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

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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-

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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 trifluroacetic 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