1 **Effect of poly(ethylene glycol) content and formulation parameters on**

2 **particulate properties and intraperitoneal delivery of insulin from PLGA**

3 **nanoparticles prepared using the double-emulsion evaporation**

4 **procedure**

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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.

65　**Key words** insulin, nanoparticles, diblock copolymers, encapsulation efficiency,

66　intraperitoneal bioactivity

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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

91 leads to hypoglycaemic shock (11). However, the primary concern is poor stability of

92 insulin after exposure to formulation conditions present during the emulsification and

93 solvent removal-based processes (12). The primary emulsification phases are of particular

94 concern (13). Emulsification of the primary aqueous protein solution with an immiscible

95 solvent, such as dichloromethane, facilitates protein aggregation at the aqueous–organic

96 interfacial boundary (14). The end result is incomplete release (15) or low encapsulation

97 efficiencies (16).

98 There is a need to improve the sustained release kinetics of insulin and biologically

99 active peptides from biodegradable carriers, whilst preserving activity during fabrication.

100 Formulation studies using potent peptides is often hampered given that available quantities

101 of peptide are often low. Using model drugs, such as insulin, is a common strategy which

102 gathers preliminary data. Formulation of insulin-loaded NP by the double-emulsion

103 technique is commonly done by shearing the system with either sonication or

104 homogenisation (17). Encapsulating insulin using sonication gives high insulin burst

105 release and low entrapment efficiency as troublesome consequences (18). Emulsification

106 using homogenising maintains a linear release profile and higher encapsulation efficiency

107 of hydrophilic drugs that cannot be achieved in particles fabricated by sonication (17), but

108 details of stability are less well documented. Therefore, in this study, sustained release NP

109 for parenteral insulin delivery were evaluated for stability and biological activity during

110 both fabrication steps and release. The formulation strategy used was a modified double-

111 emulsion solvent evaporation technique adopting either homogenisation or sonication to

112 optimise the entrapment efficiency and the initial release of insulin from PLGA and its

113 diblock copolymers containing 5% and 10% PEG. The effect of polymer type,

114   insulin/polymer loading ratio and concentration of poly(vinyl alcohol) in the internal

115   aqueous phase, on the physicochemical characteristics of insulin-loaded NP, together with

116   *in vitro* release profiles and stability were investigated. Furthermore, we evaluated *in vivo*

117   insulin activity using the IP route, as administration is relatively straightforward in the

118   murine model. It is also relevant to the development of continuous intraperitoneal insulin

119   infusion. IP insulin has been shown to provide adequate glycaemic control, which appears

120   superior to that seen following treatment with conventional SC insulin (19). However, the

121   approach is not without its clinical difficulties and more data is needed to assess long-term

122   safety, which will include evaluation of novel delivery strategies, such as nanoparticulate

123   platforms (19).

124

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.

170

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.

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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.

190

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.

11

214 **2.8. *In vivo* studies**

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216 ***Experimental animals***

217 Young (8-week-old) male National Institutes of Health Swiss mice (18–20 g) (Harlan, UK)

218 were age matched, divided into four groups and housed individually in an air-conditioned

219 room at 22 ± 2 °C with a 12:12 hour light-dark cycle (08:00–20:00) and free access to a

220 standard diet and water *ad libitum*. To induce Type I diabetes, weight-matched mice

221 received a single intraperitoneal injection of streptozotocin (STZ, 150 mg kg^{-1}) dissolved

222 in citrate buffer (pH 4.5). Mice with fasting blood glucose level >8 mmol l^{-1} 72 hours after

223 STZ administration were considered diabetic and included in the study. All animal

224 experiments were carried out in accordance with the UK Animals (Scientific Procedures)

225 Act of 1986 and Ulster University's Animal Ethics Committee guidelines.

226

227 ***In vivo* bioactivity of insulin formulation**

228 Four groups (n=6) of insulin-deficient STZ-induced diabetic mice were used for this study.

229 Insulin-loaded NP and free insulin were suspended in sterile PBS and injected

230 intraperitoneally at a dose of 25 U kg^{-1} body weight. The two control groups comprised

231 mice treated with saline or blank NP. Blood samples were withdrawn by tail vein puncture

232 prior to injection and at predetermined time points thereafter. Blood glucose and insulin

233 were measured. Blood glucose concentrations were determined instantaneously by the

234 glucose oxidase method using a handheld Ascencia Counter meter (Bayer Healthcare, UK)

235 (25), while plasma insulin was measured by radioimmunoassay (22), as described

236 previously.

237

2.9 Statistical analysis

239 Results are presented as mean $\pm$ SD and mean $\pm$ SEM for *in vitro* and *in vivo* studies,

240 respectively. Comparative analyses between groups were carried out using one-way

241 analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Area-under-the-curve

242 (AUC) analysis was performed using the trapezoidal rule with baseline correction. A value

243 of $p < 0.05$ was considered statistically significant.

244

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.

268   PEGylation resulted in a significant increase (p<0.05) in insulin loading and

269   encapsulation efficiency. PEG-PLGA is an amphiphilic polymer and creates a benign

270   environment for encapsulating hydrophilic molecules, like insulin, through its hydrophilic

271   PEG chains. This slows peptide diffusion to the external aqueous phase and results in high

272   loading efficacy (27). The higher encapsulation efficiency of F7 compared to F1 and F4

273   is due to the lower solubility of 10% PEG-PLGA in DCM, which resulted in faster polymer

274   precipitation that prevented insulin escape to the outer phase and consequently increased

275   its entrapment efficiency (28).

276   *In vitro* release profiles of F1, F4 and F7 are shown in Fig. 2. Insulin-loaded NP

277   prepared with PEGylated polymers displayed an initial burst release, followed by a steady

278   release profile. The burst release from insulin-loaded PLGA NP was smaller in comparison

279   to PEGylated polymers, followed by a more sustained phase. The burst release of insulin

280   is associated with its peripheral loading, which enables it to diffuse quickly towards the

281   release medium (29) and is more prevalent in the PEGylated types. The burst release was

282   faster and significantly higher from F4 and F7 when compared to F1 (p<0.05), with greater

283   cumulative release after 24 hours. Results showed sustained release of insulin from F1,

284   whereas release from F4 and F7 approached complete release over a 7-day period. The

285   release of insulin from PEGylated NP is governed by its diffusion through a swollen inner

286   structure formed after contact with the aqueous release medium due to the presence of

287   hydrophilic PEG moieties in the polymer matrix (20).

288

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

16

formation of water-filled channels that allow subsequent elution of the core-residing insulin (31, 32).

3.3 Effect of internal aqueous phase stabiliser concentration

PVA was used as a stabilising agent in this study and set at three different concentrations, namely 0.00%, 1.25% and 2.50% w/v. The resulting physicochemical properties of different NP formulations of PLGA (F3, F10 and F11), 5% PEG-PLGA (F6, F12 and F13) and 10% PEG-PLGA (F9, F14 and F15) are shown in Table 1. The NP size was significantly decreased ($p < 0.01$) by PVA addition to the primary emulsion. Increasing PVA concentration caused further decreases in the mean NP size. There are differing views in the literature on whether the primary or secondary emulsion phases have the greater influence on particle size. It is proposed that PVA stabilises the inner water droplets against coalescence, which produces smaller primary emulsion droplets and resultantly smaller NP sizes (31). However, this is in contrast to findings of Bilati *et al*. (33) who concludes that the secondary emulsification step effectively determines the final particle size in double-emulsion techniques. In this current study, particle size reduction was more significant in PEGylated NP when compared to PLGA NP ($p < 0.05$). Since PLGA is more hydrophobic than a diblock copolymer like PEG-PLGA, the interactions between PVA and PEGylated polymers will be stronger than those to PLGA alone. Therefore, our results support the role of the primary emulsion on determining final particle size, especially if PEGylated polymers are used.

PDI values were significantly decreased by adding PVA to the internal phase, whereas a negligible effect on the surface charge was observed. Addition of internal phase

334   stabiliser had a no significant impact on the encapsulation efficiency in all types of NP,

335   although a decreasing trend was observed. This may be due to the decreasing particle size,

336   resulting in a larger interfacial area for mass transfer (34). These results are not observed

337   in other work, which show a sharp increase in encapsulation efficiency by adding PVA (or

338   other stabilisers), possibly by improved stabilisation of the primary emulsion (36) or

339   payload-PVA interactions (31, 35). Clearly, further detailed study is required to determine

340   the exact effect of PVA on encapsulation efficiency.

341   The effect of internal aqueous phase stabiliser on the insulin release from different

342   NP formulations is shown in Fig. 6. A significant increase ($p < 0.05$) in the release rate after

343   PVA addition was observed, and the amount of initial release also increased concomitantly.

344   The *in vitro* release profile of F11, F13 and F15 with 2.5% w/v PVA was significantly

345   faster than the release from NP prepared with 1.25% w/v PVA and NP without PVA. Initial

346   bursts of 49%, 61% and 68% were observed from F11, F13 and F15, respectively,

347   compared to 27%, 42% and 43% from F3, F6 and F9, respectively. The results in Fig. 6

348   indicate that increasing PVA concentration increased the early stage release, possibly by

349   way of enhanced peripheral accumulation of insulin close to the NP surface. In addition,

350   higher concentrations of PVA enable porosity to form in the NP matrix, which facilitates

351   outwards diffusion of insulin (34, 36).

352

353   **3.4 Comparison between homogenisation and sonication**

354   In this work, minimising NP size and maximising %EE was considered as an outcome from

355   optimisation of the formulation variables. Formulation F9, which was prepared by

356   homogenisation using 10% PEG-PLGA, contained NP that were significantly lower in size

357 and higher in %EE (P value <0.05) compared to formulations fabricated by other types of

358 polymers under similar conditions. This formulation was, therefore, chosen for

359 comparison to methods that used sonication instead of homogenisation. It is also important

360 to note that these optimised NP (size < 400 nm and encapsulation efficiency > 90%) are

361 clearly distinguishable from other published work, such as that of Liu *et al.* (37).

362 Insulin-loaded NP (F16) prepared by sonication resulted in a decrease in %EE

363 compared to NP prepared by homogenisation (F9). Size and PDI values were significantly

364 (p<0.01) higher. The release profile of F16 showed significantly higher initial and higher

365 total amounts of insulin released (p<0.05) when compared to similar data from F9 (Fig. 7).

366 Release data for F9 and F16 follow a profile that is typical of drug delivery systems that

367 display Higuchi kinetics. Indeed, fitting cumulative percentage drug release data in Fig. 7

368 to the square root of time (data not shown) confirmed this assumption.

369

370 **3.5 Scanning electron microscopy**

371 Fig. 8 shows a micrograph of the optimised insulin-loaded NP (F9). NP were of spherical

372 shape with a size distribution in approximate agreement with light scattering data. The

373 smooth NP surface was free from void and pores. After 7 days of *in vitro* release, there

374 was little evidence of the presence of spherical structures. Instead, the recovered polymeric

375 material was irregular and sheet-like (Fig. 8B). As F9 has a high PEG content, pore

376 formation during exposure to aqueous media is expected, which may explain poor retention

377 of spherical shape and the enhanced release observed in Fig. 7 (20).

378

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,

402 inactivation or irreversible aggregation. In addition, the high shear stress used for effective

403 emulsification may influence the three-dimensional structure of insulin, which is essential

404 for its therapeutic activity (41).

405   Aggregation is a major concern for protein-loaded PLGA microspheres, caused by

406 the acidic environment when polymer degradation products accumulate during the release

407 phase (42). To investigate the possibility of insulin aggregation after formulation and *in*

408 *vitro* release, the integrity of the insulin released after seven days was evaluated. The three

409 single lines in the gels (Fig. 10), representing the insulin band of the naked and

410 encapsulated insulin released from F9 and F16, provided evidence that the entrapped

411 insulin did not suffer any significant aggregation during its formulation and after *in vitro*

412 release (36). Although use of a stability indicating method, such as HPLC, would highlight

413 possible degradation, quantification by radioimmunoassay in this work supported the lack

414 of degradation or aggregation of encapsulated insulin. This stability is attributed to co-

415 polymerised PEG, which prevents an acidic micro-environment within particulate systems

416 (43). Furthermore, the hydrophobicity of the PLGA surface is masked by PEG, reducing

417 insulin re-adsorption and preventing possible aggregation (44). Both insulin-loaded NP

418 prepared by homogenisation or sonication maintained insulin integrity after fabrication and

419 *in vitro* release.

420

421 **3.8 *In vivo* bioactivity**

422 It is well-documented that formulation steps inactivate or otherwise denature a proportion

423 of encapsulated insulin (45). If this occurs, then bioactivity is compromised and careful *in*

424 *vivo* assessment is required. Therefore, in this work, we determined the bioactivity of an

425   optimised NP formulation in insulin-deficient Type 1 diabetic mice. The effect of a single

426   intraperitoneal injection of (25 U kg^{-1}) of free insulin and insulin-loaded NP (F9) on blood

427   glucose levels in type I diabetic mice is shown in Fig. 11A-11C. Free insulin significantly

428   decreased plasma glucose (P <0.001) directly after injection, compared to the blank NP

429   and saline groups. Blood glucose concentrations decreased by 69.1% (P <0.001) 2 hours

430   after administration.   However, following this initial reduction, blood glucose

431   concentrations in free insulin-injected mice returned rapidly to control diabetic levels. This

432   is in accordance with the short half-life of parenterally delivered insulin (20).

433   Insulin-loaded NP induced a significant and sustained glucose lowering effect, as

434   expected from *in vitro* release data. At the 2-hour time point, insulin-loaded NP had

435   reduced blood glucose levels by 48.3% in our Type 1 diabetic subjects, and these mice had

436   significantly lower glucose levels compared to free insulin-treated mice at 3 hours, post-

437   injection. The clear difference between the glycaemic statuses of the two groups of mice

438   was apparent throughout the entire 24-hour observation period, highlighted by significantly

439   lowered blood glucose concentrations in mice treated with insulin-loaded NP at 24 hours.

440   Encouragingly, blood glucose levels remained within the normal range in insulin-loaded

441   NP treated mice (5-7 mmol l^{-1}) throughout the 24-hour period, with no obvious signs of

442   hypoglycaemia. This is a foremost advantage for any insulin-containing formulation (20).

443   Our results showed a clear effect on blood glucose concentration, which is in contrast to

444   the results of Liu *et al*. (37), who showed insignificant decreases in blood glucose levels

445   following insulin-loaded NP administration within the first 12 hours.

446   Plasma insulin profiles closely mirrored blood glucose levels in all groups of mice,

447   confirming that the initial burst release and subsequent sustained release of insulin for the

448 NP were key features of the improved glycaemic status (20). Plasma insulin levels in mice

449 injected with free insulin (Fig. 12A) revealed a mean peak for plasma insulin concentration

450 (C_{max}) of 21.3 ng ml^{-1} observed after one hour, with levels returning to basal, 3 hours after

451 administration. Mice injected with insulin-loaded NP showed a peak insulin concentration

452 of 14.5 ng ml^{-1}, which occurred 3 hours after injection and was significantly (P <0.001)

453 higher than the free insulin group. Plasma insulin levels remained elevated in insulin-

454 loaded NP treated mice, and were still significantly (P<0.01) greater than free insulin

455 injected mice 24 hours post-injection. AUC analysis (Fig. 11C and 12A) confirmed the

456 sustained and enhanced bioactive profile of released insulin, confirming preservation of its

457 biological activity. Although further studies are required, it is feasible that the remaining

458 insulin within the NP (over 50%) would be slowly released to further sustain

459 normoglycaemic status beyond 24 hours.

460

461 ## 4. Conclusions

462 The aqueous solubility of insulin hinders entrapment efficiency and gives rise to a

463 pronounced burst release from particulate systems. High encapsulation efficiency, low

464 initial release and maintaining stability are essential requirements for sustained insulin

465 delivery from biodegradable formulations. In this current study, we modified key features

466 of the double emulsion technique, such as pH of the internal phase, the ratio of internal

467 phase to external phase and homogenisation speed. After optimisation of the formulation

468 conditions, the %EE of insulin reached 91.5% and the burst release within the first 24 hours

469 was restricted to 28%. An optimised insulin-loaded NP was fabricated by homogenisation

470 from 10% PEG-PLGA with low insulin loading and without any surfactants in the primary

471   emulsion. Insulin-loaded NP prepared by homogenisation showed lower particle size,

472   lower PDI, higher encapsulation efficiency and lower initial release compared to NP

473   prepared by sonication. PEGylated (10%) PLGA polymer exerted a protective effect on

474   insulin stability by suppressing adsorption phenomena and preventing insulin aggregation.

475   The optimised insulin-loaded NP controlled *in vitro* insulin release for 168 hours. Both *in*

476   *vitro* and *in vivo* data were in close agreement. Intraperitoneal administration showed

477   effective and pronounced glycaemic control, which verified preservation of biological

478   activity and confirmed the sustained release profiles determined during release studies.

479

480   ## Declaration of interest

481   The authors report no conflicts of interest. The authors alone are responsible for the content

482   and writing of this article.

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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

Primary Emulsion

2.0 ml

Polymer solution

← Polymer type

Secondary Emulsion

50 ml

PVA solution
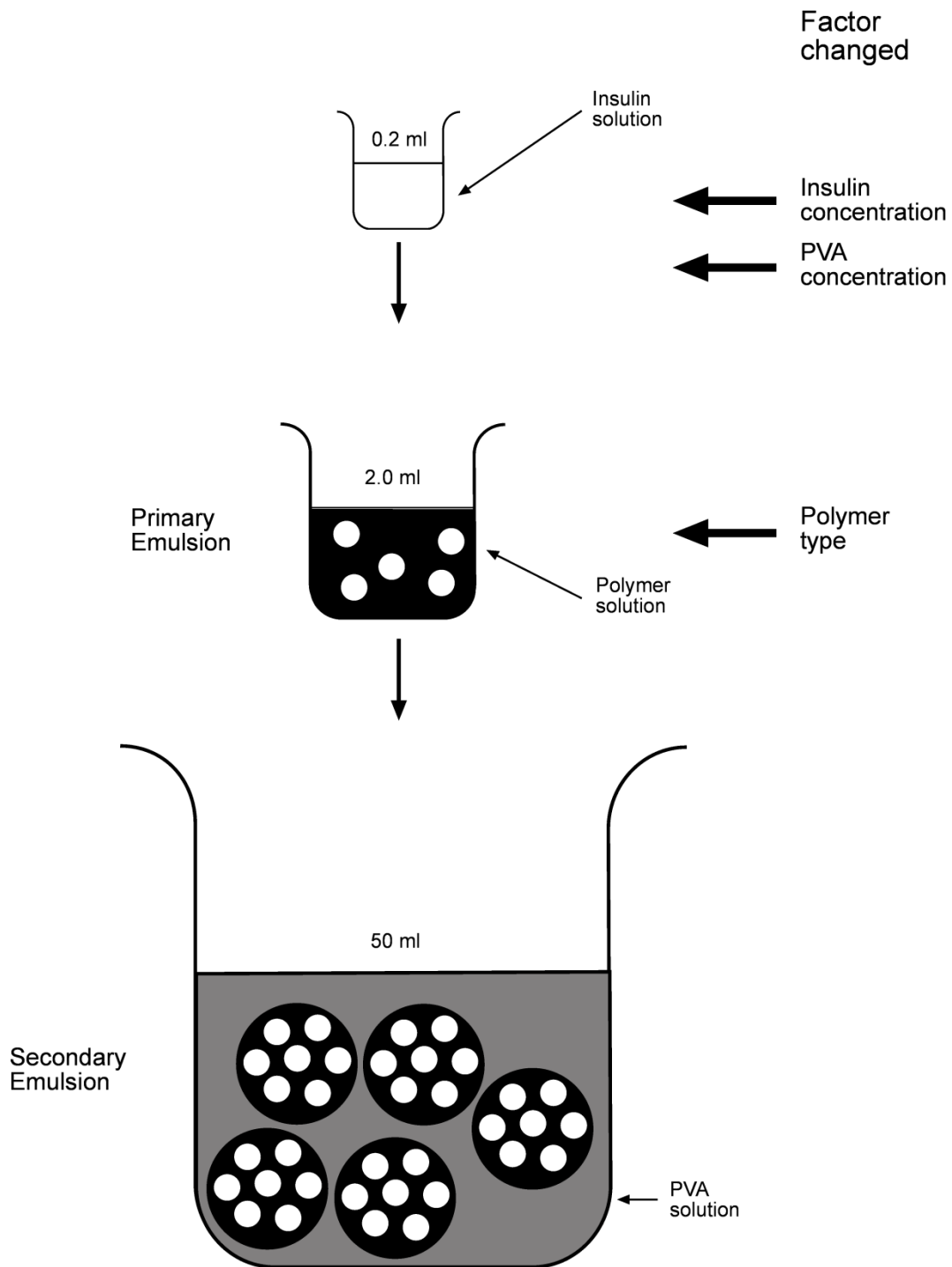
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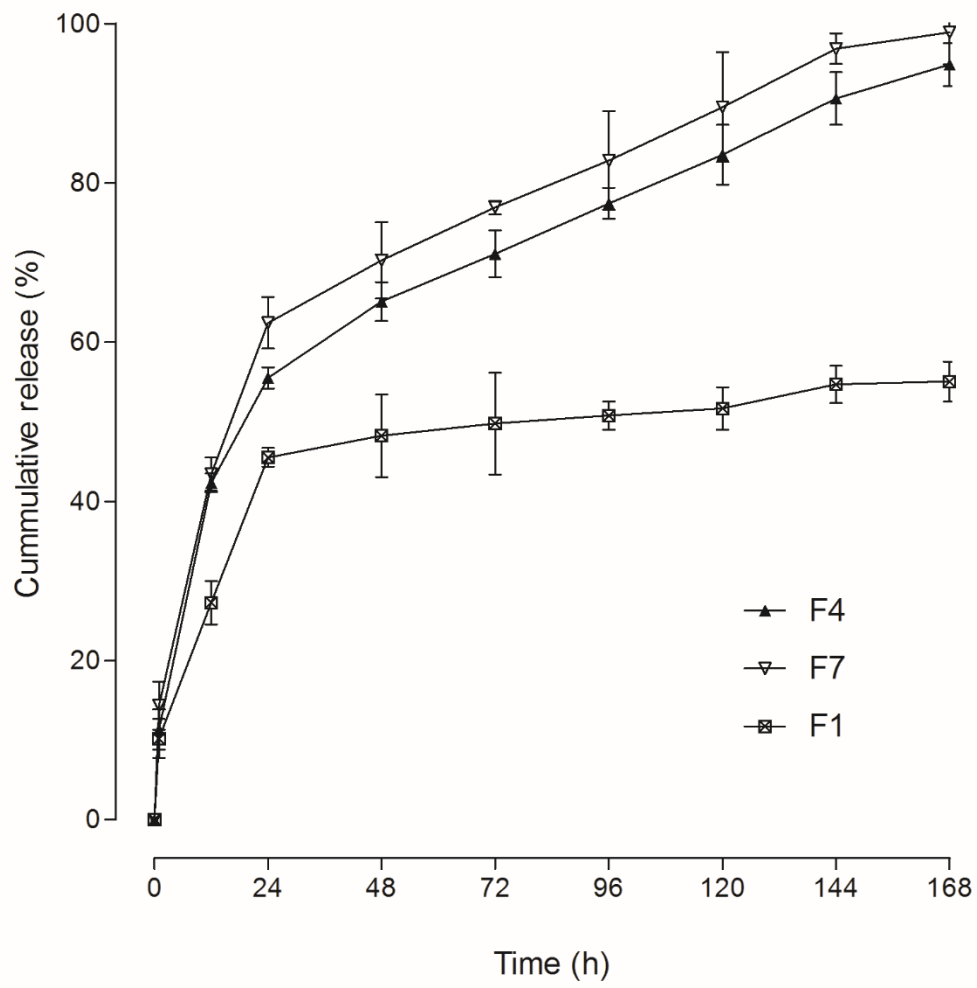
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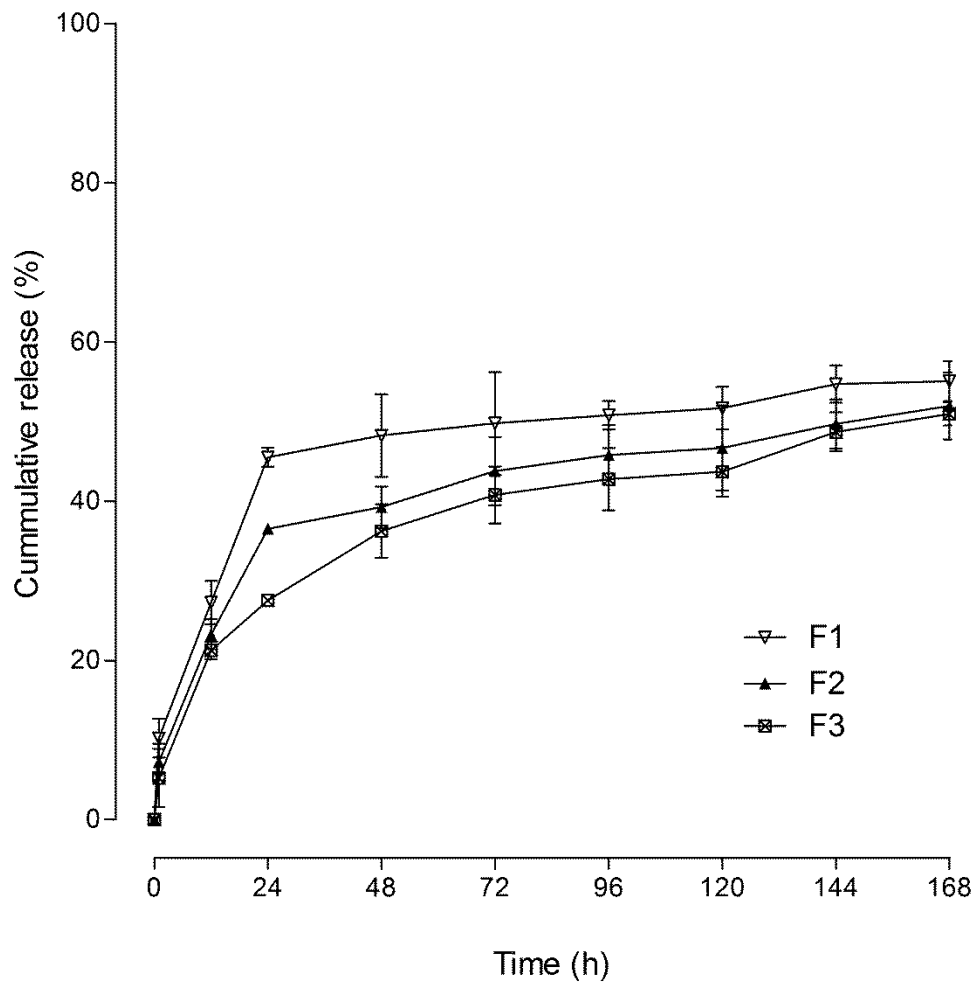
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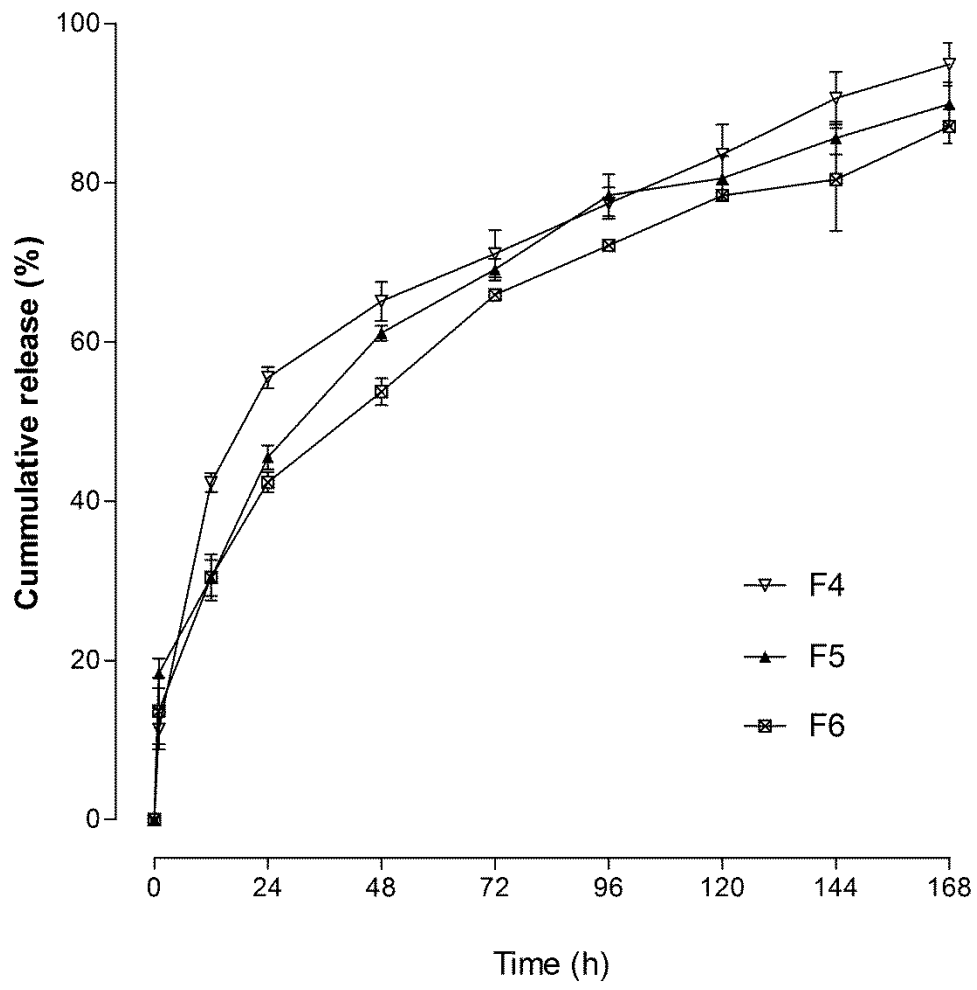
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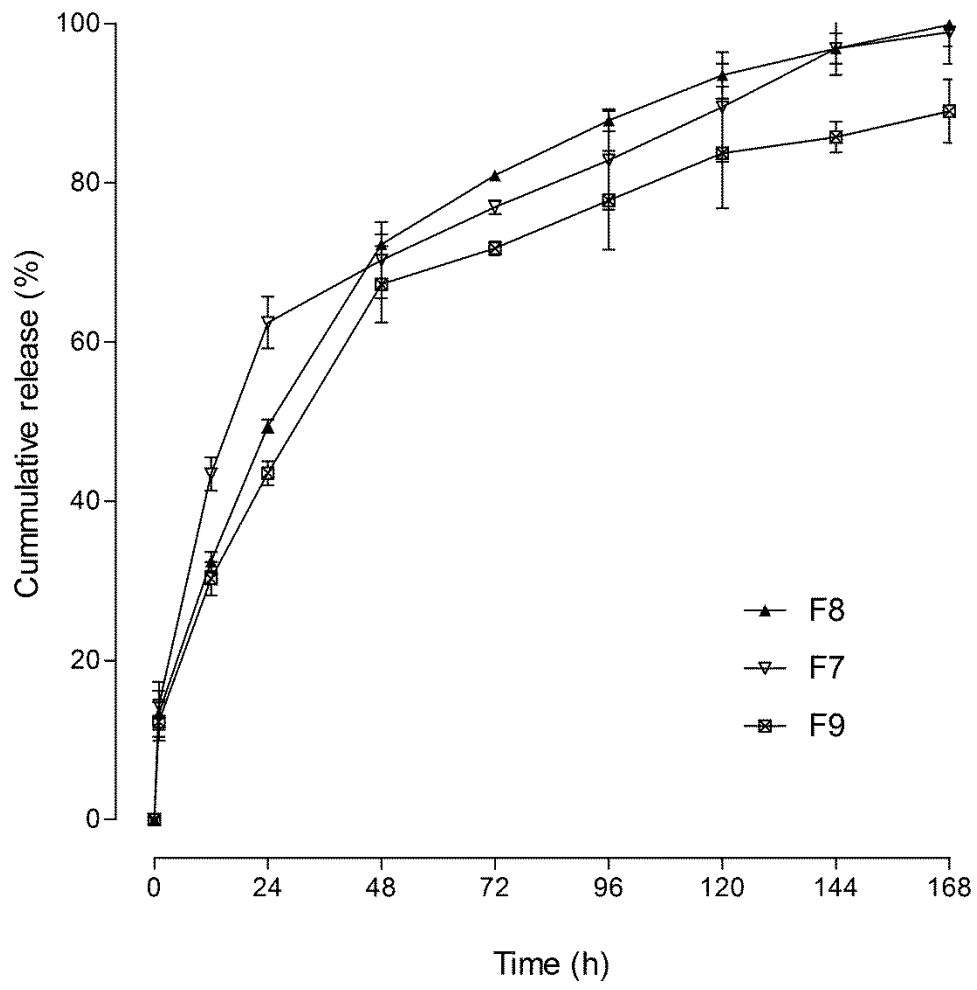
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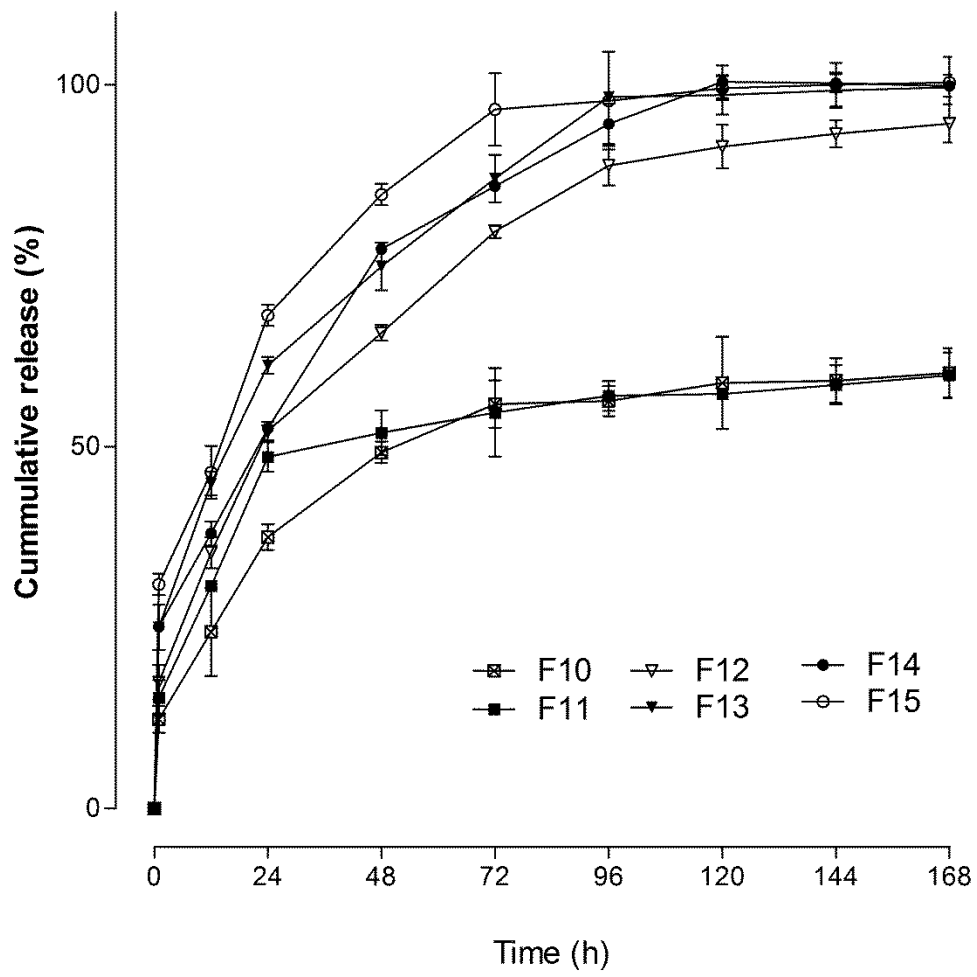
Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

(A)

(B)

(C)

Fig. 12

(A)
■ Free Insulin
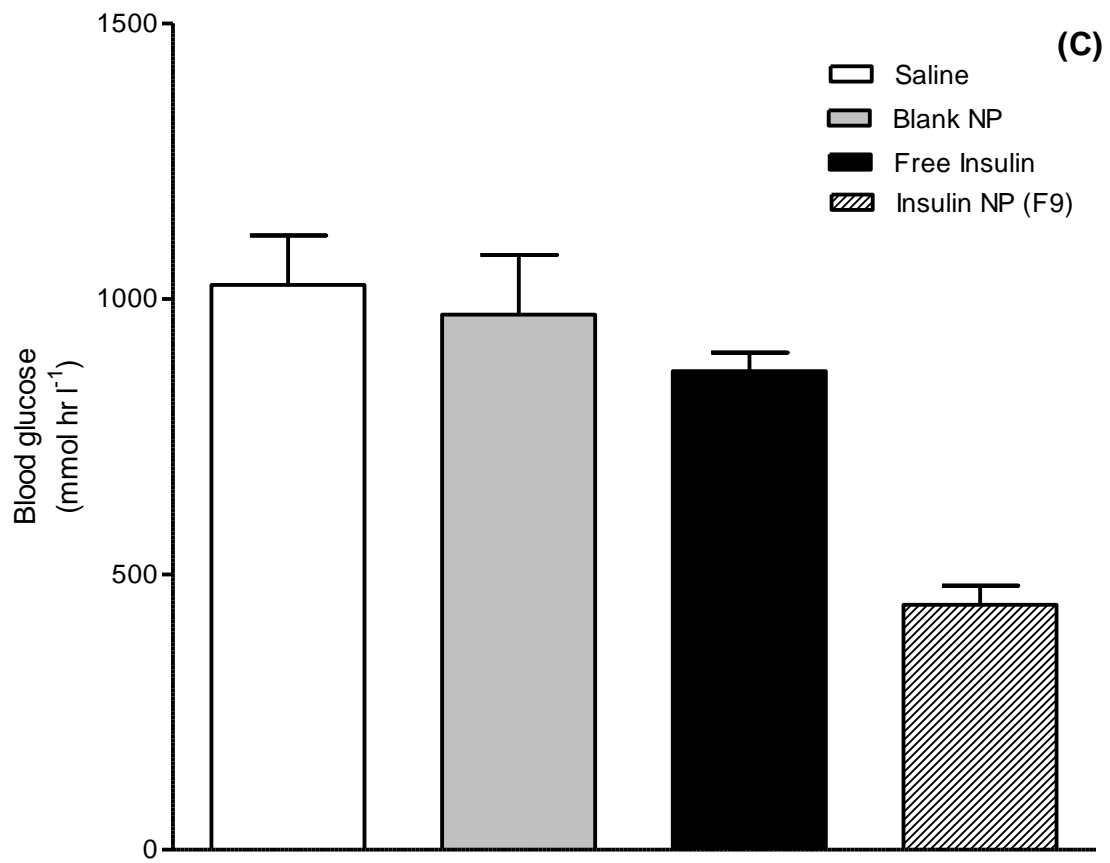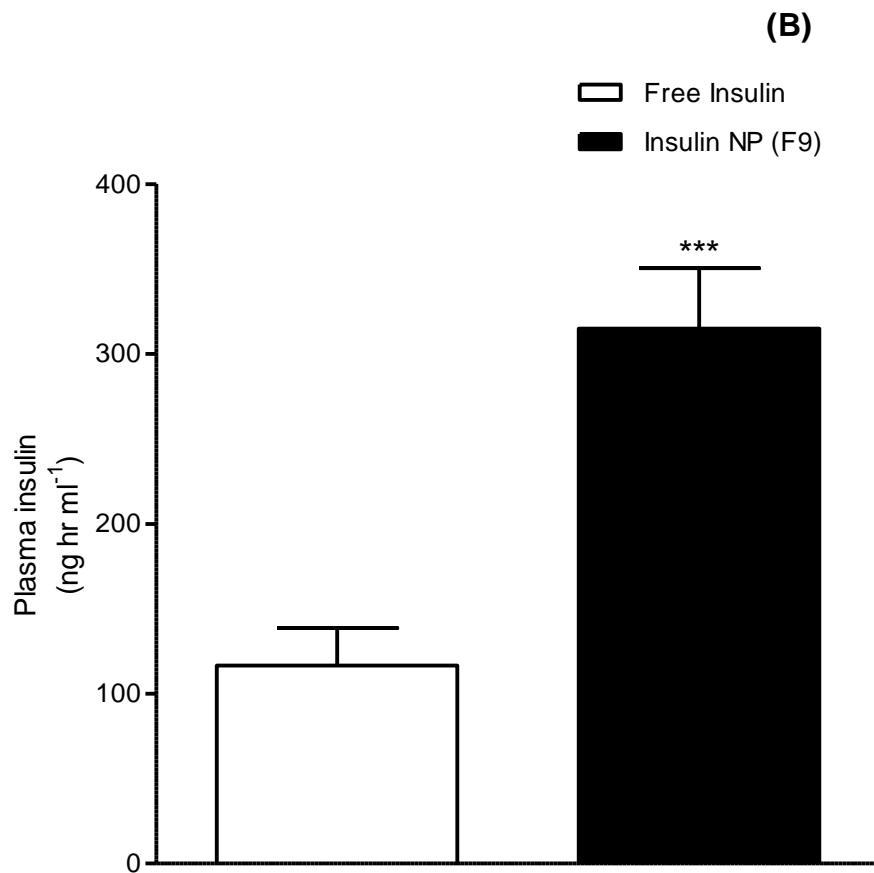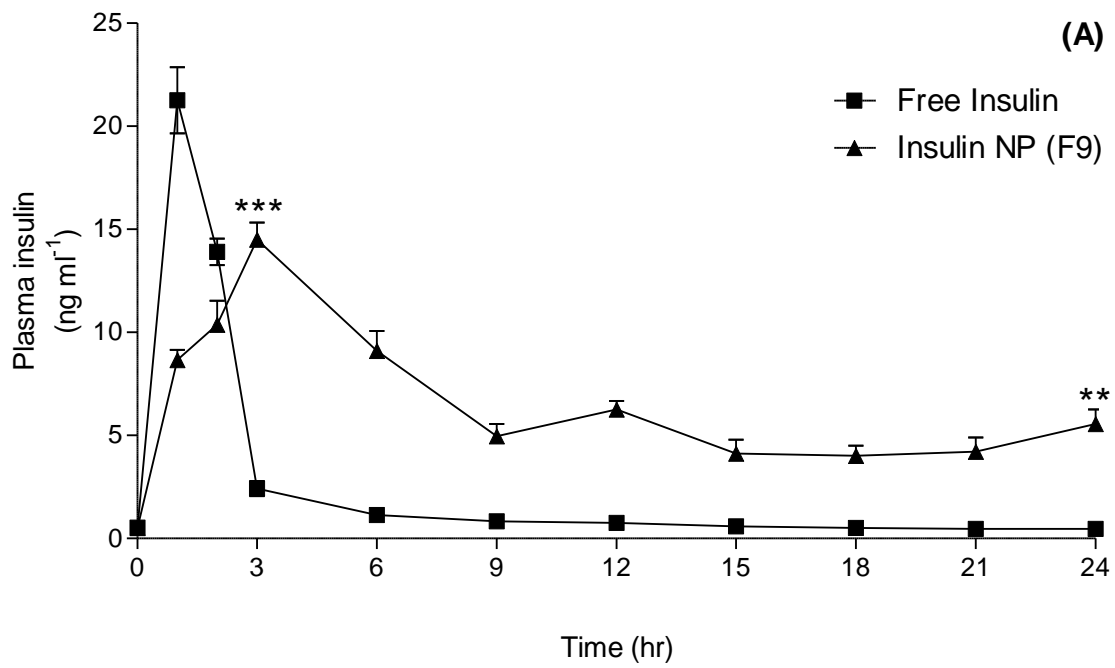▲ Insulin NP (F9)

(B)
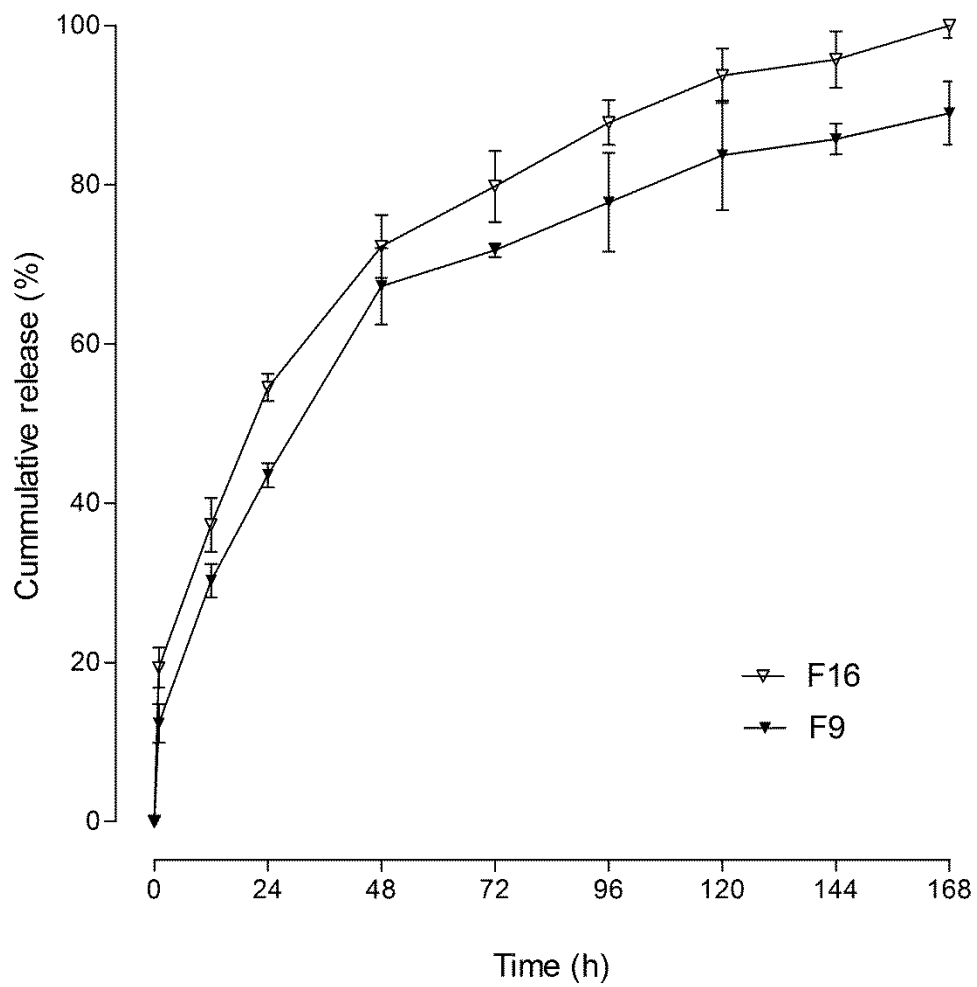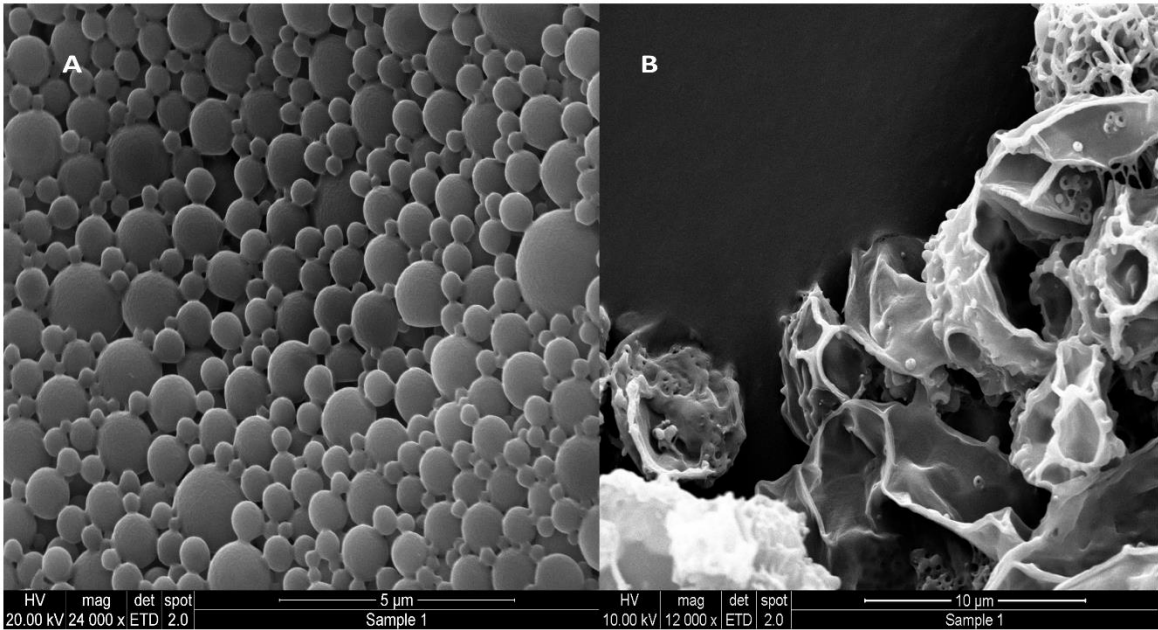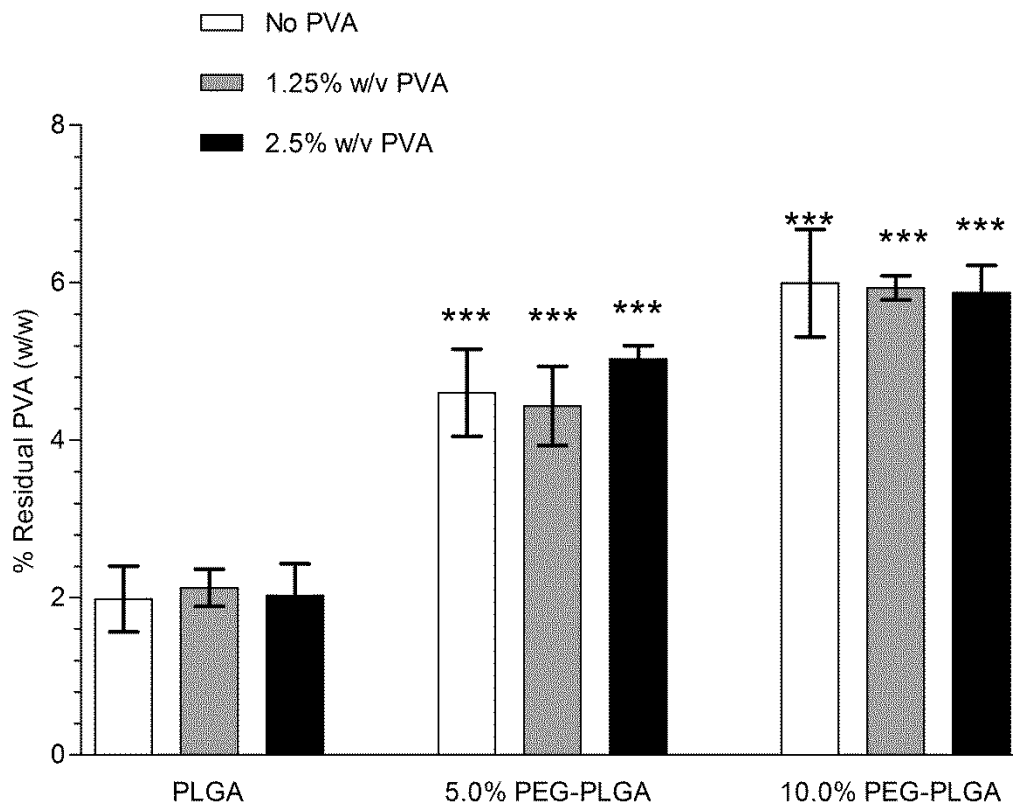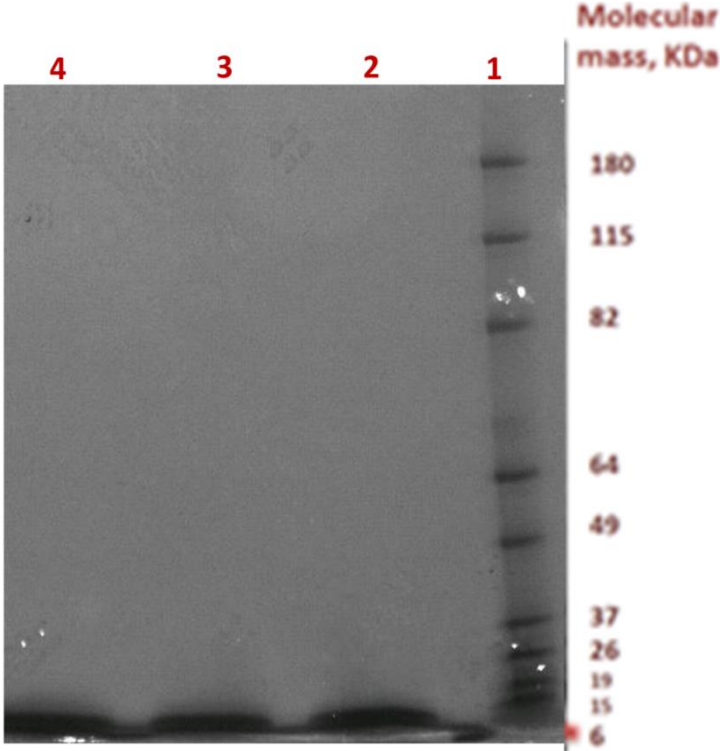
□ Free Insulin
■ Insulin NP (F9)
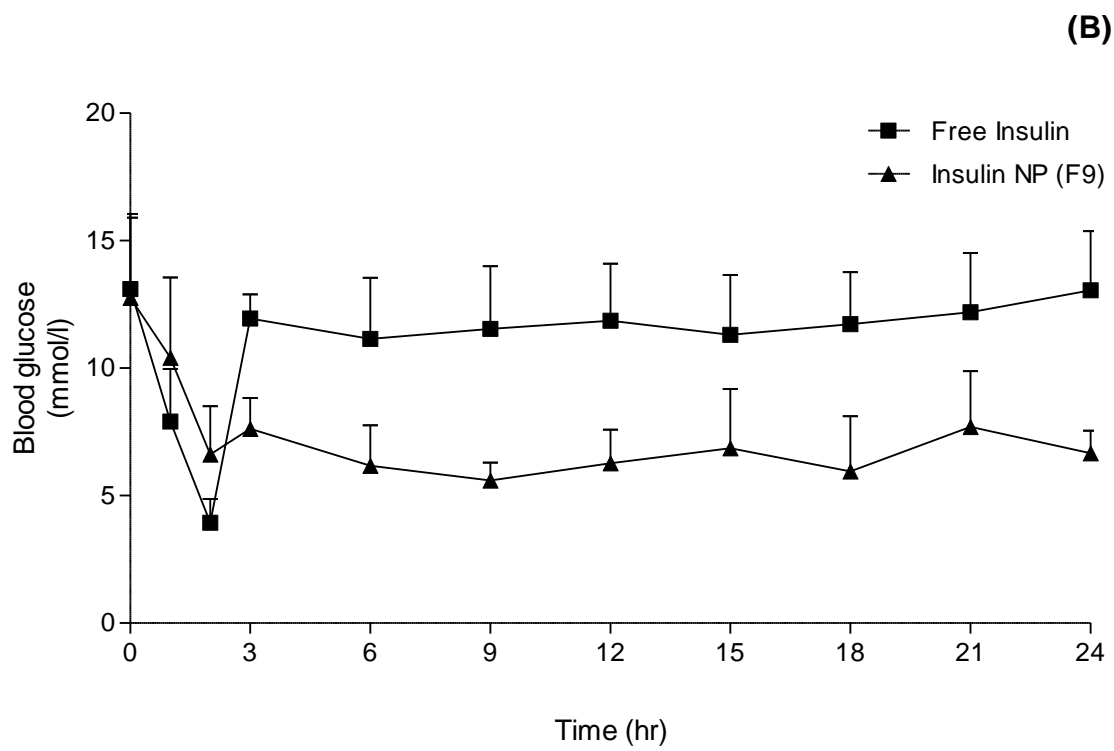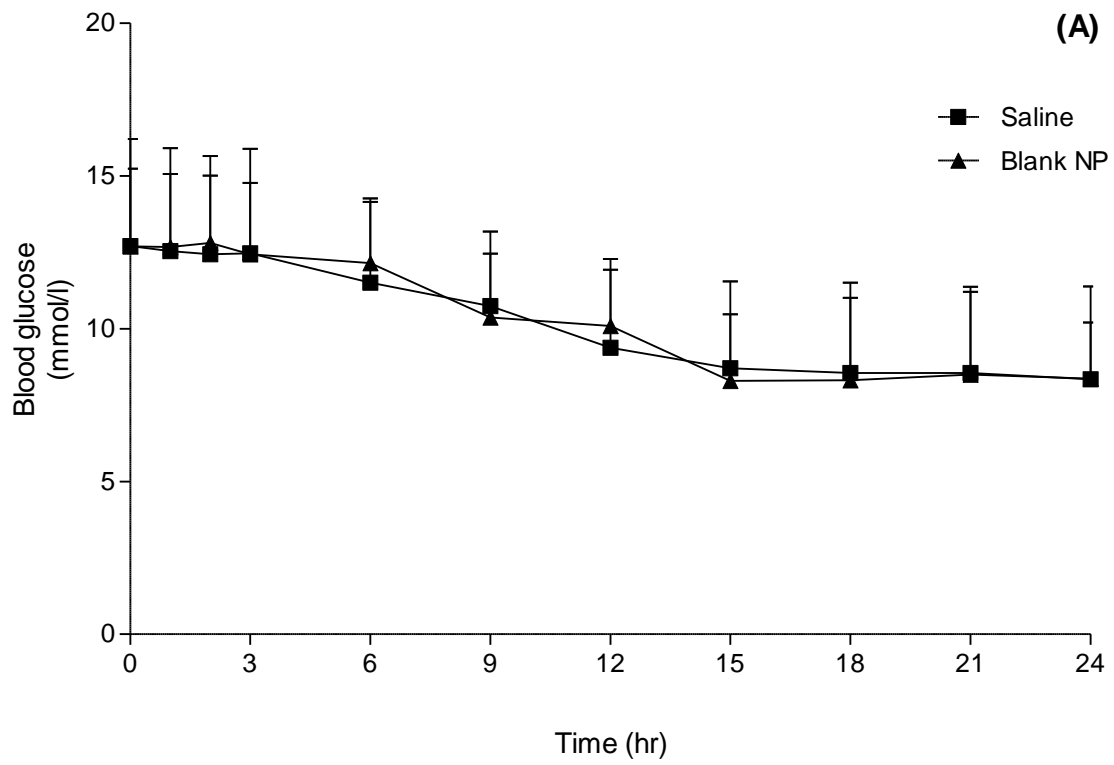
Legend to Figures

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.