Integrity and Bioactivity of Insulin Loaded PLGA Nanoparticles Prepared by a Novel Aqueous Method and its Comparison to Emulsion Solvent Evaporation Method

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Abstract
Poly lactic-coglycolic acid polymer nanoparticles of insulin were prepared by a novel aqueous based mixed micelle (MM) method and traditional emulsion solvent evaporation method that use organic solvents. The particle size and entrapment efficiency and insulin release from the nanoparticles were determined. The integrity of the entrapped insulin, bioactivity and immunogenicity were investigated using MALDI MS, cell viability assay, and ELISA tests. The pharmacodynamic activity of the entrapped insulin in nanoparticles was compared with its subcutaneous administration in diabetic rats. The nanoparticles released 50% of insulin immediately at pH 7.4, followed by slow release of the remaining entrapped insulin. At pH 1.2, complete release of insulin occurred within 5 minutes. At a pH closer to PI of insulin, the burst release decreased to 8%. The molecular mass, cell viability and Elisa test showed that insulin retained its integrity and activity. The blood glucose levels in rats showed sustained reduction following the administration of insulin loaded nanoparticles suggesting that insulin activity in nanoparticles prepared by MM and ESE methods has remained intact.

Keywords: Bioactivity, Immunogenicity, Insulin integrity, Mixed micelle, Nanoparticles

Introduction
Recently, considerable progress has been made in developing biodegradable nanoparticles as effective vehicles for the delivery of proteins and peptides (1). These polymer drug delivery systems offer many advantages as they can carry and deliver the drug to a target site, have the ability to deliver proteins, peptides and genes, increase the therapeutic benefits and minimize the side effects of the drug (2,3). Also, they can control the release of a pharmacologically active component at the therapeutically optimal rate and dose regimen and help to increase the stability of drugs and proteins (4,5). The PLGA polymers, being biocompatible, have been used as controlled release delivery systems for parenteral and implantable applications (6). A successful PLGA nanoparticulate system should have a high drug loading capacity as it allows a small quantity of the carrier during a single administration. Insulin is the most effective drug in the treatment of advanced-stage diabetes. Despite the significant advancement in the field of pharmaceutical research, development of a proper insulin delivery system remained a challenge (7). The biological half life of peptides is short and need frequent and multiple administrations. Their transport across biological barriers is also poor, due to poor diffusivity and lower partition coefficients. In this
respect, biodegradable nanoparticulate delivery systems have been proposed for the safe and controlled parenteral administration of peptides (8). PLGA polymers possess various unique properties for the design of sustained release drug delivery application (9-11). An aqueous dispersion of insulin loaded PLGA nanospheres showed complete bioactivity after pulmonary administration in guinea pigs (12). The bioactivity of the entrapped protein need to be ascertained, as high sheer force, organic solvents and surfactants used in the nanoparticle preparation have potential to inactivate them. Homogenization or sonication used to obtain a stable primary w/o emulsion in w/o/w method can provoke cavitation stress that may destroy proteins (13-14). Insulin encapsulated in various polyester and polyanhydride nanospheres was found to retain its bioactivity (15). A modified o/o solvent evaporation method used for the preparation of insulin loaded PLGA micro-particles using homogenous single phase in which insulin and PLGA are dissolved (16). This method also depends on volatile organic solvents which have harmful effects on both human and environment. So, it is important to avoid the w/o interface and organic volatile solvents during preparation of protein loaded nanoparticles. A novel aqueous based method (mixed micelle method- MM method) for the preparation of proteins entrapment in PLGA nanoparticles was developed in our laboratory (17). Insulin is incorporated into mixed micelles of PEG/tween dissolving PLGA polymer then polymer precipitation in aqueous conditions. This method is similar to the nanoprecipitation technique but without the use of organic solvents(14). It has the advantage of avoiding organic solvents and easy to prepare at room temperature. However, the integrity and biological activity of protein nanoparticles prepared by MM method is not known. In this study, the bioactivity of insulin PLGA nanoparticles prepared by aqueous based MM method and other organic solvent based methods were compared. The release profiles of insulin nanoparticles at different pH were also investigated. Additionally, the integrity, immunogenicity, and biological activity of insulin entrapped in nanoparticles prepared by different methods were evaluated.

Material and Methods

Poly (lactic-co-glycolic acid) (PLGA), 50:50 DL2A, M.W., 10 kDa was obtained from Alkermes, Inc. (Cincinnati, OH). Polyvinyl alcohol (PVA, M.W. 30-70 kDa), Bovine Insulin (M.W. 5.7335 kDa), and sulphorhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology Inc. (Rockford, IL). Dulbecco’s Modified Eagle’s Medium (DMEM) was obtained from Gibco BRL (Grand Island, NY). Streptozotocin purchased from Calbiochem, a division of EMD Biosciences, Inc. (La Jolla, CA). All other chemicals and solvents used were of analytical grade.

Nanoparticle preparation

ESE method: To 100 µL of insulin suspension (100 mg/mL) in distilled water, 10 mL of PLGA solution in methylene chloride or ethyl acetate (4% w/v) was added. The first water/oil (W/O) and solid/oil (S/O) emulsions were generated by a high-speed homogenizer at 22,000 rpm for 1 min (Polytron® PT-MR 2100, Switzerland by Kinematica AG). This primary emulsion was added to 40 mL of ice cooled 1% w/v aqueous PVA solution and homogenized at 22,000 rpm for 15 minutes to form the multiple emulsions (both W1/O/W2 and S/O/W2). The emulsion was stirred at room temperature in a hood with a magnetic stirrer adjusted to 500 rpm overnight (Tekstir ® 20, Tekpro/American Hospital supply corp., Evanston, IL), to remove the organic solvent.
**MM technique:** The PLGA polymer was dissolved into 10 mL of methoxy-PEG 350 in 4% w/v. A stabilizer such as Tween 20 was added as 1% v/v of the final volume (50 mL). One hundred microlitres of Insulin suspension (100 mg/mL) in distilled water were added while stirring at 500 rpm for one minute, and then about 40 mL of PVA solution (1% w/v) was poured while stirring at 500 rpm. Nanoparticles were formed at once and no further stirring was required. The procedure was performed at ambient room temperature.

**Nanoparticles purification:** The nanoparticles were collected and purified by centrifugation and washing by distilled water two times at 17,300 g for 20 minutes. The purified suspension of nanoparticles was then frozen and dried using freeze dryer (FTS systems, Corrosion resistant freeze dryer, Stone Ridge, New York, USA).

**Protein entrapment efficiency:** The amount of insulin loaded into nanoparticles was calculated indirectly as the difference between the total amount of initial insulin added and the amount of insulin determined in the supernatants obtained during the purification step (18). The results were confirmed by dissolving 5 mg of the dried nanoparticles into 3 mL of cold acetone and centrifuged at 6,000 rpm to separate the precipitated protein. The precipitated protein was dried under vacuum at room temperature for 3 hours and then dissolved into 1 mL of distilled water.

BCA protein assay kit was used to determine the amount of protein entrapped and the method termed as direct method of determining the EE%. A standard calibration curve was established using serial protein concentrations from 10 to 150 µg/mL and the color produced was estimated using UV-Visible spectrophotometer (UV-1601 – Shimadzu) at wave length of 562 nm. The protein entrapment efficiency (EE %) was defined as the percentage of protein loaded relative to the initial amount of the insulin.

**Particle size determination:** Particle size was determined using a photon correlation spectroscopy (PCS) with a Zetasizer 3000HS (Malvern Instruments Ltd., UK). Each blank nanoparticle batch was appropriately diluted with double distilled water just after production and after freeze drying. Mean size was measured three times for each batch.

**Zeta potential determination:** Zeta potential of each nanoparticle composition was determined by Zetasizer 3000HS (Malvern Instruments Ltd., UK). Zeta potential was measured by diluting a sample of formulation 1:16, with phosphate buffer of different pH values (6.8, 7, and 7.4). An average of three readings was recorded.

**Nanoparticle morphology:** Nanoparticle shape and morphology were analyzed by scanning electron microscopy (SEM) (Hetachi 26100, Japan). Freeze dried nanoparticles were placed on a metal stub coated with gold under vacuum. Concentrated nanoparticle dispersions prepared by the MM method were finely spreading over the metal stub and left to dry under vacuum then coated with gold.

**In vitro release studies:** The freeze dried nanoparticles were suspended in phosphate buffer (pH 7.4 or 5.6) or in pH 1.2 buffer as 1 mg/mL in 20 mL glass vials. The suspension was placed in a shaking water bath at 37°C in dark conditions. From this, 1 mL samples were withdrawn at 0.08, 0.5, 1, 6, and 24 h after incubation. Samples were centrifuged as mentioned before and supernatants were assayed by reversed phase HPLC. The percentage of insulin released was plotted as a function of time (19).

**Quantitative analysis of insulin:** The amount of insulin released into the medium was quantified using an HPLC equipped with UV detector (SPD-10A- UV-VIS- Shimadzu), waters TM 600 pump
and WatersTM 717 plus autosampler (Millipore, USA). The mobile phase consisted of distilled water (70%) and acetonitril (30%) with trifluroacetic acid (0.1%) and 0.6 mmol sodium sulfate. Chromatography was performed using C18 reversed phase column (Nova-Pak C18, 3.9X150 mm, WatersTM, Ireland) at a flow rate of 1.5 mL/min and the eluent was monitored at 220 nm. The concentration of the insulin was determined by integration of the peak area using the external standardization method. All measurements were conducted in triplicate.

**Insulin Integrity:** Insulin released from nanoparticles was characterized using MALDI-TOF MS to assess the molecular mass (20). Nanoparticles were dissolved in acetone, dried at room temperature under vacuum. These samples containing insulin were reconstituted in 1 mL of 0.01 N HCl. Reference insulin solution was also prepared the same way by dissolving into 0.01 NHCl. Aliquots of 2 µL samples were then mixed with 8 µL of the matrix (α-CHCA) in solution (10 mg/ml in 0.1% TFA and 50% acetonitrile solution) and 2 µL of the mix was allowed to dry on a plate in a solid spot under vacuum. The plate was then inserted into the mass spectrometer.

MALDI-TOF MS experiments were conducted on a MALDI-TOF instrument (Shimadzu, Japan) using a 337-nm nitrogen laser. Spectra were acquired in positive ion linear mode (Acceleration voltage 33 kV).

**Immunogenicity of insulin:** The immunogenicity of insulin was assayed by an ELISA test (Mercodia, Uppsala, Sweden). Accurately weighed amounts of nanoparticles were dissolved into acetone and centrifuged to precipitate the protein and the supernatant containing PLGA polymer was discarded. Samples were washed twice by acetone and centrifuged to separate the pure insulin. Samples were then vacuum dried and re-dissolved in distilled water in the concentration range of (1–200 mU/L) using the manufacture’s protocol and its relative bioactivity was calculated by comparison with values obtained by BCA analysis of the same aliquots (21).

**Cell viability assay:** MCF-7 cells were grown into 96 well plates in 5,000 cells per well for 24 h at 37 °C, 5% CO2 in DMEM + 5% heat inactivated fetal bovine serum, non-essential amino acids, and L-glutamine (200 mM). The cells were serum and insulin starved for another 24 h and then incubated with insulin or nanoparticles carrying insulin in concentrations of 7.5, 10, and 12 µg/mL for 48 hours. Control wells were treated similarly and PBS of pH 7.4 was added instead of insulin. Cell viability was then estimated using SRB assay (22).

**In vivo effects of insulin loaded nanoparticles**

**Animals / Conditioning:** Male Sprague–Dawley (SD) rats, body weight ranging 160–200 g were used in the study. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of the Pacific. The animals were grouped in standard polypropylene cages and maintained under controlled room temperature (22±2 °C) and humidity (55±5% RH) with 12:12h light and dark cycle. All the rats are provided with commercially available rat normal pellet diet and water ad labium. If the weight of the animals increases beyond 250 g, they were discontinued from the study.

**Induction of Diabetes:** Diabetes was induced in male rats (250 ± 30g) by an intra-peritoneal injection of streptozotocin (65 mg/kg) in a 10 mM citrate buffer at pH 4.5 (23). Streptozotocin solutions were used within 30 min. Rats were considered diabetic when blood glucose level was higher than 300 mg/dL, a week after streptozotocin treatment (24).
Blood glucose levels after subcutaneous administration of insulin nanoparticles: Insulin Loaded nanoparticles (10 IU/kg) prepared by the above different methods were injected to fed diabetic rats. Free insulin (10 IU/kg), unloaded nanoparticles and PBS (pH 7.4) were also administered in control animals. Samples of 1 drop of blood were withdrawn from the tail vein at different time intervals and evaluated for the glucose levels using OneTouch Ultra 2 glucometer before injection and 0.5, 1, 1.5, 2, 3, 6, 8, 10, 12, 24, 48, and 72 hour post injection. Rats were maintained fasted during the experiment up to 12 h and fed thereafter.

Statistical analysis: The mean and the standard errors were calculated for each group. For group comparisons a one-way analysis of variance followed by the least significant difference (LSD) as a post-hoc test was applied, using SPSS program version 9 software. When P<0.05, the difference was considered significant.

Results
Nanoparticles size, morphology and insulin entrapment efficiency: In the present investigation, insulin nanoparticles were prepared by two different methods. An aqueous solvent based method (MM) was found to be easier and faster method for preparing PLGA nanoparticles.

As shown in Table 1, the size of the nanoparticles produced using MM method were smaller (247 nm) than those prepared by solvent evaporation method using dichloromethane (DCM) solvent (472.63 nm). ESE (Et-Ac) method produced nanoparticles of 257.9 nm which considered close to that produced by MM method. Morphological analysis of nanoparticles by SEM showed a homogenous distribution of spheroid particles with different particle sizes and no insulin crystals were observed. SEM photographs (Fig. 1) are clearly showing the difference in particle size according to the initial solvent used (each solvent corresponds a different method of preparation). All methods of preparation resulted in greater than

Table 1: Size, yield, and EE% of insulin nanoparticles prepared by different methods. (n=3).

<table>
<thead>
<tr>
<th>Method of preparation</th>
<th>Before freeze drying Particle size (µm) (mean±SD)</th>
<th>Yield(%) (mean±SD)</th>
<th>EE(%) (mean±SD)</th>
<th>Zeta potential</th>
</tr>
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<tbody>
<tr>
<td>ESE (DCM; PLGA 75:25)</td>
<td>863.90±11.50</td>
<td>72.93±2.80</td>
<td>99.22±0.10</td>
<td>N/A</td>
</tr>
<tr>
<td>ESE (DCM; PLGA 50:50)</td>
<td>472.63±22.92</td>
<td>67.58±3.50</td>
<td>87.67±8.85</td>
<td>-8.1±5.00</td>
</tr>
<tr>
<td>MM (M-PEG-350; PLGA 50:50)</td>
<td>247.00±5.30</td>
<td>51.23±1.20</td>
<td>84.75±0.75</td>
<td>-3.8±4.3</td>
</tr>
<tr>
<td>ESE(Et-Ac; PLGA 50:50)</td>
<td>257.90±3.20</td>
<td>65.67±2.75</td>
<td>90.95±13.57</td>
<td>-1.3±1.1</td>
</tr>
<tr>
<td>Nanoprecipitation (Acetone; PLGA 50:50)</td>
<td>248.40±2.20</td>
<td>56.67±4.25</td>
<td>89.00±1.83</td>
<td>-9.5±7.4</td>
</tr>
</tbody>
</table>

Fig. 1. Scanning electron micrographs of PLGA nanoparticles prepared by ESE method using DCM (A, PLGA 50:50; and Et-Ac (B), and mixed micelle method using methoxy Peg 350 as solvent (C).
84% entrapment efficiency (EE) as shown in Table 1. Of the different methods of preparation, MM method resulted in lower EE%. However, there was no significant difference in Insulin EE% compared to nanoparticles prepared by ESE methods (p>0.05). All nanoparticles prepared by different methods showed negative zeta potential at pH 7.4.

**Insulin Release from nanoparticles prepared by different methods**: The release of insulin from nanoparticles prepared by different methods was found to be biphasic (Fig. 2). The initial rapid release (burst effect) was high, where, 52%, 52.4, and 54.2% of the entrapped insulin was released from nanoparticles prepared by ESE (DCM), MM, ESE (ET-AC), respectively. This burst release in the first 5 minutes was followed by about 8% release of insulin after 30 minutes in case of ESE (DCM) and MM methods. On the other hand, about 16% of insulin was released from nanoparticles of ESE (ET-AC) method following the burst release. After the first 30 minutes, very slow release of minute amounts of insulin was observed over 24 hours.

To use nanoparticles via the oral route, it is important to test the release of insulin at different pH range. Insulin release from nanoparticles of ESE (DCM) method was selected for the test as they show no significant differences in release compared to MM method (P>0.05). The release of insulin was significantly affected by the external pH. Complete release of insulin occurred in 5 minutes at pH 1.2 as shown in Figure 3. However, the initial burst release was only 8% at pH 5.6 and 52% at pH 7.4. After 24 hours, only 42.5% of insulin was...
found released at pH 5.6, whereas 63.5% and 96% of insulin was released in pH 7.4 and 1.2 media, respectively.

**Effect of Method of preparation on Insulin Integrity and Bioactivity:** The integrity of insulin during the preparation of nanoparticles (organic solvent, homogenization, surfactants, freeze drying steps), was evaluated by extracting insulin from nanoparticles and analyzing the molecular weight by MALDI-TOF Mass Spectrometry. The mass spectra showed only a molecular peak at m/z 2866.4 (doubly charged, molecular weight of bovine insulin is 5733.5 Da) for samples prepared by both methods as well as the insulin solution (Fig. 4). This indicated that insulin retained its integrity and insulin was not degraded in the preparation process in all the methods evaluated.

The immunogenicity of insulin after encapsulation into nanoparticles by different methods was evaluated using ELISA test. The data showed that the antigen activity of insulin remained particularly unaffected by DCM significantly reduced ($P<0.05$) when Tween-20 concentration was 0% suggesting the plausible reason for the reduced antigenicity of insulin nanoparticles is MPEG-350 and not Tween-20 as shown in Table 2.

Cell viability assay showed that all insulin carrying nanoparticles had increased the growth of MCF-7 cells, regardless of the method of preparation (Fig. 6). Increasing standard insulin concentration from 7.5 to 12 µg/mL had increased cell viability from 110% to 154.85% compared to insulin starved cells. The nanoparticles carrying insulin prepared by ESE (DCM) method showed (102.46% ± 19%), or ET-AC (97.63%± 18.18%) in ESE method. However, only 28.18±13.38% insulin antigenicity was retained from nanoparticles prepared by MM method (Fig. 5).

Tween-20 and MPEG-350 were used in the preparation of insulin nanoparticles using MM method. To differentiate which of these two surfactants reduced antigenicity of insulin, ELISA assays were conducted using nanoparticles prepared at different Tween-20 concentrations. The antigenicity of insulin remained constant in the Tween-20 range of 1%-4% while it is significantly reduced ($P<0.05$) when Tween-20 concentration was 0% suggesting the plausible reason for the reduced antigenicity of insulin nanoparticles is MPEG-350 and not Tween-20 as shown in Table 2.

Cell viability assay showed that all insulin carrying nanoparticles had increased the growth of MCF-7 cells, regardless of the method of preparation (Fig. 6). Increasing standard insulin concentration from 7.5 to 12 µg/mL had increased cell viability from 110% to 154.85% compared to insulin starved cells. The nanoparticles carrying insulin prepared by ESE (DCM) method showed

**Table 2:** Effect of tween 20 concentration on immunogenicity of insulin. Data represented as the mean±SD. (n=3).

<table>
<thead>
<tr>
<th>Tween 20 Concentration (%)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigenicity (%)</td>
<td>23.11±0.02</td>
<td>29.51±0.72</td>
<td>27.61±0.16</td>
<td>28.18±13.38</td>
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</table>

**Fig. 6.** Cell Viability (%) in the presence of insulin solution and insulin nanoparticles. Data presented as the mean±SD. (n=3).

**Fig. 7.** Blood glucose concentrations following subcutaneous administration of insulin solution, insulin loaded nanoparticles prepared by different methods. PBS (pH 7.4) or empty nanoparticles of MM method (nanoparticles control). Insulin was administered at the concentration of 10IU/kg. Before the injection glycemia was 485.40±116.84 mg/dl. Results are expressed as means ±S.D. (n=6 per group).
increased cell viability from 148.55% to 199.61% as the concentration of insulin increased from 7.5 to 12 µg/ml, respectively. The same behavior was observed when preparing nanoparticles by ESE (ET-Ac) technique. Also, Nanoparticles prepared by MM method increased cell viability from 168.56% to 191.4% as insulin content increased from 7.5 to 12 µg/ml, respectively.

**In vivo effects of nanoparticles loaded with insulin on diabetic rats**

In contrast to the in vitro release results, where about 60% to 70% of insulin was released in the first 30 minutes, insulin nanoparticles have prolonged the hypoglycemic effect of the drug more than 24 hours compared to control insulin solution of the same dose (Figure 7). This clearly showed lack of in vitro/in vivo correlation for insulin containing nanoparticles. After the subcutaneous administration of 10 IU of insulin into diabetic rats as solution (in PBS, pH 7.4) and encapsulated in nanoparticles prepared by ESE (DCM), ESE (Et-Ac), and MM methods, the blood glucose levels decreased by 14.44%, 35.50%, 19.70% and 22.12%, respectively after 30 minutes. However, only nanoparticles of ESE (DCM) showed significantly lower blood levels compared to the remaining formulations and the controls (p<0.05) except nanoparticles of MM method (p>0.05). At 3 hours, blood glucose levels decreased by 69.30%, 63.81%, 56.33%, and 77.54% using insulin solution, nanoparticles of ESE (DCM), ESE (Et-Ac), and MM methods, respectively, and differences between the groups are not statistically significant (P>0.05). Blood glucose levels were significantly lower (p<0.05) in rats injected with insulin containing preparations when compared to control preparations 6 hours post-administration. On the other hand, only insulin nanoparticles prepared by MM method provided of significantly lower blood glucose levels than insulin solution (P<0.05). After 8 hours, all the insulin nanoparticle (except ET-AC- ESE) exhibited significantly lower blood glucose levels when compared to insulin solution in rats (P<0.05). All insulin loaded nanoparticles were effective in reducing the blood glucose levels even after 12 hours of administration when compared to insulin solution (P<0.05). Insulin nanoparticles prepared by MM method resulted in significantly lower blood glucose levels (P<0.05) compared to all insulin formulations and the controls except nanoparticles of ESE (DCM) method (P>0.05). During the 12 hours post-administration, nanoparticles of ESE (DCM), ESE (ET-AC), and MM methods showed a plateau phase where blood glucose levels were constant (Figure 7). Statistical analysis showed a significant difference between insulin nanoparticles and solution forms 24 hours after administration (P<0.05). After 48 and 72 hours of administration, the rats recovered completely from the insulin effect and there was no difference in blood glucose levels between the control and test groups (P>0.05). The effect of insulin on the reduction of blood glucose levels lasted between 12-25 hours from nanoparticles preparation. However, only insulin nanoparticles prepared by MM method could keep blood glucose levels at < 50% of control group over the 24 hours.

**Discussion**

The data showed that the method of preparation affected both size and EE% for insulin (25-27). The ESE (DCM) method resulted in largest particle size nanoparticles due to water immiscibility of DCM and formation of larger emulsion globules. The partial water miscibility of Et-Ac and the complete water miscibility of MPEG-350 lead to the formation of much smaller nanoparticles. The thermodynamic parameters, such as diffusion coefficients (D sw, D ws), exchange ratio (R = D sw/D ws), and solvent–polymer interaction parameter (χ) were studied from PLGA nanoparticles prepared by emulsification-diffusion method. They have found
that R was proportional to the size of the PLGA nanoparticles, while χ was inversely proportional to it (28). The size distributions of the PLGA nanospheres prepared with different water immiscible, water miscible and partially water miscible solvents when PVA (1%) was used as the stabilizer were also studied (29). There was no significant difference in particle size between partially water soluble like ET-AC or fully water soluble solvent like acetone. Also, the study concluded that the larger particle size of nanoparticles prepared using DCM was due to its immiscibility with water causing significant aggregation of emulsion droplets.

The zeta potential results showed that insulin nanoparticles were negatively charged. It is well known that insulin above its PI (5.5) acquires a partial negative charge and because insulin’s relatively smaller molecular size (6 kDa) when compared to other peptides, it might diffuse to the nanoparticle surface and impart negative charge to nanoparticles at the pH of 7.4 (30,31). The drug present on the surface of the nanoparticles gets released quickly upon exposure to media, resulting in the initial burst of insulin (32). Previous studies have shown that the particle size of microparticle did not significantly affect the drug in-vitro release profiles (33). Hence, the variability in the release profiles may not be due to variability in the nanoparticle size.

The release of insulin was found to be similar from nanoparticles prepared by aqueous MM method and by ESE (DCM) method. Both methods showed lower burst release of insulin compared to ESE (ET-AC) method. A plausible explanation is that an increase in the hydrophilicity of ET-Ac enhanced the migration of insulin molecules to the surface of nanoparticles and insulin subsequently adsorbs to the nanoparticles surface after the evaporation of the solvent (34,35). On the other hand, higher viscosity of MPEG, resulted in the accumulation of smaller insulin molecules on the nanoparticle surface by MM method. Thus, the drug release is largely controlled by the weak affinity between the insulin and the polymer, which resulted in a higher burst release (36).

The solubility of insulin varies at different pH conditions: >10 mg/ml, <1mg/mL, and about 5 mg/mL at pH 1.2, 5.6 (close to IP of insulin), and 7.4 (37). Insulin release from the nanoparticles was found to depend on the pH of the release media as insulin partitioning into the aqueous release depended on its solubility (38). This result suggested the nanoparticles are not useful for oral delivery.

Insulin fragments could be detected using MALDI TOF MS as a stability indicating assay (25). The mass spectra of insulin showed in Figure 4, exhibited only one identical monoisotopic peak corresponding to its molecular ion. No evidence of covalent aggregation or degradation peaks of disamido, dimmers, trimers or single chains insulin was noticed by MALDI TOF MS. The MALDI TOF MS analysis can prove unfolding of the C-terminal region of the α subunit in the folding intermediate (39). Also, this analysis was effectively used to prove the signal of insulin PEG conjugates and detect insulin degradation fragments. The resulting degradation products must exhibit two molecular weight distributions showing a shift in the MW distribution due to α and β chains of insulin (40). Figure 4 showed only a peak of 100% intensity which correspond insulin extracted from nanoparticles with no differences compared to ideal insulin solution. The result suggested extreme integrity of insulin molecule entrapped in nanoparticles by MM method and solvent evaporation technique.

ELISA method has very high sensitivity and specificity as the assay depends on the reaction of antigen with antibody. The method
was considered an appropriate means for detection of potential changes of the antigenic activity. It can confirm that the receptor binding epitopes on insulin were maintained after encapsulation (16). This could be true whenever the antigenicity is close to 100 %, however, in this study, reduction in antigenicity as tested by ELISA was not detrimental on receptor binding epitopes. Results showed that nanoparticles prepared by ESE method had 100% immunogenicity, whereas, nanoparticles prepared by MM method showed only 28.18% insulin immunogenicity based on ELISA testing. As shown in Table 2, the presence of minute amounts of methoxy PEG 350 after purification of nanoparticles was responsible for the reduced immunogenicity, while Tween-20 did not show any such effect. It is commonly known that PEGs could interfere with antibody antigen interaction. This effect is presumably the result of reduced exposure of hydrophobic parts of the protein in the presence of PEG (41). Low PEG residues on the nanoparticle surface, being more hydrophilic, decreased their opsonization, and reduced immune system recognition (42). This implied that the organic solvent/water interfaces, surfactants (PVA and Tween 20) and homogenization steps used in the preparation of nanoparticles did not alter insulin integrity. However, low immunogenicity did not result lower bioactivity obtained from cell viability assay.

The insulin present in the supplemented serum free medium seemed to be responsible for the enhanced cell growth (43). Insulin has potentiated lysophosphatidic acid to stimulate MCF-7cell cycle progression and DNA synthesis (44). Hence, cell viability assay performed to elucidate the insulin bioactivity on growth of MCF-7 cells was studied using nanoparticles prepared by ESE and novel MM methods. Higher cell viability was evident from nanoparticles when compared to standard insulin solution at the same concentrations. The intimate contact of nanoparticles to cells increased the concentration of insulin closure to cellular membranes enhancing cell viability. Since, the nanoparticles prepared by MM method exhibited good bioactivity while reducing the immunogenicity, this method of nanoparticle preparation seems to have advantages.

The biological effects of insulin encapsulated into nanoparticles were also proved by the in vivo studies. Nanoparticles preparation methods that involved organic solvents and surfactants showed prolonged reduction in blood glucose levels of diabetes induced rats, further suggesting that the biological activity of insulin is not affected by preparation methods. The blood glucose profiles of insulin nanoparticles showed 4 phases as shown in Figure 7. In the first phase a rapid reduction in blood glucose levels was observed that peaked at 3 hours, which correlated with insulin absorbed from the burst effect in vitro release media. In the second phase, blood glucose levels have increased as the absorbed insulin rapidly cleared from the rats between 3rd and 6th hours. The third plateau phase after 12 hours could be due to slow release of insulin from nanoparticles. A fourth phase of blood glucose levels recovery back to 100% and continued at that level at 48 and 72 hours revealed complete clearance of insulin from the rats. The reduced blood levels observed between 12 and 24 hours might from the insulin release due to the degradation of the polymer (19). On the other hand, insulin solution showed only first 2 phases shown by nanoparticles. This data clearly showed the unique advantages of nanoparticulate delivery systems, which include offering protection from degradation by enzymes in the body (44). A similar study showed lowered blood glucose levels in diabetic rats over 24 hours following intraperitoneal injection of PLGA nanoparticles (45). In that study, blood glucose level showed a minimum of 10 mmol/L at 1 hour after nanoparticle

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administration which might be due to burst release of insulin, and a later prolonged hypoglycemic effect due to insulin continuous release from nanoparticles. In another study, the aqueous dispersion of PLGA nanospheres prepared by organic solvent based method and administered pulmonary route to guinea pig via nebulization have reduced the blood glucose level significantly for over 48 hours when compared to nebulized aqueous solution of insulin as a control (46). About 85% of insulin release from nanospheres was found to occur during the initial burst, followed by a prolonged release of the remaining drug in saline solution at 37 °C. Current study clearly indicated that encapsulation of insulin into biodegradable nanoparticles preserved its biological activity and prolonged its pharmacological activity over extended period of time. This might help to reduce the frequent multiple injections required to maintain blood glucose level at normal concentration and inhibit hyperglycemic side effects.

Conclusion

MM method is a promising technique for preparing PLGA nanoparticles of good physicochemical properties compared to conventional methods. MM method has better potential than existent methods as PLGA nanoparticles can be prepared using aqueous based solvent with low shear stress or energy at room temperature resulting in the retention of biological activity with lower immunogenicity. Finally, in vivo study results showed prolonged blood glucose reduction in diabetes induced rats suggesting that insulin bioactivity was retained.

References


Integrity and Bioactivity of Insulin Loaded PLGA


