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Preparation and *in vivo* evaluation of insulin-loaded biodegradable nanoparticles prepared from diblock copolymers of PLGA and PEG

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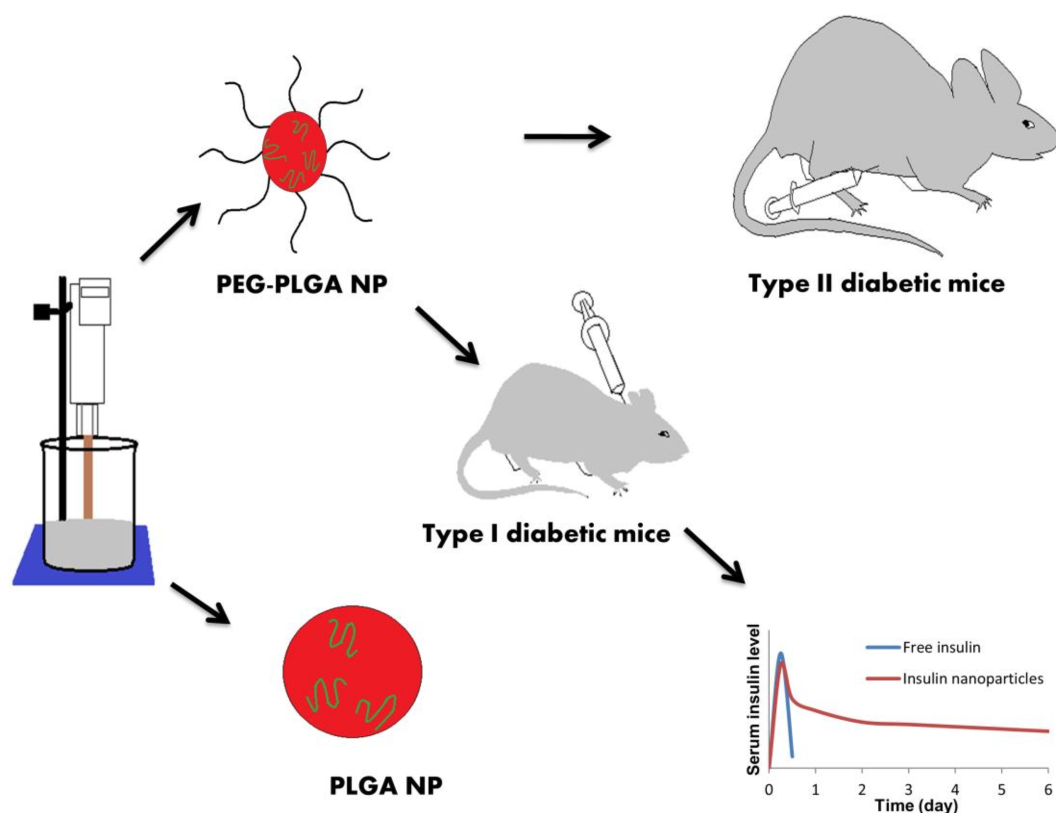
Graphical abstract



ABSTRACT

The aim of this study was to design a controlled release vehicle for insulin to preserve its stability and biological activity during fabrication and release. A modified, double emulsion, solvent evaporation, technique using homogenisation force optimised entrapment efficiency of insulin into biodegradable nanoparticles (NP) prepared from poly (dl-lactic-*co*-glycolic acid) (PLGA) and its PEGylated diblock copolymers. Formulation parameters (type of polymer and its concentration, stabiliser concentration and volume of internal aqueous phase) and physicochemical characteristics (size, zeta potential, encapsulation efficiency, *in vitro* release profiles and *in vitro* stability) were investigated. *In vivo* insulin sensitivity was tested by diet-induced type II diabetic mice. Bioactivity of insulin was studied using Swiss TO mice with streptozotocin-induced type I diabetic profile. Insulin-loaded NP were spherical and negatively charged with an average diameter of 200–400 nm. Insulin encapsulation efficiency increased significantly with increasing ratio of co-polymeric PEG. The internal aqueous phase volume had a significant impact on encapsulation efficiency, initial burst release and NP size.

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Optimised insulin NP formulated from 10% PEG-PLGA retained insulin integrity *in vitro,* insulin sensitivity *in vivo* and induced a sustained hypoglycaemic effect from 3 hours to 6 days in type I diabetic mice.

Keywords:

Insulin, PEG-PLGA, Nanoparticles, stability, controlled delivery, diabetes.

1. INTRODUCTION

Diabetes is a common cause of morbidity and mortality, afflicting about 30 million children and adults in the United States alone (King et al., 1998). Maintaining strict glycaemic control with insulin administration is a vital treatment option for both type I and type II diabetic patients (UKPDS, 1998). Type I diabetes, is characterized by an absolute deficiency of insulin however the majority of diabetic patients have type II disease, which is characterized by reduced sensitivity of cells to insulin action beside a relatively insulin deficiency (Steil, 1999).

Various novel insulin delivery approaches have been described, but parenteral subcutaneous injection is still the mainstay (Agarwal and Khan, 2001). Novel approaches include use of polymeric NP (Richardson et al., 2001) and the careful choice of the encapsulating polymer provides a biodegradable and biocompatible formulation (Mundargi et al., 2008). Biodegradable polyesters are ideal precursors for making NP that encapsulate smaller peptides and proteins, leading to improved pharmacokinetic profiles and reduced frequency of administration (Chan et al., 2009), but they are not without difficulty (Takenaga et al., 2002). The major problem is the inherent instability of therapeutic proteins when exposed to the harsh formulation conditions prevailing during conventional emulsion/solvent removal techniques (Sah, 1999). Conformational instability results in incomplete release from the matrix (Lu and Park, 1995). Other difficulties include a high initial burst release of drug and low encapsulation efficiencies (Yeo and Park, 2004). Further limitations arise due to the innate polyester hydrophobicity. Insertion of poly (ethylene glycol) (PEG) into the polymeric structure, as a block copolymer, is a simple way to alleviate this problem, spawning various formulations, such as NP, hydrogels, micelles and injectable systems possessing more favourable pharmacokinetic parameters (Jeong et al., 1997).

The double emulsion technique is used frequently to process PLGA-PEG block copolymers, producing practical yields and more efficient peptide and protein encapsulation than other methods. It involves dissolution of peptide in an aqueous medium, followed by either sonication-assisted dispersal or homogenisation into an immiscible organic polymer solution. This primary emulsion is then transferred into an aqueous medium containing a stabiliser, which creates a secondary emulsion, again assisted by either sonication or homogenisation. Residual organic solvent can be removed by heat and vacuum (Li et al., 2001).

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Protein microencapsulation using this methodology reveals that the emulsification step is potentially detrimental to the biological activity of the incorporated protein (Li et al., 2000). Upon homogenisation of aqueous protein solutions with dichloromethane, for example, shear stress facilitates the formation of insoluble protein aggregates at the aqueous–organic interface of the primary emulsion (Diwan and Park, 2001). Alternatively, the use of sonication as the source of agitation to form the two emulsion types preserves the physical and chemical stability of insulin during NP fabrication (Fonte et al., 2012; Sarmento et al., 2007; Sheshala et al., 2009). However, low encapsulation efficiency (Sarmento et al., 2007; Sheshala et al., 2009) and high initial burst effect (Fonte et al., 2012; Ibrahim et al., 2005) are observed.

Recently, it is reported that the double emulsion based technologies are widely used for the preparation of micro/nanoparticles of various classes of substances that are pharmaceutically and biopharmaceutically active. Examples include, but are not limited to Leuprolide, Hepatitis B surface antigen, Recombinant human erythropoietin (rhEPO), Tetanus toxoid, Leutinizing hormone releasing hormone (LHRH) antagonist, and Exenatide, all of which exhibited biological activity following *in vivo* administration (Giri et al, 2013).

It has been observed that the inclusion of PEG segments in PLGA polymers played an important role on the properties of micro/nanoparticles. Diblock copolymer PLA-mPEG yielded a more stable interfacial layer at the interface of oil and water phase compared to PLGA, and thus was more suitable to stabilize primary emulsion and protect coalescence of inner droplets and external water phase, resulting in high encapsulation efficiency and stability (Wei et al, 2008).

PLGA microspheres containing peptide and protein drugs and already being marketed is limited to parenteral delivery of leuprolide (LHRH) analogues, human growth hormone and octreotide acetate peptide which were formulated using double emulsion technique (Almeida and Souto, 2007).

The formulation plan used in this study was based on a modified double emulsion solvent evaporation technique, using homogenisation to optimise the entrapment efficiency of insulin and to modify its initial burst from biodegradable NP prepared from PLGA and diblock copolymer variants containing PEG. The influence of various formulation parameters, such as the polymer type, its concentration, stabiliser concentration and volume of internal aqueous phase on the physicochemical characteristics of the NP, *in vitro* release profiles, *in vitro* stability, *in vivo* sensitivity and bioactivity of encapsulated insulin were investigated. Here, we show that an optimised fabrication method was developed to prepare a stable long-acting insulin NP formulation providing basal insulin delivery requirement via a single weekly subcutaneous injection.

2. MATERIALS AND METHODS

2.1 MATERIALS

PLGA (Resomer® RG 503H) with a lactic:glycolic ratio of 50:50 (MW 34 kDa) and block copolymers of poly[(d,l-lactide-*co*-glycolide)-co-PEG] (Resomer® RGP d 5055 (5% PEG, 5

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kDa) and Resomer® RGP d 50105 (10% PEG, 5 kDa)) were purchased from Boehringer-Ingelheim Ltd. (Ingelheim, Germany). Bovine insulin (51 amino acids, MW 5734 Da), poly (vinyl alcohol) (PVA, 87-89% hydrolysed, MW 31000-50000) and phosphate buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, USA). MicroBCA Kit was obtained from Pierce (Rockford, IL). Dichloromethane was of HPLC grade and other reagents were of analytical grade or higher. All water used in this study was produced to Type 1 standard (Milli-Q®, 18.2 MΩ cm at 25 °C).

2.2 Preparation of insulin-loaded NP

The modified, double emulsion, solvent evaporation method used in this work is shown in Figure 1. Insulin (2.0 mg) was dissolved in 0.1 M HCl to form the internal aqueous phase and mixed with 2.0 ml of dichloromethane (DCM) containing different polymers and emulsified (Silverson L5T Homogeniser, Silverson Machines, England) at 6,000 rpm for 2 minutes. The first emulsion (W1/O) was injected directly into 50 ml poly (vinyl alcohol) (PVA) solution under agitation and emulsification continued at 10,000 rpm for 6 minutes to produce a W1/O/W2 emulsion using the same homogeniser. The system was stirred (24 hours) under vacuum to evaporate DCM and prevent pore formation on the surface of the NP. Once formed, NP were centrifuged at 10,000 rpm for 30 minutes at 4 °C using centrifugation (Sigma 3-30K, Germany), washed three times with distilled water and 2% w/v sucrose solution and freeze dried (LABCONCO, Kansas city, Missouri 64132,USA). The final product was stored in a desiccator at room temperature.

2.3 NP Characterisation

Particle size, zeta potential and surface analyses

Lyophilised NP samples (5.0 mg) were diluted with Milli-Q-water to a suitable concentration and suspended with vortex for 5 minutes. The mean diameter and size distribution were analysed by photon correlation spectroscopy (PCS) using a Malvern Zetasizer 5000 (Malvern Instruments, UK). All measurements were performed in triplicate.

Zeta potential analysis was performed on lyophilised NP samples following dilution and adjustment of conductivity using 0.001 M KCl. Electrophoretic mobility was measured using laser doppler anemometry (Malvern Zetasizer 5000, Malvern Instruments, UK). All measurements were performed in triplicate. Surface morphology was studied using scanning electron microscopy (FEI Quanta 400 FEG SEM) following coating with a gold layer under vacuum.

Determination of insulin loading and encapsulation efficiency.

Insulin content was determined by both direct extraction from intact NP and by an indirect procedure based on determination of non-encapsulated insulin. The direct method began by dissolving NP of known mass into 0.5 ml of 1 M sodium hydroxide and incubating overnight at 37 °C. The solution was then neutralised (1 M HCl), centrifuged for 5 minutes at 10000 rpm and the supernatant analysed for total protein content using bicinchoninic acid detection of copper reduction (MicroBCA protein assay kit) (Carino et al., 2000). From this result, the

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percentage loading (w/w, insulin content per unit mass of dry NP) was determined. Encapsulation efficiency was expressed as a ratio of actual insulin loading to the theoretical loading. The indirect method was based on determining non-encapsulated free insulin content in the supernatant using radioimmunoassay(Flatt and Bailey, 1981). The concentrations of insulin in unknown samples were extrapolated using a standard curve of bovine insulin prepared over a concentration range of 3.9x10-3 to 20.0 ng ml^{-1}. Encapsulation in the NP was calculated by the difference between the initial amount of insulin added and the non-entrapped insulin remaining in the external phase after NP formation. Each sample was assayed in triplicate and the average of the two assay method results was represented as the % insulin encapsulation efficiency.

2.4 *In vitro* release studies

Insulin-loaded NP (5.0 mg) were dispersed in 1.0 ml of phosphate buffered saline (PBS, pH 7.4) solution and incubated at 37 °C using a reciprocal shaking water bath at 100 rpm. Samples were taken at predetermined time intervals of 1, 12, 24, 48, 72, 96, 120, 144 and 168 hours and replaced with fresh medium maintained at the same temperature. Samples were centrifuged for 5 minutes at 10000 rpm and the insulin content in the supernatant determined in triplicate by direct analysis and radioimmunoassay, as described previously in section 2.3.

2.5 *In vitro* stability studies

SDS-PAGE analysis was performed using a BioRad Mini Protean II gel apparatus (Hercules, CA). Sampling was performed on the receiver phase of the *in vitro* release experiment. The sample was prepared under non-reducing conditions for application on a gel consisting of 4% and 12% stacking and resolving gel, respectively. Coomassie brilliant blue fixative solution was employed to reveal the separated protein bands. Insulin dispersed in PBS was used as control. Electrophoresis was run in constant current mode of 50 mA with constant voltage modes of 60 V and 120 V during stacking and running, respectively(Park et al., 1998) .

2.6 *In vivo* studies

Experimental animals

Young (8-week-old) male National Institutes of Health Swiss mice (Harlan UK) were age matched, divided into three 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) with access to water and food *ad libitum*. To induce type I diabetes, weight-matched mice received a single intraperitoneal injection of streptozotocin (STZ, 150 mg kg^{-1}) dissolved in phosphate buffer (pH 7.4). For induction of type II diabetes, mice were placed on a special high-fat diet containing 45% kcal from fat, 20% kcal from protein and 35% kcal from carbohydrate (Total energy 19.5 kJg^{-1}, Dietex International Ltd, Witham, UK) for 12 weeks prior to the experiment. Mice with fasting glucose level >8 mmol l^{-1} at 72 hours post-STZ administration were considered diabetic and included in the study. Moreover, animals fed a high fat diet showed clearly manifested features of obesity-diabetes prior to the commencement of the study. All animal experiments were carried

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out in accordance with the UK Animals (Scientific Procedures) Act of 1986 and the University of Ulster's Animal Ethics Committee guidelines.

In vivo sensitivity of insulin formulations

Glycaemic responses of Swiss TO mice, with diet-induced obesity-diabetes, to intraperitoneal injection of the test insulin formulation were assessed. Overnight fasted mice were given saline (control) or 25 U kg^{-1} body weight (ip) of free insulin and insulin-loaded NP suspended in sterile PBS. Blood glucose was measured using an Ascencia Counter meter (Bayer Healthcare, UK) from blood samples collected by tail vein puncture prior to and at 30, 60, 120 and 180 minutes post insulin injection (Trinder, 1969).

In vivo bioactivity of insulin formulation

Four groups (n=5) of diabetic mice were used for this study. Insulin-loaded NP and free insulin were suspended in sterile PBS and injected subcutaneously into the neck region 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 as described previously (Flatt and Bailey, 1981). All blood samples were withdrawn in the morning following an overnight fast, except for samples taken at 0.5, 6 and 12 hours after dosing. Blood glucose concentrations were determined by the glucose oxidase method (Trinder, 1969), while serum insulin was measured by radioimmunoassay (Flatt and Bailey, 1981).

2.7 Statistical analysis

Results are presented as mean ± standard error mean (SEM). Particle size, zeta potential, entrapment efficiency and values of *in vitro* release profiles were treated statistically using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For *in vivo* studies, values were compared using one-way ANOVA followed by Student-Newman-Keuls *post hoc* test. Area under the curve (AUC) analysis was performed using the trapezoidal rule with baseline correction. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

This study investigated the outcome of variation in four key formulation parameters, as illustrated in table 1, on the physicochemical characteristics of insulin-loaded NP. The polymer type and its concentration in the organic phase of the primary emulsion were investigated. The concentration of PVA in the continuous phase of the secondary emulsion was also examined, together with the volume of aqueous phase used to dissolve the insulin at the beginning of the procedure. Experimental responses, such as particle size, surface charge, morphology, encapsulation efficiency and *in vitro* release were determined.

3.1 Effect of polymer type

The physicochemical properties of three different formulations of insulin NP (F1, F4 and F7) (Table 1) made by varying the polymer type, are presented in Figure 2. NP were characterised

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by a larger diameter relative to those prepared by other reported techniques, such as nanoprecipitation or the single emulsion methods (Mora-Huertas et al., 2010). However, All NP formulations showed low polydispersity index (PDI) ranging from 0.185 to 0.442. PLGA NP (F1) was significantly bigger in size (P value < 0.05) than those prepared from 5% PEG-PLGA (F4) and 10% PEG-PLGA (F7) diblock copolymers (Figure 2.A). Increasing the percentage of PEG in the polymer and using a lower molecular weight (compared to the PLGA polymer) caused a decrease in diameter, a finding explained by copolymeric PEG decreasing polymer association during NP formation (Beletsi et al., 2005) . F1 exhibited negative zeta potential values of −20.9 mV, which was significantly higher (P value < 0.001) than that of the PEGylated PLGA (F4 and F7) types (Figure 2.B). PEG is known to be amphiphilic, decreasing measurable zeta potential by shielding the native PLGA surface charge (Mora-Huertas et al., 2010). Insulin loading and encapsulation efficiency were significantly increased (P value < 0.01) in PEGylated PLGA NP (F4 and F7) compared to PLGA (F1). Increasing the % PEG in the polymer resulted in further significant increases (P value < 0.05). The amphiphilic nature of PEG-PLGA facilitates micelle formation in the primary emulsion and enhances the encapsulation of hydrophilic molecules (Locatelli and Comes Franchini, 2012). The higher encapsulation efficiency seen with 10% PEGylated PLGA NP (Figure 2.C) can be explained by considering its lower solubility in DCM compared to both PLGA and 5% PEGylated PLGA. Unwanted diffusion of hydrophilic drugs into the continuous phase of the secondary emulsion is most likely to occur during the polymer solidification stage, which is normally during the first 10 minutes of emulsification. PEGylated polymers with low solubility in DCM will solidify quicker during this crucial phase and give rise to higher encapsulation efficiencies (Mehta et al., 1996). *In vitro* release profiles for F1, F4 and F7 showed evidence of a burst release, with the rate significantly higher from F4 and F7, which released approximately 66% and 72% of insulin, respectively, within the first 24 hours, when compared to 38% of insulin released from the F1 (Figure 2.D). Burst release phenomenon is attributed to drug attached on or close to the surface of the NP (Essa et al., 2010). These results suggest that there is a higher amount of insulin attached to the PEGylated NP surface compared to PLGA NP because of presence of PEG chains. Moreover, PEG chains are hydrophilic and can be easily hydrated in an aqueous release medium and this facilitates water permeation into the NP matrix, allowing insulin to be released. A higher content of PEG accelerated surface dissolution because water was taken up more readily when compared with the PLGA NP (Locatelli and Comes Franchini, 2012).

3.2 Effect of PVA concentration in the external aqueous phase

PVA is a commonplace stabiliser utilised in the formulation of PLGA NP, producing desirable and tightly dispersed size distributions (Sahoo et al., 2002). PVA concentration in the external aqueous phase of the secondary emulsion has been shown to control NP size (Figure 3.A), which was a finding also observed in this work (Jeffery et al., 1993). This was attributed to increased viscosity of the continuous phase and enhanced emulsion stability during homogenisation. Increasing the emulsion stability prevented premature emulsion coalescence and maintained a small mean and tight size distribution following NP maturation (Zambaux et al., 1998). Increasing the PVA concentration resulted in a significant decrease in zeta potential

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(P value < 0.05) across all three polymer types, as seen in Figure 3.B. This drop is explained by coating of the NP with a residual PVA layer, thereby attenuating surface charge. Moreover, a significant increase in insulin loading (P value < 0.05) and encapsulation efficiency were obtained by increasing PVA concentration (Figure 3.C). This is explained on the basis of increased viscosity of the continuous phase in the secondary emulsion and interfacial effects, shown elsewhere to resist insulin diffusion out of polymer-rich phases, leading to higher protein loadings (Zambaux et al., 1998). The release profiles of different insulin-loaded NP prepared with 1.25%, 2.5%, and 5% PVA are shown in Figure 3.D. The release of F1, F4 and F7 prepared with 1.25% PVA in the external phase showed a significantly lower initial burst release (P value < 0.05) compared to those for F3, F6 and F9 prepared with 5% PVA. If the burst release is considered to be diffusion primarily from peripheral drug, then any parameter that reduces mean NP size, such as PVA concentration, is expected to increase surface area and the rate of release. The results in this work and the findings of others support that contention (Fude et al., 2005).

3.3 Effect of polymer concentration

The effect of polymer concentrations on the physicochemical properties of insulin-loaded NP is shown in Figure 4. Three different formulations for each type of polymer were used (F1, F10 and F11), (F4, F12 and F13) and (F7, F14 and F15) (Table 1). NP size was increased significantly (P value < 0.01) by increasing polymer concentration in DCM from 2.5% w/v to 7.5% w/v (Figure 4.A). This arises due to increased viscosity of the organic phase and the formation of larger dispersed droplets in the secondary emulsion under conditions of constant shear (Fude et al., 2005; Lamprecht et al., 2000). However, increasing the polymer concentration from 2.5% w/v to 5% w/v did not increase PLGA NP size significantly (F1, F10) (P value > 0.05), but did when 5% PEG-PLGA and 10% PEG-PLGA were used (P value <0.05) at these intermediate levels (F4, F12 and F7, F14, respectively). It was also shown that increasing the polymer concentration had no effect on the surface charge (P value > 0.05) (Figure 4.B). Significant increases in insulin encapsulation efficiency were demonstrated by increasing the different polymer concentration from 2.5% w/v (F1, F4 and F7) to 7.5% w/v (F11, F13 and F15) (Figure 4.C) but in a similar pattern observed with NP size, increases of 2.5% w/v to 5% w/v PLGA did not enhance insulin loading (F1 and F10). These patterns of enhanced entrapment efficiency are attributed to the higher viscosity of the DCM phase as more polymer amount is present and the ensuing larger size of the primary emulsion droplets. Furthermore, higher polymer concentrations give rise to more rapid polymer deposition as the DCM is removed from the system, which is expected to retard any unwanted insulin dissolution into the continuous phase of the secondary emulsion (Rafati et al., 1997).

The influence of different polymer concentrations on the release profile is shown in Figure 4.D. The initial burst release was decreased by increasing the polymers concentrations. PLGA NP (F11) showed the lowest burst effect of 23.6% of insulin released in 24 hours. Similar patterns were observed after increasing the polymer concentration of the PEGylated variants, A decrease in burst release from 66% to 42% and from 72% to 52% was observed with 5% PEG-PLGA NP and 10% PEG-PLGA NP, respectively, The decrease in initial release is explained by rapid solidification of the polymer-rich phase due to higher polymer concentrations in the

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primary emulsion. This process leads to a dense matrix that offers low permeability to entrapped protein and a more tortuous structure because of the chain entanglement(Yang et al., 2001).

3.4 Effect of internal aqueous phase volume

The influence of the internal aqueous phase volume on the physicochemical characteristics is shown in Figure 5. In this work, the volume of organic phase was held constant and so increases in the internal aqueous phase volume would lead to an increase in the mean diameter of the dispersed phase in the secondary emulsion (Figure 5.A) (Crotts and Park, 1995). Variation in internal aqueous phase volume did not affect zeta potential (Figure 5.B). However, significant effects (P value <0.05) were observed on the encapsulation efficiency (Figure 5.C). A small internal phase volume of 0.2 ml enhanced loading, although this finding is not always observed (Crotts and Park, 1995). The results in this work could be explained by the effectiveness of the deposited polymer network during DCM evaporation from the secondary emulsion. Increases in the internal aqueous phase whilst the organic phase remains constant means that the thickness of a nascent polymer film is thinner and is less effective in preventing premature leakage of insulin and other potential payloads into the continuous phase of the secondary emulsion (Li, 1999). Insulin release profiles are shown in Figure 5.D with the initial burst release from NP with the lowest internal water volume (F16, F18 and F20) (Table 1) significantly lower (P value <0.05) than those of higher internal phase volumes (F17, F19 and F21). It has been shown that increases in internal phase volume produce NP with discernible surface pores, leading to a particulate matrix that is receptive to release medium ingress and payload dissolution (Crotts and Park, 1995). The data in Table 2 show the formulations considered to be optimised for further investigation. These data detail NP with low mean size and high encapsulation efficiency. Increasing the polymer concentration (10% PEG-PLGA) to 5% and 7.5% w/v, in order to decrease the initial burst release, was achieved in F22 and F23. F22 was used as the optimised formulation for further *in vitro* and *in vivo* characterisation based on its lower NP size and lower PDI compared to F23 besides higher encapsulation efficiency and lower burst release compared to F20.

3.5 Scanning electron microscopy

Figure 6.A shows an SEM image of F22. The insulin NP had a spherical appearance with a narrow size distribution. The nanoparticles' surface is free from pores. Figure 6.B was obtained after 7 days of release and show insulin-loaded NP displaying poor retention of spherical shape with increased evidence of erosion. The high content of PEG in the block polymer resulted in the formation of large pores and voids, which subsequently favoured insulin diffusion from the NP (Kang and Singh, 2001). This is consistent with release data showing approximately 90% of insulin released in the first 4 days of *in vitro* analysis.

3.6 *In vitro* stability and *in vivo* insulin sensitivity

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Encapsulation of insulin in polymer constructs is problematic for a number of reasons. Its surface activity leads to adsorption at interfaces, resulting in instability caused by unfolding, inactivation or irreversible aggregation. Furthermore, high rates of shear, often associated with emulsification based techniques, affect the three-dimensional structure of insulin, maintenance of which is essential for therapeutic activity (Manoharan and Singh, 2009; Perez et al., 2002). Formation of insulin loaded NP by a double emulsion technique uses either sonication or homogenisation as a source of high shear agitation (Blum and Saltzman, 2008). In the current work, homogenisation was used to create both primary and secondary emulsions, due to the following reasons. It is an effective means to form (w/o/w) double emulsion, resulting in NP with high reproducibility, particle size uniformity and low polydispersity (Li et al., 2008). Additionally, there is evidence that microparticles prepared using homogenisation gave higher encapsulation efficiencies with desirable linear release profiles of hydrophilic drugs, such as DNA, when compared to the same particles fabricated using sonication, which resulted in a lower DNA encapsulation efficiency and a higher burst release (Blum and Saltzman, 2008). Given its susceptibility to instability, insulin aggregation during formulation and after *in-vitro* release were analysed as part of this study. Analysis using SDS PAGE, as shown in Figure 7, shows the insulin band of the naked and encapsulated insulin. Entrapped insulin did not suffer significant aggregation following formulation and during *in vitro* release. It could be argued that the surface activity of PEGylated PLGA during formation of the primary emulsion impedes insulin adsorption at the DCM-water interface and helps prevent possible aggregation. To investigate conformational stability and sensitivity of insulin encapsulated in PEG-PLGA, Swiss TO mice with diet-induced obesity-diabetes (type II) were used. This diabetic animal model is closely aligned to diabetic obesity found increasingly in humans consuming high-fat and energy-rich diets (McClean et al., 2007). The high-fat diet resulted in glucose intolerance and impaired insulin sensitivity in animals used in this study (Bailey et al., 1986; Flatt et al., 1990). Results in Figure 8(A&B) showed that the optimised insulin NP (F22) significantly decreased plasma glucose level (P value <0.01) directly after intraperitoneal injection, compared to the saline group. It showed similar hypoglycaemic control compared to free insulin at the same dose of 25 Ukg^{-1} during the first three hours after injection. However, the decrease in blood glucose level occurred after treatment with the free insulin was higher than insulin released from the NP due to only initial burst release during the first three hours. These results demonstrated the sensitivity of the encapsulated insulin, which in turn validated its conformational stability.

3.7 *In vivo* insulin bioactivity

The effect of a single subcutaneous administration of insulin NP (F22) on blood glucose level of streptozotocin-induced type I diabetic mice is shown in Figure (9.A-9.C). Streptozotocin uptake via the cell membrane GLUT2 glucose transporter, causing DNA alkylation and eventual β cell death, mimics autoimmune T-lymphocytes-related insulitis that characterizes type 1 diabetes in humans (Rossini et al., 1977; Deeds et al, 2011). The appropriateness of chemically-induced diabetes in investigations involving drug testing or in therapies working via non-beta cell dependent pathways, such as new insulin formulation tested in this study, has been widely reported (Jederstrom et al., 2005; Sheshala et al., 2009).

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Following subcutaneous injection of free insulin (25 Ukg^{-1}), blood glucose level decreased significantly by 58 % (P value <0.05) after 30 minutes. The plasma glucose level decreased further to its lowest level of 21% of the original value, 3 hours after administration. This effect was maintained up to 3 hours, then glycaemia increased sharply and plasma glucose values started to return to its control level before the injection. The glycaemic profile of the same dose of insulin NP showed only a 12% reduction in blood glucose levels after the first 30 minutes. After 3 hours, the maximum decrease in plasma glucose was similar to what was observed for free insulin. A significant difference between the two groups was noted after 6 hours post injection with insulin NP maintaining a significant decrease in blood glucose level (P value <0.05) for up to 144 hours after the injection. Beyond this period, no significant difference was observed in blood glucose level in mice injected with free insulin or insulin NP (P value >0.05). During the first 6 days following injection, the blood glucose level of mice treated with F22 did not return to the original level compared with control mice or mice injected with free insulin. These observations indicate that the NP had the ability to hold and release insulin in a sustained manner. Plasma insulin levels in mice injected with saline, free insulin or encapsulated insulin (Figure 10.A) revealed a mean peak for serum insulin concentration (Cmax) of 16$ngml^{-1}$ achieved after 30 minutes of injection in mice treated with free insulin. This was followed by a 97% (P<0.001) decreased after 3 hours. However, mice injected with encapsulated insulin showed a peak insulin concentration 2 hours after injection followed by a 79% decrease in serum insulin level after 24 hours. The therapeutic insulin level in the latter mice remained between 3.36 and 0.6 ng ml^{-1} for up to 6 days post injection. Moreover, a significant difference in blood glucose level (P value <0.001) was observed in the insulin NP group for up to 144 hours (Figure 10.B). It was clear that insulin NP prolonged the hypoglycaemic effect of the free insulin from 3h to 6 days. Parenteral administration of PEG-PLGA insulin NP resulted in a depot injection at the site of administration that could release the encapsulated insulin by gradual diffusion across the polymeric matrix.

Though oral delivery of insulin remains a challenge due to its limited oral absorption resulting from rapid degradation by proteolytic enzymes in the gastrointestinal tract and inadequate transport across the intestinal epithelium, encapsulation of insulin into nanoparticles has been identified as a promising means of facilitating oral insulin delivery due to its ability to promote greater insulin transport across the intestinal mucosa (Fonte et al., 2013). While it is not yet clear if the encapsulation reported in this study prevented proteolytic degradation of insulin, our data highlight a sizeable difference between the slower insulin release kinetics *in vivo* compared to *in vitro* results. The major reason for the slower *in vivo* kinetics might be attributed to lower water availability in the body tissues compared with the *in vitro* conditions, in which insulin NP are incubated in aqueous release media at 37 °C with continuous shaking (Blanco-Prieto et al., 2004). Based on this, the tissue micro-environment surrounding the insulin NP will retard *in vivo* release.

4. CONCLUSIONS

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Optimising the entrapment efficiency and release kinetics of insulin using a modified double emulsion solvent evaporation technique utilising homogenisation while preserving stability and biological activity during fabrication and release can be achieved through adjustment of process variables. Of particular importance is the ratio of the internal aqueous phase to the volume of organic solvent that comprising the primary emulsion. Insulin-loaded 10% PEGylated PLGA NP with improved physicochemical properties was superior to conventional PLGA NP based on *in vitro* characterisation. The amphiphilic nature of the PLGA-PEG block copolymers allow for better dispersion of the insulin-containing aqueous phase into the double emulsion, thus improving encapsulation efficiency. Moreover, PEG residues in the matrix preserve insulin stability during manufacture, minimising aggregation and inactivation, as confirmed by *in vivo* results. A single weekly injection of PEGylated insulin NP sustained the delivery of basal insulin requirement in streptozotocin-induced mice.

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Figure captions

Figure 1: Sequence of NP manufacturing steps involving formation of primary and secondary emulsions. The experimental factors changed in the study comprised variation in internal aqueous phase volume, polymer type, polymer concentration and PVA concentration.

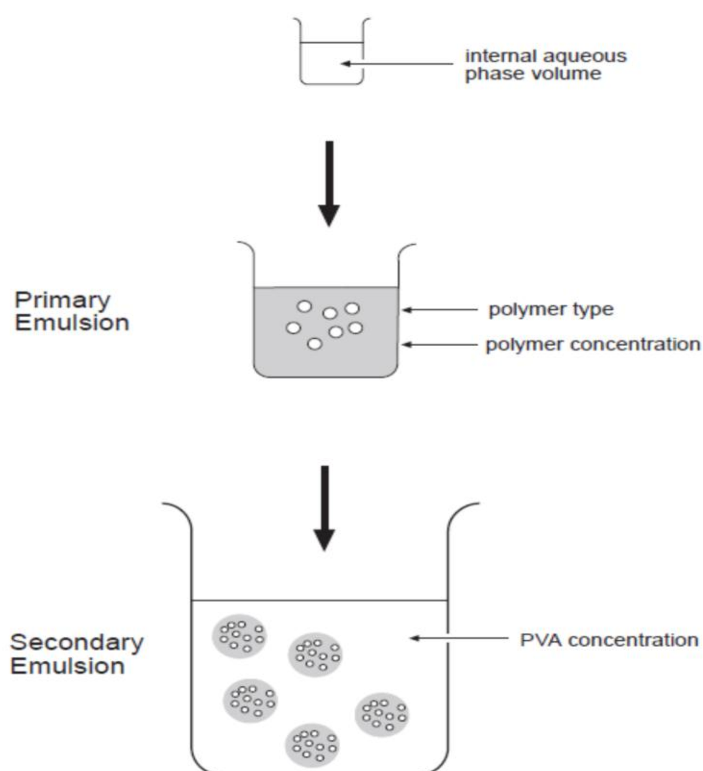

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Figure 2: Effects of polymer type on insulin nanoparticle size (A), zeta potential (B), encapsulation efficiency (C) and insulin *in vitro* release (D). Values are mean ± SEM with n=3. For 1A-1C, *P<0.05, **P<0.01, ***P<0.001 compared with PLGA. $^{\Delta}$P<0.05 compared with 5% PEG-PLGA.

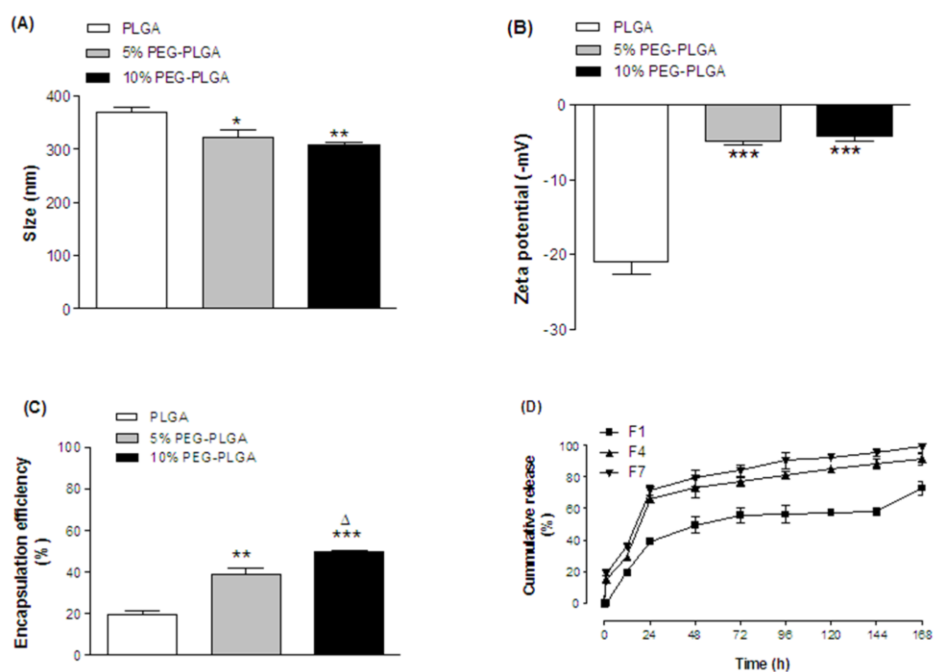

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Figure 3: Effects of PVA concentration in the external aqueous phase on insulin nanoparticle size (A), zeta potential (B), encapsulation efficiency (C) and insulin *in vitro* release (D). Values are mean ± SEM with n=3. For 1A-1C, *P<0.05, **P<0.01, ***P<0.001 compared with 1.25% PVA for each polymer type. $^{\Delta}$P<0.05, $^{\Delta\Delta}$ P<0.01, $^{\Delta\Delta\Delta}$P<0.001 compared with 2.5% PVA for each polymer type.

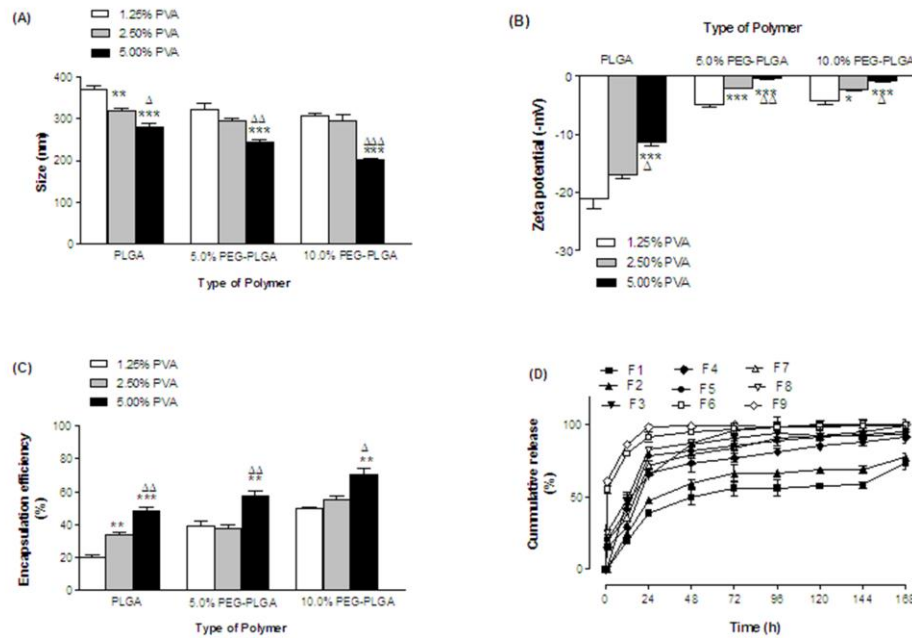

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Figure 4: Effects of polymer concentration on insulin nanoparticle size (A), zeta potential (B), encapsulation efficiency (C) and insulin in vitro release (D). Values are mean ± SEM with n=3. For 1A-1C, *P<0.05, **P<0.01, ***P<0.001 compared with 2.5% polymer concentration for each polymer type. $^{\Delta}$P<0.05, $^{\Delta\Delta}$ P<0.01, $^{\Delta\Delta\Delta}$P<0.001 compared with 5% polymer concentration for each polymer type.

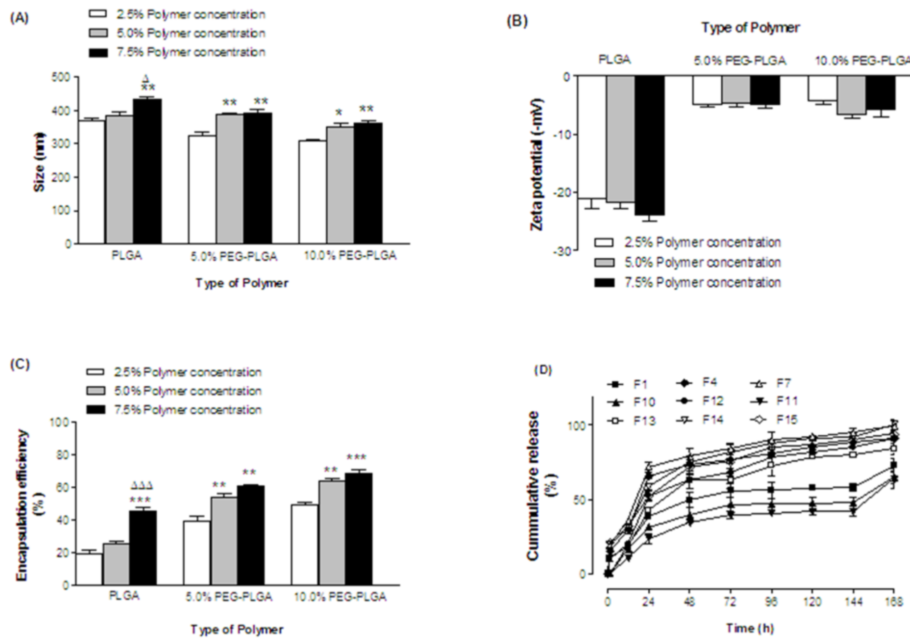

Figure 5: Effects of internal water volume on insulin nanoparticle size (A), zeta potential (B), encapsulation efficiency (C) and insulin in vitro release (D). Values are mean ± SEM with n=3. For 1A-1C, *P<0.05, **P<0.01, ***P<0.001 compared with 0.2ml for each polymer type. $^{\Delta}$P<0.05, $^{\Delta\Delta}$ P<0.01, $^{\Delta\Delta\Delta}$P<0.001 compared with 0.5ml for each polymer type.

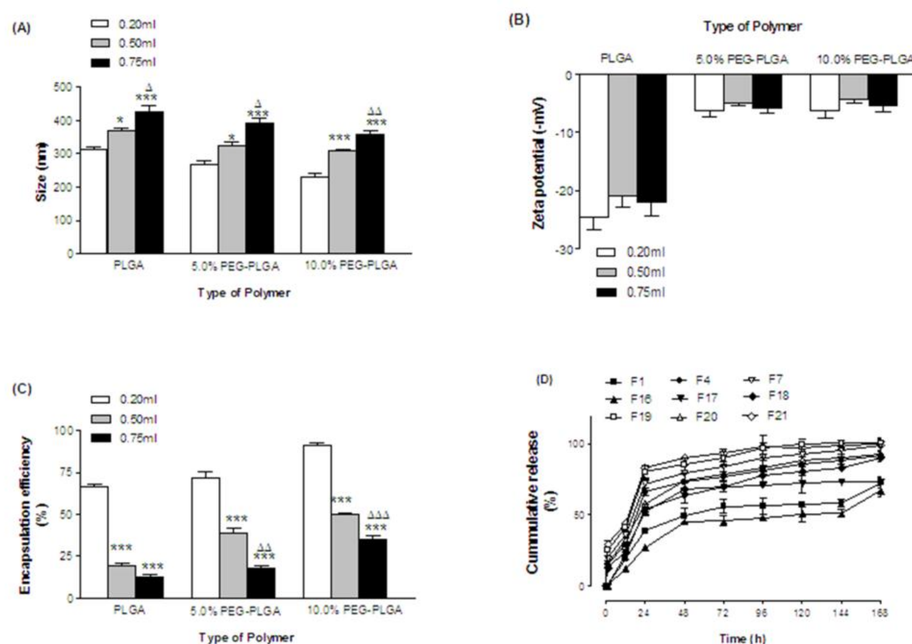

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Figure 6: Scanning electron micrographs of F22 after formulation (A) and after 7 days of *in vitro* release (B).

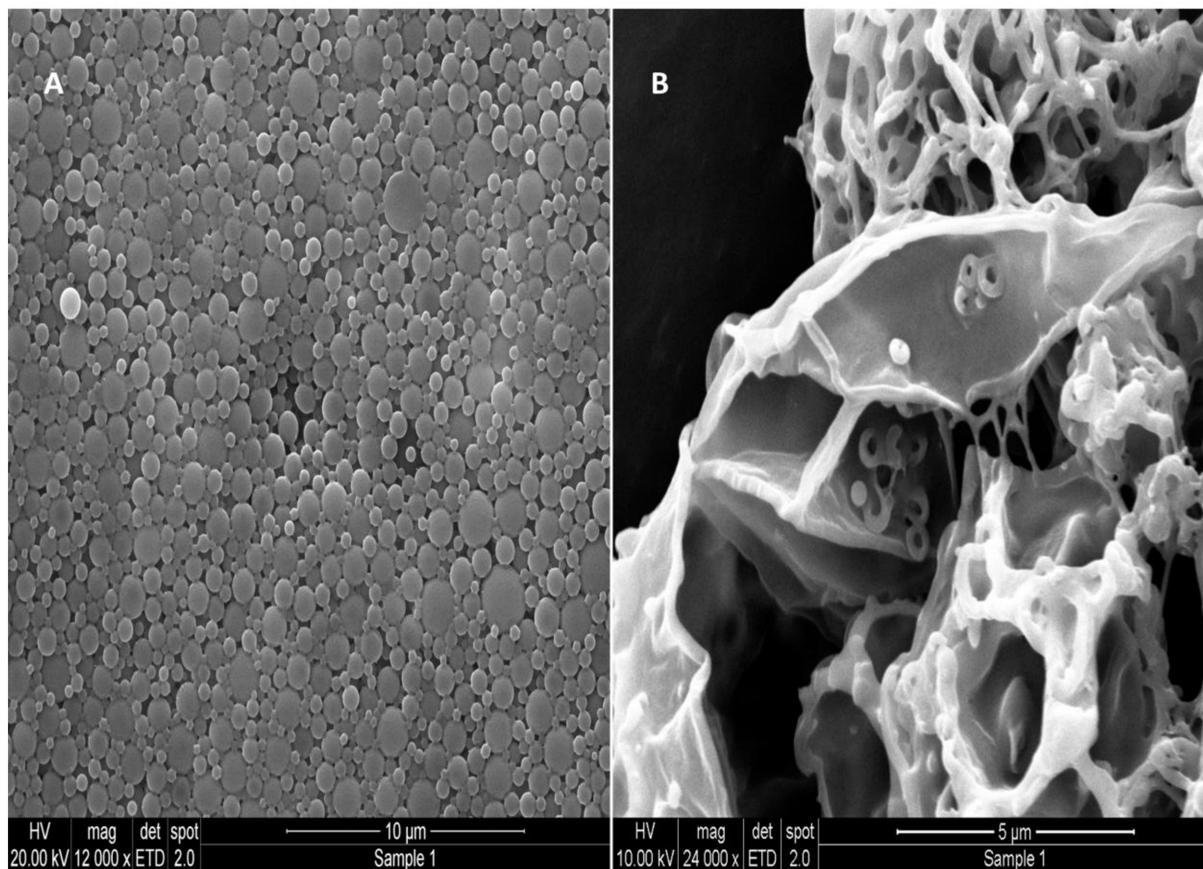

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Figure 7: SDS PAGE images of free insulin and an insulin sample released from NP formulation (F22) after 7 days of *in vitro* analysis. Molecular weight markers (lane 1), Free insulin dispersed in PBS (lane 2), Insulin released from F22 (lane 3).

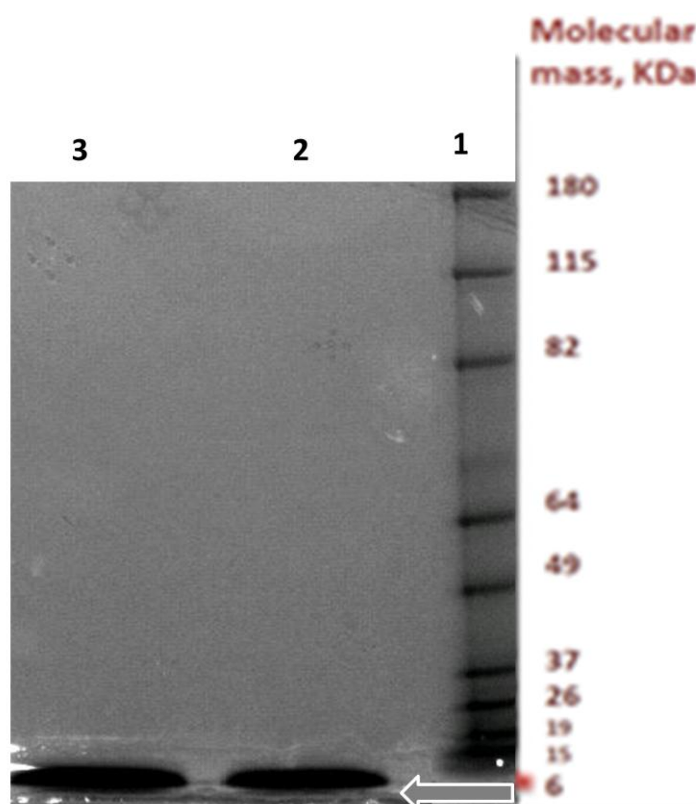

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Figure 8: Insulin sensitivity in Swiss TO mice with diet-induced obesity-diabetes expressed as line graph (A) and area under the curve (B). Values are mean ± SEM with n = 4. Plasma glucose was measured prior to and at 30, 60, 120 and 180 minutes after intraperitoneal injection of saline, insulin or nanoparticle encapsulated insulin. **P<0.01 compared with the saline group. Insulin NP is F22.

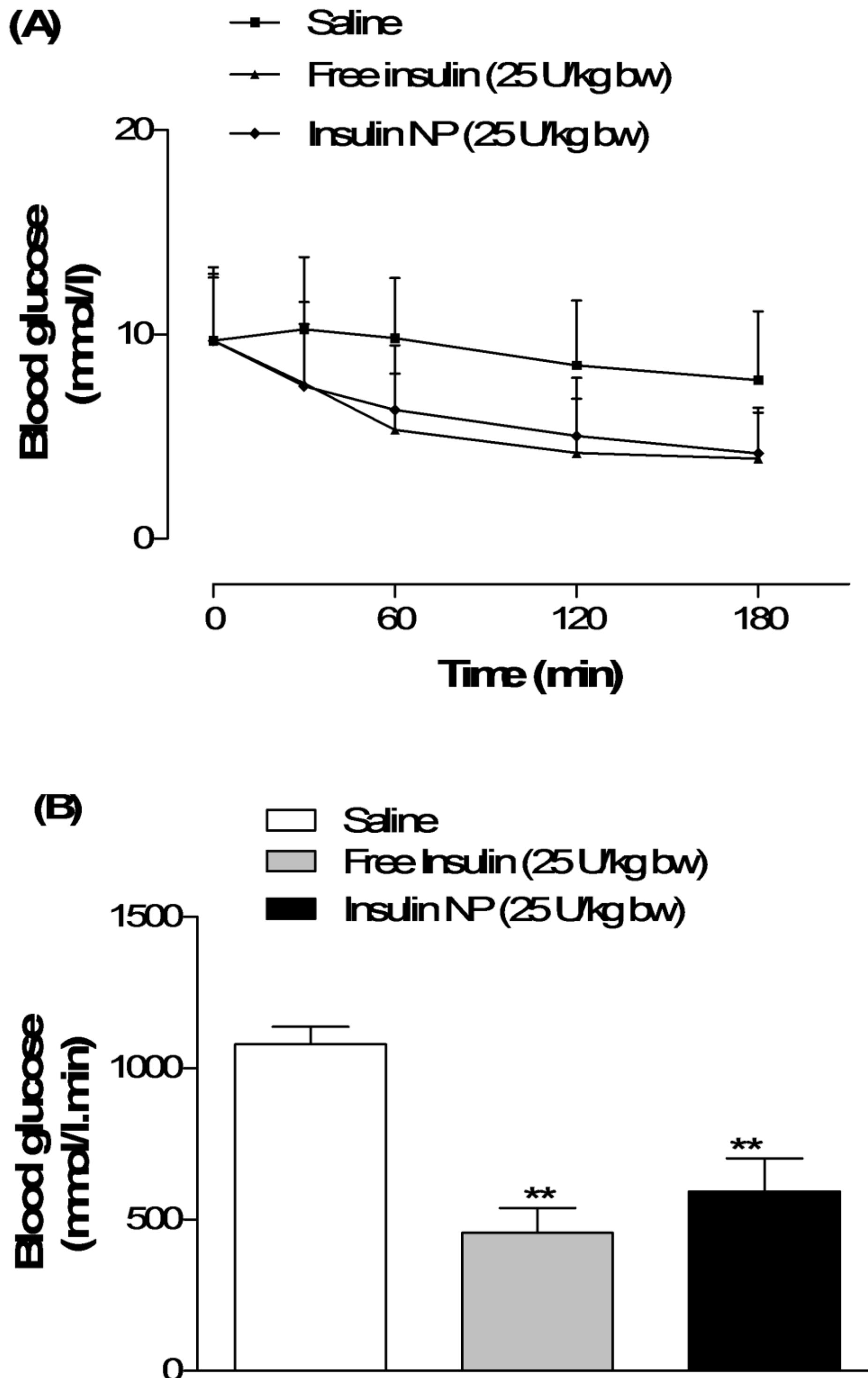

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Figure 9: Effects of insulin and insulin NP on blood glucose concentrations in streptozotocin-induced diabetic Swiss TO mice expressed as line graph (A&B) and area under the curve (C). Blood glucose concentrations were measured prior to and after intraperitoneal injection of insulin or insulin NP (25U/kg bw) in streptozotocin-induced mice for 144 hours. Values are Mean ± SEM for 6 mice. *P<0.05, **P<0.01compared to saline. $^{\Delta}$P<0.05 compared to free insulin. Insulin NP is F22.

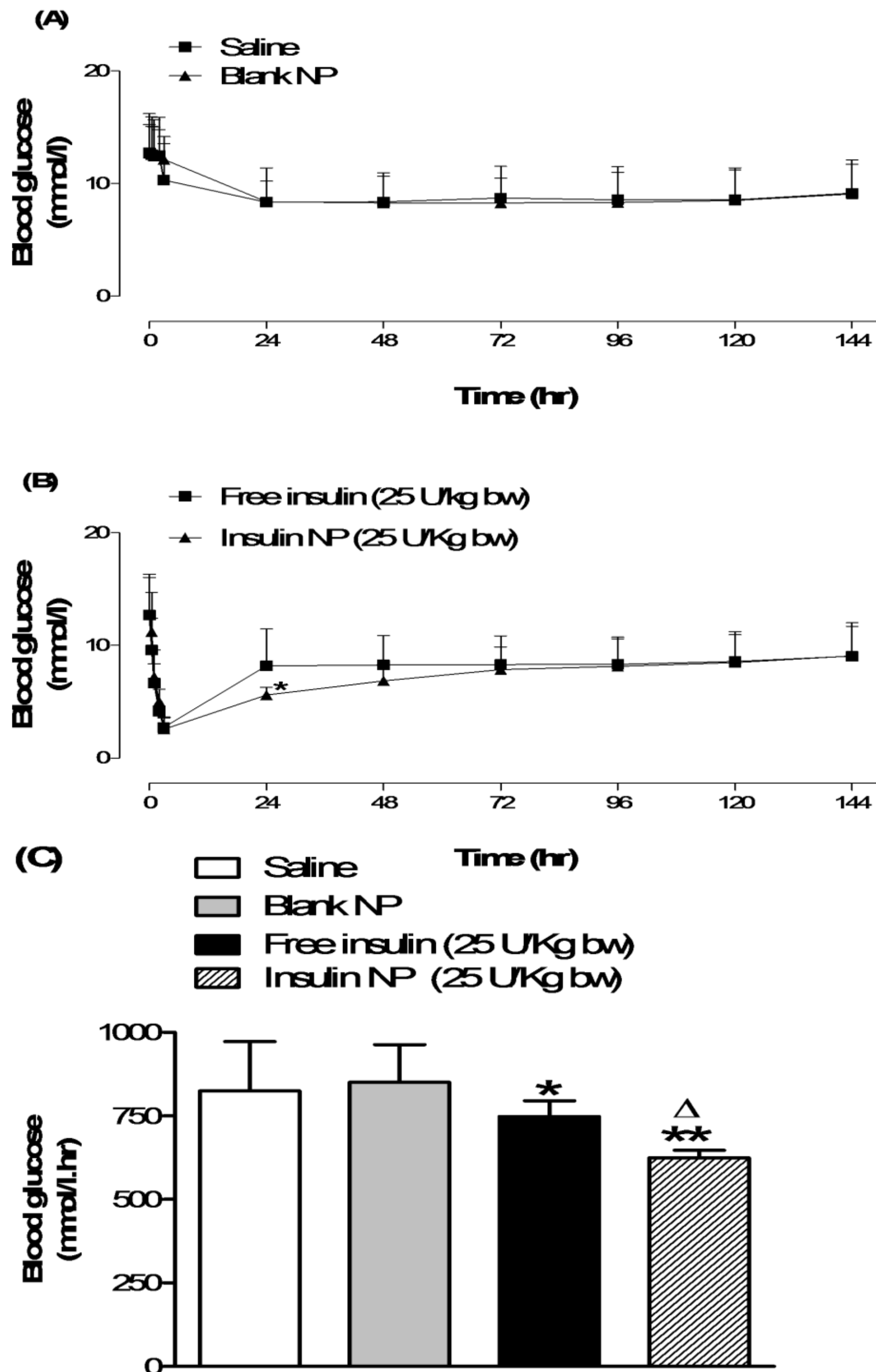

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Figure 10: Effects of insulin and insulin NP on plasma insulin concentrations in streptozotocin-induced diabetic Swiss TO mice expressed as line graph (A) and area under the curve (B). Plasma insulin concentrations were measured prior to and after intraperitoneal injection of insulin or insulin NP (25U/kg bw) in streptozotocin-induced mice for 144 hours. Values are Mean ± SEM for 6 mice. *P<0.05, **P<0.01, ***P<0.001 compared with free insulin. Insulin NP is F22.

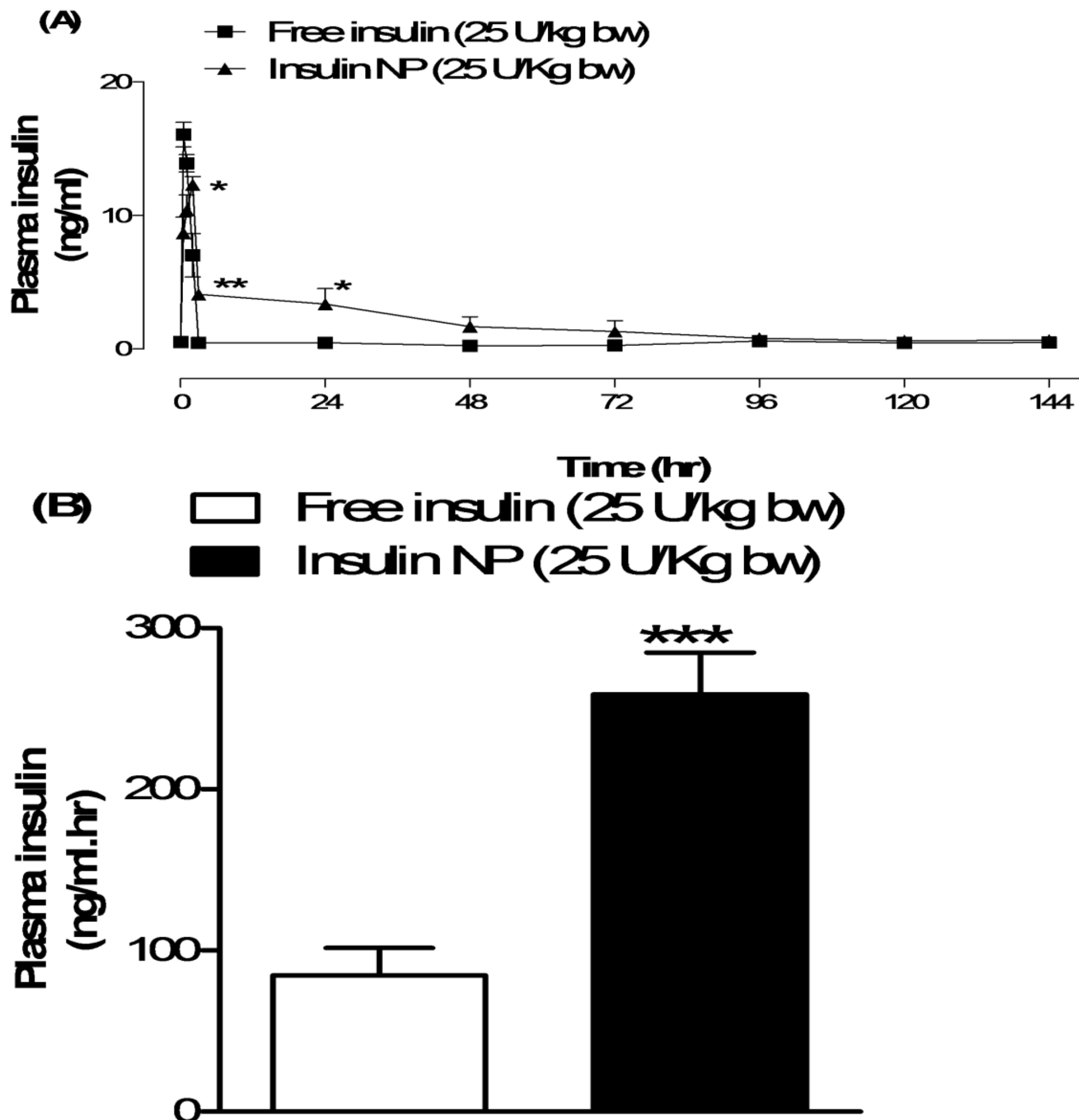

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Table 1 Formulation identification and parameters used to produce insulin NP

Formulation ID	Polymer type	Polymer conc. (% w/v)	PVA conc. (%w/v)	Internal aqueous phase volume (ml)
F1	PLGA	2.5	1.25	0.50
F2	PLGA	2.5	2.50	0.50
F3	PLGA	2.5	5.00	0.50
F4	5%PEG-PLGA	2.5	1.25	0.50
F5	5% PEG-PLGA	2.5	2.50	0.50
F6	5% PEG-PLGA	2.5	5.00	0.50
F7	10%PEG-PLGA	2.5	1.25	0.50
F8	10%PEG-PLGA	2.5	2.50	0.50
F9	10%PEG-PLGA	2.5	5.00	0.50
F10	PLGA	5.0	1.25	0.50
F11	PLGA	7.5	1.25	0.50
F12	5%PEG-PLGA	5.0	1.25	0.50
F13	5%PEG-PLGA	7.5	1.25	0.50
F14	10%PEG-PLGA	5.0	1.25	0.50
F15	10%PEG-PLGA	7.5	1.25	0.50
F16	PLGA	2.5	1.25	0.20
F17	PLGA	2.5	1.25	0.75
F18	5% PEG-PLGA	2.5	1.25	0.20
F19	5% PEG-PLGA	2.5	1.25	0.75
F20	10%PEG-PLGA	2.5	1.25	0.20
F21	10%PEG-PLGA	2.5	1.25	0.75
F22	10%PEG-PLGA	5.0	1.25	0.20
F23	10%PEG-PLGA	7.5	1.25	0.20

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Table 2. Physicochemical properties of optimised insulin NP

Formula ID	Size(nm)	PDI	Zeta potential (mV)	EE %[1]	Burst release after 24 hours[2]
F20	231.50±19.00	0.378±0.52	-6.15±2.70	90.98±2.40	57.60±2.40
F22	304.00±16.40***	0.234±0.34*	-6.13±1.40	95.50±0.90*	44.63±2.50**
F23	379.75±13.70***ΔΔΔ	0.397±0.47ΔΔ	-5.23±1.50	96.85±1.40*	42.49±4.00**

[1]encapsulation efficiency

[2]expressed as a % of total theoretical release

*P<0.05, **P<0.01, ***P<0.001 compared with F20.

ΔP<0.05, ΔΔ P<0.01, ΔΔΔP<0.001 compared with F22.