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Haggag, Yusuf A., Faheem, Ahmed, Tambuwala, Murtaza M., Osman, Mohamed A., El-Gizawy, Sanaa A., O'Hagan, Barry, Irwin, Nigel and McCarron, Paul A. (2017) 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. *Pharmaceutical Development and Technology*. pp. 1-12. ISSN 1083-7450

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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**

5

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42 **Abstract**

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

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

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

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

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

64

65 **Key words** insulin, nanoparticles, diblock copolymers, encapsulation efficiency,
66 intraperitoneal bioactivity
67

68 **1. Introduction**

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

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

89 Studies describing encapsulation of insulin into biodegradable polymeric devices
90 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

125 **2. Materials and Methods**

126 **2.1 Materials**

127 PLGA (Resomer[®]RG 503H) with an average molecular weight of 34 kDa and a lactide-to-
128 glycolide ratio of 50:50 was purchased from Sigma Chemical Co. (St. Louis, USA). Two
129 PEG-PLGA diblock copolymers (Resomer[®] RGP d 5055 (5% PEG) and Resomer[®] RGP d
130 50105 (10% PEG) with PEG molecular weight of 5 kDa, were purchased from Boehringer-
131 Ingelheim (Ingelheim, Germany). Bovine insulin (51 amino acids, MW5.734 kDa),
132 poly(vinyl alcohol) (PVA) 87-89% hydrolysed (MW 31.000-50.000) and phosphate
133 buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, USA). A Micro
134 BCA[®] Kit was obtained from Pierce Ltd. (Rockford, IL). Dichloromethane was of HPLC
135 grade and all other reagents were of analytical grade or higher purity. Milli-Q-water was
136 used throughout the study.

137

138 **2.2 Preparation of insulin-loaded NP**

139 A modified, double-emulsion, solvent evaporation technique was used in this work, as
140 illustrated schematically in Fig. 1 (20). Insulin was dissolved in 0.2 ml of internal aqueous
141 phase (0.1 M HCl), which was then emulsified at 6,000 rpm (Silverson L5T, Silverson
142 Machines Ltd., Buckinghamshire, UK) for 2 minutes into 2.0 ml of dichloromethane
143 (DCM) containing 10% w/v of the polymer type under investigation. The primary
144 emulsion (w/o) was injected directly into 50 ml of PVA solution (external aqueous phase)
145 under agitation. Emulsification continued at **10,000 rpm for 6 minutes** to produce the
146 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

171 **2.4 Determination of insulin loading and entrapment efficiency.**

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

181

182 **2.5 *In vitro* release studies**

183 Lyophilised insulin-loaded NP (5.0 mg) were suspended in a release medium of 1.0 ml
184 PBS (pH 7.4) and incubated at 37 °C using a reciprocal shaking water bath at a fixed speed
185 of 100 rpm. Samples (100 µl) were taken at predetermined time intervals of 1, 12, 24, 48,
186 96, 120, 144 and 168 hours and replaced with fresh PBS at the same temperature. The
187 collected samples were centrifuged for 5 minutes at 10,000 x g and the insulin content in
188 the supernatant determined using the indirect radioimmunoassay, as described in section
189 2.4. Each experiment was performed in triplicate.

190

191 **2.6 Determination of residual PVA**

192 The residual mass of PVA attached to the NP surface after washing was determined by a
193 colorimetric method based on the reaction with iodine. A sample of freeze-dried NP (2.0
194 mg) was incubated in 0.5 M NaOH for 15 minutes at 60 °C, neutralised by addition of 1.0
195 M HCl and the final volume adjusted to 5.0 ml with water. To each NP sample, 3.0 ml of
196 iodine reagent (0.65 M solution of boric acid, 0.5 ml of a solution of I₂/KI (0.05 M/0.15
197 M)) and 1.5 ml of water were added. The absorbance of each sample was measured using
198 visible spectroscopy (Agilent 8453, Agilent Technologies, Palo Alto, CA, USA) at 690 nm
199 after 15 minutes incubation and compared to a standard plot of known PVA concentration
200 (23). Residual PVA was expressed as a percentage mass fraction of the total NP mass.

201

202 **2.7 *In vitro* stability and protein integrity studies**

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

213

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

215

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

238 **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

245 **3. Results and discussion**

246 **3.1 Effect of polymer type on 9% insulin-loaded NP (F1, F4 and F7)**

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

258 PLGA NP exhibited higher negative zeta potential values when compared to
259 PEGylated NP (F4 and F7), but no significant difference between the two latter types was
260 observed. PEG chains on the NP surface are expected to mask surface charge arising from
261 PLGA (7). This reduction of surface charge is a drawback for the PEGylated types, given
262 that NP are notoriously difficult to stabilise when dispersed or suspended in aqueous media.
263 Attractive forces cause NP to agglomerate or aggregate, and surface charges play an
264 important role in preventing this effect. However, during this work, no discernible effects
265 relating to aggregation were observed. To obtain long-term stable NP samples, the
266 dispersions were freeze-dried and NP were re-dispersed in a suitable medium prior to the
267 *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

289 3.2 Effect of insulin/polymer loading ratio

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

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

306 The *in vitro* release profiles and the initial bursts were directly related to the degree
307 of theoretical insulin loading, as shown in Fig. 3, 4 and 5. There was a positive correlation
308 between insulin loading and the extent of the initial burst phase. High insulin burst and
309 rapid release rate could be attributed to the drug concentration gradient between the NP
310 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

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

379 **3.6 Residual PVA**

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

394

395 **3.7 *In vitro* stability and protein integrity**

396 Preserving secondary and tertiary structures of proteins and peptides is essential in
397 maintaining therapeutic efficacy. Stability issues are affected by different formulation
398 conditions with attention focused on the harsh fabrication stress employed during
399 emulsification-based methods (40). Encapsulation of insulin using the double-emulsion
400 technique enables it to act as a surface active molecule, which tends to adsorb at the primary
401 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

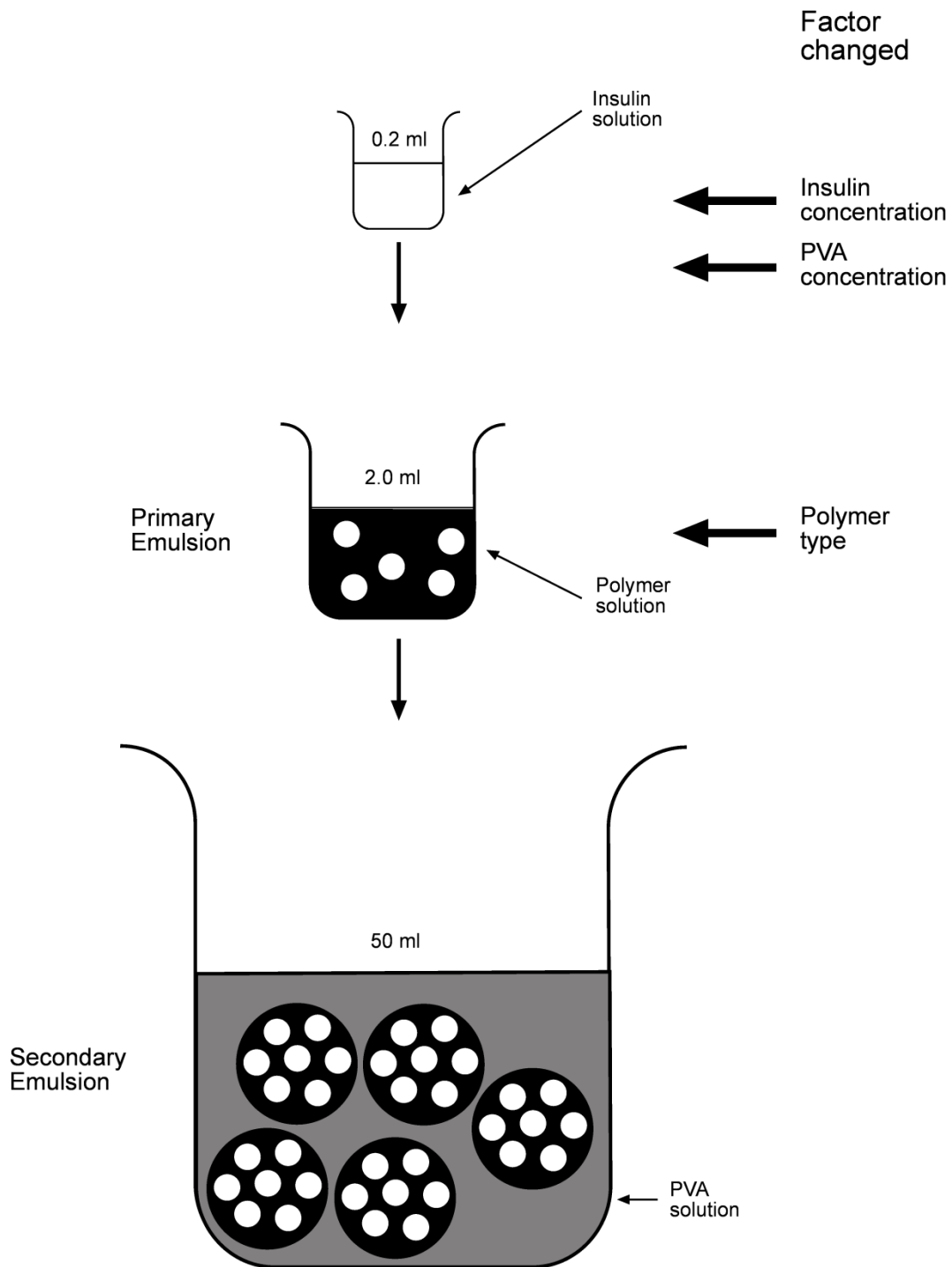


Fig. 2

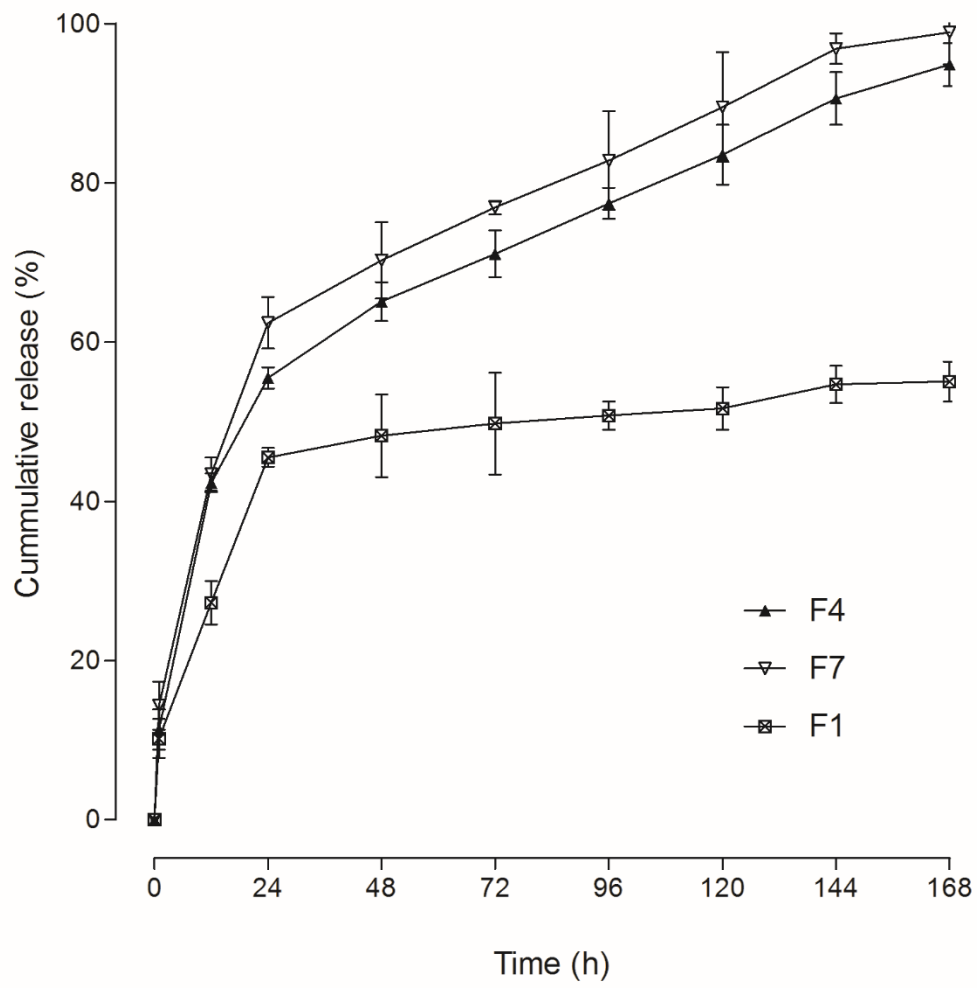


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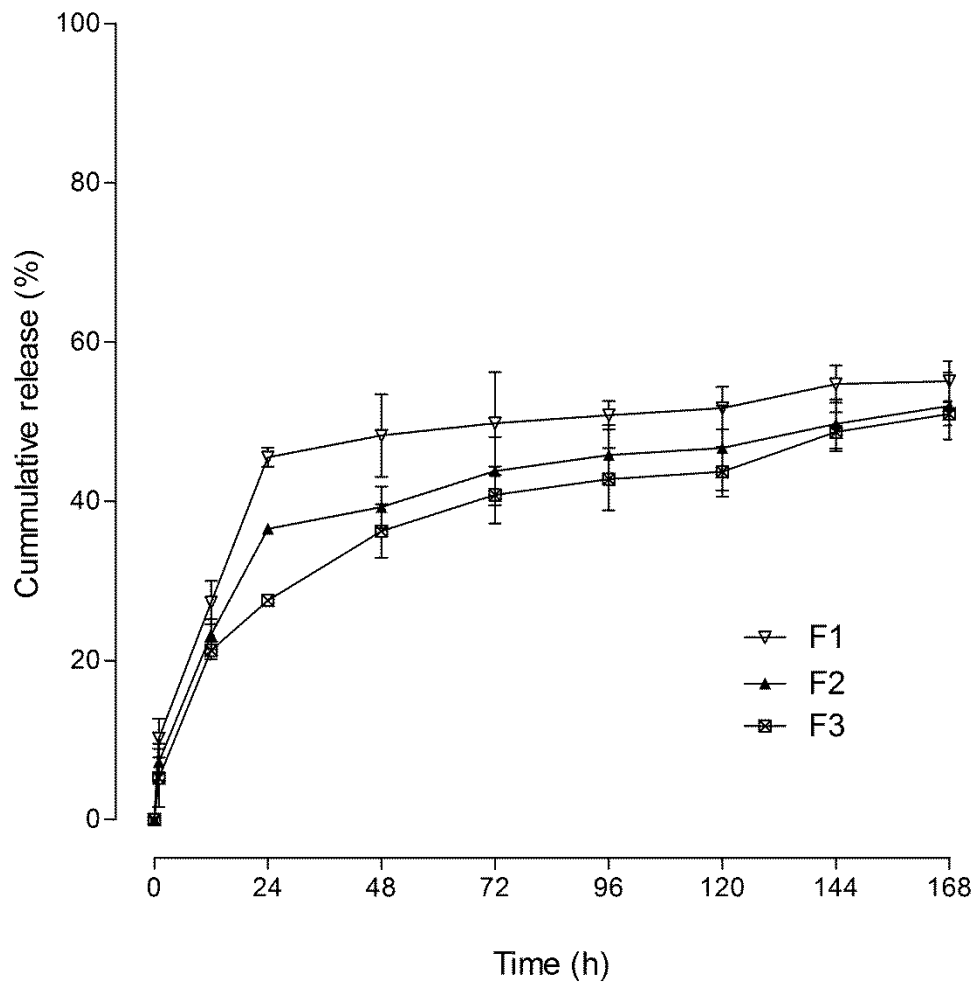


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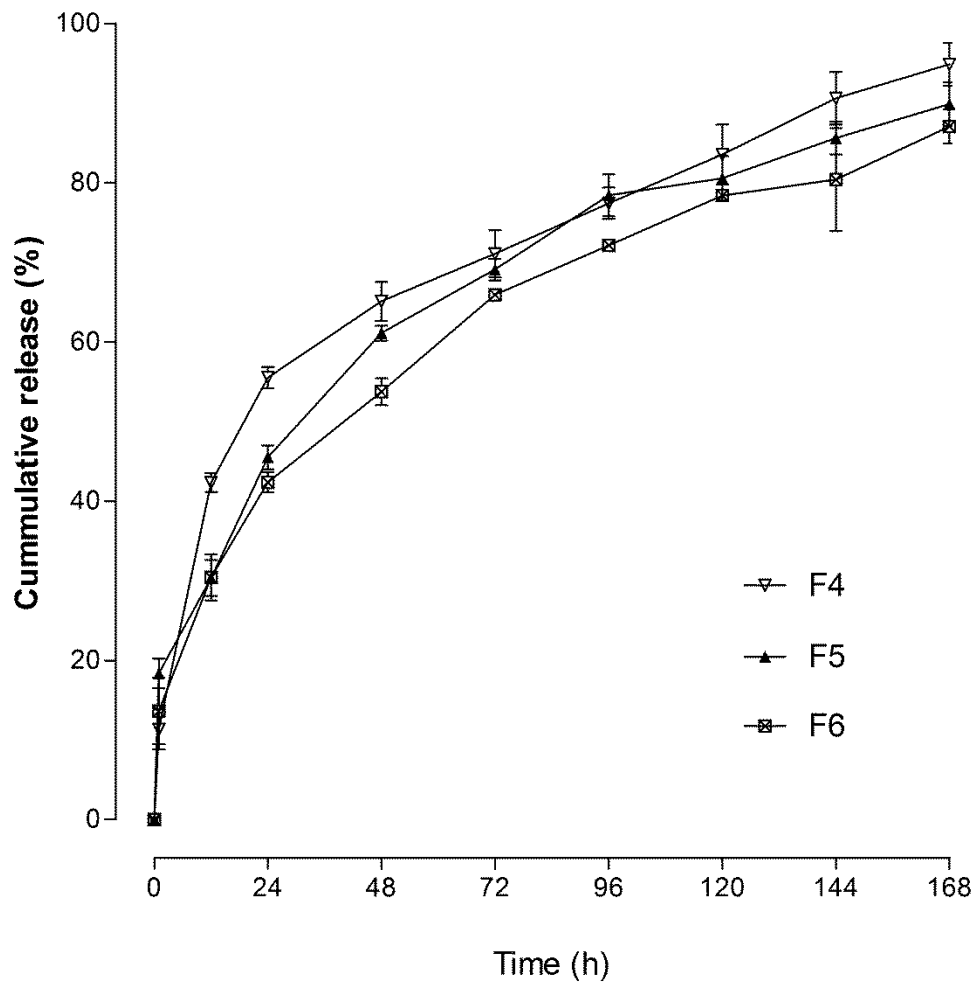


Fig. 5

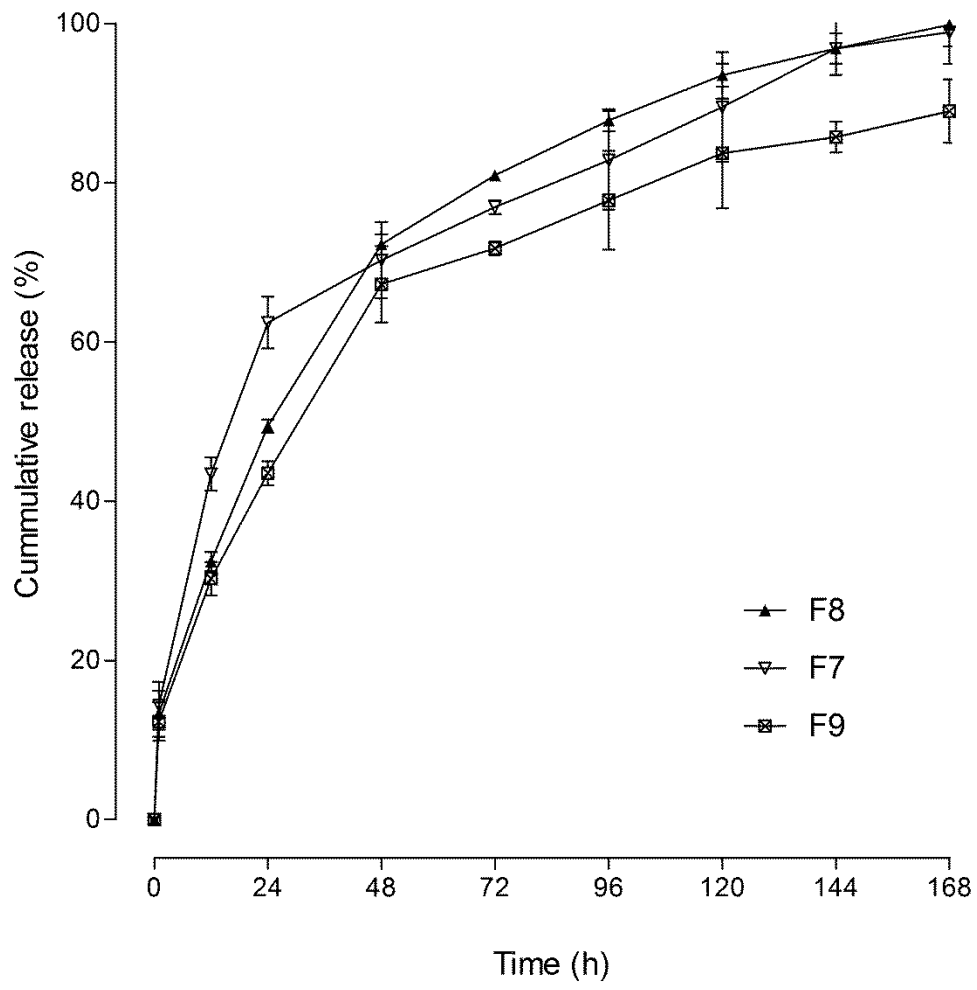


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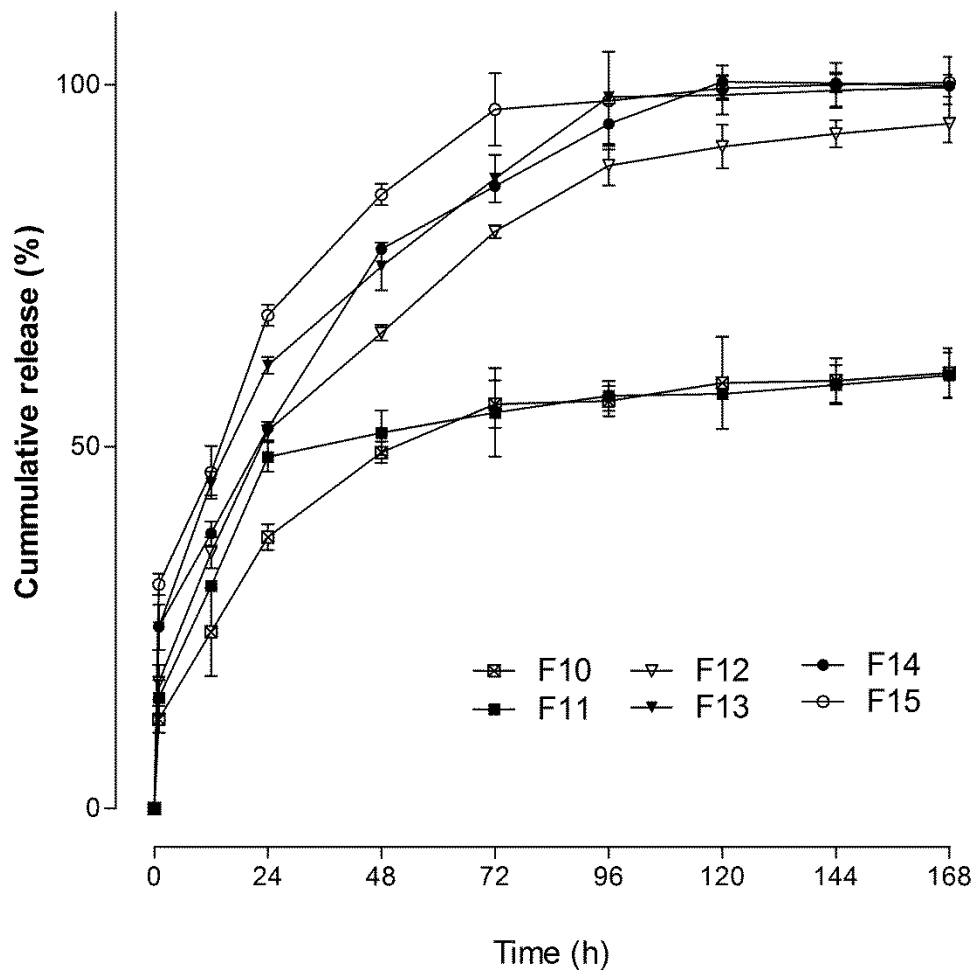


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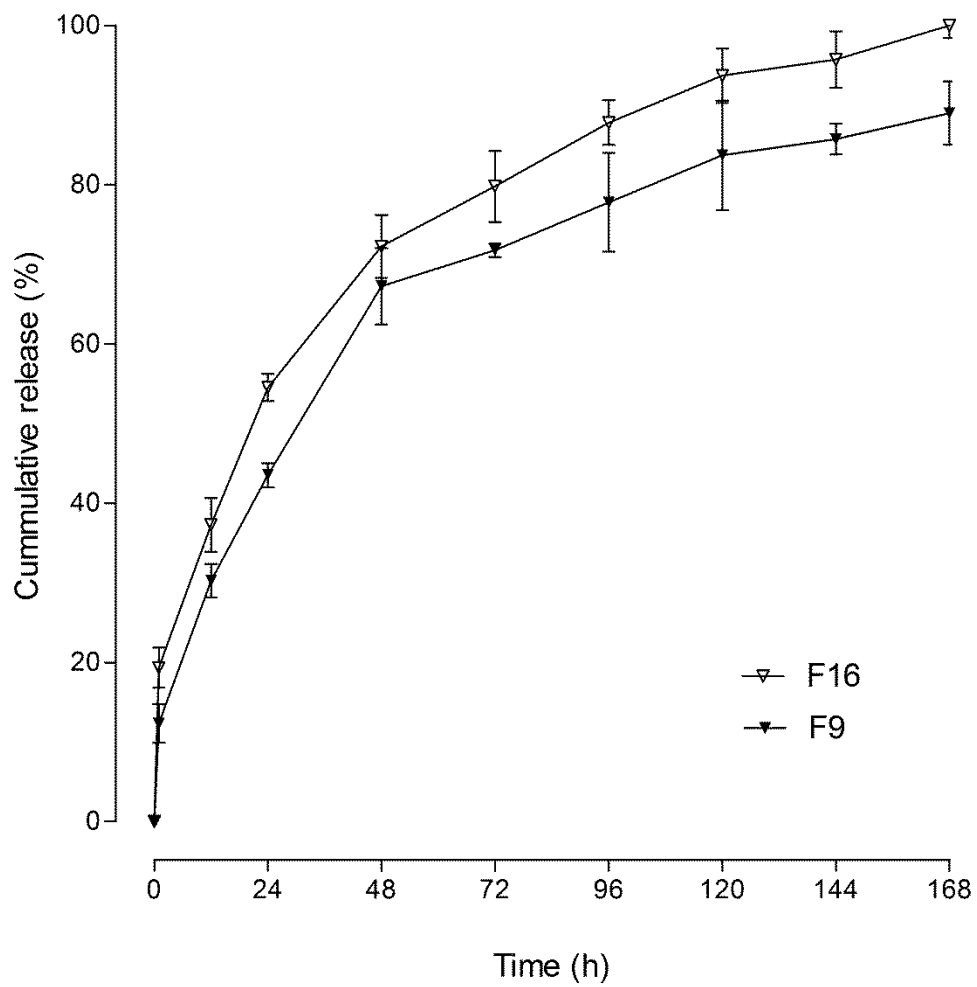


Fig. 8

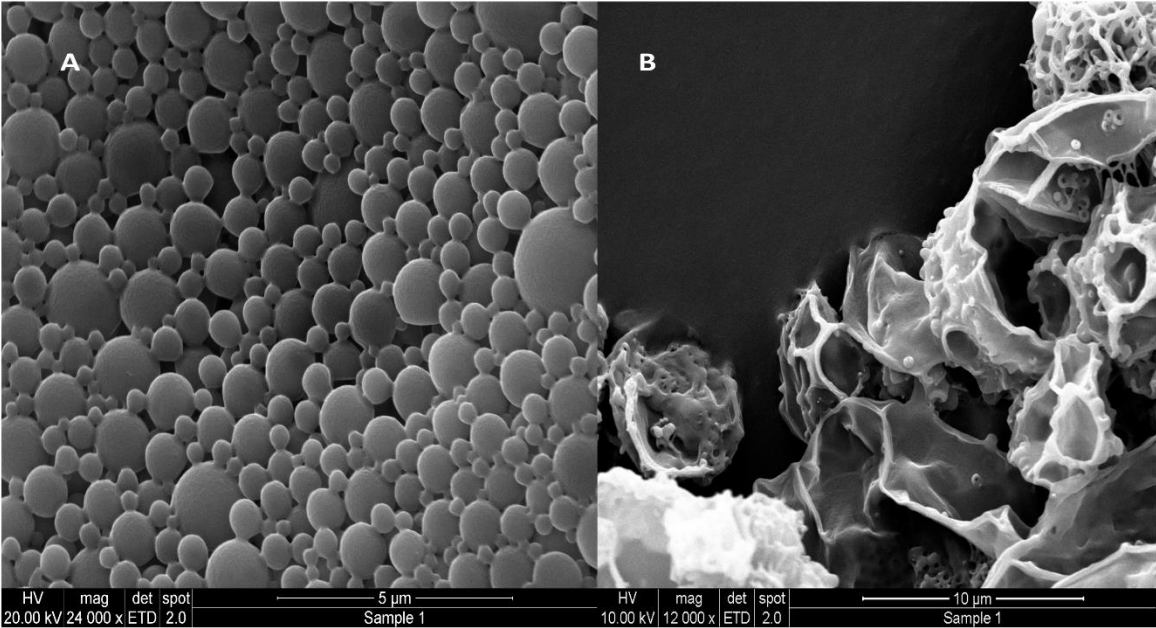


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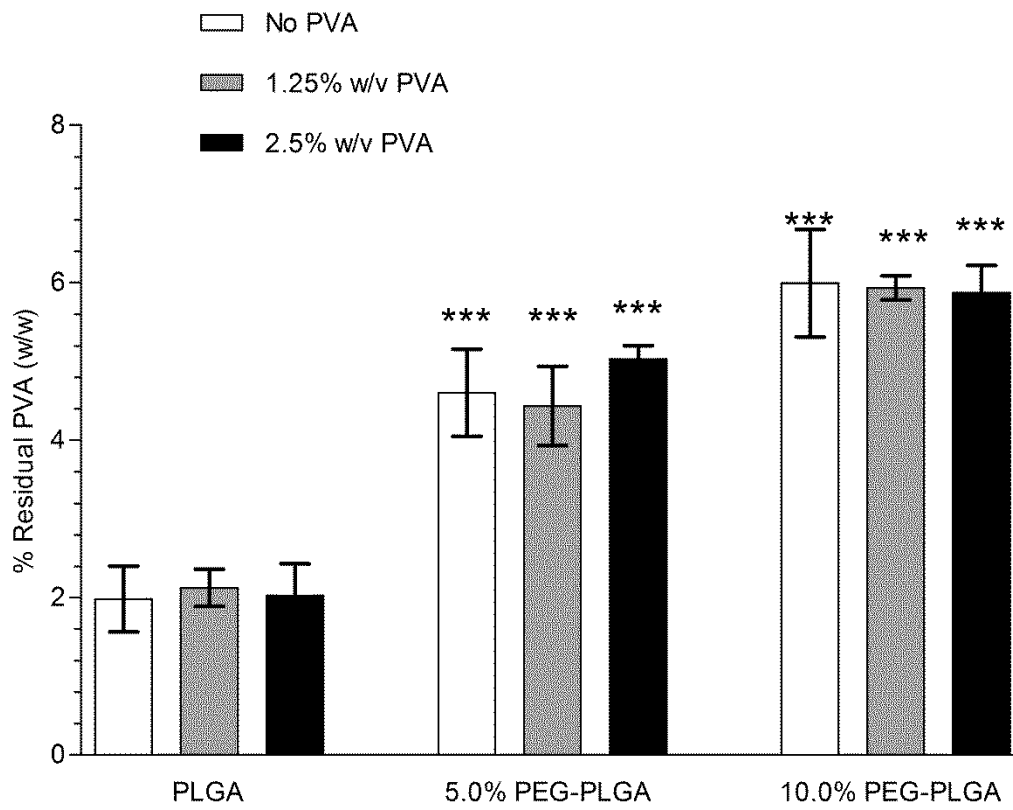


Fig. 10

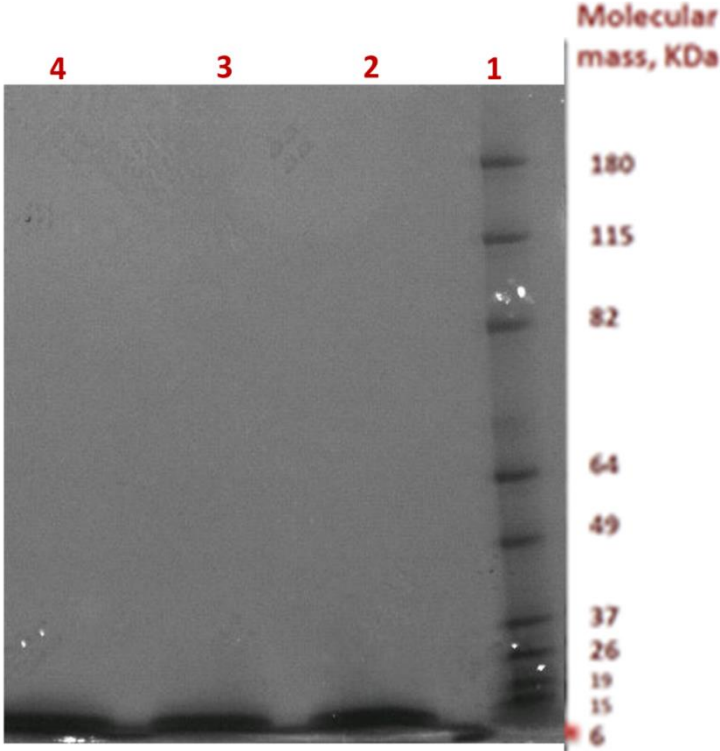
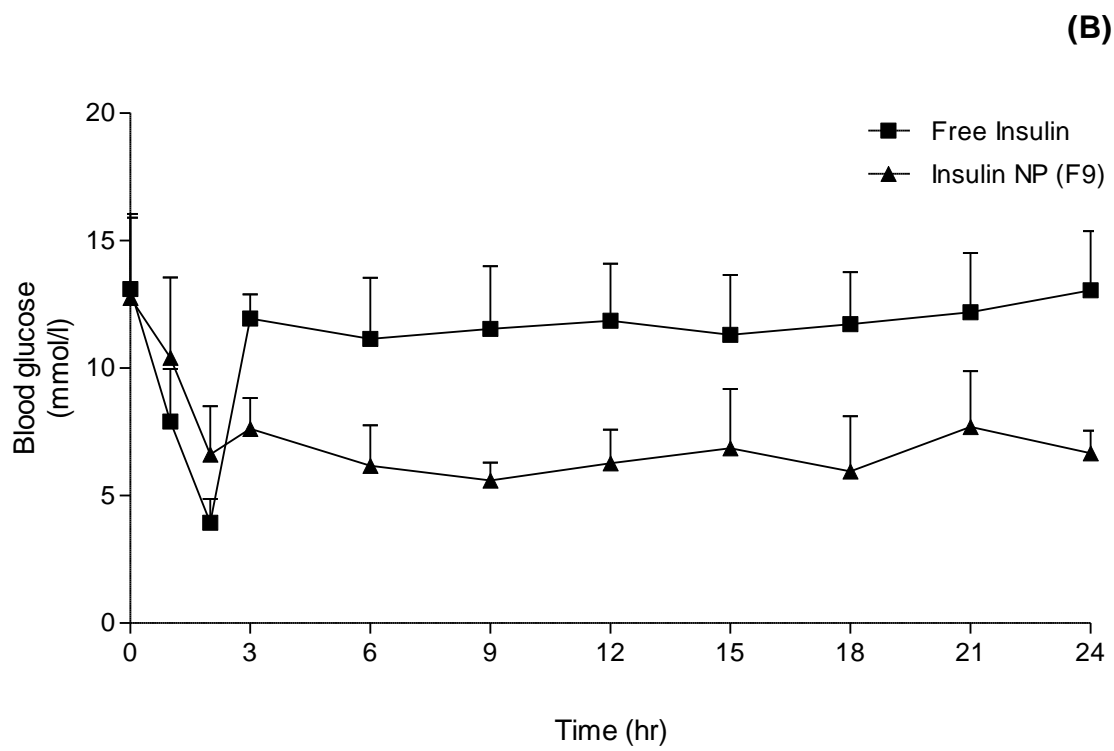
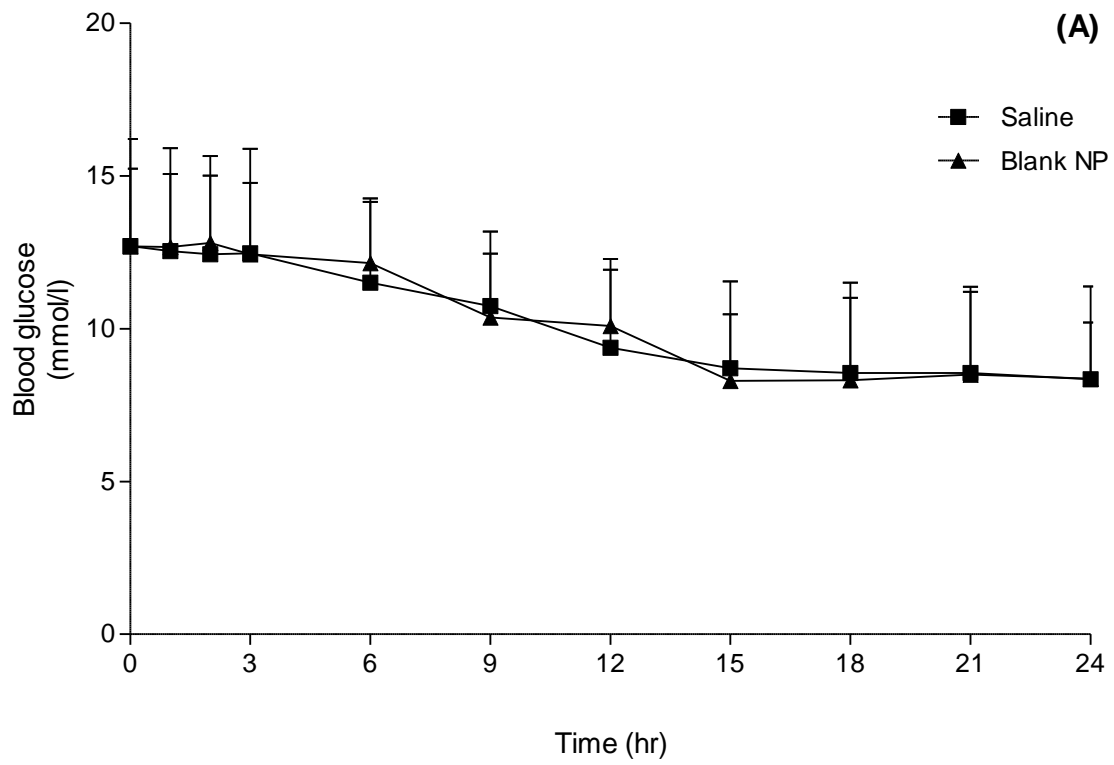


Fig. 11



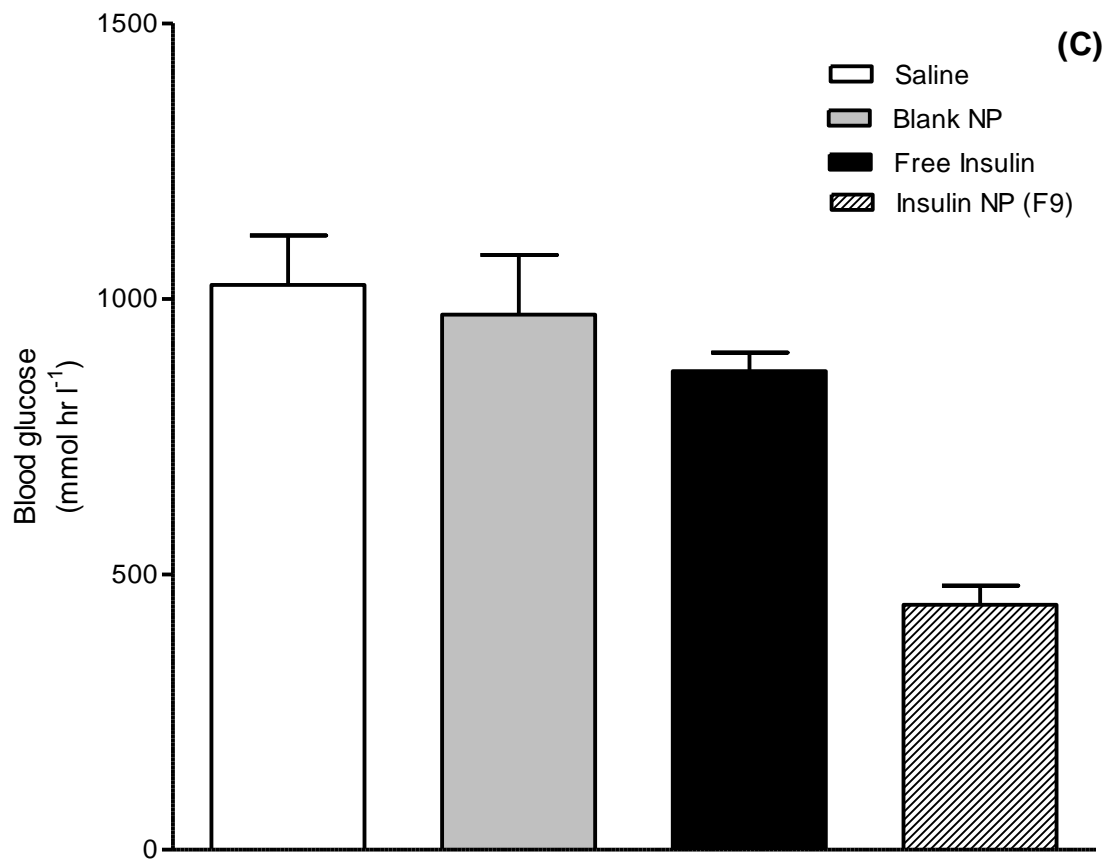
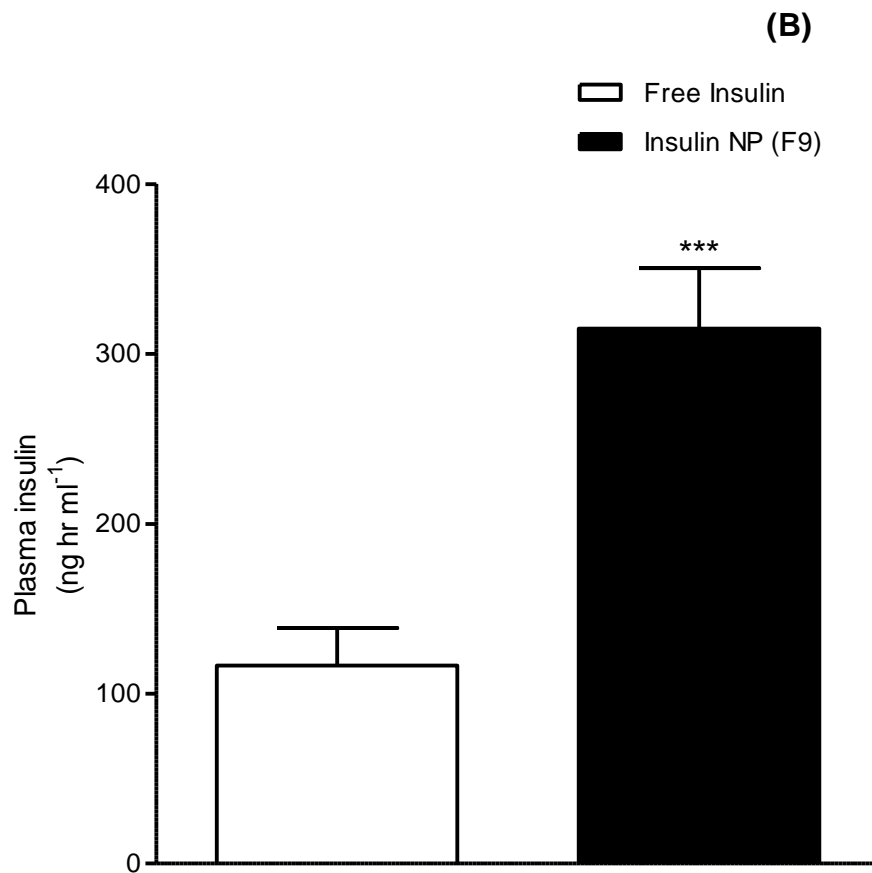
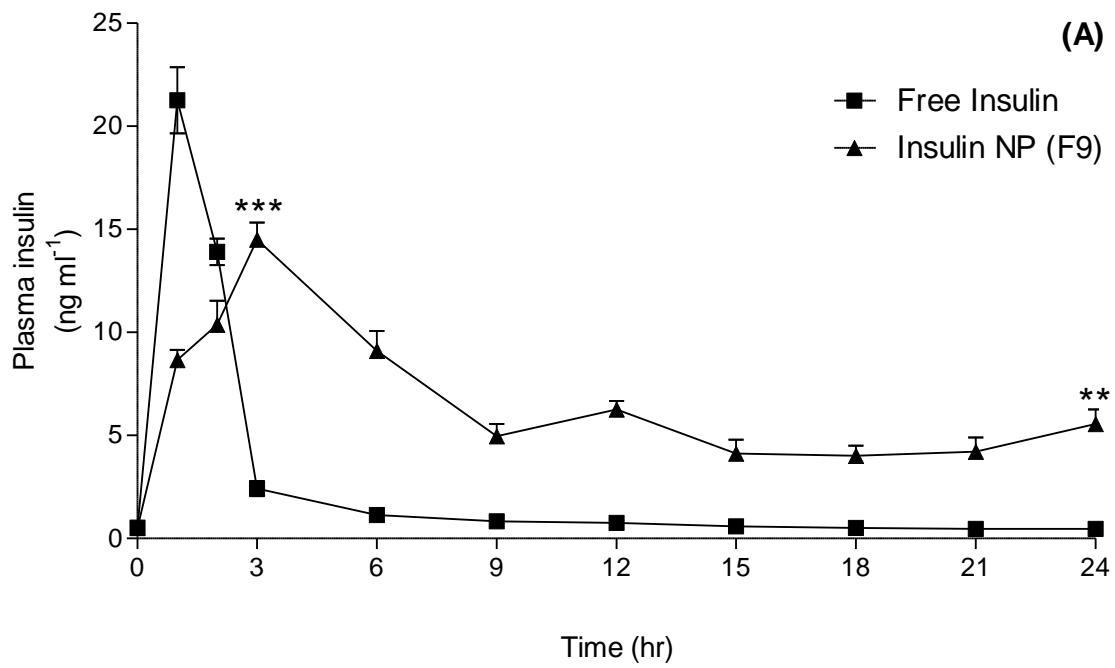


Fig. 12



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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 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 \pm SEM for 6 mice. **P < 0.01, ***P < 0.001 compared with free insulin.