like PEG-PLGA, the interactions between PVA and PEGylated
329 polymers will be stronger than those to PLGA alone. Therefore, our results support the
330 role of the primary emulsion on determining final particle size, especially if PEGylated
331 polymers are used.
332 PDI values were significantly decreased by adding PVA to the internal phase,
333 whereas a negligible effect on the surface charge was observed. Addition of internal phase

stabiliser had a no significant impact on the encapsulation efficiency in all types of NP, although a decreasing trend was observed. This may be due to the decreasing particle size, resulting in a larger interfacial area for mass transfer (34). These results are not observed in other work, which show a sharp increase in encapsulation efficiency by adding PVA (or other stabilisers), possibly by improved stabilisation of the primary emulsion (36) or payload-PVA interactions (31, 35). Clearly, further detailed study is required to determine the exact effect of PVA on encapsulation efficiency.

The effect of internal aqueous phase stabiliser on the insulin release from different NP formulations is shown in Fig. 6. A significant increase ($p<0.05$) in the release rate after PVA addition was observed, and the amount of initial release also increased concomitantly. The *in vitro* release profile of F11, F13 and F15 with 2.5% w/v PVA was significantly faster than the release from NP prepared with 1.25% w/v PVA and NP without PVA. Initial bursts of 49%, 61% and 68% were observed from F11, F13 and F15, respectively, compared to 27%, 42% and 43% from F3, F6 and F9, respectively. The results in Fig. 6 indicate that increasing PVA concentration increased the early stage release, possibly by way of enhanced peripheral accumulation of insulin close to the NP surface. In addition, higher concentrations of PVA enable porosity to form in the NP matrix, which facilitates outwards diffusion of insulin (34, 36).

3.4 Comparison between homogenisation and sonication

In this work, minimising NP size and maximising %EE was considered as an outcome from optimisation of the formulation variables. Formulation F9, which was prepared by homogenisation using 10% PEG-PLGA, contained NP that were significantly lower in size

and higher in %EE (P value <0.05) compared to formulations fabricated by other types of polymers under similar conditions. This formulation was, therefore, chosen for comparison to methods that used sonication instead of homogenisation. It is also important to note that these optimised NP (size < 400 nm and encapsulation efficiency > 90%) are clearly distinguishable from other published work, such as that of Liu *et al*. (37).

Insulin-loaded NP (F16) prepared by sonication resulted in a decrease in %EE compared to NP prepared by homogenisation (F9). Size and PDI values were significantly ($p<0.01$) higher. The release profile of F16 showed significantly higher initial and higher total amounts of insulin released ($p<0.05$) when compared to similar data from F9 (Fig. 7). Release data for F9 and F16 follow a profile that is typical of drug delivery systems that display Higuchi kinetics. Indeed, fitting cumulative percentage drug release data in Fig. 7 to the square root of time (data not shown) confirmed this assumption.

3.5 Scanning electron microscopy

Fig. 8 shows a micrograph of the optimised insulin-loaded NP (F9). NP were of spherical shape with a size distribution in approximate agreement with light scattering data. The smooth NP surface was free from void and pores. After 7 days of *in vitro* release, there was little evidence of the presence of spherical structures. Instead, the recovered polymeric material was irregular and sheet-like (Fig. 8B). As F9 has a high PEG content, pore formation during exposure to aqueous media is expected, which may explain poor retention of spherical shape and the enhanced release observed in Fig. 7 (20).

3.6 Residual PVA

PVA is an emulsifier, widely used in the preparation of PLGA NP, and popular because of its ability to produce uniform NP with a size easily dispersed in aqueous media. Importantly, residual PVA associated with PLGA NP can modify their bio-physicochemical properties (38). The addition of PVA to the internal aqueous phase had no impact on the residual PVA attached to all types of NP. Residual PVA determined in different types of NP was less than 7%, being higher for PEGylated NP (Fig. 9). The higher adsorption of PVA to the surface of PEGylated NP is attributed to the amphiphilic characters of these polymers due to PEG chains, with possible PVA interaction. The presence of PEG chains increases the surface area of the NP and consequently increase the probability of hydrophilic molecules, like PVA, to attach to its surface (39). These results contradict those of others (29) who found a reduction in PVA adsorption when PEG is used to modify PLA during NP formation. Thus, it is clear that the alteration in NP surface hydrophilicity using co-polymerised PEG does not produce a clear trend when PVA adsorption is considered.

3.7 *In vitro* stability and protein integrity

Preserving secondary and tertiary structures of proteins and peptides is essential in maintaining therapeutic efficacy. Stability issues are affected by different formulation conditions with attention focused on the harsh fabrication stress employed during emulsification-based methods (40). Encapsulation of insulin using the double-emulsion technique enables it to act as a surface active molecule, which tends to adsorb at the primary emulsion water/oil interfaces. This adsorption may lead to instability by unfolding,

inactivation or irreversible aggregation. In addition, the high shear stress used for effective emulsification may influence the three-dimensional structure of insulin, which is essential for its therapeutic activity (41).

Aggregation is a major concern for protein-loaded PLGA microspheres, caused by the acidic environment when polymer degradation products accumulate during the release phase (42). To investigate the possibility of insulin aggregation after formulation and *in vitro* release, the integrity of the insulin released after seven days was evaluated. The three single lines in the gels (Fig. 10), representing the insulin band of the naked and encapsulated insulin released from F9 and F16, provided evidence that the entrapped insulin did not suffer any significant aggregation during its formulation and after *in vitro* release (36). Although use of a stability indicating method, such as HPLC, would highlight possible degradation, quantification by radioimmunoassay in this work supported the lack of degradation or aggregation of encapsulated insulin. This stability is attributed to co-polymerised PEG, which prevents an acidic micro-environment within particulate systems (43). Furthermore, the hydrophobicity of the PLGA surface is masked by PEG, reducing insulin re-adsorption and preventing possible aggregation (44). Both insulin-loaded NP prepared by homogenisation or sonication maintained insulin integrity after fabrication and *in vitro* release.

3.8 *In vivo* bioactivity

It is well-documented that formulation steps inactivate or otherwise denature a proportion of encapsulated insulin (45). If this occurs, then bioactivity is compromised and careful *in vivo* assessment is required. Therefore, in this work, we determined the bioactivity of an

optimised NP formulation in insulin-deficient Type 1 diabetic mice. The effect of a single intraperitoneal injection of (25 U kg^{-1}) of free insulin and insulin-loaded NP (F9) on blood glucose levels in type I diabetic mice is shown in Fig. 11A-11C. Free insulin significantly decreased plasma glucose ($P < 0.001$) directly after injection, compared to the blank NP and saline groups. Blood glucose concentrations decreased by 69.1% ($P < 0.001$) 2 hours after administration. However, following this initial reduction, blood glucose concentrations in free insulin-injected mice returned rapidly to control diabetic levels. This is in accordance with the short half-life of parenterally delivered insulin (20).

Insulin-loaded NP induced a significant and sustained glucose lowering effect, as expected from *in vitro* release data. At the 2-hour time point, insulin-loaded NP had reduced blood glucose levels by 48.3% in our Type 1 diabetic subjects, and these mice had significantly lower glucose levels compared to free insulin-treated mice at 3 hours, post-injection. The clear difference between the glycaemic statuses of the two groups of mice was apparent throughout the entire 24-hour observation period, highlighted by significantly lowered blood glucose concentrations in mice treated with insulin-loaded NP at 24 hours. Encouragingly, blood glucose levels remained within the normal range in insulin-loaded NP treated mice (5-7 mmol l^{-1}) throughout the 24-hour period, with no obvious signs of hypoglycaemia. This is a foremost advantage for any insulin-containing formulation (20). Our results showed a clear effect on blood glucose concentration, which is in contrast to the results of Liu *et al*. (37), who showed insignificant decreases in blood glucose levels following insulin-loaded NP administration within the first 12 hours.

Plasma insulin profiles closely mirrored blood glucose levels in all groups of mice, confirming that the initial burst release and subsequent sustained release of insulin for the

NP were key features of the improved glycaemic status (20). Plasma insulin levels in mice injected with free insulin (Fig. 12A) revealed a mean peak for plasma insulin concentration (C_{max}) of 21.3 ng ml^{-1} observed after one hour, with levels returning to basal, 3 hours after administration. Mice injected with insulin-loaded NP showed a peak insulin concentration of 14.5 ng ml^{-1}, which occurred 3 hours after injection and was significantly ($P < 0.001$) higher than the free insulin group. Plasma insulin levels remained elevated in insulin-loaded NP treated mice, and were still significantly ($P<0.01$) greater than free insulin injected mice 24 hours post-injection. AUC analysis (Fig. 11C and 12A) confirmed the sustained and enhanced bioactive profile of released insulin, confirming preservation of its biological activity. Although further studies are required, it is feasible that the remaining insulin within the NP (over 50%) would be slowly released to further sustain normoglycaemic status beyond 24 hours.

4. Conclusions

The aqueous solubility of insulin hinders entrapment efficiency and gives rise to a pronounced burst release from particulate systems. High encapsulation efficiency, low initial release and maintaining stability are essential requirements for sustained insulin delivery from biodegradable formulations. In this current study, we modified key features of the double emulsion technique, such as pH of the internal phase, the ratio of internal phase to external phase and homogenisation speed. After optimisation of the formulation conditions, the %EE of insulin reached 91.5% and the burst release within the first 24 hours was restricted to 28%. An optimised insulin-loaded NP was fabricated by homogenisation from 10% PEG-PLGA with low insulin loading and without any surfactants in the primary

emulsion. Insulin-loaded NP prepared by homogenisation showed lower particle size, lower PDI, higher encapsulation efficiency and lower initial release compared to NP prepared by sonication. PEGylated (10%) PLGA polymer exerted a protective effect on insulin stability by suppressing adsorption phenomena and preventing insulin aggregation. The optimised insulin-loaded NP controlled *in vitro* insulin release for 168 hours. Both *in vitro* and *in vivo* data were in close agreement. Intraperitoneal administration showed effective and pronounced glycaemic control, which verified preservation of biological activity and confirmed the sustained release profiles determined during release studies.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

1. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. Can Med Assoc J 1922;12:141-6.
2. Hanas R, Ludvigsson J. Experience of pain from insulin injections and needle-phobia in young patients with IDDM. Practical Diabetes International. 1997;14:95-9.
3. Sheeja VS, Reddy MH, Joseph J, Reddy ND. Insulin therapy in diabetes management. Int J Pharm Sci Rev Res 2010;2:98-105.
4. Pisal DS, Kosloski MP, Balu-Iyer SV. DELIVERY OF THERAPEUTIC PROTEINS. J Pharm Sci 2010;99:2557-75.
5. Haggag YA, Faheem AM. Evaluation of nano spray drying as a method for drying and formulation of therapeutic peptides and proteins. Frontiers in Pharmacology. 2015;6:1-5.
6. Cai Q, Shi G, Bei J, Wang S. Enzymatic degradation behavior and mechanism of poly(lactide-co-glycolide) foams by trypsin. Biomaterials. 2003;24:629-38.
7. Xiao RZ, Zeng ZW, Zhou GL, Wang JJ, Li FZ, Wang AM. Recent advances in PEG-PLA block copolymer nanoparticles. Int J Mol Med 2010;5:1057-65.
8. Li Y, Pei Y, Zhang X, Gu Z, Zhou Z, Yuan W, et al. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. J Control Release 2001;71:203-11.
9. Han K, Lee KD, Gao ZG, Park JS. Preparation and evaluation of poly(L-lactic acid) microspheres containing rhEGF for chronic gastric ulcer healing. J Control Release 2001;75:259-69.
10. Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials 2000;21:2475-90.
11. Takenaga M, Yamaguchi Y, Kitagawa A, Ogawa Y, Mizushima Y, Igarashi R. A novel sustained-release formulation of insulin with dramatic reduction in initial rapid release. J Control Release 2002;79:81-91.

12. Ibrahim MA, Ismail A, Fetouh MI, Göpferich A. Stability of insulin during the erosion of poly(lactic acid) and poly(lactic-co-glycolic acid) microspheres. J Control Release 2005;106:241-52.
13. Li X, Zhang Y, Yan R, Jia W, Yuan M, Deng X, et al. Influence of process parameters on the protein stability encapsulated in poly-DL-lactide-poly(ethylene glycol) microspheres. J Control Release 2000;68:41-52.
14. Diwan M, Park TG. Pegylation enhances protein stability during encapsulation in PLGA microspheres. J Control Release 2001;73:233-44.
15. van de Weert M, van't Hof R, van der Weerd J, Heeren RM, Posthuma G, Hennink WE, et al. Lysozyme distribution and conformation in a biodegradable polymer matrix as determined by FTIR techniques. J Control Release 2000;68:31-40.
16. Jiang G, Thanoo BC, DeLuca PP. Effect of osmotic pressure in the solvent extraction phase on BSA release profile from PLGA microspheres. Pharm Dev Technol 2002;7:391-9.
17. Blum JS, Saltzman WM. High loading efficiency and tunable release of plasmid DNA encapsulated in submicron particles fabricated from PLGA conjugated with poly-L-lysine. J Control Release 2008;129:66-72.
18. Fonte P, Soares S, Costa A, Andrade JC, Seabra V, Reis S, et al. Effect of cryoprotectants on the porosity and stability of insulin-loaded PLGA nanoparticles after freeze-drying. Biomatter. 2012;2:329-39.
19. Almalki MH, Altuwaijri MA, Almehthel MS, Sirrs SM, Singh RS. Subcutaneous versus intraperitoneal insulin for patients with diabetes mellitus on continuous ambulatory peritoneal dialysis: meta-analysis of non-randomized clinical trials. Clin Invest Med 2012;35:132-143.
20. Haggag Y, Abdel-Wahab Y, Ojo O, Osman M, El-Gizawy S, El-Tanani M, et al. Preparation and in vivo evaluation of insulin-loaded biodegradable nanoparticles prepared from diblock copolymers of PLGA and PEG. Int J Pharm 2016;499:236-46.

21. Kang F, Singh J. Preparation, in vitro release, in vivo absorption and biocompatibility studies of insulin-loaded microspheres in rabbits. AAPS Pharm Sci Tech 2005;6:E487-E94.

22. Flatt PR, Bailey CJ. Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. Diabetologia 1981;20:573-7.

23. Joshi DP, Lan-Chun-Fung YL, Pritchard JG. Determination of poly(vinyl alcohol) via its complex with boric acid and iodine. Anal Chim Acta 1979;104:153-60.

24. Park TG, Yong Lee H, Sung Nam Y. A new preparation method for protein loaded poly(d,l-lactic-co-glycolic acid) microspheres and protein release mechanism study. J Control Release 1998;55:181-91.

25. Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol 1969;22:158-61.

26. Beletsi A, Panagi Z, Avgoustakis K. Biodistribution properties of nanoparticles based on mixtures of PLGA with PLGA-PEG diblock copolymers. Int J Pharm 2005;298:233-41.

27. Locatelli E, Comes Franchini M. Biodegradable PLGA-b-PEG polymeric nanoparticles: synthesis, properties, and nanomedical applications as drug delivery system. J Nanopart Res 2012;14:1-17.

28. Mehta RC, Thanoo BC, Deluca PP. Peptide containing microspheres from low molecular weight and hydrophilic poly(d,l-lactide-co-glycolide). J Control Release 1996;41:249-57.

29. Essa S, Rabanel JM, Hildgen P. Effect of polyethylene glycol (PEG) chain organization on the physicochemical properties of poly(D, L-lactide) (PLA) based nanoparticles. Eur J Pharm Biopharm 2010;75:96-106.

30. Lamprecht A, Ubrich N, Hombreiro Perez M, Lehr C, Hoffman M, Maincent P. Influences of process parameters on nanoparticle preparation performed by a double emulsion pressure homogenization technique. Int J Pharm 2000;196:177-82.

31. Yang YY, Chung TS, Ng NP. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 2001;22:231-41.

32. Hora MS, Rana RK, Nunberg JH, Tice TR, Gilley RM, Hudson ME. Release of human serum albumin from poly(lactide-co-glycolide) microspheres. Pharm Res 1990;7:1190-4.
33. Bilati U, Allemann E, Doelker E. Sonication parameters for the preparation of biodegradable nanocapsules of controlled size by the double emulsion method. Pharm Dev Technol 2003;8:1-9.
34. Li X. Investigation on process parameters involved in preparation of poly-co-lactide-poly(ethylene glycol) microspheres containing Leptospira Interrogans antigens. Int J Pharm 1999;178:245-55.
35. Mobarak DH, Salah S, Elkheshen SA. Formulation of ciprofloxacin hydrochloride loaded biodegradable nanoparticles: optimization of technique and process variables. Pharm Dev Technol 2014;19:891-900.
36. 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.
37. Liu J, Zhang SM, Chen PP, Cheng L, Zhou W, Tang WX, et al. Controlled release of insulin from PLGA nanoparticles embedded within PVA hydrogels. J Mater Sci-Mater M 2007;18:2205-10.
38. Sahoo SK, Panyam J, Prabha S, Labhasetwar V. Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release 2002;82:105-14.
39. Simón-Yarza T, Formiga FR, Tamayo E, Pelacho B, Prosper F, Blanco-Prieto MJ. PEGylated-PLGA microparticles containing VEGF for long term drug delivery. Int J Pharm 2013;440:13-8.
40. Bummer PM. Chemical considerations in protein and peptide stability. 2 ed. E.J. McNally JEH, editor: Informa Healthcare; 2007.
41. Perez C, Castellanos IJ, Costantino HR, Al-Azzam W, Griebenow K. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. J Pharm Pharmacol 2002;54:301-13.

42. Schwederman SP, Cardamone M, Brandon MR, Klibanov A, Langer R. Stability of proteins and their delivery from biodegradable microspheres. S. Cohen HB, editor. New York: Marcel Dekker; 1996.
43. Jiang W, Schwendeman SP. Stabilization and controlled release of bovine serum albumin encapsulated in poly(D, L-lactide) and poly(ethylene glycol) microsphere blends. Pharm Res 2001;18:878-85.
44. Lucke A, Teßmar J, Schnell E, Schmeer G, Göpferich A. Biodegradable poly(d,l-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers: structures and surface properties relevant to their use as biomaterials. Biomaterials 2000;21:2361-70.
45. Carino GP, Jacob JS, Mathiowitz E. Nanosphere based oral insulin delivery. J Control Release 2000;65:261-9.

Table 1. Formulation variables for insulin nanoparticles

Formulation ID	Polymer type	Insulin loading (w/w %)	Internal aqueous phase PVA concentration (% w/v)	Size (nm)	*PDI	Zeta potential (mV)	%EE
Homogenisation							
F1	PLGA	9	0.00	396.5±20.9	0.43±0.05	-21.98±2.82	42.89±3.01
F2	PLGA	6	0.00	357.0±14.4	0.36±0.01	-20.15±1.96	56.73±3.45
F3	PLGA	3	0.00	322.5±15.0	0.29±0.02	-20.03±0.95	70.33±5.99
F4	5%PEG-PLGA	9	0.00	357.5±14.4	0.39±0.06	-6.11±1.04	52.17±3.02
F5	5% PEG-PLGA	6	0.00	310.3±16.7	0.32±0.11	-5.66±1.53	68.89±3.28
F6	5% PEG-PLGA	3	0.00	273.5±8.5	0.25±0.01	-6.21±1.91	80.84±4.23
F7	10%PEG-PLGA	9	0.00	317.5±16.5	0.40±0.04	-5.21±0.98	61.18±3.82
F8	10%PEG-PLGA	6	0.00	272.5±17.	0.33±0.01	-6.05±1.85	73.88±4.16
F9	10%PEG-PLGA	3	0.00	239.3±13.0	0.25±0.03	-5.36±1.70	91.52±4.59
F10	PLGA	3	1.25	300.5±11.8	0.22±0.01	-19.26±1.47	63.76±3.06
F11	PLGA	3	2.50	277.5±14.7	0.21±0.01	-20.15±1.69	61.66±2.87
F12	5%PEG-PLGA	3	1.25	240.8±15.1	0.21±0.02	-5.76±0.72	74.39±2.79
F13	5%PEG-PLGA	3	2.50	200.3±8.5	0.22±0.01	-5.91±1.12	71.23±5.27
F14	10%PEG-PLGA	3	1.25	197.5±6.8	0.21±0.02	-5.41±0.83	82.45±5.08
F15	10%PEG-PLGA	3	2.50	160.0±15.7	0.21±0.01	-5.80±1.06	80.88±5.42
Blank NP	PLGA	0	0.00	269.5±13.5	0.31±0.06	-19.58±1.78	-
Blank NP	5%PEG-PLGA	0	0.00	211.5±17.6	0.24±0.07	-6.77±0.97	-
Blank NP	10%PEG-PLGA	0	0.00	187.8±9.8	0.21±0.04	-5.01±1.43	-
Sonication							
F16	10%PEG-PLGA	3	0.00	283.8±16.3	0.40±0.05	-5.93±1.33	84.01±5.72

*polydispersity index

Fig. 1

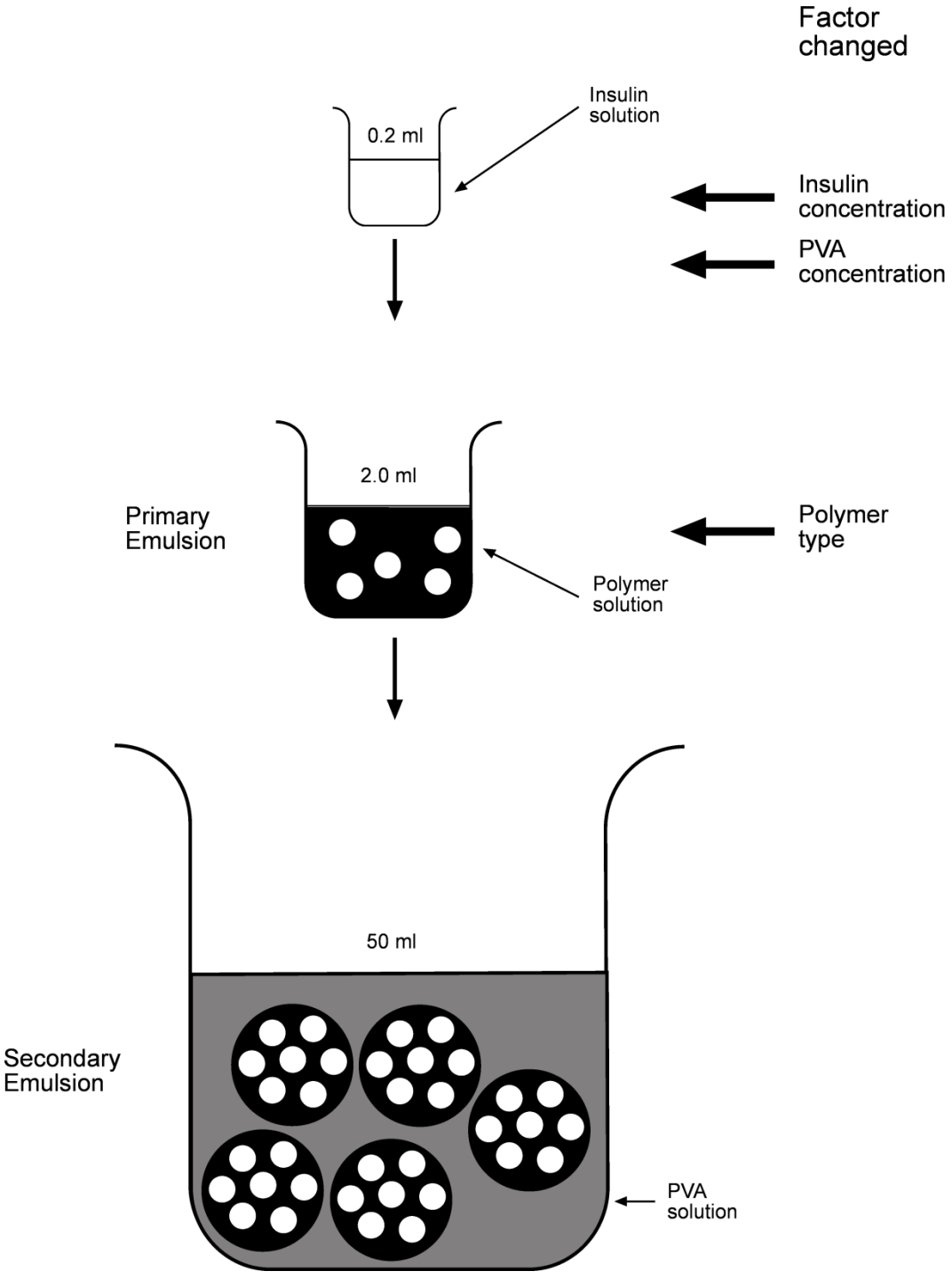

Factor changed
Insulin solution
0.2 ml
Insulin concentration
PVA concentration
2.0 ml
Primary Emulsion
Polymer type
Polymer solution
50 ml
Secondary Emulsion
PVA solution

Fig. 2

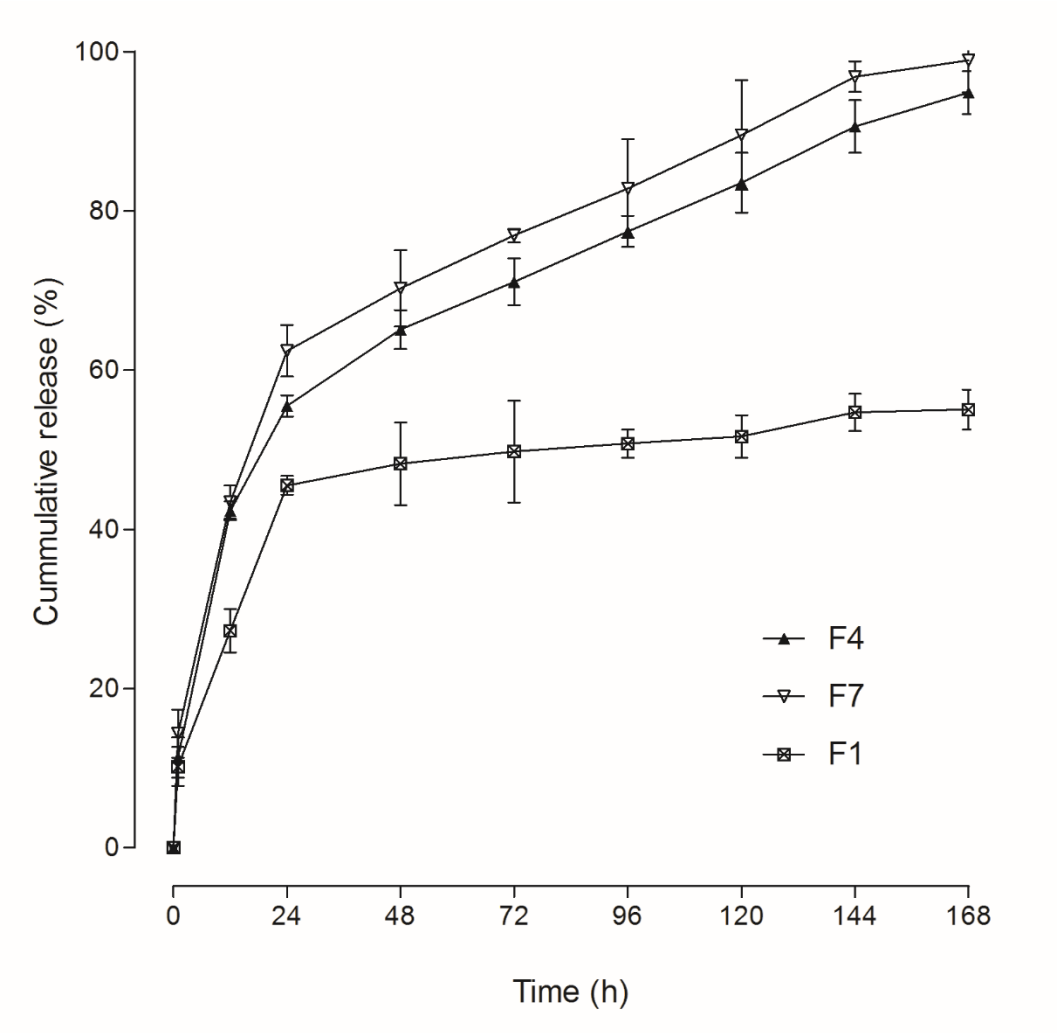

100
80
60
40
20
0
Cummulative release (%)
0
24
48
72
96
120
144
168
Time (h)
F4
F7
F1

Fig. 3

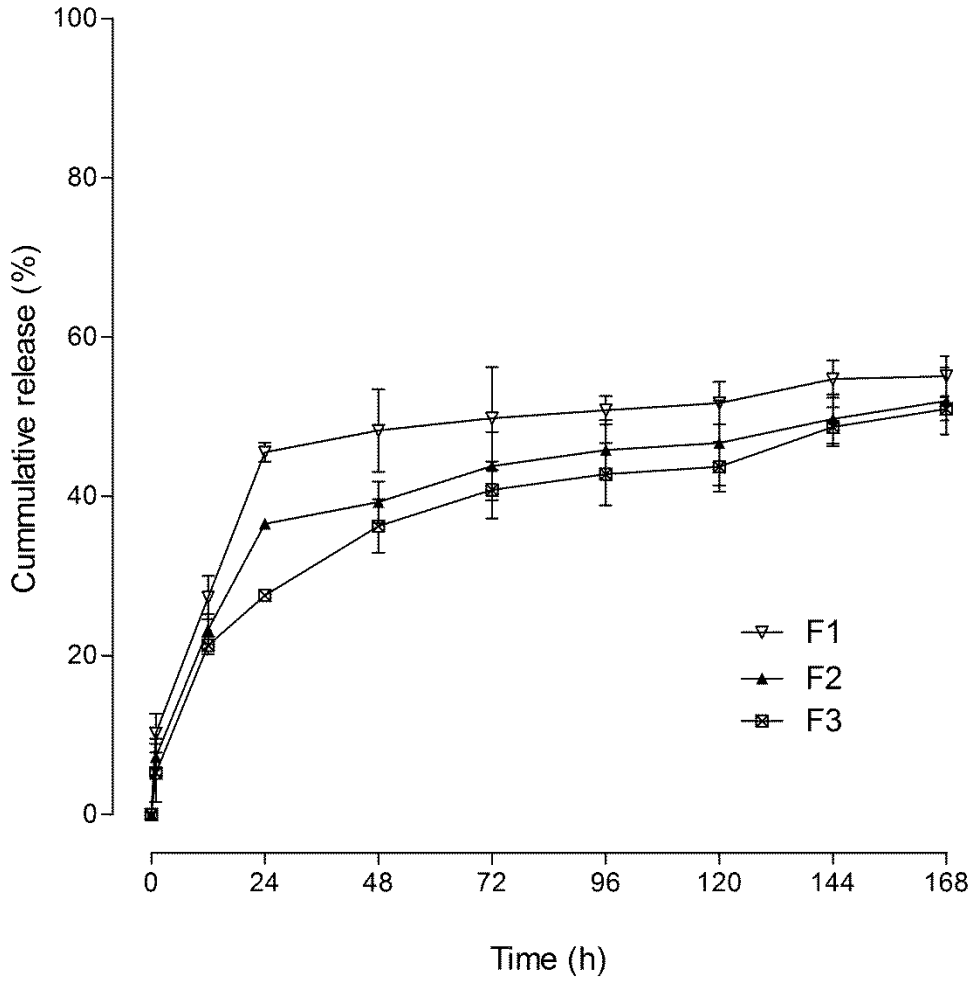

100
80
60
40
20
0
Cummulative release (%)
0
24
48
72
96
120
144
168
Time (h)
F1
F2
F3

Fig. 4

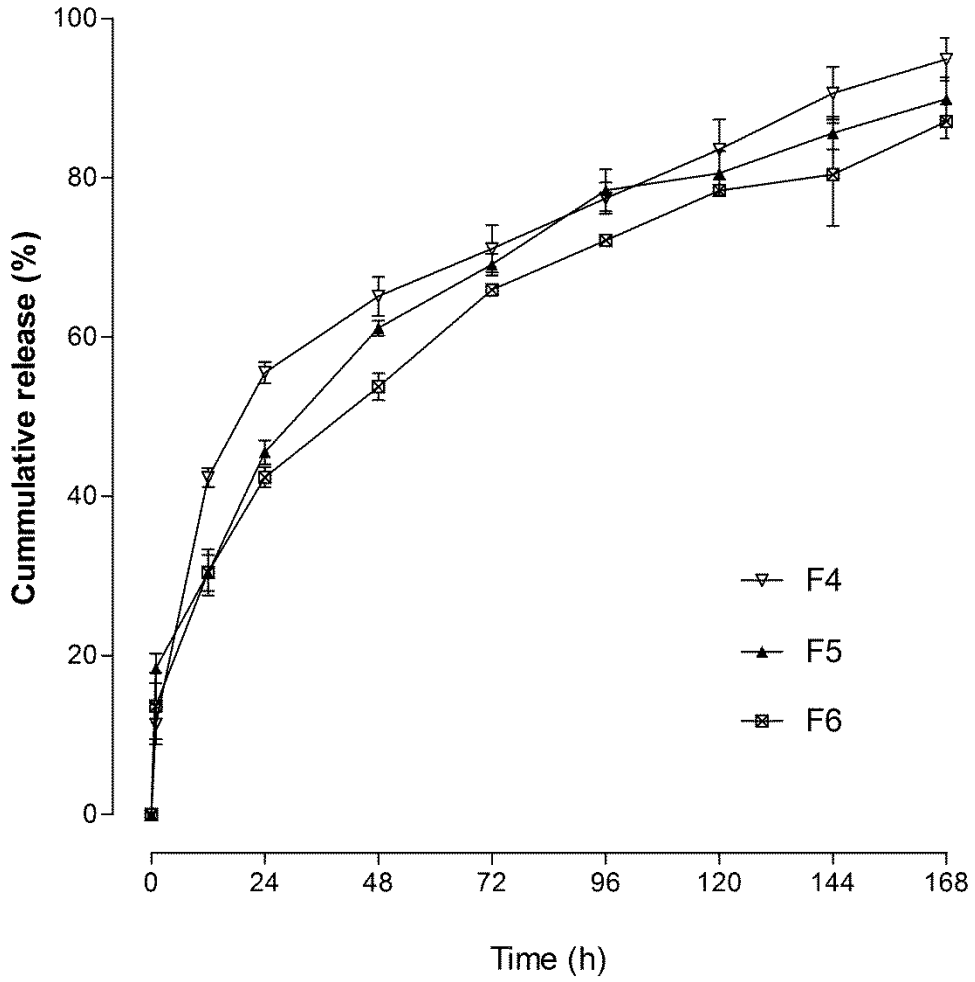

Cummulative release (%)
100
80
60
40
20
0
0
24
48
72
96
120
144
168
Time (h)
F4
F5
F6

Fig. 5

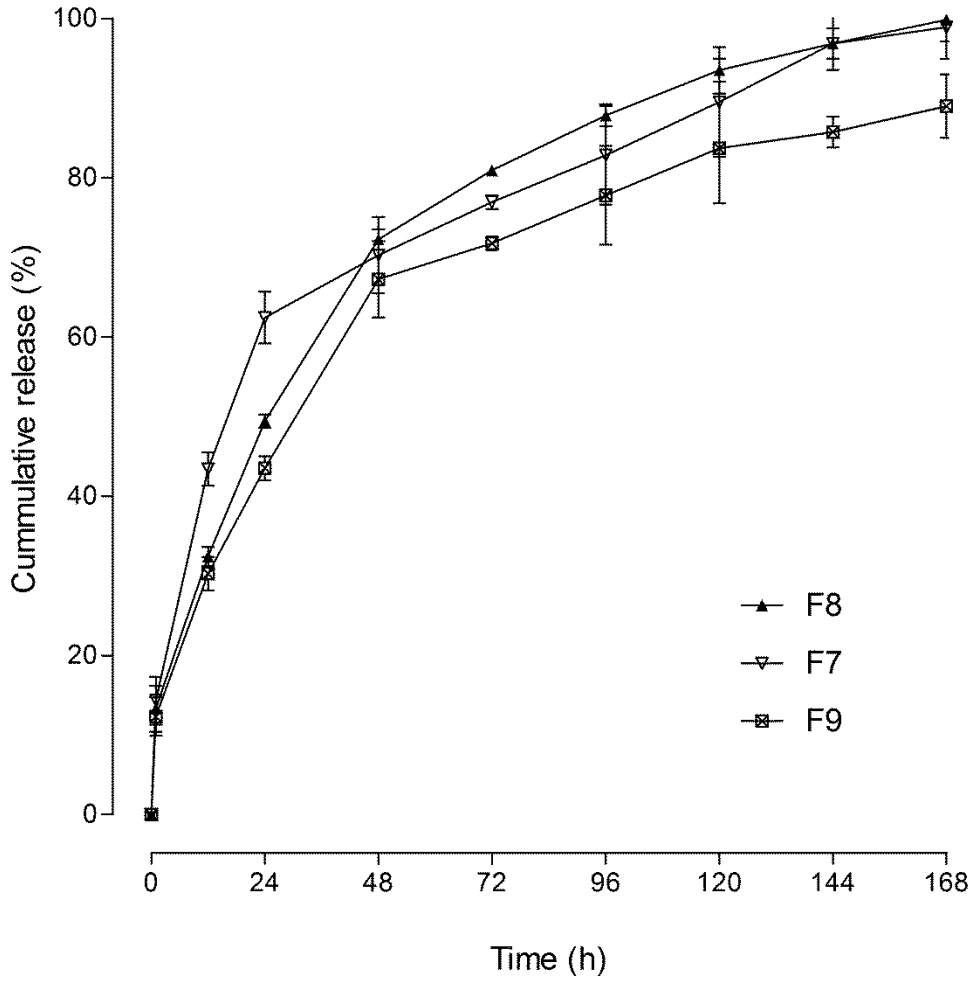

Cummulative release (%)
100
80
60
40
20
0
0
24
48
72
96
120
144
168
Time (h)
F8
F7
F9

Fig. 6

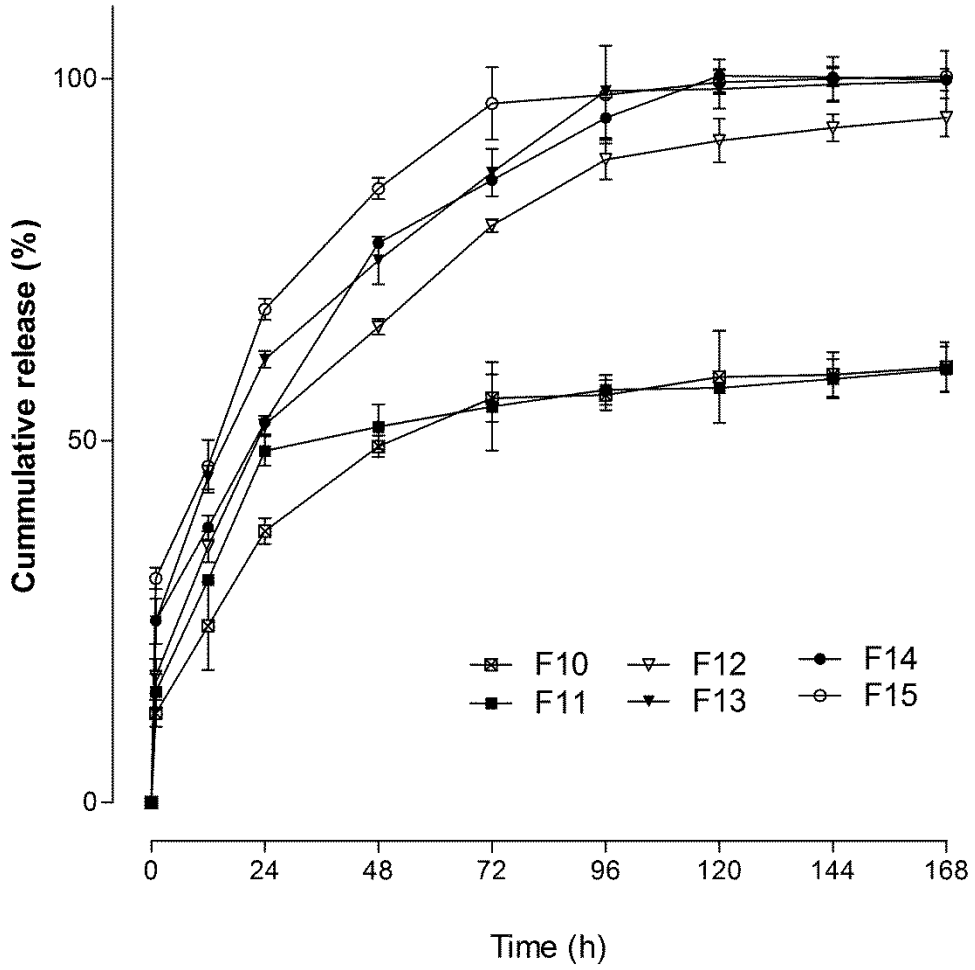

Cummulative release (%)
100
50
0
0
24
48
72
96
120
144
168
Time (h)
F10
F11
F12
F13
F14
F15

Fig. 7

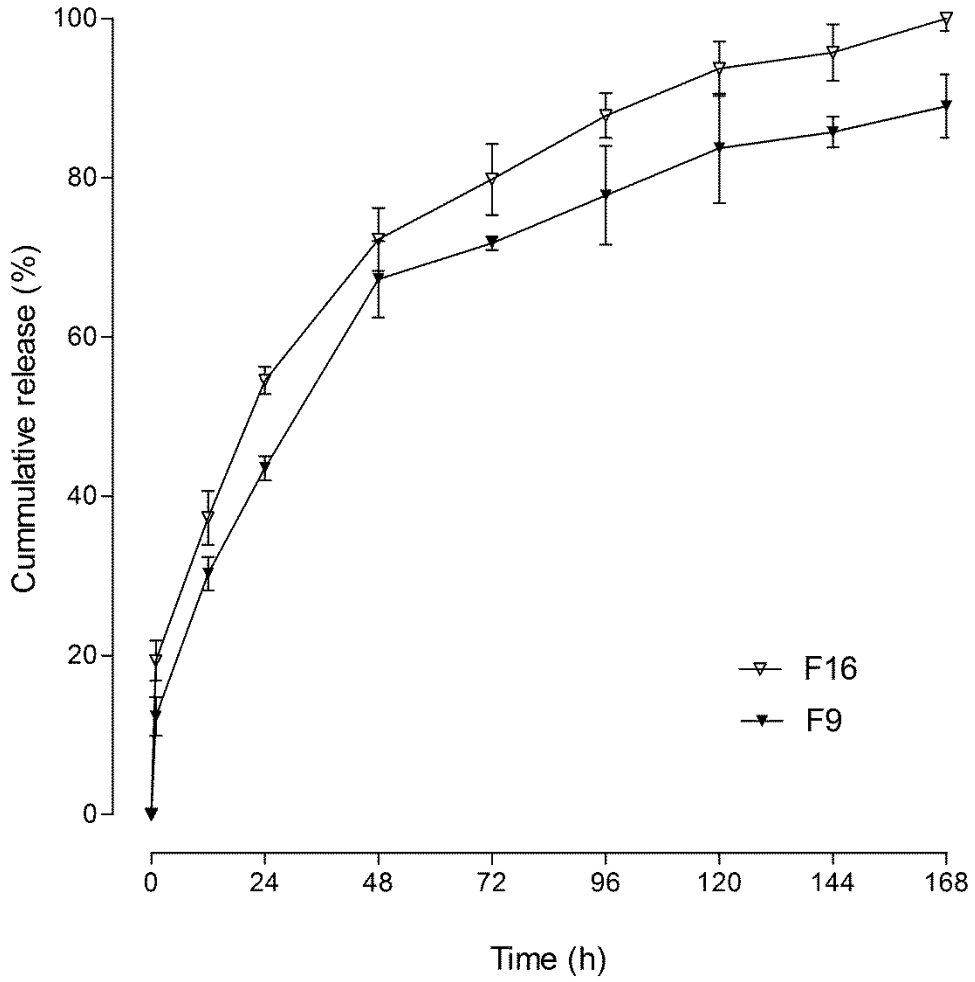

100
80
60
40
20
0
Cummulative release (%)
0
24
48
72
96
120
144
168
Time (h)
F16
F9

Fig. 8

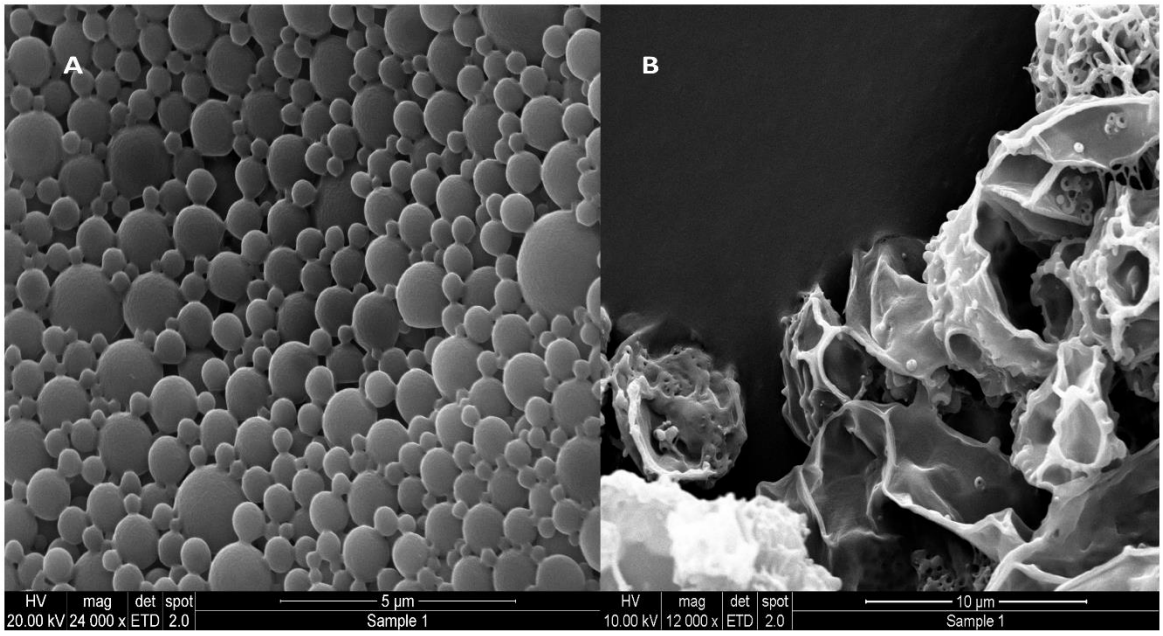

A
B
HV
mag
det
spot
5 µm
20.00 kV
24 000 x
ETD
2.0
Sample 1
HV
mag
det
spot
10 µm
10.00 kV
12 000 x
ETD
2.0
Sample 1

Fig. 9

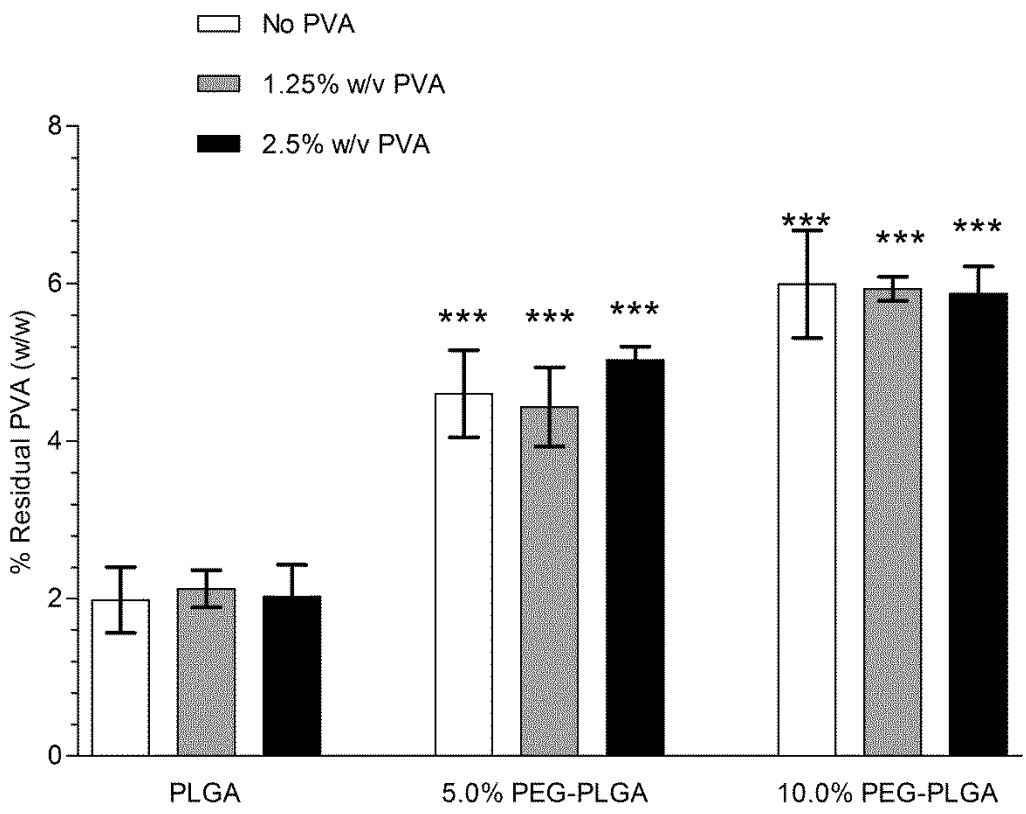

No PVA
1.25% w/v PVA
2.5% w/v PVA
% Residual PVA (w/w)
0
2
4
6
8
***
***
***
***
***
***
PLGA
5.0% PEG-PLGA
10.0% PEG-PLGA

Fig. 10

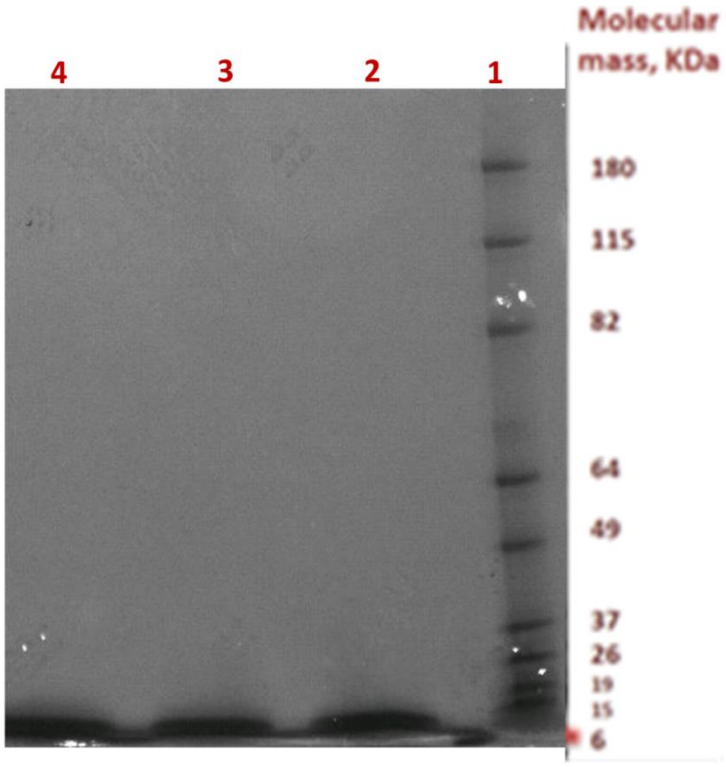
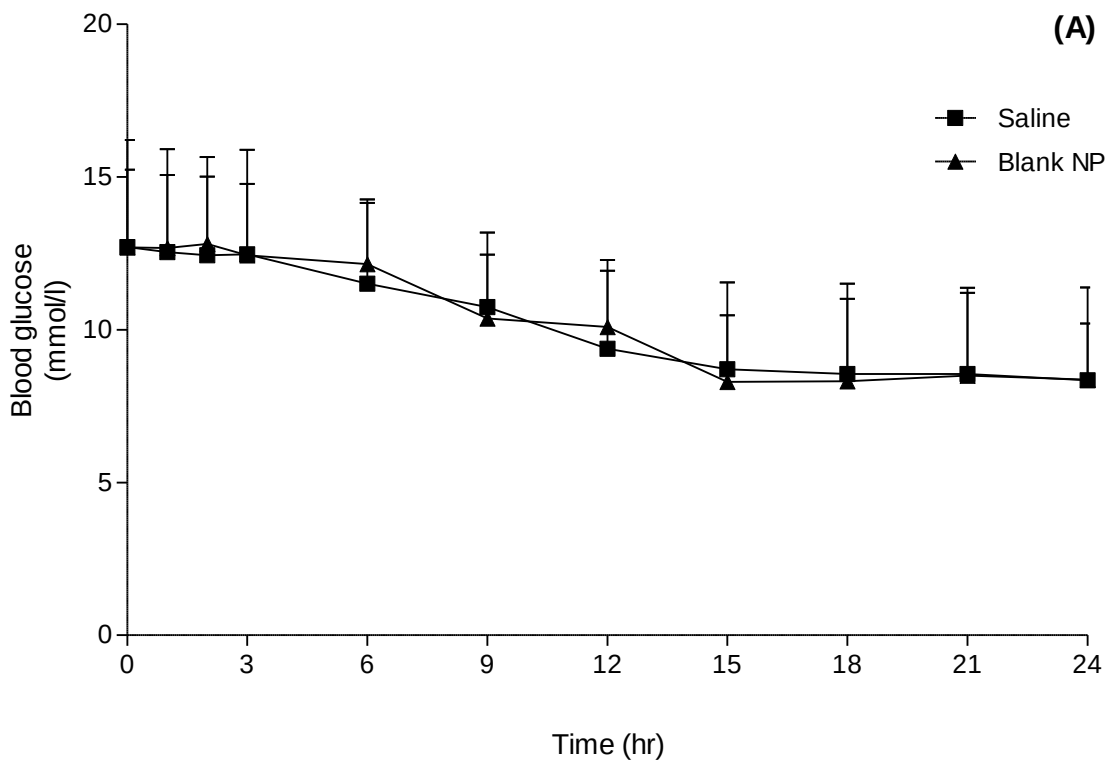
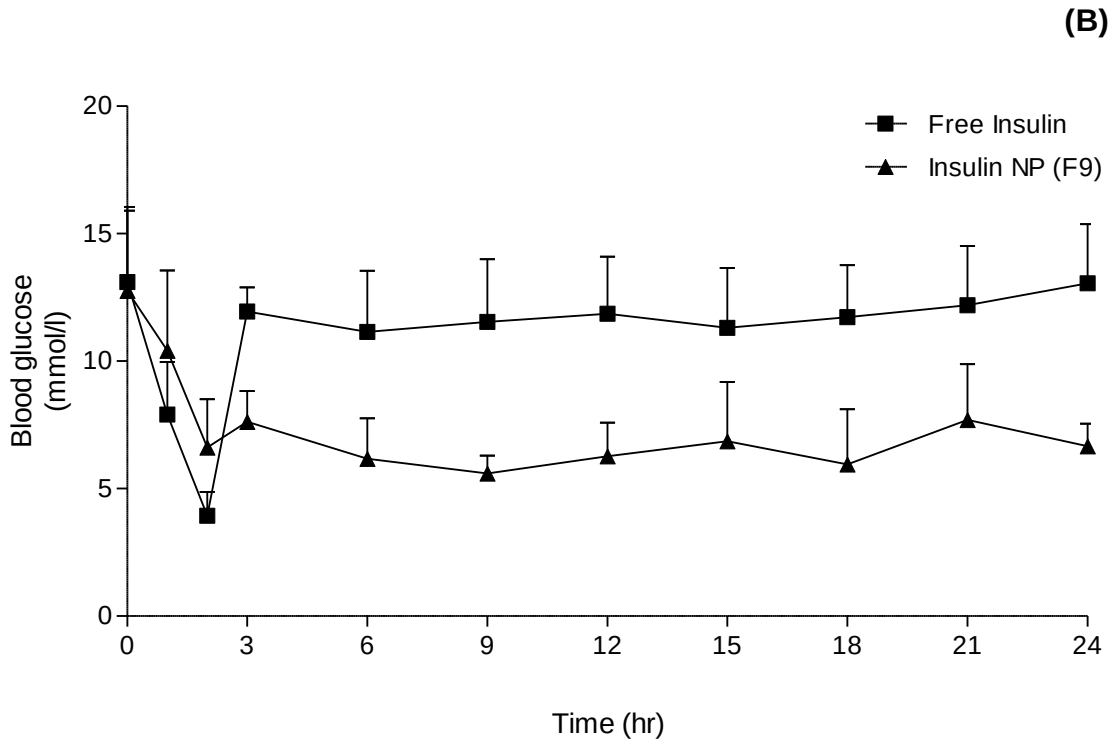


Fig. 11


(A)
Saline
Blank NP
Blood glucose (mmol/l)
Time (hr)
0
5
10
15
20
0
3
6
9
12
15
18
21
24


(B)
Free Insulin
Insulin NP (F9)
Blood glucose (mmol/l)
Time (hr)
0
5
10
15
20
0
3
6
9
12
15
18
21
24

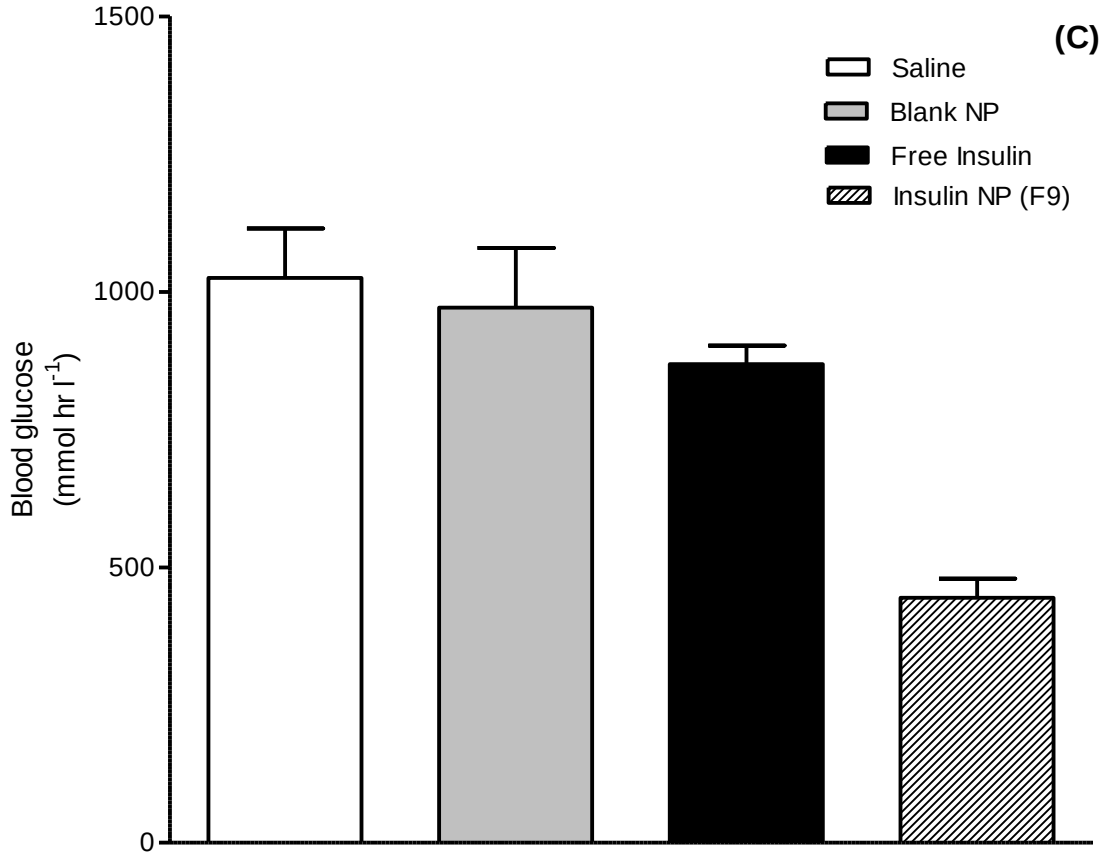
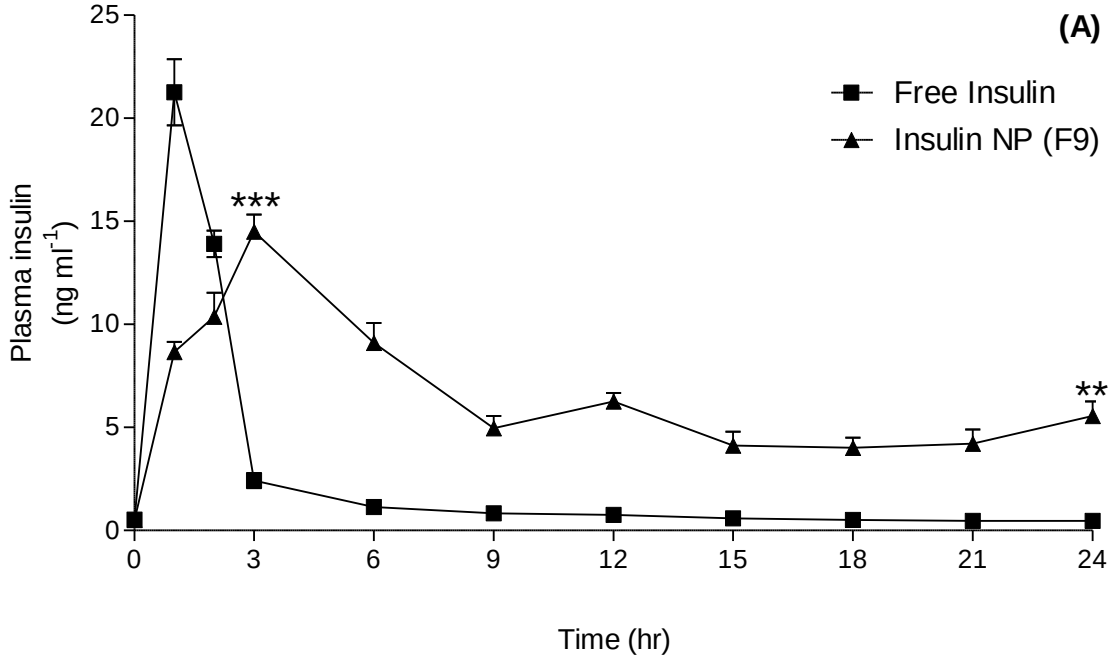
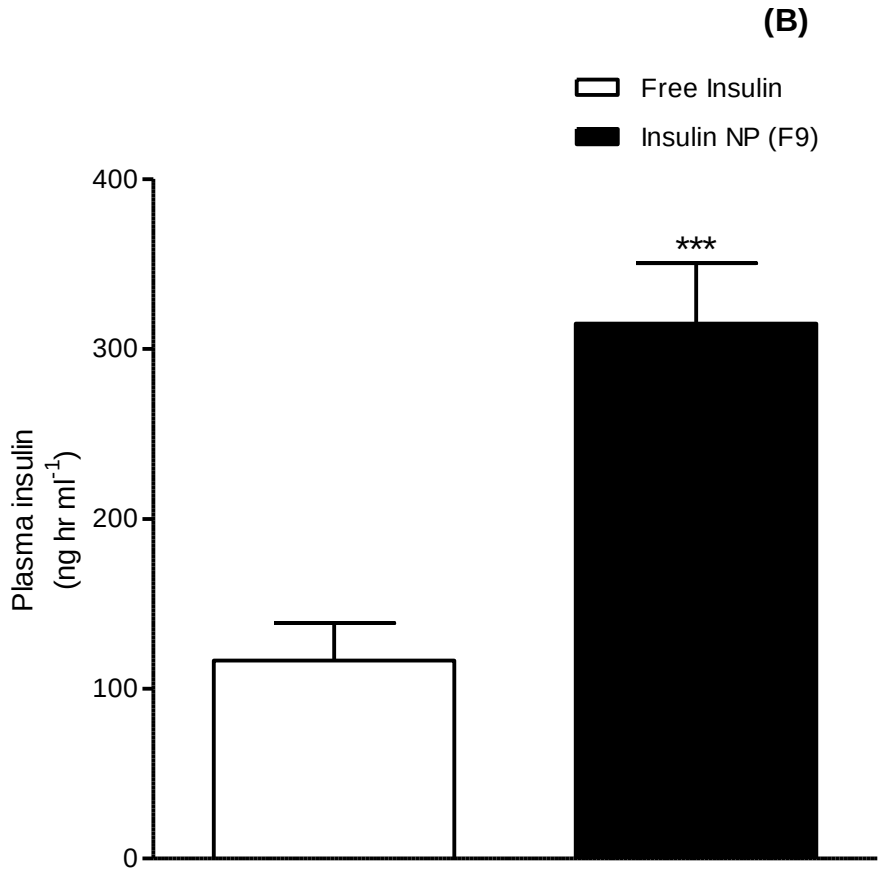

(C)
Saline
Blank NP
Free Insulin
Insulin NP (F9)
Blood glucose
(mmol hr l-1)
1500
1000
500
0

Fig. 12


(A)
Free Insulin
Insulin NP (F9)
Plasma insulin (ng ml-1)
25
20
15
10
5
0
***
**
0
3
6
9
12
15
18
21
24
Time (hr)


(B)
Free Insulin
Insulin NP (F9)
Plasma insulin (ng hr ml-1)
400
300
200
100
0
***

Legend to Figures

Fig. 1. Sequence of steps comprising the double-emulsion, solvent evaporation technique. The factors investigated in this study were the insulin concentration, polymer type and PVA concentration in the primary emulsion.

Fig. 2. *In vitro* release profiles of NP prepared using PLGA (F1), 5%PEG-PLGA (F4) and 10%PEG-PLGA (F7) with insulin loading kept constant at 9% and no PVA in the secondary emulsion. Results are mean ± standard deviation (n=3).

Fig. 3. *In vitro* release profiles of NP prepared using PLGA with insulin loading of 9% (F1), 6% (F2) and 3% (F3), with no PVA in the secondary emulsion continuous phase. Results are mean ± standard deviation (n=3).

Fig. 4. *In vitro* release profiles of NP prepared using 5%PEG-PLGA with insulin loading of 9% (F4), 6% (F5) and 3% (F6), with no PVA in the secondary emulsion continuous phase. Results are mean ± standard deviation (n=3).

Fig. 5. *In vitro* release profiles of NP prepared using 10%PEG-PLGA with insulin loading of 9% (F7), 6% (F8) and 3% (F9), with no PVA in the secondary emulsion continuous phase. Results are mean ± standard deviation (n=3).

Fig. 6. *In vitro* release profiles of NP prepared using 1.25% PVA (F10, F12 and F14) and 2.50% PVA (F11, F13 and F15) in the secondary emulsion continuous phase with 3% insulin loading. Results are mean ± standard deviation (n=3).

Fig. 7. *In vitro* release profiles of NP prepared using homogenisation (F9) and sonication (F16) with no PVA in the secondary emulsion continuous phase and 3% insulin loading. Results are mean ± standard deviation (n=3).

Fig. 8. Scanning electron micrographs of F9 after formulation (A) and after 7 days of *in vitro* release (B).

Fig. 9. The percentage of residual PVA on PLGA, 5% PEG-PLGA and 10% PEG-PLGA insulin-loaded NP. Values are mean ± SD with n=3. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with PLGA NP.

Fig. 10. SDS-PAGE of free insulin and insulin samples released from formulation F9 & F16 after 7 days of *in vitro* incubation. Molecular weight markers (lane 1); free insulin dispersed in PBS (lane 2); insulin released from F9 (lane 3) and insulin released from F16 (lane 4).

Fig. 11. Effects of free insulin and insulin NP (F9) on blood glucose concentrations in insulin-deficient type I diabetic NIH Swiss mice expressed as a time-resolved plot (A and B) and AUC data (C). Blood glucose concentrations were measured prior to and after intraperitoneal injection of free insulin or insulin NP (25 U/kg bw) in insulin-deficient type I diabetic mice for 24 hours. Values are mean ± SEM for 6 mice.

Fig. 12. Effects of insulin and insulin NP (F9) on plasma insulin concentrations in insulin-deficient type I diabetic NIH Swiss mice expressed as line graph (A) and AUC data (B). Plasma insulin concentrations were measured prior to and after intraperitoneal injection of insulin or

insulin NP (25U/kg bw) in insulin-deficient type I diabetic mice for 24 h. Values are mean ±SEM for 6 mice. **P < 0.01, ***P < 0.001 compared with free insulin.