Abdelkader, D H, El-Gizawy, Sanaa A, Faheem, Ahmed, McCarron, Paul A and Osman, Mohamed A (2018) Effect of process variables on formulation, in-vitro characterisation and subcutaneous delivery of insulin PLGA nanoparticles: An optimisation study. Journal of Drug Delivery Science and Technology, 43. pp. 160-171. ISSN 17732247

Downloaded from: http://sure.sunderland.ac.uk/id/eprint/8523/

Usage guidelines

Please refer to the usage guidelines at http://sure.sunderland.ac.uk/policies.html or alternatively

contact sure@sunderland.ac.uk.

Effect of process variables on formulation, in-vitro characterisation and subcutaneous delivery of insulin PLGA nanoparticles: An optimisation study

Dalia H. Abdelkader[a,b,*], Sanaa A. El-Gizawy[b], Ahmed M. Faheem[c], Paul A. McCarron[a], Mohamed A. Osman[b]

[a] School of Pharmacy and Pharmaceutical Sciences, Saad Centre for Pharmacy and Diabetes, Ulster University, Cromore Road, Coleraine, Co. Londonderry, BT52 1SA, UK
[b] Faculty of Pharmacy, Pharmaceutical Technology Department, Tanta University, Tanta, 31111, Egypt
[c] Sunderland Pharmacy School, University of Sunderland, Sunderland, SR1 3SD, UK

ARTICLE INFO

Keywords:
Human insulin
PLGA
Nanoparticles
Poly (ethylene glycol)
Sustained delivery
Acute diabetes

ABSTRACT

This study was initiated to investigate the effect of PLGA concentration, PVA concentration, internal to external phase ratio (IEPR), PEG molecular weight and concentration on mean particle size, zeta potential, polydispersity index (PDI), percentage drug entrapment and in vitro release profile. Using PLGA (50:50) as the carrier, insulin nanoparticles (NP) were prepared using double emulsion solvent evaporation technique. The particle size was analysed by dynamic light scattering (DLS) and the geometrical shape was examined using scanning electron microscopy (SEM). Mean particle size was highly dependent on the combined effect of PLGA and PVA concentrations. Drug entrapment would be greatly controlled by PLGA concentration and internal to external phase ratio. Addition of PEG could modulate in vitro release behavior of insulin with initial burst at the first 12 h and sustain the drug release for 6 days. Insulin integrity was assessed in vitro using MALDI-TOF mass spectroscopy. The optimised NP formulation, had particle size of 202.60 nm and percent entrapment efficiency (EE) equal to 67.72%, was tested in vivo to examine its hypoglycemic effect after subcutaneous injection. Insulin NP had a significant hypoglycemic effect comparing to free insulin ($p < 0.01$) and insulin zinc suspension ($p < 0.05$).

1. Introduction

Insulin is a peptide hormone with multiple physiological roles. Basically, it regulates blood glucose level and has an additional beneficial role in wound healing. Many pathological changes would occur if insulin level deviated from its normal range [1]. Diabetes is a metabolic disease categorised into two types; Type I, an autoimmune pathologic process occurs in the pancreatic islets which affects negatively on insulin secretion. Type II, the cause is a combination of resistance to insulin action and a suboptimum compensatory insulin secretory response [2].

Recently, several drug delivery systems have been developed to formulate human insulin in biocompatible nanocarriers such as nanoparticles, liposomes, dendrimers and other micellar systems to overcome its premature degradation by encapsulation within polymeric matrix [3]. Special focus is paid on nanoparticles for the delivery of insulin via using several natural and synthetic polymers to manufacture polymeric nanoparticles. Generally, polymeric materials could help in alteration of physicochemical characteristics (hydrophobicity, zeta potential) of the therapeutic agents, drug release properties (delayed, prolonged, triggered) and biological action (bio-adhesion, improved cellular uptake) of the NP, also, it increases macromolecules (peptides, protein) stability and enhances the solubility of hydrophobic drugs [4].

Poly (lactic-co-glycolic acid) (PLGA) has been widely used in nanotechnology applications because it is biocompatible, biodegradable, commercially available and commonly used in pharmaceutical formulations approved by the FDA. Poly (ethylene glycol) (PEG) might be added as a co-block polymer with PLGA or physically mixed during NP preparation. PEG could enhance drug encapsulation and modulate drug release profile to get an initial burst with better sustained effect. Several encapsulation techniques have been used to encapsulate the bioactive drugs that can be divided into; chemically polymerisation process (emulsion, mini-emulsion and interfacial) and physically incorporation method (spray drying, multiple emulsion and solvent diffusion). The optimum selection of the method would result in a proper particulate size and adequate drug encapsulation [3].

Double emulsion solvent evaporation technique (w/o/w) plays an important role in encapsulating hydrophilic drugs [5]. The water-

soluble molecules were solubilised in the inner w_1 phase then the oily organic phase was added using a shear force resulting from homogenisation, and in some circumstances sonication might be used to protect highly sensitive peptide from the damage of its dimensional structure. The external aqueous medium containing a stabiliser was added using the same shear force to form the second emulsion (w/o/w). This method exhibits sustained drug release, minimum toxic effects and high encapsulation efficiency of the active agent. For this reason, proteins have been extensively encapsulated by w/o/w emulsion system. The physicochemical properties including the stability, globular size, surface charge and release properties of double emulsions can be highly controlled by several processing parameters (polymer concentration, surfactant concentration, ratio between internal and external aqueous medium and PEG concentration) that will be discussed in this study.

Prolonged control of hyperglycemia over several days is a beneficial gain could be obtained from polymeric insulin NP after subcutaneous administration. Most of the marketed products can control the glucose level for maximally 24 h, frequent administration is a significant drawback that should be overcome for patient convenience. The optimised insulin NP, selected for in vivo study, can maintain the glucose level within normal range via single weekly injection with significant difference compared to marketed insulin zinc suspension.

2. Materials and methods

2.1. Materials

Insulin, recombinant human, dry powder and poly (D,L-lactide-co-glycolide, acid terminated, lactide:glycolide 50:50, MW 24,000–38,000) were purchased from Sigma Aldrich, UK. Hydrochloric acid (HCl), acetic acid and trifluoroacetic acid (TFA) solutions were purchased from Fluka, Sigma Aldrich, UK. Poly (vinyl alcohol) (MW = 31,000–50,000, 87–89% hydrolysed), poly (ethylene glycol) (PEG) flakes (MW 200, 2000 and 5000 Da), sucrose powder and potassium chloride (KCl) were all purchased from Sigma Aldrich, UK. Sodium acetate, 2-(N-cyclohexylamino) ethanesulfonic acid (CHES) and triethylamine were all also, purchased from Sigma Aldrich, UK. A bicinchoninic acid Protein Assay Kit (BCA) was purchased from Thermo Fisher Scientific, Pierce Biotechnology Inc., USA. Dichloromethane (DCM), trifluoracetic acid (TFA), acetonitrile (MeCN) and methanol were of HPLC grade. All other reagents and solvents were of appropriate laboratory standard and used without further purification.

2.2. PLGA NP preparation

Insulin-loaded NP were prepared using a double-emulsion, solvent evaporation technique, adapted with minor modifications from the method described by Feczkó T et al., [6]. Briefly, X ml of an internal aqueous insulin solution (5 mg, dissolved in a mixture of 0.1 M HCL and PVA 2.5% w/v, pH ≈ 1–2) was added drop-wise to an organic phase (4 ml DCM) containing Y mg of PLGA. This organic phase contained variations (Z) of PEG content, both in concentration and molecular weight. The primary emulsion (w_1/o) was homogenised in an ice bath for 2 min at 1000 rpm using a low speed homogeniser (Ultra-Turrax® T10 Basic Disperser, IKA® Works, VWR®International, UK) before drop-wise addition to 50 ml of an external aqueous phase containing Q% w/v PVA [7] as defined by the formula codes in Table 1. The secondary emulsion (w_1/o/w_2) was stirred continuously for 6 min at 10,000 rpm using a high speed homogeniser (model L5M-A Silverson Ltd., UK). DCM was evaporated using magnetic stirring for 6–8 h. NP were collected by centrifugation (3-30k, Sigma Laboratory Centrifuge Henderson Biomedical Ltd., Germany) at 11,000 × g for 30 min at 4 °C and washed with 2% w/v sucrose solution [8]. The pellet was frozen at −20 °C for 4–6 h and then lyophilised (4.5 Plus, Labconco Ltd., USA) for 48 h. NP were stored in a desiccator at room temperature for in vitro characterisation.

Table 1
Formulae identification and composition of insulin-PLGA NP.

Formula code	PLGA concentration (%w/v)	PVA concentration in aqueous external phase (%w/v)	Internal - external phase volume ratio (IEPR)	PEG content in primary emulsion M_w (% w/w)
M1	2.50	1.25	0.01	–
M2	2.50	1.25	0.15	–
M3	2.50	1.25	0.02	–
M4	2.50	2.50	0.01	–
M5	2.50	2.50	0.15	–
M6	2.50	2.50	0.02	–
M7	2.50	5.00	0.01	–
M8	2.50	5.00	0.15	–
M9	2.50	5.00	0.02	–
M10	5.00	1.25	0.01	–
M11	5.00	1.25	0.15	–
M12	5.00	1.25	0.02	–
M13	5.00	2.50	0.01	–
M14	5.00	2.50	0.15	–
M15	5.00	2.50	0.02	–
M16	5.00	5.00	0.01	–
M17	5.00	5.00	0.15	–
M18	5.00	5.00	0.02	–
M19	7.50	1.25	0.01	–
M20	7.50	1.25	0.15	–
M21	7.50	1.25	0.02	–
M22	7.50	2.50	0.01	–
M23	7.50	2.50	0.15	–
M24	7.50	2.50	0.02	–
M25	7.50	5.00	0.01	–
M26	7.50	5.00	0.15	–
M27	7.50	5.00	0.02	–
F1	2.50	1.25	0.002	–
F2	2.50	1.25	0.002	200Da (5%)
F3	2.50	1.25	0.002	200Da (10%)
F4	2.50	1.25	0.002	2 kDa (5%)
F5	2.50	1.25	0.002	2 kDa (10%)
F6	2.50	1.25	0.002	5 kDa (5%)
F7	2.50	1.25	0.002	5 kDa (10%)

2.3. Particle size and zeta potential measurements

An appropriate amount of lyophilised NP were dispersed in distilled water or 1.0 Mm KCL solution and then stirred for 3 min using the vortex. Particulate size (diameter, nm) and polydispersity index (PDI) were determined by dynamic light scattering (DLS) (ZetaSizer Nano series, Malvern Instruments, Worcestershire, UK). Surface charge (zeta potential, mV) was determined by measuring electrophoretic mobility. Measurements were performed in triplicate at 25 °C.

2.4. Morphological characterisation

Lyophilised NP (intact or cracked after exposure to release media) and free insulin were coated with a mixture of gold and palladium under vacuum for 3 min and examined for morphology using scanning electron microscopy at 20 kV (Zeiss, Oberkochen, Germany).

2.5. Human insulin analysis

2.5.1. High pressure liquid chromatography (HPLC)

Reversed phase HPLC (Shimadzu Corporation, Kyoto, Japan) method was used to analyse human insulin. A Luna® C18 column (5 μm, 150 × 4.6 mm, Phenomenex, CA, USA) was utilised as a stationary phase. The mobile phase composed from a binary mixture of 0.1% TFA in water (solution A) and 0.1% TFA in MeCN (solution B) [9] was gradient eluted by increasing solution B concentration from 10% to 35% over a 15-min period at λ_{max} = 210 nm with a flow rate of

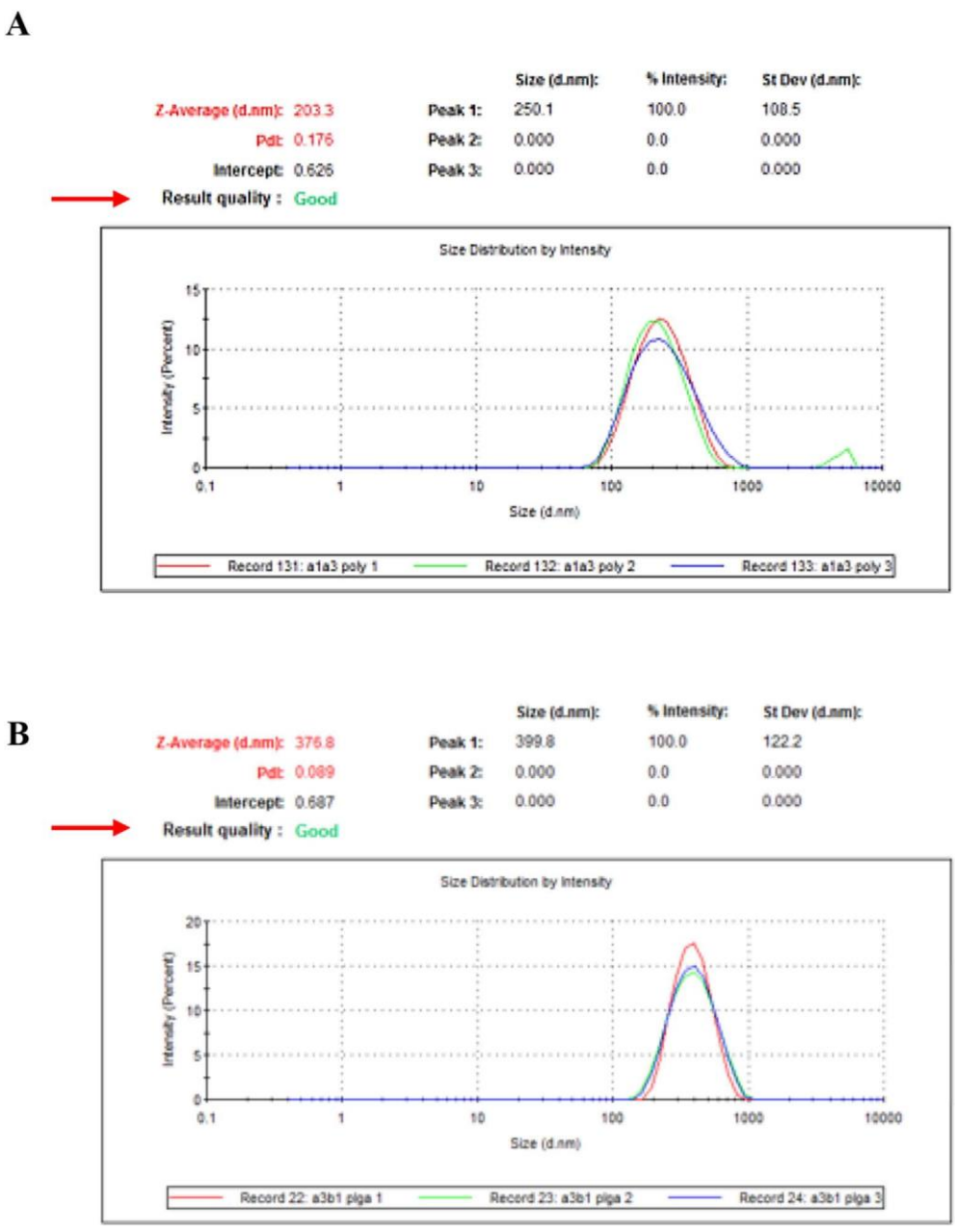


Fig. 1. Representative graphs showing particle size and PDI measurements using Malvern DlS v.6.20 software. Reported graph showed good quality (red arrows) of insulin NP prepared with (A) PLGA 2.5% w/w, (B) PLGA 5% w/w. (For interpretation of the references to colour in this fi gure legend, the reader is referred to the web version of this article.)

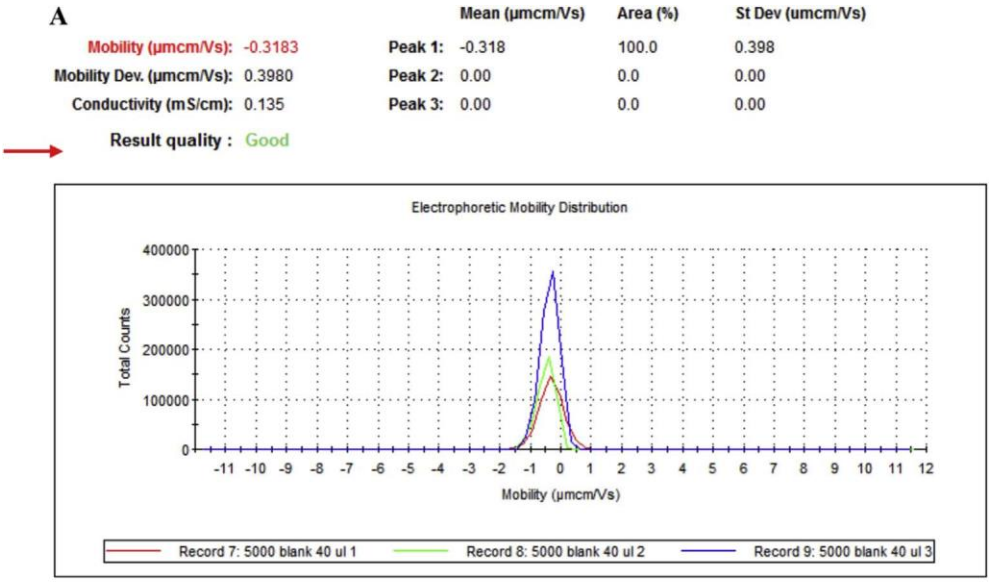
1.1 ml min^{-1}. Analysis was conducted at ambient temperature and peak area released at predetermined retention time was used to quantify the analyte.

2.5.2. Capillary electrophoresis (CE)

CE analysis was performed on Agilent 3DCE G1600AX capillary electrophotography (Agilent, West Lothian, UK) controlled by Chemstation B.02.01 (Agilent, West Lothian, UK). The capillaries were from fused-silica (Agilent, West Lothian, UK) with a standard length of 48.5 cm and with an internal diameter 50 μm. The pressure injection mode was set for 4 s with 50 mbar at λ_{max} = 210 nm and migration time equal to 6 min. The separation voltage was 30 kV and the resulting current was approximately equal to 35 μA. Fused silica capillaries were fi rstly conditioned by fl ushing of 1 M NaOH at > 950 mbar at 40 °C for 20 min followed by equilibration with running buff er for 40 min applying the above-mentioned separation conditions. A buff er system comprised from 50 mmol/l acetate, 850 mmol/l 2-(N-cyclohexylamino) ethanesulfonic acid (CHES) and 10% acetonitrile was used [10]. The virtual pH of the buff er was adjusted to 7.8.

2.6. Direct and indirect entrapment effi ciency (EE)

Insulin loading within NP matrix was determined directly by extraction from lyophilised NP or indirectly by analysing the supernatant (non-entrapped fraction during manufacturing process). The direct EE was calculated as a ratio of mass of the drug found in NP to mass of the drug initially added before NP fabrication. The extraction of insulin was applied by incubating an appropriate amount of NP with 1.0 M NaOH for 2–3 h at 37 °C followed by neutralisation with 1.0 M HCl [6]. This extract was analysed by bicinchoninic acid protein assay (BCA) kit to quantitate the amount of insulin directly entrapped in NP. Insulin concentration in the supernatant (indirect EE) was determined using RP-HPLC and CE, as described in section 2.5. The indirect EE (%) was calculated using the following Eq [11].



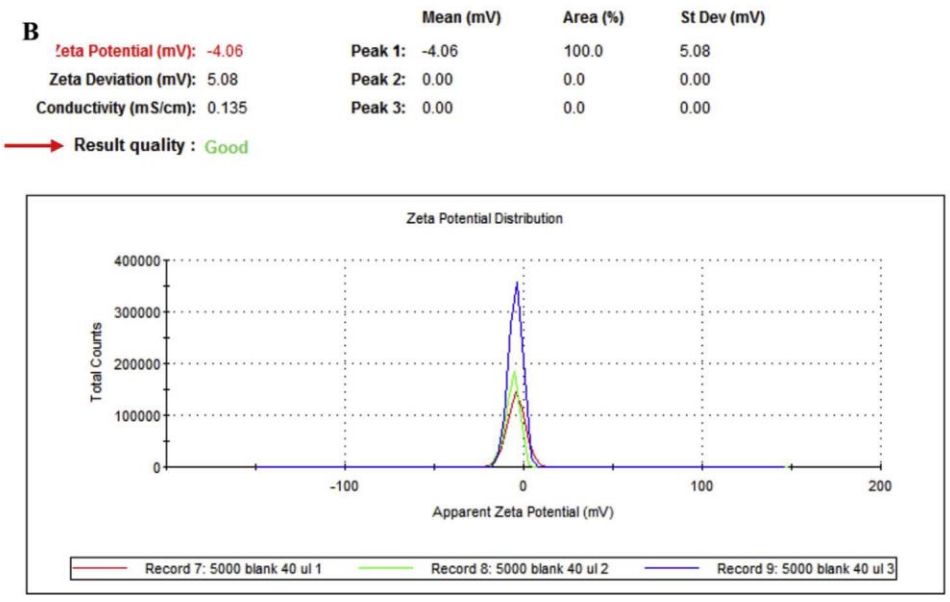


Fig. 2. Representative graphs showing zeta potential measurements of PLGA NP using Malvern DlS v.6.20 software. Reported graph showed good quality (red arrows) of nanoparticles prepared with (A) PLGA 2.5% w/w, (B) PLGA 7.5% w/w. (For interpretation of the references to colour in this fi gure legend, the reader is referred to the web version of this article.)

$$\textit{Indirect \%EE} = \frac{\textit{Total mass of drug used (mg)} - \textit{mass of drug in supernatant (mg)}}{\textit{Total mass of drug used (mg)}} \; x\ 100$$

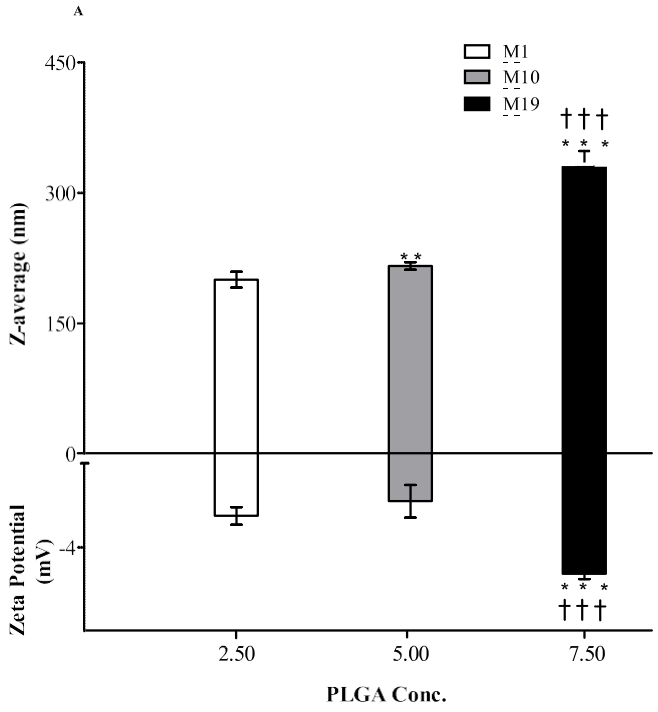
2.7. In vitro drug stability

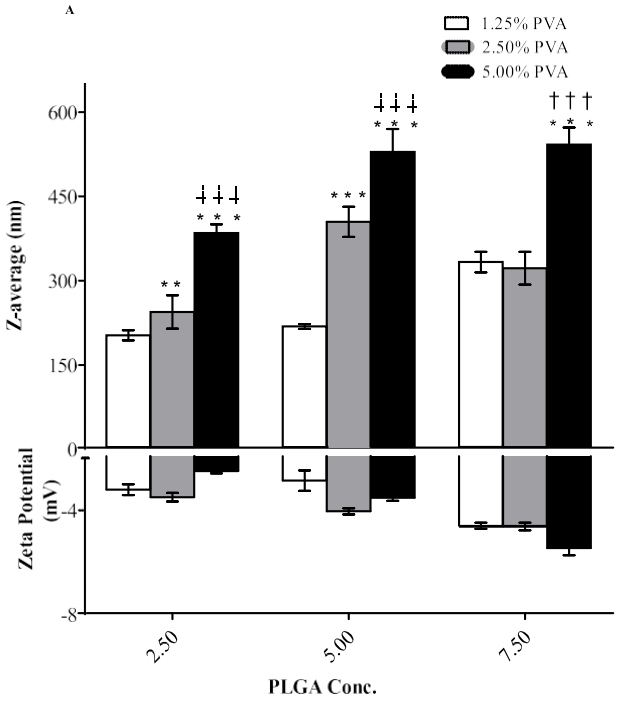
MALDI-TOF MS (Matrix assisted laser desorption ionisation time of fl ight mass spectrometry).

MALDI-TOF MS was performed using a PerSeptive Biosystems Voyager-DE Biospectrometer (Hertfordshire, UK) equipped with a 1 m time-of- fl ight tube. A 1.5 µL aliquot of insulin standard solution (30 µl ml^{-1}) in 0.01 M HCl and insulin extracted from NP (obtained after dissolving 1 mg of insulin-NP by 200 µl DCM and addition of 500 µl 0.01 M HCl to extract the entrapped insulin) were pipette onto a predefi ned well of a 100-well stainless steel plate and allowed to dry at room temperature. A 1.5 µL aliquot of CHCA (α-cyano-4-hydroxycinnamic acid) solution (10 mg/ml) was prepared in MeCN/H_2O/TFA (80:20:0.1%) and added to the sample spot to enhance the ionisation of protein [12]. All measurements collected in linear positive ionisation mode using 50 laser shots/spectrum and the accelerating voltage was maintained at 20,000 V. The nitrogen laser was set at 337 nm and directed toward the concentrated area of the sample/matrix spot. The mass/charge ratio (m/z) was plotted against relative abundance.

2.8. In vitro release kinetics

The lyophilised insulin-loaded NP (15 mg) were dispersed in 1.0 ml phosphate-buffered saline solution (PBS, pH 7.4). The samples were incubated at 37 °C with agitation using a rotating mixer (Stuart Rotator Drive STR4, Bibby Scientific Ltd., UK) at 100 rpm. The release medium was withdrawn at predetermined time intervals for 6 days after centrifugation at 5500 × g (Mini-Spin Eppendorf, Davidson & Hardy Ltd., UK) for 5 min. A fresh release media (1.0 ml) was replaced at each time interval [13]. The supernatant was analysed using RP-HPLC, as described previously in section 2.5.





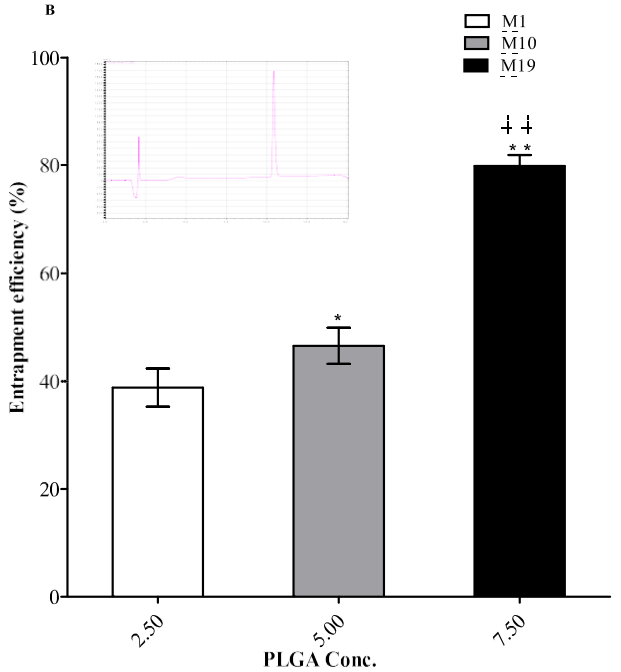


Fig. 3. Eff ect of PLGA concentration on insulin NP; (A) particle size and zeta potential,
(B) entrapment efficiency measured indirectly by HPLC (Representative peak was shown in the upper left side of the graph). (Formulation codes shown in Table 1). Results show mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with 2.50% PLGA concentration. †P < 0.05, ††P < 0.01, †††P < 0.001 compared with 5.00% PLGA concentration.

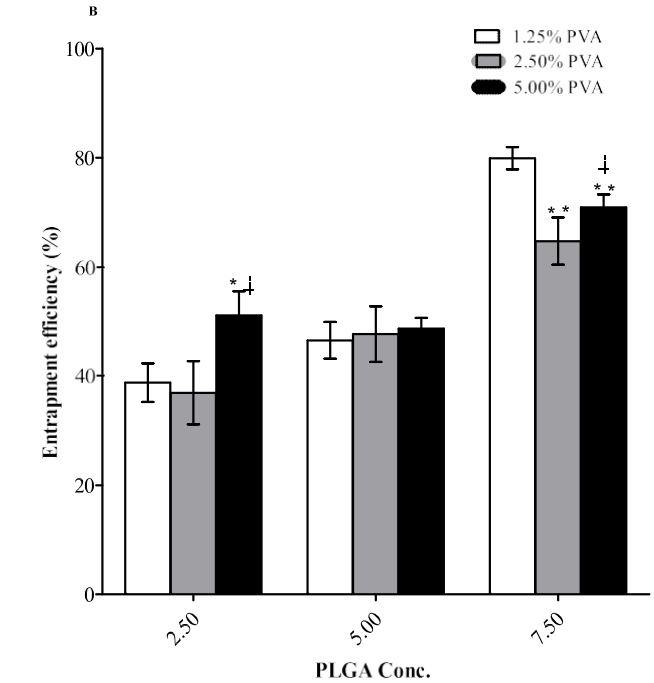


Fig. 4. Eff ect of PVA concentration on insulin NP; (A) particle size and zeta potential, (B) entrapment efficiency measured indirectly by HPLC. Results show mean ± SD (n = 3).
*P < 0.05, **P < 0.01, ***P < 0.001 compared with 1.25% PVA. †P < 0.05, ††P < 0.01, †††P < 0.001 compared with 2.50% PVA.

2.9. In vivo study

This research protocol was approved by Ulster University's Animal Ethics Committee in accordance with the UK Animals (Scientifi c Procedures) Act of 1986.

2.9.1. Induction of type I diabetes

Diabetes was induced by intraperitoneal injection (I.P) of a single dose of streptozotocin (STZ) in 0.1 M sodium citrate buff er (pH 4.5) at a dose of 50–60 mg kg^{-1} body weight [14]. The freshly prepared solution was injected immediately. Eighteen Male Sprague–Dawley rats (12-week-old) weighing 250–300 g were fasted for 6 h prior to injection. A blood glucose measurement was performed 48 h after STZ injection

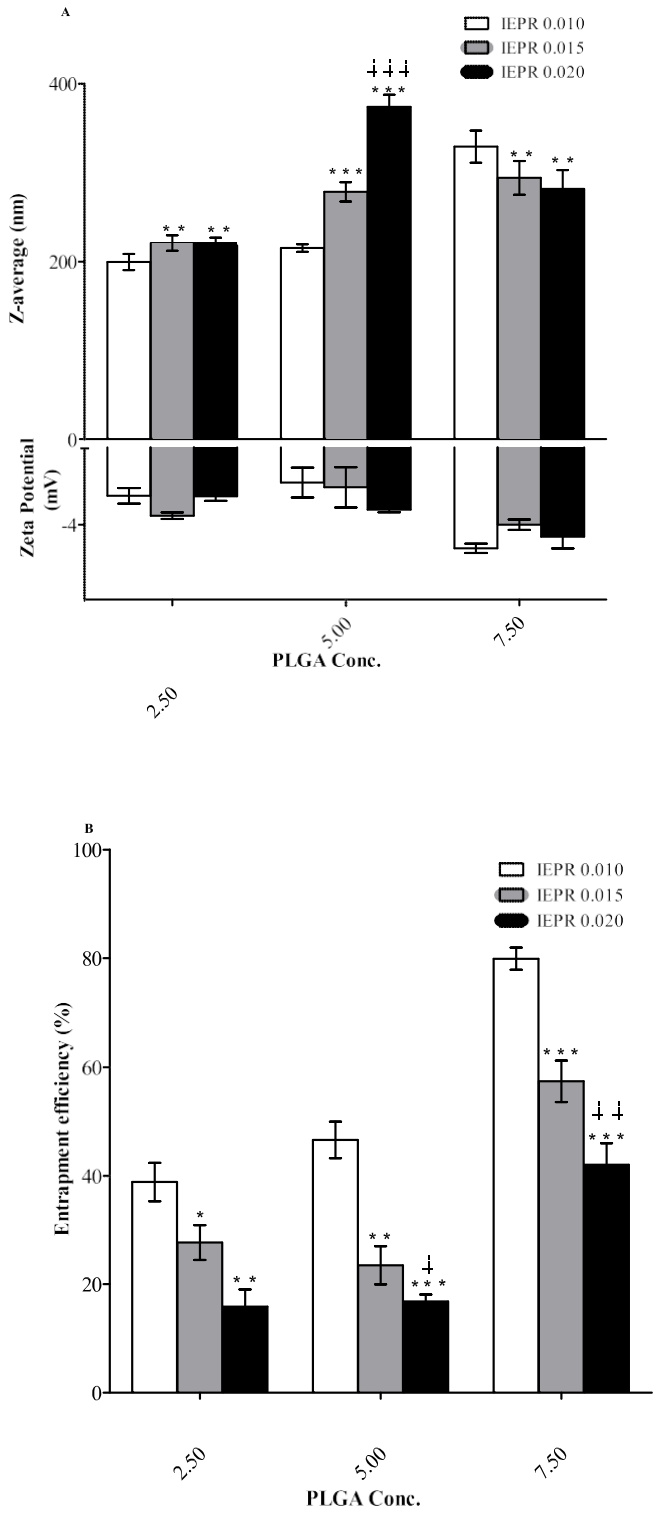


Fig. 5. Eff ect of IEPR on insulin NP; (A) particle size and zeta potential, (B) entrapment efficiency measured indirectly by HPLC. Results show mean ± SD (n = 3). *P < 0.05,
**P < 0.01, ***P < 0.001 compared with 0.010 IEPR. †P < 0.05, ††P < 0.01, †††P < 0.001compared with 0.015 IEPR.

[15]. Blood was drawn from the tail vein and the glucose level determined using a glucometer (Lever Chek®, TalDoc Tech Corp, Germany). Rats with blood glucose levels > 250 mg dl^{-1} were considered to be in a diabetic state.

2.9.2. Experimental groups

This study was conducted using three groups (n = 6) of diabetic rats to be the same (25 IU kg^{-1}). Glycaemia, in blood samples withdrawn from the tail vein, was measured before injection and 0.5, 3, 6, 12, 24, 48, 72, 96, 120 and 144 h. Rats were maintained fasted for the fi rst 12 h
of the experiment and fed thereafter [16].

2.10. Statistical analysis

Data are presented as the mean ± standard deviation. Statistical analysis was performed using Prism 5 (Graph-Pad Software). A one-way ANOVA, followed by a pairwise comparison post-hoc test, was conducted wherever appropriate. The signifi cance level for rejecting the
null hypothesis was 5% ($p < 0.05$). Student's t-test was used to compare the hypoglycemic eff ect of the subcutaneous administration of
insulin PLGA NP, free insulin and insulin zinc suspension.

3. Results and discussion

In this study, several key processing parameters were investigated during fabrication of insulin- PLGA NP, as demonstrated in Table 1. Four independent factors, polymer concentration, the concentration and the molecular weight of PEG in the organic phase, PVA (surface modi fi er) concentration in the external aqueous phase and the volume
ratio of internal to external aqueous phases (IEPR) were examined to evaluate their infl uences on the mean particle size, zeta potential, percentage EE, surface morphology and in-vitro release pro fi le. The
optimised insulin NP formulation was selected to examine its hypoglycaemic eff ect comparing with free insulin and insulin-zinc sus-
pension after subcutaneous injection in experimental rats.

3.1. E ff ect of process formulation variables

(Male Sprague–Dawley) injected subcutaneously with: (i) free human insulin, (ii) insulin PLGA NP (F4), (iii) insulin zinc suspension. Free insulin and insulin NP were suspended in PBS and homogenously dispersed before subcutaneous injection into the neck region at a dose of 25 IU kg^{-1} body weight. The dose of insulin zinc suspension was kept

3.1.1. PLGA concentration

The mean particle size of formulations prepared using 2.5%, 5.0%, or 7.5% (formulations M1 M10 and M19 as mentioned in Table 1) of PLGA resulted in 199.8 nm, 215.5 nm and 329.9 nm, respectively (Fig. 3A). The unimodal symmetric size distribution was seen in Fig. 1 with low polydispersity index values (PDI). Increasing the polymer concentration, while keeping the volume of organic phase constant at 4 ml, the resistance of the organic phase to flow is elevated, which leads to increase in the consistency of droplet and higher shear force to be broken, thus bigger oil droplets are formed with a significantly increasing in particle size [17].

PLGA being a negative charged polymer imparts anionic nature to nanoparticles which depends on their molecular weight and concentration as it is shown in Fig. 2 [18]. It was shown that increasing the polymer concentration from 2.5 to 5.0% of PLGA had no effect on the surface charge ($p > 0.05$) (Fig. 3A), whereas increasing the polymer concentration to 7.5% led to significant increase in the negative value of zeta potential which might have a good impact on the stability of NP due to increase the electrostatic repulsion and more protection from aggregation [19]. Increasing PLGA concentration by 2.5% increments from 2.5% to 7.5% resulted in a significant increase in the percent of entrapment efficiency from 38.8% to 79.9% (Fig. 3B). The increase in polymer concentration increases the organic phase viscosity, which prevents the diffusion of drug molecules out to aqueous phase during homogenisation step, thereby better drug entrapping within NP matrix [17].

3.1.2. PVA concentration

In this study, PVA, a water-soluble polymer with a high biocompatibility and biodegradability [20] was selected and used as the surfactant added to the external aqueous phase during NP preparation to enhance their stability and redispersibility [21]. PVA (2.5% w/v) was also, added to the internal aqueous phase to promote the emulsification of insulin solution in the organic phase (1^{ry} emulsion). The effect of increasing PVA concentration varied from 1.25, 2.50 and 5.00% w/v on

Table 2
Physicochemical properties of insulin-PLGA NP formulations (F1-F7). Data represent mean values ± SD of at least three measurements.

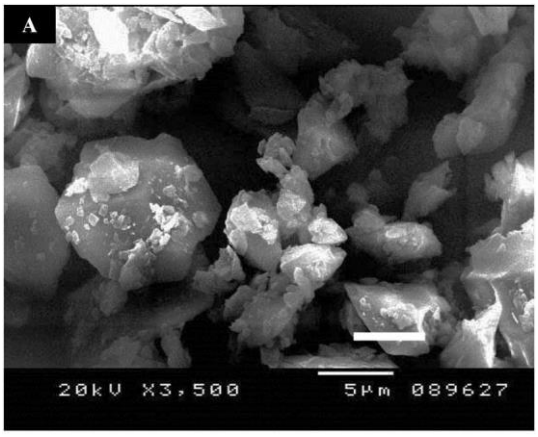
Formula Code	Z-average (nm)	Polydispersity index (PDI)	Zeta potential (mV)	% EE (Direct)[a]	%EE (Indirect)[b]
F1	297.82 ± 18.85	0.37 ± 0.06	−3.94 ± 0.02	56.94 ± 10.70	69.17 ± 0.60
F2	459.07 ± 65.40	0.42 ± 0.26	−5.62 ± 0.28	–	–
F3	484.40 ± 75.53	0.49 ± 0 .04	−6.81 ± 0.13	–	–
F4	202.60 ± 20.60	0.38 ± 0.06	−5.71 ± 0.17	67.72 ± 5.42	69.50 ± 3.33
F5	186.93 ± 14.03	0.26 ± 0.04	−5.75 ± 0.03	71.20 ± 7.68	69.60 ± 5.66
F6	243.57 ± 16.51	0.35 ± 0.04	−7.52 ± 0.07	63.45 ± 8.28	73.74 ± 3.22
F7	255.67 ± 28.94	0.38 ± 0.02	−8.76 ± 0.17	60.53 ± 9.83	69.73 ± 3.01

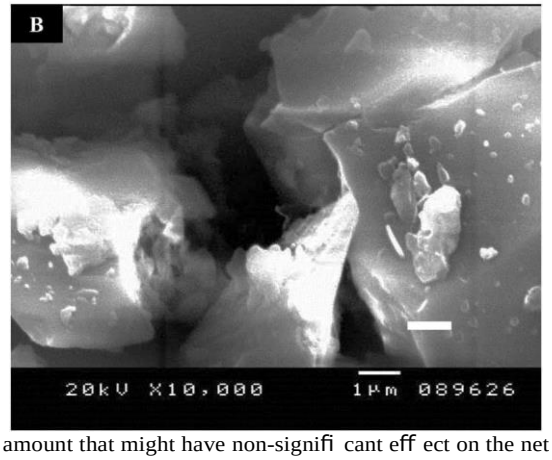
[a] Direct EE is measured by BCA.
[b] Indirect EE is measured by HPLC. Non-signifi cant diff erence between direct and indirect entrapment effi ciency.

Table 3
Comparison between the percent entrapment effi ciency (% EE) results of insulin- PLGA NP measured directly by BCA or indirectly by HPLC and CE.

Formula Code	Percent entrapment effi ciency (% EE) measured by		
	BCA	***HPLC	***CE
M1	20.10 ± 1.88	38.80 ± 3.53	55.85 ± 1.29
M2	11.92 ± 0.71	27.65 ± 3.22	23.74 ± 4.37
M3	03.81 ± 0.39	15.85 ± 3.17	21.04 ± 5.16
M4	10.25 ± 0.80	36.92 ± 5.79	48.31 ± 7.54
M5	05.94 ± 0.15	18.53 ± 4.73	17.84 ± 2.43
M6	09.99 ± 0.11	15.86 ± 6.15	16.32 ± 1.41
M7	10.97 ± 0.18	51.13 ± 4.40	55.06 ± 5.58
M8	03.97 ± 0.50	25.30 ± 2.11	27.07 ± 3.17
M9	02.39 ± 0.37	24.53 ± 2.43	30.84 ± 5.83
M10	21.77 ± 3.35	46.55 ± 3.37	39.05 ± 4.50
M11	06.46 ± 0.15	23.48 ± 3.51	17.57 ± 5.47
M12	00.91 ± 0.05	16.81 ± 1.27	10.14 ± 2.45
M13	21.19 ± 0.37	47.70 ± 5.10	36.52 ± 0.37
M14	10.55 ± 0.45	27.41 ± 1.54	09.44 ± 4.63
M15	06.12 ± 0.66	24.61 ± 0.22	07.75 ± 5.14
M16	20.24 ± 2.15	48.71 ± 1.93	35.42 ± 3.21
M17	16.86 ± 1.10	47.37 ± 4.27	30.11 ± 5.64
M18	11.24 ± 0.14	40.33 ± 2.41	24.32 ± 4.34
M19	49.99 ± 3.14	79.92 ± 2.04	76.99 ± 2.77
M20	39.76 ± 3.09	57.35 ± 3.81	53.15 ± 3.07
M21	30.12 ± 0.63	41.99 ± 3.95	35.91 ± 2.82
M22	41.83 ± 1.97	64.72 ± 4.33	62.01 ± 1.63
M23	22.76 ± 1.42	43.19 ± 1.18	39.33 ± 1.75
M24	13.82 ± 0.89	31.63 ± 2.25	26.05 ± 0.84
M25	39.28 ± 1.21	70.91 ± 2.38	73.39 ± 2.67
M26	28.14 ± 0.82	58.13 ± 2.14	55.70 ± 2.98
M27	21.03 ± 0.34	32.04 ± 2.81	28.54 ± 4.19

***P < 0.001, a signifi cant diff erence between direct EE measured by BCA and indirect EE measured by HPLC and CE. Non-signifi cant diff erence between HPLC and CE results.





the properties of insulin NP was seen in Fig. 4. Increasing PVA concentration resulted in a signifi cant increase in the mean particles size of
NP at each polymer concentration used (Fig. 4A). Doelker et al. [22] and Quintanar-Guerrero et al. [23] reported that a decrease in size for increasing stabiliser concentration, PVA prevents oil droplets coalescence and maintains $w_1/o/w_2$ emulsion stability following NP preparation. Zweers et al. [21] mentioned that increasing PVA concentration decreases NP size till an optimum concentration at maximum 2.00% w/v and exceeding this level might aff ect negatively on particle
size. Hydrocarbon branches of PVA chains are possibly adsorbed on the surface of PLGA NP via hydrophobic bonding, and most of hydroxyl groups of PVA at high concentration (> 2% w/v) could be hydrated at the surface so, strong hydrogen bonds via hydroxyl groups will be formed between inter- or intra-molecules of PVA. This might produce aggregated NP leading to bigger particle size [24]. During NP preparation, pellets were collected and exposed to a washing process with three sequential steps using an optimum concentration of the cryoprotectant. PVA should be removed during this step leaving small residual amount that might have non-signifi cant eff ect on the net surface

Fig. 6. Scanning electron micrographs of naked free insulin before NP processing, (A) observed under low magnifi cation and (B) at higher magnifi cation. Bar in A represents
5 μm and in B represents 1
μm.

charge of the lyophilised NP (Fig. 4A).

Increasing PVA concentration had variable e ff ects on entrapment effi ciency depending on the concentration of PLGA used (Fig. 4B). At 2.5% w/v of PLGA, increasing PVA concentration led to a signifi cant increase in entrapment effi ciency, this might be explained in terms of
increased viscosity of the continuous phase with higher concentrations of PVA which resists drug di ff usion from the internal to the external
aqueous phase resulting in higher drug loading [19], whereas, at 5.00% w/v of PLGA, there is no signifi cant eff ect for increasing PVA con- centration. At 7.50% w/v of PLGA, entrapment effi ciency started to signi fi cantly decrease with increasing PVA concentration. Insulin being
a hydrophilic drug gets entrapped inside the PLGA nanoparticles (1^{ry} emulsion) and PVA stabilizes the nanoparticles by di ff using out the
water molecules forming the polymer rich coacervate [18]. At higher

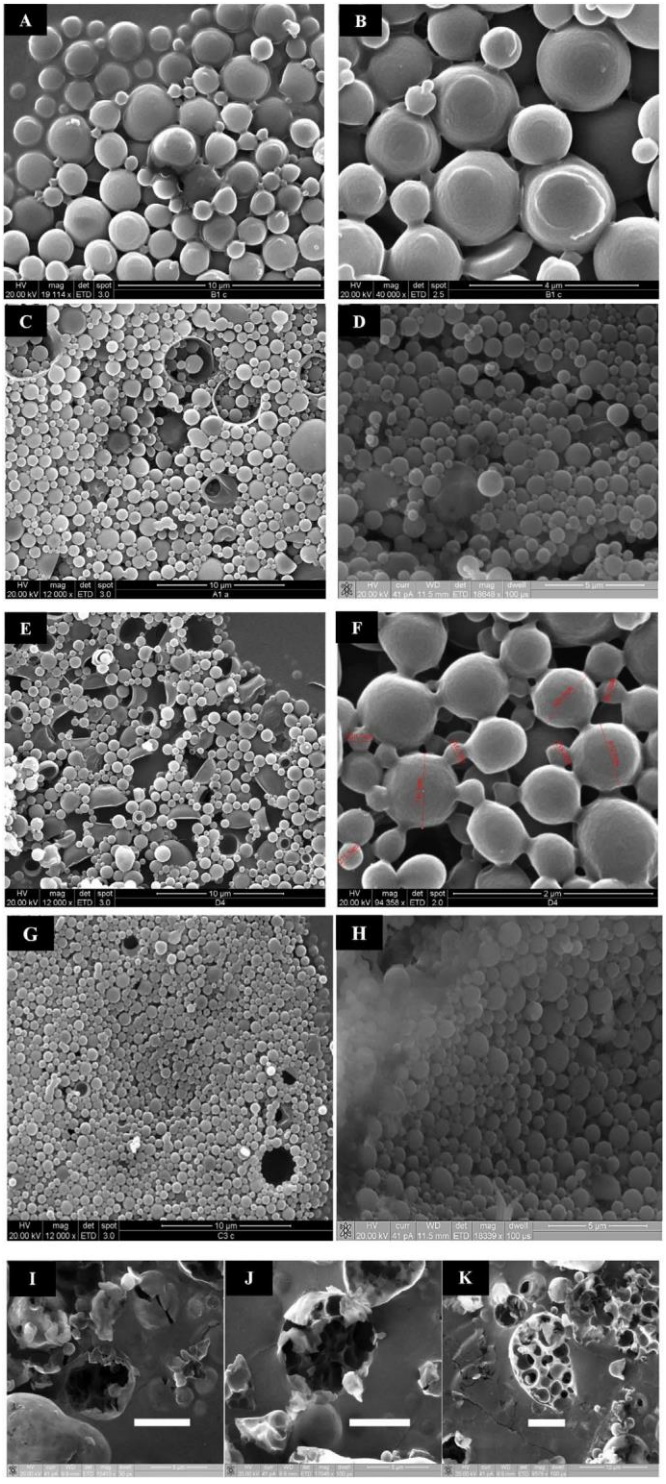


Fig. 7. Scanning electron micrographs of insulin-loaded NP. (M1: A & B), (M10: C & D), (F2: E & F) and (F4: G & H). A, C, E and G observed under low magnification. B, D, F and H observed at higher magnification. Images in I-K show polymeric structures of NP formula (F4) with voids following exposure to release phase media. Bars in I, J and K represent 5 μm.

concentration of PLGA (7.5%w/v), increasing concentration of PVA exhibited a higher aqueous solubility of drug by squeezing the nanoparticles which in turn increased the partition of drug out to the external aqueous phase and thereby resulted in decrease in entrapment of insulin inside the polymeric vesicle [25].

3.1.3. Internal to external phase volume ratio (IEPR)

In order to study this factor, the volumes of the organic and external aqueous phases were held constant, whereas, the volume of internal aqueous phase in which the drug was dissolved varied from 0.50, 0.75 and 1.00 ml. IEPR was calculated as a ratio between the volumes of internal to external (50 ml) aqueous phases. Fig. 5A showed the effect of IEPR on the zeta potential and particle size, increasing IEPR has no significant effect on zeta potential. At PLGA concentrations of 2.50 and 5.00 %w/v, particle size increase with increasing IEPR. The lower volume of internal aqueous phase resulted in a well-dispersed, efficient homogenisation and stable w/o emulsion, which could probably reduce the frequency of collisions during the formation of NP in the second emulsification step, leading to a decrease in the particle size [26], higher volumes of internal aqueous phase led to bigger particle size. Increasing the viscosity of primary emulsion at high PLGA concentration (7.5% w/v) led to decrease the particle size with increasing IEPR. Increasing the volume of internal aqueous phase might balance the resistance of viscous emulsion to shear stress produced from homogenisation. NP size can be controlled, by the inner aqueous phase volume via the influence on emulsion viscosity [27].

It was found that IEPR has a significant effect on the entrapment efficiency (Fig. 5B). Increasing IEPR resulted in decreasing % EE at each polymer concentration used. Higher volume of internal aqueous phase in proportional to a constant organic phase volume led to increase the tendency of the drug to migrate out the polymeric matrix to the external aqueous phase that might decrease drug loading [28]. Li et al. mentioned in his work that, the protein entrapment efficiency is highly dependent on the thickness of the organic phase surrounding the aqueous solution of the drug to form the primary emulsion, increasing the volume of the internal aqueous phase might lead to weaken the tighten of organic phase and facilitate escaping of drug molecules to the external aqueous phase [26].

3.1.4. PEG molecular weight and concentration

PEG is a non-ionic polymer with good biocompatibility. The presence of PEG rich layer as a periphery on NP reduces protein adsorption on the surface, which for PLGA-based NP will delay degradation and increase stability [29]. In this study, three different grades of PEG (200 Da, 2 and 5 kDa) with concentrations of 5.0 and 10.0 %w/w were used as shown in Table 1. PEG molecules are linked to NP via several methods that include covalent bonding, direct addition during NP preparation, or surface adsorption. Here, the second approach was employed and PEG was mixed with PLGA in the organic phase during NP preparation. Table 2 showed the effect of PEG content on NP characterisation in terms of particle size, PDI, zeta potential and entrapment efficiency. Molecular weight of PEG has a significant effect on the particle size. It was found that PEG with the lowest molecular weight (200 Da) has a negative impact on particle size leading to bigger diameter. PEG with the molecular weight of 2 kDa has more significant effect ($p < 0.05$) on particle size reduction than PEG with higher molecular weight (5 kDa). The addition of PEG modifies the association of polymers during the formation of NP, which leads to a decrease in the resulting particle size, as observed in the literature of Beletsi et al., [30]. PEG with molecular weight higher than 1000 Da has better solubility in organic solvent such as DCM than PEG with molecular weight

of 200–800 Da [31], which led to poor miscibility occurred between PEG (200 Da) and DCM during NP preparation and unexpected increase in the particle size of NP would exhibit.

It was found that increasing PEG content (density and/or molecular weight) had no significant effect on values of zeta potential. This might

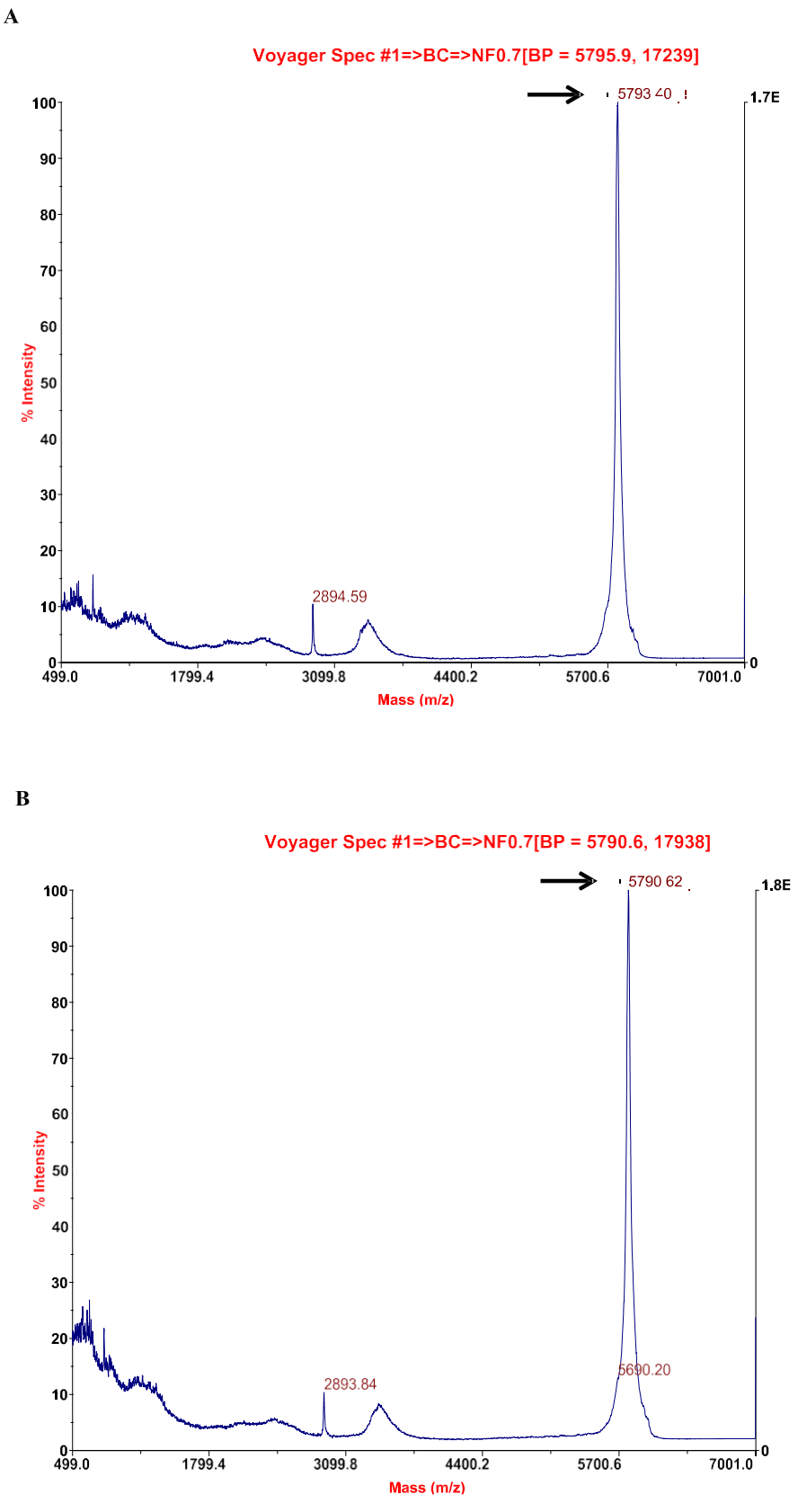


Fig. 8. MALDI-TOF mass spectrum of (A) an insulin standard and (B) insulin released from NP.

de due to physically addition of PEG to PLGA as we did not formulate PEG as a co-block with PLGA. As shown in Table 2, the direct and indirect EE of F2 and F3 were not determined because of their big particle sizes and asymmetric size distribution (high PDI values), NP formulations (F2 and F3) were not used to complete further characterisation. The presence of PEG (2 and 5 kDa) resulted in a significant increase in the direct EE with a greater effect for PEG with molecular weight of 2 kDa ($p < 0.05$). PEG chains at the interface between the

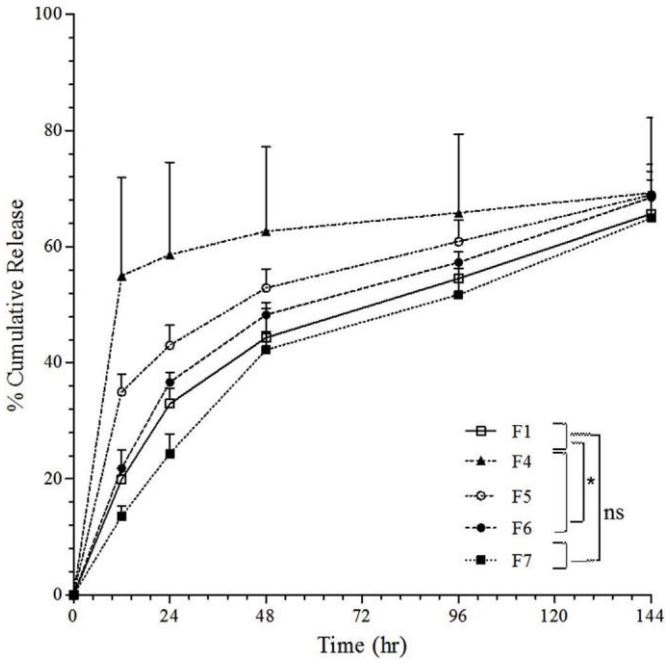


Fig. 9. In vitro drug release profiles from insulin-loaded PLGA NP (Formulation codes shown in Table 1). For clarity, data are shown as mean + SD (n = 3). * Statistical significance ($p < 0.05$) between (F4-F7) and F1, ns (non-significant difference) between FVII and FI.

internal phase and the organic phase prevent drug molecules from migrating towards the external aqueous phase, which might result in the higher encapsulation efficiencies [32].

3.2. Direct and indirect entrapment efficiency

The choice of method used to measure entrapment efficiency had a bearing on the estimate of entrapped drug. As demonstrated in Table 3, % EE measured directly by BCA was significantly ($p < 0.05$) less than % EE measured indirectly by HPLC or CE, whereas non-significant difference was detected between indirect % EE values measured by HPLC and CE ($p > 0.05$). Higher values of % EE resulted in the indirect EE methods comparing to direct EE method could be attributed that the indirect method for estimating EE (%) depends on detecting drug concentration in the supernatant and is, therefore, not a direct measure of particulate content. Indeed, further processing, such as washing and centrifugation, will remove loosely bound drug and so a preliminary analysis of the supernatant immediately following NP formation may be an overestimation. Although significantly different, the data in Table 2 does show reasonably good agreement between both methods for the NP formulations (F4-F7), so it can be concluded that the incorporated insulin is firmly entrapped within the NP and not loosely bound to its surface. The EE of NP formulations (F2-F3) were not determined as mentioned in section 3.1.4.

3.3. Morphological imaging

Scanning electron microscopy was performed on naked free insulin and insulin NP to investigate the effect of double emulsion solvent evaporation technique on the geometric shape and particle size. As it is shown in Fig. 6, free insulin has asymmetric irregular shape and bigger particle size with 4–5 μm range. Whereas, NP displayed a spherical geometry with smooth and pore-free surfaces (Fig. 7). The effect of increasing PLGA concentration from 2.5% (Fig. 7 A & B) to 5.0% (Fig. 7C & D) resulted in a significant increase in the particle size which showed a close matching with DLS results.

The effect of adding PEG is highly dependent on the molecular weight and concentration used. PEG with the lowest molecular weight (F2-F3) showed a significant bigger particle size and non-uniform size distribution (Fig. 7 E & F) as demonstrated in Table 2. We observed under scanning electron microscopy that PEG (200 Da) appeared as separate units with different shape containing several voids and bigger size (F2) which led to suboptimal in vitro characterisation of NP formulations (non-uniform geometrical shape, high PDI value and bigger particle size).

The effect of PEG (2 kDa), as represented by F4, decreased particle size and tightened the size distribution (Fig. 7 G & H) compared with M1 (Fig. 7 A & B). The residual appearance of NP following drug extraction after exposing to release medium for 48 h (Fig. 7I–K) showed large pores and voids with losing the spherical shape, which suggesting

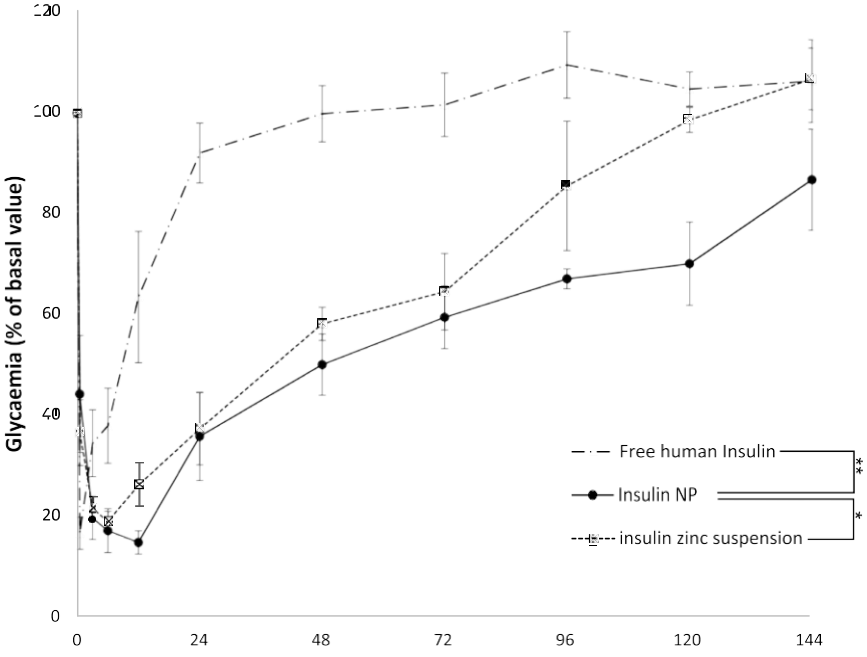


Fig. 10. Profiles of glycaemia after a subcutaneous administration of free human insulin (triangles), insulin-loaded nanoparticles (circles) and insulin zinc suspension (squares) in diabetic rats. Insulin was administered at the concentration of 25 IU/kg. Before the injections, average blood glucose level was 520.28 ± 58.60 mg/dL. Results are expressed as means ± SD. (n = 6 per group). Insulin NP was statistically different from free human insulin (**$p < 0.01$) and insulin zinc suspension (*$p < 0.05$).

Time (hr)

a gradual erosion of polymer matrix to diff use drug molecules out to the surrounding release medium. We found in this current work that the initial burst release in the fi rst 12 h was likely attributed to diff usion of the drug bound to the surface of polymer and the sustained release phase was due to gradual erosion of the polymer matrix which capsulated the drug molecules [33].

3.4. In vitro drug stability

MALDI-TOF MS was used to compare the molecular weight of insulin standard (pure) to insulin extracted from NP. As it is shown in Fig. 8, the measured molecular weight of the standard (5793.40 Da) and the sample (5790.62 Da) are almost equal, confi rming that the method of preparation would protect the drug against any risk of changing the architecture of insulin molecules following NP processing and storage.

3.5. In vitro release profi le

In vitro release experiment was performed for NP formulations (F1 & F4 - F7). Incorporation of PEG with optimum molecular weights and concentrations led to higher EE values with smaller particle sizes with 200–250 nm range. As it is shown in Fig. 9, an initial burst release was observed within the fi rst 12 h followed by a sustained release profi le over 144 h. Larger particles have a lesser initial burst and more sustained release than smaller particles. In addition, higher drug loadings typically produce a higher initial burst and a faster release rate [29].

In vitro release profi les for F4, F5 and F6 showed evidence of a burst release, with higher signifi cant eff ect for F4 and F5 than F6, which released approximately 65% and 34% of insulin, respectively, within the fi rst 12 h, when compared to 19% of insulin released from F1 (Fig. 9). Formulations containing PEG (F4-F6) had greater drug loadings, which resulted in signifi cant higher initial burst releases ($p < 0.05$). This initial burst release could be explained by the diff usion of drug molecules bound on the surface of the NP, which was enhanced by the addition of PEG [34]. PEG are hydrophilic chains, increases the porosity of the NP and improves water permeation to NP core which facilitates rapid insulin release in the fi rst 12 h [35].

3.6. Subcutaneous delivery of insulin NP (In vivo study)

A comparison between free insulin, insulin zinc suspension and insulin NP was performed in vivo using STZ induced diabetic rats. An optimised insulin NP (F4) was chosen based on their in vitro release profi le which showed the highest initial burst peak with the maximum % cumulative release after 6 days, also, F4 has a high EE value with optimum particle size and low PDI. As shown in Fig. 10, the initial burst of insulin released from NP resulted in signifi cantly hypoglycemic eff ect in the fi rst 12 h to 14.76%, compared to 26.14% and 63.50% for insulin zinc suspension and free insulin respectively. Indeed, after the subcutaneous injection of free insulin (25 $IU.kg^{-1}$), glycaemia decreased maximally by 16.59% after 30 min and started to increase after 3 h to 34.48%. The continuous increase in the glycaemia level was maintained up to 12 h and zero time values were reached 24 h after the injection. Insulin zinc suspension (25 $IU.kg^{-1}$) decrease the glycaemia signifi cantly ($p < 0.001$) to 36.57% after 30 min, the maximal decrease (18.90%) being reached after 6 h, the glucose level started to increase again restoring its initial value at 48 h post injection. Insulin NP prolonged the hypoglycemic eff ect with signifi cantly decrease in the glucose level. Glucose level started to slightly increase at 48 h and till the end of experiment after 6 days, glucose level did not reach to its initial value and the hypoglycemia eff ect was signifi cantly lower (86.80%) than insulin zinc suspension (106.80%, $p < 0.01$) and free insulin (106.36%, $p < 0.01$).

Thus, the hypoglycemic eff ect of free insulin disappeared after

30 min, whereas encapsulated insulin and insulin zinc suspension re- mained active for more than 12 h after the injection. Insulin NP has
signifi cantly hypoglycemic eff ect ($p < 0.05$) than insulin zinc sus-
pension throughout the six days of the experiment. Nanoparticles encapsulation technique maintains insulin activity for longer time [16]. Addition of PEG would increase the residence time of insulin in the blood circulation via scaping mononuclear phagocyte systems (MPS)
[34] which off ers an advantage for insulin NP over insulin zinc sus-
pension.

4. Conclusion

Double emulsion solvent evaporation technique is an optimum method used to prepare nanoparticles while maintaining stability and biological activity of protein or peptide during processing and release as
confi rmed by MALDI- TOF MS technique. Optimisation of the process
variables during NP manufacturing is the crucial key to control in vitro characterisation of NP. Addition of PEG resulted in better entrapment effi ciency, smaller particle size and sustained in vitro release profi le for 6 days with initial burst at the fi rst 12 h. A single weekly subcutaneous injection of optimised NP formulation is effi cient to sustain the delivery
of insulin in streptozotocin induced diabetic rats.

Acknowledgement

The authors would like to acknowledge fi nancial support from the cultural aff airs and mission sector, Egyptian government, Egypt.

References

[1] D.H. Abdelkader, M.A. Osman, S.A. El-Gizawy, A.M. Faheem, P.A. McCarron, The role of insulin in wound healing Process: mechanism of action and pharmaceutical applications, J. Anal. Pharm. Res. 2 (2016) 1–6, http://dx.doi.org/10.15406/japlr.2016.02.00007.

[2] A.D. Association, Diagnosis and classi fi cation of diabetes mellitus, Diabetes Care 37 (2014) 81–90, http://dx.doi.org/10.2337/dc14-S081.

[3] M. Iqbal, N. Zafar, H. Fessi, A. Elaissari, Double emulsion solvent evaporation techniques used for drug encapsulation, Int. J. Pharm. 496 (2015) 173–190, http://dx.doi.org/10.1016/j.ijpharm.2015.10.057.

[4] R. Goyal, L.K. Macri, H.M. Kaplan, J. Kohn, Nanoparticles and nanofi bers for topical drug delivery, J. Control. Release 240 (2015) 77–92, http://dx.doi.org/10.1016/j.jconrel.2015.10.049.

[5] V.R. Sinha, A. Trehan, Biodegradable microspheres for protein delivery, J. Control. Release 90 (2003) 261–280.

[6] T. Feczkó, J. Tóth, J. Gyenis, Comparison of the preparation of PLGA–BSA nano- and microparticles by PVA, poloxamer and PVP, Coll. Surf. A Physicochem. Eng. Asp. 319 (2008) 188–195, http://dx.doi.org/10.1016/j.colsurfa.2007.07.011.

[7] T. Feczkó, J. Tóth, G. Dósa, J. Gyenis, Optimization of protein encapsulation in PLGA nanoparticles, Chem. Eng. Process. Process Intensif. 50 (2011) 757–765, http://dx.doi.org/10.1016/j.cep.2011.06.008.

[8] W. Abdelwahed, G. Degobert, S. Stainmesse, H. Fessi, Freeze-drying of nanoparticles: formulation, process and storage considerations☆, Adv. Drug Deliv. Rev. 58 (2006) 1688–1713, http://dx.doi.org/10.1016/j.addr.2006.09.017.

[9] M.H. Mj, K. Ah, M. Mohd, A. Mmr, S. Liza, S. Mj, T.J. Be, A simple and sensitive Hplc method for the determination of insulin in rat plasma and its application in pharmacokinetic study, Int. J. Pharm. Pharm. Sci. 5 (2013) 1–5.

[10] Annette Kunkel, Stefan Gunter, Christoph Dette, Hermann Waitzig, Quantitation of insulin by capillary electrophoresis and high- performance liquid chromatography method comparison and validation 1, J. Chromatogr. A 781 (1997) 445–455.

[11] M. García-Díaz, C. Foged, H.M. Nielsen, Improved insulin loading in poly(lactic-co- glycolic) acid (PLGA) nanoparticles upon self-assembly with lipids, Int. J. Pharm. 482 (2015) 84–91, http://dx.doi.org/10.1016/j.ijpharm.2014.11.047.

[12] A.C. Nøhr, M.A. Shehata, A.S. Hauser, V. Isberg, K.B. Andersen, D.S. Pedersen, D.E. Gloriam, H. Bräuner-Osborne, J. Mokrosinski, I.S. Farooqi, The orphan G protein-coupled receptor GPR139 is activated by the peptides: adrenocorticotropic hormone (ACTH), α-, and β-melanocyte stimulating hormone (α-MSH, and β-MSH), and the conserved core motif HFRW, Neurochem. Int. 102 (2017) 105–113, http://dx.doi.org/10.1016/j.neuint.2016.11.012.

[13] J. Liu, S.M. Zhang, P.P. Chen, L. Cheng, W. Zhou, W.X. Tang, Z.W. Chen, C.M. Ke, Controlled release of insulin from PLGA nanoparticles embedded within PVA hydrogels, J. Mater. Sci. Mater. Med. 18 (2007) 2205–2210, http://dx.doi.org/10.1007/s10856-007-3010-0.

[14] C. Qian, C. Zhu, W. Yu, X. Jiang, F. Zhang, High-fat diet/low-dose streptozotocin-induced type 2 diabetes in rats impacts osteogenesis and Wnt signaling in bone marrow stromal cells, PLoS One 10 (2015) 1–15, http://dx.doi.org/10.1371/journal.pone.0136390.

[15] S. Apikoglu-Rabus, F.V. Izzettin, P. Turan, F. Ercan, Eff ect of topical insulin on cutaneous wound healing in rats with or without acute diabetes, Clin. Exp. Dermatol 35 (2010) 180–185, http://dx.doi.org/10.1111/j.1365-2230.2009.03419.x.

[16] C. Damgé, P. Maincent, N. Ubrich, Oral delivery of insulin associated to polymeric nanoparticles in diabetic rats, J. Control. Release 117 (2007) 163–170, http://dx.doi.org/10.1016/j.jconrel.2006.10.023.

[17] N. Sharma, P. Madan, S. Lin, Eff ect of process and formulation variables on the preparation of parenteral paclitaxel-loaded biodegradable polymeric nanoparticles: a co-surfactant study, Asian J. Pharm. Sci. 11 (2016) 404–416, http://dx.doi.org/10.1016/j.ajps.2015.09.004.

[18] D. Sharma, D. Maheshwari, G. Philip, R. Rana, S. Bhatia, M. Singh, R. Gabrani, S.K. Sharma, J. Ali, R.K. Sharma, S. Dang, Formulation and optimization of polymeric nanoparticles for intranasal delivery of lorazepam using box-behnken design:in vitro and in vivo evaluation, Biomed. Res. Int. 2014 (2014) 1–14.

[19] M.F. Zambaux, F. Bonneaux, R. Gref, P. Maincent, E. Dellacherie, M.J. Alonso, In fl uence of experimental parameters on the characteristics of poly (lactic acid) nanoparticles prepared by a double emulsion method, J. Control. Release 50 (1998) 31–40.

[20] D.H. Abdelkader, M.A. Osman, S.A. El-Gizawy, A.M. Faheem, P.A. McCarron, Characterisation and in vitro stability of low-dose, lidocaine-loaded poly(vinyl alcohol)-tetrahydroxyborate hydrogels, Int. J. Pharm. 500 (2016) 326–335, http://dx.doi.org/10.1016/j.ijpharm.2016.01.046.

[21] M.L.T. Zweers, D.W. Grijpma, G.H.M. Engbers, J. Feijen, The preparation of monodisperse biodegradable polyester nanoparticles with a controlled size, J. Biomed. Mater. Res. - Part B Appl. Biomater. 66 (2003) 559–566, http://dx.doi.org/10.1002/jbm.b.10046.

[22] E. Allémann, R. Gurny, E. Doelker, Preparation of aqueous polymeric nanodispersions by a reversible salting-out process: infl uence of process parameters on particle size, Int. J. Pharmacol. 87 (1992) 247–254.

[23] D. Quintanar-Guerrero, H. Fessi, E. Allémann, E. Doelker, Infl uence of stabilizing agents and preparative variables on the formation of poly(D,L-lactic acid) nanoparticles by an emulsifi cation- diff usion technique, Int. J. Pharmacol. 143 (1996) 133–141.

[24] H. Takeuchi, M. Kobayashi, Infl uence of the degrees of hydrolyzation and polymerization of poly (vinylalcohol) on the preparation and properties of poly(DL-lactide-co-glycolide) nanoparticle, Int. J. Pharm. 149 (1997) 43–49.

[25] U. Seju, A. Kumar, K.K. Sawant, Development and evaluation of olanzapine-loaded PLGA nanoparticles for nose-to-brain delivery: in vitro and in vivo studies, Acta Biomater. 7 (2011) 4169–4176, http://dx.doi.org/10.1016/j.actbio.2011.07.025.

[26] Xiaohong Li, Xianmo Deng, Minglong Yuan, Chengdong Xiong, Zhitang Huang, Yanhua Zhang, Wenxiang Jia, Investigation on process parameters involved in preparation of poly-?-lactide-poly(ethylene glycol) microspheres containing Leptospira Interrogans antigens, Int. J. Pharm. 178 (1999) 245–255, http://dx.doi.org/10.1016/S0378-5173(98)00379-2.

[27] G. Crotts, T.G. Park, Preparation of porous and nonporous biodegradable polymeric hollow microspheres, J. Control. Release 35 (1995) 91–105.

[28] K. Narayanan, V.M. Subrahmanyam, J. Venkata Rao, A fractional factorial design to study the eff ect of process variables on the preparation of hyaluronidase loaded PLGA nanoparticles, Enzyme Res. 2014 (2014), http://dx.doi.org/10.1155/2014/162962.

[29] M. Hans, A. Lowman, Biodegradable nanoparticles for drug delivery and targeting, Curr. Opin. Solid State Mater. Sci. 6 (2002) 319–327, http://dx.doi.org/10.1016/S1359-0286(02)00117-1.

[30] a. Beletsi, Z. Panagi, K. Avgoustakis, Biodistribution properties of nanoparticles based on mixtures of PLGA with PLGA–PEG diblock copolymers, Int. J. Pharm. 298 (2005) 233–241, http://dx.doi.org/10.1016/j.ijpharm.2005.03.024.

[31] A.G.C. Ozdemir, Solubility profi les of poly (ethylene glycol)/solvent systems, I: qualitative comparison of solubility parameter approaches, Eur. Polym. J. 43 (2007) 3068–3093, http://dx.doi.org/10.1016/j.eurpolymj.2007.02.022.

[32] E. Sah, H. Sah, Recent trends in preparation of poly (lactide- co -glycolide) nanoparticles by mixing polymeric organic solution with antisolvent, J. Nanomater 2015 (2015) 1–23.

[33] F. Danhier, E. Ansorena, J.M. Silva, R. Coco, A. Le Breton, V. Préat, PLGA-based nanoparticles: an overview of biomedical applications, J. Control. Release 161 (2012) 505–522, http://dx.doi.org/10.1016/j.jconrel.2012.01.043.

[34] M. Noori Koopaei, M.R. Khoshayand, S.H. Mostafavi, M. Amini, M.R. Khorramizadeh, M. Jeddi Tehrani, F. Atyabi, R. Dinarvand, Docetaxel loaded PEG-PLGA nanoparticles: optimized drug loading, in-vitro cytotoxicity and in-vivo antitumor eff ect, Iran. J. Pharm. Res. IJPR 13 (2014) 819–833 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4177642&tool=pmcentrez&rendertype=abstract.

[35] B. Zanetti-Ramos, M. Soldi, V. Soldi, E. Lemos-Senna, The eff ect of polyethylene glycol on drug content, particle morphology, and carbamazepine release profi les of sustained release microspheres prepared from cellulose acetate butyrate, Acta Farm. Bonaer. 25 (2006) 177–183.