Novel Aqueous solvent based method for protein based nanoparticles

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Abstract

The objective of the present study is to develop a novel method for the preparation of poly(D,L-lactide-co-glycolide)(PLGA) nanoparticles for protein/peptide delivery, and compare this method with traditional solvent evaporation methods for nanoparticle particle size and entrapment efficiency. The nanoparticles were prepared by three different methods namely: w/o/w emulsification-solvent evaporation method (ESE), nanoprecipitation method, and novel aqueous mixed micelle (MM) method. The prepared nanoparticles were evaluated as drug carrier systems using bovine serum albumin (BSA) as a model peptide. The physicochemical characteristics of the nanoparticles; morphology, particle size, zeta potential, and the effect of different parameters (method of preparation, time of stirring, drug concentration, and polymer concentration) on particle size and protein encapsulation efficiency percent (EE%) were studied. Severe aggregation of nanoparticles was noticed after freeze drying, especially for MM method. To reduce or completely prevent nanoparticle aggregation during freeze drying, three sugar cryoprotectants (glucose, trehalose, and mannitol) were evaluated. Results showed that all the preparation methods yielded greater than 90% EE of BSA into nanoparticles. Increasing the time of stirring during preparation decreased the particle size and was of a little effect on EE% in case of ESE and nanoprecipitation methods. On the other hand, the time of stirring has no effect on the particle size and EE% in case of MM method. Increasing the drug concentration and PLGA concentration was found to increase both particle size and EE%. Both glucose and trehalose protected nanoparticles from aggregation during freeze drying while mannitol had increased their aggregation. In conclusion, this study suggested the potential of preparing PLGA nanoparticles using aqueous solvent that can be used for loading peptide drugs.

Key words: Emulsification, Nanoprecipitation, Mixed micelle, Nanoparticles, PLGA

Introduction

Nanoparticles offer an attractive way to deliver pharmaceutical and biotechnological agents (1). These nanoparticles are commonly prepared by emulsion processes (emulsification solvent evaporation; ESE) (2). Emulsions are typically prepared by simple mixing (e.g., magnetic stirring), homogenization, and ultrasonication. Polymer nanoprecipitation (or solvent displacement method) is another method of preparation for nanoparticles that was orginally developed and patented by Fessi and co-workers (3,4). This technique is based on the dissolution of the polymer in a solvent (acetone for PLGAs), followed by its dispersion in a continuous external phase, in which the polymer is insoluble. The main difference between the emulsion and nanoprecipitation technique is the miscibility of the organic and aqueous phases. In ESE method, a solvent with limited solubility in water is used...
resulting in the formation of oil-in-water emulsions. This will be followed by precipitation of the polymer in water phase, when the solvent is evaporated. In case of nanoprecipitation method, the solvent is completely miscible with the aqueous phase, resulting in immediate polymer precipitation. Consequently, neither evaporation nor extraction of the solvent is required for polymer precipitation. After nanoparticle formation, the solvent is eliminated by evaporation. These methods have several difficulties such as the use of toxic solvents and problem with scale up, limiting their use (5).

Development of a new method for PLGA nanoparticles that uses aqueous solvents would solve these problems and also will be useful for loading peptide drugs. PLGA is shown to have good solubility in methoxy-PEG 350. Moreover, it has been reported that tween 20 and tween 80 can form mixed micelles of higher hydrophobic environment with PEGs (6).

The therapeutic use of proteins and peptides is limited by their rapid clearance necessitating the administration of high doses (7). The high doses may cause toxic effects, forcing to stop the continued therapy. A plausible solution to this problem is the development of biodegradable nanoparticle delivery systems for targeted administration and controlled or sustained release of protein drugs. Aliphatic polyesters such as poly (L-lactic acid) and its copolymers with D-lactic and glycolic acid of poly (D,L-lactic-co-glycolic acid) [PLGA] have been used as microparticulate carriers for small molecular weight drugs, peptides and proteins (8, 9). A successful commercialized drug delivery product is PLGA microspheres with leutenizing hormone (LH-RH) analogs (10). Also, triblock copolymer PLGA-PEG-PLGA has been studied as a delivery system for continuous release of insulin (11).

The objective of the present study is to develop a new method that does not use organic solvents for the preparation of PLGA nanoparticles. The effect of nanoparticles size, zeta potential and encapsulation efficiency towards BSA as a model protein was compared amongst different methods of preparation.

**Material and Methods**

Poly (lactic-co-glycolic acid) (PLGA), 50:50 DL2A, 10 kDa Mol. Wt., was obtained from Alkermes, Inc. (Cincinnati, Ohio). D-(+)- Glucose, Polyvinyl alcohol (PVA, Mol. Wt. 30-70 kDa) and Bovine serum albumin (BSA, Mw 65 kDa) were purchased from Sigma-Aldrich (St. louis, MO). D-(+)-Trehalose dihydrate, and Mannitol were purchased from VWR (Westchester, PA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce biotechnology Inc. (Rockford, IL).

**Nanoparticle preparation**

1- **ESE technique**

Briefly, 100 µL of BSA solution (100 mg/mL) in distilled water was added to 10 mL of PLGA solution in organic solvent (methylene chloride or ethyl acetate) of 1, 2, or 4 % w/v. The W/O emulsion was generated by a high-speed homogenizer (Polytron® PT-MR 2100, Switzerland by Kinematica AG) at 22,000 rpm for 1 minute. This primary emulsion was added to ice cooled 40 mL of 1% w/v aqueous PVA and homogenized at 22,000 rpm for 0, 5, 10, or 15 minutes to form the multiple emulsion (W/1/O/w2). The emulsion was stirred overnight at room temperature in a hood with a magnetic stirrer (Tekstir® 20, Tekpro/American Hospital supply corp., Evanston, IL, USA) adjusted to 500 rpm to remove the organic solvent. The nanoparticles were collected and purified by centrifugation (Eppendorf centrifuge 5810R, Germany) and washing twice with distilled water at 17,300 g for 20 minutes. The purified suspension of nanoparticles was then freeze dried (FTS systems, Corrosion resistant freeze dryer, Stone Ridge, New York, USA).
2- Nanoprecipitation technique

The PLGA polymer was dissolved in acetone at a concentration of 4% w/v. To this, about 100 µL of BSA solution (100 mg/mL in distilled water) was dispersed by homogenization for 1 minute at 22,000 rpm. BSA suspension in acetone was then poured into the ice cooled dispersing phase (non-solvent, water containing 1% w/v PVA) while homogenization at 22,000 rpm for 15 minutes. The formed nanoparticles were then stirred using magnetic stirrer at 500 rpm over-night in a hood to remove acetone by evaporation. The purified suspension of nanoparticles was then freeze dried.

3- MM technique

The PLGA polymer was dissolved into 10 mL of methox-PEG 350 at 1%, 2%, and 4% w/v concentrations. Tween 20 at 4% v/v of the final volume (50 mL) was added to stabilize this solution. BSA (100 µL of 100 mg/mL solution in water) was added while stirring at 500 rpm for 1 minute, and then about 40 mL of PVA solution (1% w/v) was slowly added while stirring at 500 rpm. All the procedure was carried out at ambient temperature and nanoparticles were then freeze dried.

Entrapment efficiency

The amount of BSA loaded into nanoparticles was calculated as the difference between the amount of BSA drug added and the amount of BSA remained in the supernatants after separation of the nanoparticles (12). A standard calibration curve was established using serial BSA concentrations from 10 to 300 µg/mL and the color produced was estimated using UV-Visible spectrophotometer (UV-1601 – Shimadzu) at wave length of 562 nm. The entrapment efficiency (EE %) was defined as the percentage of BSA loaded compared to the initial amount of the drug. On the other hand, the drug loading was calculated as the ratio between the amount of BSA entrapped in nanoparticles (expressed in µg) and the nanoparticle polymer weight (expressed in mg).

Particle size determination:

Particle size was determined by photon correlation spectroscopy (PCS) using a Zetasizer 3000HS (Malvern Instruments Ltd., UK). Each nanoparticle batch was appropriately diluted with double distilled water just after preparation and after freeze drying. All the measurements were conducted in triplicate.

Zeta potential determination:

Zeta potential of each nanoparticle formulation was determined by Zetasizer 3000HS (Malvern Instruments Ltd., UK). Zeta potential was measured by diluting formulations at 1:16 ratio with phosphate buffer of different pH values (6.8, 7, and 7.4). All measurements were conducted in triplicate.

Nanoparticle morphology:

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

Results

The PLGA 50:50 nanoparticles were prepared by three different methods as described above. Both the ESE and nanoprecipitation methods are time consuming (took more than 6 hours for preparation and additional time for purification), and depend on organic solvents which are environmentally toxic and require especial treatments. On the other hand, the newly designed MM method is simple, easy to prepare, include the use of water as solvent.
Morphological analysis of nanoparticles by SEM showed a homogenous distribution of spherical particles with particle size of about 200-450 nm. SEM photographs (Figure 1) clearly showed the difference in particle size according to the initial solvent used (each solvent corresponds a different method of preparation). The method of preparation of PLGA nanoparticles of BSA had significantly influenced the particle size and BSA entrapment of nanoparticles. The particle size decreased from: ESE method > MM method > nanoprecipitation method, as shown in Table 1. All the three methods of nanoparticle preparation showed high EE% (more than 90%) for BSA as a model protein (Table 1). Of the three, nanoprecipitation and ESE methods yielded higher EE% values when compared to MM method.

Table 1: Particle size, yield, EE% and zeta potential of PLGA nanoparticles prepared using different methods (PLGA concentration was 4% of the organic phase). Results represented as the mean ± SD. (n=3).

<table>
<thead>
<tr>
<th>Method of preparation</th>
<th>Particle size before freeze drying (nm)</th>
<th>Yield</th>
<th>EE%</th>
<th>Zeta potential at different pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pH 6.8</td>
</tr>
<tr>
<td>ESE (DCM)</td>
<td>452.87 ± 38.12</td>
<td>83.1± 2.4</td>
<td>94.59±1.12</td>
<td>19.7±1.9</td>
</tr>
<tr>
<td>ESE (Et-Ac)</td>
<td>391.09±15.41</td>
<td>79.7±2.54</td>
<td>96.56±0.86</td>
<td>13.6±8.7</td>
</tr>
<tr>
<td>MM (Methoxy PEG 350)</td>
<td>336.5±82.1</td>
<td>72.49±3.76</td>
<td>91.03±2.82</td>
<td>20.5±9.6</td>
</tr>
<tr>
<td>nanoprecipitation (Acetone)</td>
<td>302.97 ± 19.00</td>
<td>75.17±2.98</td>
<td>97.37±0.15</td>
<td>13.3±3.2</td>
</tr>
</tbody>
</table>

Figure 1: Scanning electron micrographs of PLGA nanoparticles prepared by ESE method using DCM (a) and Et-Ac (b), nanoprecipitation method using acetone as a solvent (c), and mixed micelle method using methoxy Peg 350 as solvent (d).

Figure 2: Effect of time of homogenization or stirring on nanoparticle size and BSA EE%. PLGA concentration was 4% of the organic phase in case of ESE and nanoprecipitation methods and 2% of the organic phase in case of MM method.
Homogenization or magnetic stirring decreased the particles significantly from 2369.2 to 452.87 nm (using ESE method with CH$_2$Cl$_2$ solvent) and from 542.63 to 302.97 nm (nanoprecipitation method) as the time of homogenization increased from 1 to 16 minutes. In contrast, the time of stirring did not affect the particle size produced by MM method. Statistical analysis revealed no significant differences between nanoparticle sizes as the time increased from 1 to 16 minutes (P>0.05). This suggested that nanoparticle formation was rapid at fixed solvent diffusion rate and low mechanical stress of the magnetic stirrer as shown in figure 2a. As the particle size decreased with stirring time, the EE% of BSA decreased from 98.17% to about 94% as shown in figure 2b. However, in case of nanoprecipitation method a decrease in nanoparticle size did not affect the EE% of BSA. Also, in case of MM method there was no change in EE% as the stirring time increased from 1 to 16 minutes.

An increase in the polymer concentration from 1% to 4% ESE (Table 2), resulted in an increase in the particle size from 310 to 452.87 nm and a marginal increase in EE% from 90.58% to 94.59%. However an increase in polymer concentrations in MM method significantly increased the particle size from 231.03 to 336.5 nm and the EE% from 48.16% to 91.03%, respectively.

Table 2: Effect of polymer concentration on particle size and BSA EE%. Results represented as the mean ± SD. (n=3).

<table>
<thead>
<tr>
<th>PLGA % wt/organic phase volume</th>
<th>ESE method</th>
<th>MM method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particle size (nm)</td>
<td>EE%</td>
</tr>
<tr>
<td>1</td>
<td>310.2±5.1</td>
<td>90.58±1.07</td>
</tr>
<tr>
<td>2</td>
<td>363.13±15.71</td>
<td>92.42±0.31</td>
</tr>
<tr>
<td>4</td>
<td>452.87±38.12</td>
<td>94.59±1.12</td>
</tr>
</tbody>
</table>

Table 3: Effect of BSA concentration on particle size and EE% of BSA. PLGA concentration was 4% and 2% of the solvent for ESE and MM methods, respectively. Results represented as the mean ± SD. (n=3).

<table>
<thead>
<tr>
<th>BSA initial content (mg)</th>
<th>ESE method</th>
<th>MM method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particle size (nm)</td>
<td>EE%</td>
</tr>
<tr>
<td>0</td>
<td>455.2±20.10</td>
<td>-</td>
</tr>
<tr>
<td>0.1</td>
<td>436.8±7.10</td>
<td>63.18±1.01</td>
</tr>
<tr>
<td>1</td>
<td>434.2±5.20</td>
<td>81.88±3.46</td>
</tr>
<tr>
<td>10</td>
<td>452.87±38.12</td>
<td>94.59±1.12</td>
</tr>
</tbody>
</table>
The concentration of BSA was found to have no effect on the particle size in ESE method, but significantly increased the particle size in MM method (Table 3). In MM method, nanoparticle size increased from 218 to 283 nm as the BSA content increased from 0 to 1 mg. A further increase in BSA to 10 mg had no effect on particle size (267.9 nm). As the initial BSA content increased from 0.1 to 10 mg/mL, the EE% increased from 63.18% to 94.59 in ESE method, but decreased EE% from 89.21% to 70.92% in MM method.

The average zeta potential of obtained nanoparticles was affected by the pH of the dispersion medium (Table 1). The PLGA used in this study had one free carboxylic group which expected to be ionized or unionized depending on the pH changes. At acidic pH the carboxylic end group of the PLGA polymer is unionized and so nanoparticles exhibited positive potential, however, at neutral pH and at slightly basic pH (7.4), the zeta potential was negative. The lower zeta potential values of the prepared nanoparticles indicated their potential for aggregation after freeze drying. To prevent aggregation of nanoparticles, three sugars namely; glucose, trehalose, and mannitol were evaluated as cryoprotectants. The particle size of nanoparticles prepared by all the methods increased significantly as shown in Table 4. Addition of a cryoprotectant to the dispersion medium at 5% concentration before freeze drying was found to decrease the particle size of nanoparticles when compared to the freeze dried nanoparticles in the absence of cryoprotectant, except for mannitol. Mannitol resulted in a significant increase in BSA loaded nanoparticles aggregation.

Table 4: Particle size of PLGA nanoparticles prepared using different methods (PLGA concentration was 4% of the organic phase) without and with cryoprotectants after freeze drying. Results represented as the mean ± SD. (n=3).

<table>
<thead>
<tr>
<th>Method of preparation</th>
<th>Particle size after freeze drying (nm)</th>
<th>Particle size after freeze drying with cryoprotectants (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>ESE (DCM)</td>
<td>917.1±94.6</td>
<td>461.40±2.9</td>
</tr>
<tr>
<td>ESE (Et-Ac)</td>
<td>505.2±9.5</td>
<td>428.2±13.0</td>
</tr>
<tr>
<td>MM (Methoxy PEG 350)</td>
<td>6299.80±4143.40</td>
<td>376.5±6.5</td>
</tr>
<tr>
<td>nanoprecipitation (Acetone)</td>
<td>464.97±65.48</td>
<td>321±2.4</td>
</tr>
</tbody>
</table>

Discussion

Effect of method of preparation on nanoparticle size and EE% of BSA

The difference in particle size of PLGA nanoparticles may be caused by various physicochemical properties between polymer and solvents such as solubility difference of polymer in different solvents, viscosity of solvents and polymer, miscibility difference in solvent and water (13). Hydrophilicity of solvents increased in the following order: DCM < Et-Ac < Acetone < Methoxy PEG 350. As a result, DCM the immiscible solvent with water showed relatively larger nanoparticle size compared to other
solvents with partial or complete water miscibility. It has been previously demonstrated that higher the rate of diffusion, the smaller the nanoparticles and the higher the yield of polymer transformation into nanoparticles (14,15). Methoxy PEG 350 is an apparently viscous solvent than acetone, hence its diffusion delayed and the particle size increased. Higher polymer concentration was found to result in the formation of large nanoparticle aggregates which could affect the overall distribution of nanoparticle size (15).

The high BSA incorporation efficiency may be attributed to the rapid polymer precipitation on protein molecules in case of nanoprecipitation and MM methods (Table 1). On the other hand, formation of stable duel emulsions using DCM and Et-Ac solvents could reduce BSA partitioning into the aqueous phase and hence increase entrapment into nanoparticles during solvent evaporation and polymer deposition. The lower BSA entrapment efficiency using MM method compared to other methods could be attributed to the high viscosity of the solvent (methoxy PEG 350), resulting in slower polymer precipitation and increased BSA contact with the water phase enhancing protein leak out (16). In addition, it was observed that the amount of drug loading depended greatly on the rate of PLGA precipitation onto the external surface of the particles (17).

**Effect of time of stirring**

The time of stirring or homogenization was very critical in preparing nanoparticles. The decrease in particle size upon increasing time of homogenization could be due to the increased mechanical stress and homogenization pressure upon particles (18). The change in EE% using ESE method is due to the decrease in nanoparticle size with an increase in the mechanical stress. However, in case of nanoprecipitation method no significant change in particle size was observed as the polymer precipitation rapidly forming a film around BSA molecules. Also, in case of MM method, no change in EE% might be attributed to both the rapid nanoparticle formation and the fixed nanoparticle size. This suggested the advantage of MM method over other methods of preparing nanoparticles. MM method could produce nanoparticles of good morphological appearance (Figure 1d) and good drug loading at ultra short time of stirring using aqueous non-hazardous solvent, water. However, the nanoparticles prepared by this method require the use of a cryoprotectant to prevent aggregation during freeze drying.

**Effect of polymer concentration**

In order to maximize the BSA loading using the minimum amount of polymer, changing the polymer concentration at fixed amount of the drug was evaluated. An increase in the particle size with polymer concentration is probably due to the increased viscosity of the polymeric solution at higher polymer concentrations. Increasing the polymer solution viscosity could hamper both the mechanical stress of a homogenizer or the appropriate diffusion of the solvent toward the non-solvent (19,20). During the preparation of nanoparticles by MM method, at low PLGA concentration, an excess BSA resided outside the micelles dissolving greater BSA amounts in the aqueous phase and resulting in lower EE%.

**Influence of BSA initial content**

The EE% and the content of BSA into nanoparticles prepared by the ESE method increased as the initial BSA content increased. Stable w/o emulsion formed in the initial phase due to increased viscosity of the inner BSA aqueous phase may have resulted in the increased EE% (21). In case of MM method, the decrease in EE% as the initial BSA content increased could be explained based on the higher viscosity of methoxy PEG 350 exposing the BSA to water,
enabling it to leak out. Moreover, as the BSA initial content decreased, improved stabilization of BSA molecules by mixed micelles improved the EE%. Similar results were observed by Niwa et al. (22) and Govender et al. (23), while encapsulating water soluble drugs (nafarelin acetate and procaine hydrochloride) into PLGA nanoparticles by a spontaneous emulsification solvent diffusion and nanoprecipitation methods. These researchers attributed the decrease in drug EE% with increasing initial drug contents to an enhanced drug leakage into the aqueous phase at high loadings. The insignificant nanoparticle size increase using ESE method could be due to the complete solubility of BSA in water which dispersed as fine nanodroplets in the organic phase before re-emulsification in the second aqueous phase and solvent evaporation (Table 3). Constant homogenization rate and time could produce the same nanodroplet sizes irrespective of BSA amount in the aqueous phase. On the other hand, when dispersing BSA solution into acetone or methoxy PEG 350, BSA was precipitated as fine particles which dispersed either by homogenization or magnetic stirring for one minute. The increase in solid drug content of the nanoparticles with increased initial amounts of the drug in the formulation may have resulted in the increased particle size displayed (23).

**Zeta potential and the use of cryoprotectants**

The zeta potential value is an important particle characteristic, as it can influence the electrostatic repulsion between particles with the same electrical charge and prevent the aggregation of the spheres (24). It has been reported that particles could be dispersed stably when absolute value of zeta potential is above 30mV (25). Moreover, freeze-drying may generate many stresses that could destabilize colloidal suspension of nanoparticles, especially, the stress of freezing and dehydration (5,26). So, the aggregation of nanoparticles prepared using different methods seemed to result in low zeta potential values. Similar aggregations of nanoparticles were reported in a study using ciprofloxacin HCL loaded PLGA nanoparticles (27). However, in that study, use of mannitol as a cryoprotectant did not result in increased particle size. This may lead to the assumption that the effect of cryoprotectant on particle size aggregation during freeze drying is dependant on the drug entrapped within the nanoparticles. There might be drug- sugar interactions which might cause nanoparticle aggregation. Possible mechanism of nanoparticle aggregation in presence of cryoprotectants is the crystallisation of the sugar in presence of the protein, which may lead to phase separation (28). It has been observed that the crystallization of mannitol was enhanced by BSA during freezing while it was decreased with other sugars, explaining a significant increase in BSA nanoparticle size in presence of mannitol (29).

**Conclusion**

In this investigation, PLGA nanoparticles loaded with BSA at a high EE% were successfully prepared by three different methods. A new MM method utilizing aqueous solvent was found to be fast and safe procedure for the preparation of PLGA nanoparticles. The EE% and the particle size of nanoparticles were affected by the method of preparation, time of stirring, drug and polymer concentration. Both glucose and trehalose were successfully protected nanoparticles from aggregation during freeze drying. In comparison to the traditional methods for nanoparticle preparation, the new MM method is a promising technique for encapsulating protein/peptide drugs as PLGA nanoparticles using aqueous solvents.

**References**


