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Preparation of poly ε: caprolactone nanoparticles containing bovine serum albumin by emulsification technique

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Abstract
Bovine serum albumin poly ε-caprolactone nanoparticles were prepared by the double emulsion/solvent evaporation method. The effect of some preparative variables like the molecular weight of the polyester and theortical loading of the protein on the size, zeta poetntial and loading efficiency were investigated. Size wss characterized by photon correlation spectroscopy technique using Malvern zetasizer. Zeta potential is characterized by laser anemomerty technique using Malvern zetasizer. Protein loading efficiency and release rate were characterized by bicinchoninic acid assay using plate reader spectrophotometer. Morphology and size of the particles were shown by scanning electron microscopy. It was concluded that increasing the molecular weight of the poly ε-caprolactone at each bovine serum loading could increase loading efficiency which in turn augmented the nanoparticle size and release rate.

Keywords: Poly ε-caprolactone, bovine serum albumin, double emulsion, solvent evaporation

1. Introduction
Oral delivery route is the route of choice for drug administration because it avoids pain, contaminations and infections associated with injections. Biodegradable polymeric nanoparticles are of great importance from the pharmaceutical point of view for the oral protein delivery because they are highly stable in the gastrointestinal tract (GIT) and able to protect the loaded proteins from destruction in GIT (des Rieux *et al*. 2006) [6]. The use of different polymers allows the manipulation of the nanoparticles' physicochemical properties like stability and loading efficiency.

2. Materials and Methods
2.1. Material
All the chemicals used were of analytical grade and used as received from source without any further purification. poly ε-caprolactone (PCL) [10000, 45000, 80000], polyvinyl alcohol (PVA), bovine serum albumin (BSA), dichloromethane (DCM), sodium hydroxide (NaOH), potassium chloride (KC)L, hydrochloric acid (HCL) were purchased from (Aldrich, UK). Cupper sulphate (CuSo4) and bicinchoninic acid (BCA) were purchased from (Thermo-scientific, UK). Phosphate buffer saline (PBS) was purchased from (Oxoid, UK).

2.2. Methodology
2.2.1. Preparation of the nanoparticles by double emulsion/solvent evaporation (DE/SE) technique
PCL nanoparticles were prepared by a w/o/w solvent evaporation technique which comprised the drop wise addition of 1ml of 2.5% m/v aqueous solution of polyvinyl alcohol (PVA) containing theoretical bovine serum albumin (BSA) loading of 2, 5, or 10% m/m based on PCL, to 4ml of dichloromethane (DCM) containing 200 mg of the polymer and according to the experiment, based on the polymer, then homogenisation was done using the hand-held homogenizer (VWR VDI 25 Homogenizer, VWR international, UK) at a speed of 10000 revolutions per minute (rpm) for 2 minutes. The resulting w/o emulsion was subsequently added drop wise to 50ml of 1.25% m/v aqueous PVA solution followed by homogenising at a speed of 10000 rpm using Silverson L4R homogeniser (Silverson, UK) for 6 minutes. The homogenisation process was performed over ice and the resulting w/o/w emulsion was stirred continuously and allowed to evaporate overnight. The particles were then collected by centrifugation (sigma centrifugation apparatus, Sigma, UK) at 4 ^{0}C at 10,000 rpm for 30

minutes and washed with water a further 3 times using the same centrifugation parameters.
The sediment obtained was resuspended in water and freeze dried at -85 0c & 0.012 mbar (Labconco freeze dryer, Freezone 4.5 Plus, Labconco, UK) for 48h (Dash, Konkimalla 2012) [5].
Freeze drying of polymeric particles included the addition of diluted solutions of washed polymeric particles to 20 mL clear freeze-drying glass containers, covered with Parafilm M film perforated with 20 needle width holes, and frozen at -16oC for 12 hours. After that, the samples were lyophilised for 48 hours using the freeze dryer, under vacuum drawn by a high vacuum pump. Dried polymeric particles loaded with protein were stored at room temperature in desiccator (Dash, Konkimalla 2012) [5].

2.2.2 Determination of nanoparticle size by photon correlation spectroscopy (PCS)
The size of nanoparticles was determined by PCS technique using the Malvern Zetasizer nanoSeries (Malvern Instruments, UK) which is a useful for determination of the particle sizes of submicron particles in the range of 3 to 1000 nm. The theory of PCS technique is based on the use of dynamic light scattering for determination of the shape and size of particulate systems. The electric field of the incident light induces an oscillating polarisation on the particles in the sample, and then these particles whose polarity differs from the surroundings scatter the incident light. The particles in the sample are in constant motion which is called Brownian motion and this motion makes fluctuations in the detected intensity signal that can be measured digitally by PCS. The duration of the fluctuations provides information about the particles, including size and polydispersity (Akbari, Tavandashti & Zandrahimi 2011) [1].
Nanoparticles suspension was prepared by dispersing 5 mg of the nanoparticles in 1 to 2 ml distilled ddH2O, then 0.1 ml nanoparticles suspension were dispersed in 2 to 3 ml of 0.22 µm filtered ddH2O, and then measured by Malvern instruments with regard to polydispersity and Z-average diameter (Akbari, Tavandashti & Zandrahimi 2011) [1].

2.2.3 Measurement of zeta-potential of nanoparticles
Determination of zeta-potential was measured by laser anemometry in millivolts (mV) by using a Malvern zetasizer nanoseries. Zeta-potential is the electrical potential existing at the stern plane of a particle, which is an imaginary plane separating the thin layer of liquid bound to the particle surface from the rest of liquid and showing elastic and viscous behaviours. Colloidal particles are electrically charged due to their ionic characteristics and consequently, the distribution of ions in the neighbouring interfacial region will be affected by the resultant particle surface charge, and the counter ions (fixed layer) concentration will increase. A cloud-like area containing ions of opposite charges is formed outside the fixed layer. The net result is the formation of an electrical double layer in the region of the particle/liquid interface, with an inner region formed of ions strongly bound to the surface, and an outer diffuse ionic region. The potential in this region declines with the distance from the surface until at a certain distance it reaches zero (Ravi Kumar, Bakowsky & Lehr 2004, Konan *et al*. 2003) [13, 10]. When a voltage is applied to the solution in which particles are suspended, particles are attracted to the electrode of the opposite polarity, associated by the fixed layer and part of the diffuse double layer.
Nanoparticles suspension was prepared by dispersing 5 mg of the nanoparticles in 1 to 2 ml distilled H20, and then 0.5 ml of the nanoparticle suspension was diluted in 0.001 M KCL solution, which acts as a weak electrolyte, to get a sample of appropriate concentration for the measurement.

2.2.4 Determination of bovine serum albumin amounts in nanoparticles by bicinchoninic acid (BCA) protein assay method
The loading efficiency of protein was calculated by dividing the actual loading over the theoretical loading of the protein used in the preparation of the nanoparticles, as follows:
% Protein loading efficiency = (actual loading/theoretical loading) x 100
The amount of actual protein loaded per unit weight of particles was determined using the BCA assay (Bainor *et al*. 2011) [2]. The BCA protein assay includes the reduction of cupric (Cu2+) to cuprous (Cu1+) by protein in an alkaline medium followed by a highly sensitive and selective colorimetric detection of the cuprous cation (Cu1+) by bicinchoninic acid.
The first step is the protein chelation for the copper in an alkaline medium to form a blue coloured complex (biuret reaction). The second step is the colour development reaction, in which BCA; a selective and sensitive colorimetric detection reagent reacts with the cuprous cation to form a purple coloured product by the chelation of one cuprous cation with two molecules of BCA. This purple coloured product is water soluble and can be measured spectrophotometric ally at 562 nm. The Cu2+ reduction leading to BCA colour formation is highly affected by the presence of any of the four amino acid residues (tyrosine, tryptophan, cysteine and cystin) in the protein amino acid sequence. The peptide bond only is responsible for colour development at high temperatures and thus, the reaction is done at 60ºC to increase the sensitivity. This technique has the advantages of compatibility with non-ionic and ionic detergents, minimum protein-to-protein variation and limited interactions with most copper chelators and reducing agents.
A 5mg nanoparticles sample was suspended in 1 ml of 1 M NaOH at 37°C overnight for digesting the polymer. The suspension was then centrifuged and 25 µL of the supernatant was added to 3 wells. A series of calibrated BSA protein standards were prepared in distilled water and 25 µL of each standard were added to 3 wells. 200 µL of BCA reagent were added to each well. Precision and accuracy of protein absorption were determined by using of a minimum of three absorption determinations for each standard, blank and test samples. After the purple colour development, the absorbance of the contents of each well was measured at 562 nm using a plate reader.

2.2.5 Determination of protein release from polymeric nanoparticles
In vitro protein release from particles was performed to determine its release rate by incubation of 5mg of particles in 1 ml of phosphate buffered saline (PBS) having pH 7.4, containing 0.01 % sodium azide as a bacteriostatic agent and 5 mM SDS in 1.5 ml eppendorf tubes. The particles were incubated at 37°C, shaken, and then samples were withdrawn at appropriate time intervals, centrifuged and the amount of BSA in the samples was analysed using a BCA assay.

2.2.6. Scanning electron microscopy (SEM) for the determination of nanoparticles morphology

SEM microscopy is used to characterize the morphology and size of the nano/microparticles. A thin layer of nano/microparticles was spread on a circular aluminium plate using a carbon disc and the surface was then coated with a gold film using a sputter coater under an Argon atmosphere. Nano/microparticles were identified by magnification with a scanning electron microscope (Cambridge Instruments Stereoscan 90B, 25 kV, Cambridge, UK) (Todokoro, Otaka 1995) [16].

3. Results and Discussion

All results were subjected to statistical analysis using Microsoft excel 2010 software. All means were calculated as a mean ± standard error bar of the mean. All experiments were carried out in triplicate unless otherwise stated. Unpaired T tests was carried out on the data to check the significant difference when ($P< 0.05$) when compared to each other. PCL(s) Nanoparticles: The ratios of PCL(s) amounts and theoretical BSA loadings used for preparation of the various PCL(s) molecular weight (MWt) nanoparticles are shown in table 1.

Table 1: The ratios of PCL(s) amounts and BSA loading.

Polymer MWt/amount	Theoretical BSA loading
SPCL (MWt 10000) / (200 mg)	2 %
SPCL (MWt 10000) / (200 mg)	5 %
SPCL (MWt 10000) / (200 mg)	10%
MPCL (MWt 45000) / (200 mg)	2 %
MPCL (MWt 45000) / (200 mg)	5 %
MPCL (MWt 45000) / (200 mg)	10 %
LPCL (MWt 80000) / (200 mg)	2 %
LPCL (MWt 80000) / (200 mg)	5 %
LPCL (MWt 80000) / (200 mg)	10 %

On comparing the three PCL(s) according to their MWt in (table.2), LPCL showed the highest the % actual loadings (0.53, 1.79, 1.2) at all the theoretical loading levels (2, 5, 10 %) followed by the MPCL (0.51, 1.4, 0.47), and the SPCL (0.47, 0.71, 0.36) and consequently would give the same order for the % loading efficiency.

Table 2: Loading efficiency of the PCL NP(s).

Loaded polymer	Theoretical Loading, % m/m	Actual Loading, % m/m ± s.d.	Loading Efficiency, % ± s.d.
SPCl	2	0.47 ± 0.12	23.5 ± 5.77
SPCl	5	0.71 ± 0.26	14.14 ± 5.1
SPCl	10	0.36 ± 0.27	3.64 ± 2.7
MPCL	2	0.51 ± 0.10	25.5 ± 5.07
MPCL	5	1.4 ± 0.71	28 ± 14.27
MPCL	10	0.47 ± 0.14	4.70 ± 1.39
LPCL	2	0.53 ± 0.13	26.67 ± 6.25
LPCL	5	1.79 ± 0.65	35.73 ± 13.08
LPCL	10	1.2 ± 0.52	12 ± 5.21

It is clear that increasing the PCL MWt can increase the loading efficiency for each theoretical loading. The % loading efficiency has the highest value at 5 % theoretical loading and decreases when increasing theoretical loading above 5 %. BSA is characterized by having tensoactive properties and high affinity to the thermoplastic aliphatic polyesters which are considered the major determinants of the protein loading (Woodruff, Hutmacher 2010, Blanco, Alonso 1998) [19, 3]. These aliphatic polyesters have good mechanical strengths to carry a variety of drug classes such as micromolecules, vaccines, peptides and proteins (Woodruff, Hutmacher 2010) [19]. The loading efficiency is affected by several factors like the nature of the polymer, the MWt of the polymer, the surfactant type, polymer/surfactant ratio and BSA concentration. The amount and type of surfactant are responsible for the interaction between the surfactant and the protein, which protect BSA from the solvent and inhibit its dissolution in the external aqueous phase (Coccoli *et al*. 2008) [4]. The polymer concentration in the organic phase controls the emulsion viscosity which can increase BSA loading efficiency and prevent BSA diffusion towards the external aqueous (Coccoli *et al*. 2008, Youan *et al*. 1999) [4, 21]. BSA has limits of loading for the constant amounts of polymer and surfactant, above which any increase in the BSA content will decrease the BSA interaction with the polymer and the surfactant which in turn will decrease the BSA loading efficiency (Feczkó *et al*. 2011) [8].

For the current study of the PCL(s) nanoparticles, the amount of the PCL polymer is constant, PVA is used in fixed amount as a surfactant and the BSA theoretical loading is constant, so that the effect of the PCL MWt can be easily identified. It is clear that for each BSA theoretical loading, LPCL had the highest loading efficiency, SPCL has the lowest protein loading efficiency, and the MPCL has an intermediate value. This proves the validity of the concept which reveals that increasing the MWt of the polyester can increase the mechanical strength of the polyester that enhances the loading efficiency (Blanco, Alonso 1998, Wu *et al*. 2012) [3, 20]. Additionally increasing the MWt of the PCL leads to an increase in the viscosity of organic phase which reduces the BSA diffusion to the external aqueous phase before the nanoparticles hardening. Nevertheless, the results show that accepted limits of the BSA theoretical loading to enhance protein loading efficiency are from 2 to 5 %, and any increment of the BSA content above these limits sharply reduces the Loading efficiency because the BSA interactions with the available amounts of PCL and PVA decrease.

By identifying the sizes of the nanoparticles according the MWt of the PCl(s) (table 3), it is obeserved that LPCL shows the largest sizes at all the theoretical loading levels (373.63, 408, 393.43) but the sizes are reduced for the MPCL (333.4, 366.97, 341.67) and show the lowest values for the SPCL (294.67, 335.5, 307.33). It is also clear that the size of the nanopaticles increases by increasing the theoretical loading from 2 to 5 %, then declines after further rise of the theortical loading to 10 %. However, the PDI values are not dependent on the nanoparticle size or the PCL MWt. PDI starts at low values for 2 % loading of the MPCL (0.37) and LPCL (0.39), then increases by increasing the loading while it starts at high value for 2 % loading of the SPCL (0.54) then decreases at the 5 % loading (0.37) and increases again at the 10 % loading (0.39).

The size of many polyester nanoparticles is affected by several factors like amount of the loaded drug, polymer/surfactant ratio, MWt of the polymer, and method of nanoparticles' preparation (Youan *et al*. 1999, Feczkó *et al*. 2011, Wu *et al*. 2012) [21, 8, 20]. Since all the parameters are fixed except the MWt of the polymer, it is clear that increasing the molecular weight of PCL increases the nanoparticles sizes at all the BSA loading levels. Increment of the protein loading efficiency by increasing the PCL MWt is the major determinant for increasing the nanoparticles size. The PDI is mainly depending on the method of nanoparticles preparation and type of surfactant used e.g. sonication gives cyclosporine PLGA nanoparticles with better PDI than homogenization (Jain *et al*. 2010) [9] and PCL-Tween 80 copolymer gives nanoparticles with narrower PDI than PCL alone (Ma *et al*. 2011) [12]. This explains why the PDI of the PCL nanoparticles is slightly affected by the PCL MWt showing low values for 2% BSA loaded LPCL and 2% BSA loaded MPCL, one high value for the 10% BSA loaded SPCL and intermediate close values for the remaining of the PCL(s) nanoparticles.

Table 3: Size and PDI of PCl NP(s).

Loaded polymer	z- average, nm ± s.d.	Polydispersity Index ± s.d.
SPCl	294.67 ± 27.15	0.54 ± 0.06
SPCl	335.50 ± 38.43	0.52 ± 0.13
SPCl	307.33 ± 21.50	0.62 ± 0.04
MPCL	333.4 ± 13.16	0.37 ± 0.02
MPCL	366.97 ± 15.20	0.57 ±0.07
MPCL	341.67 ± 20.18	0.57 ± 0.1
LPCL	373.63 ± 15.36	0.39 ± 0.07
LPCL	408 ± 11.79	0.51 ± 0.09

The following SEM images prove that increasing the MWt of PCL increases the the size of the PCL nanoparticles. The 5% BSA loaded SPCL nanoparticles are much smaller than the 5% BSA loaded LPCL nanoparticles (fig.1 & 2).

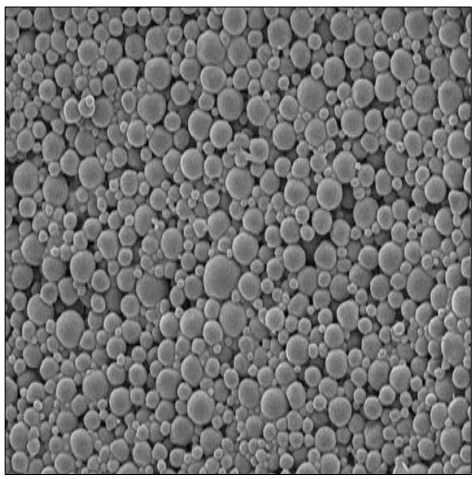

Fig 1: SEM image of 5% BSA loaded SPCL

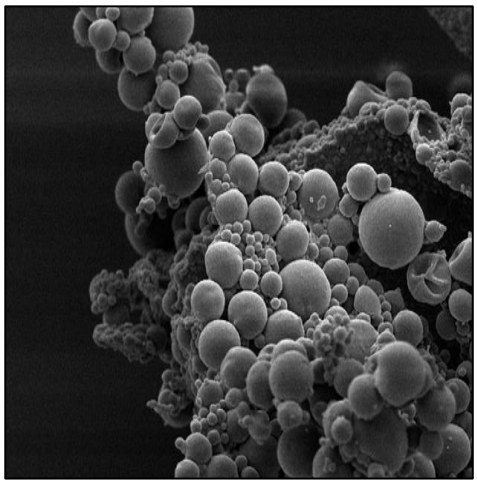

Fig 2: SEM image of 5% BSA loaded LPCL

It is obvious that LPCL has the highest zeta potential values at 2, 5 and 10 % loadings (- 9.19, -10.02, -6.06) followed by the MPCL (-7.04, -7.57, -5.98) and SPCL (-6.01, -6.12, -5.62) (table. 4). Additionally, zeta potential values increase on increasing the loading from 2 to 5 %, then decreases on increasing the loading to 10 %. It has been reported that the zeta potential of the polyesters nanoparticles e.g. PCL, PLGA without any surfactant like PVA is high due to the presence of uncapped end carboxylic groups of the polyester at the particle nanoparticle surface which provide high negative charge (Krishnamachari 2011, Sahoo *et al*. 2002) [11, 15]. On adding PVA to the polyester, it forms a layer at the surface of the polyester nanoparticles that shields the negative surface charge of the polyesters in an amount dependent manner (Sahoo *et al*. 2002) [11]. All the PCL(s) nanoparticles are prepared using the PVA as a surfactant in constant amount, so it is strongly concluded that increasing the molecular weight of PCL can reduce the shielding effect of the PVA for the surface negative charges of the PCL end carboxylic groups allowing the LPCL to achieve the highest negative charge of zeta potential. The high zeta potential either by negative or positive is beneficial to the stabilization of particles suspension and prevention of the particles aggregation as the charged particles can repel one another (Vandervoort, Ludwig 2002, Dubey *et al*. 2012) [18, 7], therefore LPCL nanoparticles suspension achieves the highest stability.

Table 4: Zeta poetical of PCL NP(s).

Loaded polymer	Zeta- potential mV ± s.d.
SPCl	-6.01 ± 0.19
SPCl	-6.12 ± 0.62
SPCl	-5.62 ± 0.09
MPCL	-7.04 ± 0.96
MPCL	-7.57 ± 2.88
MPCL	-5.98 ± 1.57
LPCL	-9.19 ± 3.13
LPCL	-10.02 ± 3.80
LPCL	-6.06 ±1.77

According to the protein release rates (figures 3, 4 & 5), it is

obvious that they are obeying the order of the % loading efficiency such that the LPCL having the highest loading efficiency shows the highest release followed by the MPCL and SPCL. Since the BSA loading efficiency is higher at the 5% loading followed by the 2 % and 10%, the BSA release rate is higher at the 5% loading followed by the 2 % and 10%. PCL is characterized by acting as a reservoir in which the drug molecules can be loaded through physical, chemical or electrostatic interactions according to their physicochemical properties. Additionally, PCL has a good permeability for the release of different drugs and the drugs release rates from PCL are affected by several factors like technique of preparation, PCL type, PCL content, and loading efficiency of the drug loaded within the the micro/nanoparticles (Woodruff, Hutmacher 2010) [19]. On using the same amount of PCL to prepare PCL nanoparticles by DE/SE under the same conditions but increasing the MWt of the PCL, the protein loading efficiency at all the theoretical loadings increases which in turn increases the protein release rates in the same order showing the highest protein release rates for the LPCL followed by the MPCL and SPCL.

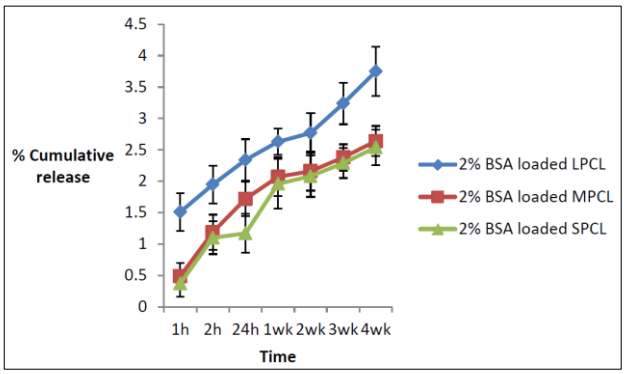

Fig 3: Release of BSA from 2% BSA loaded PCL(s) nanoparticles.

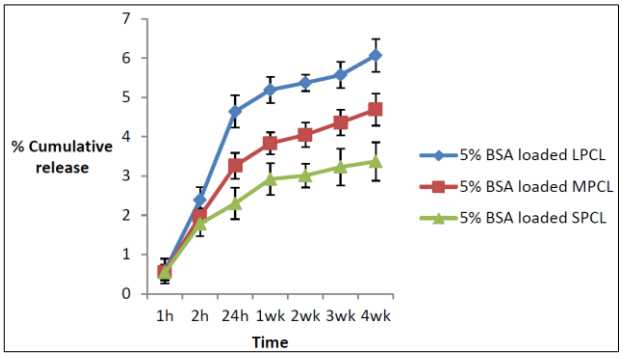

Fig 4: Release of BSA from 5% BSA loaded PCL(s) nanoparticles.

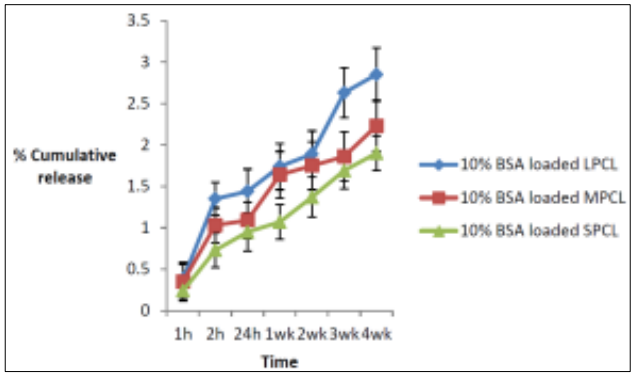

Fig 5: Release of BSA from 10% BSA loaded PCL(s) nanoparticles.

4. Conclusions

We successfully demonstrated the formulation and characterization of various PCL nanoparticles. LPCL nanoparticles have higher protein loading efficiencies, higher protein release rates, larger sizes and higher zeta potentials than MPCL and SPCL nanoparticles. In conclusion, it is clear that 5% BSA loaded LPCL nanoparticles have the advantages of the highest loading efficiency, highest release, and highest zeta potential.

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