

# THE ELECTROSPRAYED INSULIN-LOADED POLYCAPROLACTONE MICROPARTICLES AS A DRUG CARRIER

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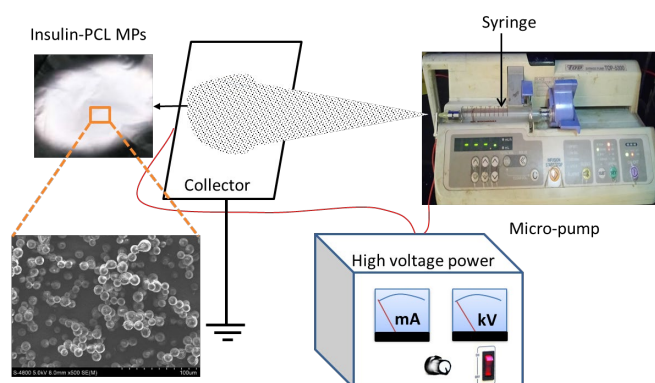
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## Graphical abstract



## Abstract

Drug and protein encapsulated polymeric microparticles have been considered as the most effective delivery in pharmaceutical biotechnology. In this work, we report the fabrication of polycaprolactone microparticles (PCL MPs) containing insulin via an electrospaying method. The morphology, chemical composition, physicochemical properties, insulin encapsulation and release efficiency, degradation of PCL MPs, and cytotoxicity are systematically characterized and analyzed. The results indicate that insulin do not incorporate with PCL matrix leading to the deformation of PCL MPs' structure. In addition, insulin can be loaded into the PCL MPs with high concentration up to 25 % while it remains chemical properties when releasing from the PCL MPs. Moreover, insulin demonstrates high burst release within the first day, subsequently the release become more stable during 2-7 days. The 80% viability of the cell suggests that PCL MPs are biocompatible to cells. As a consequence, the above mentioned material is proved to be has high potential for insulin carrier in controlled release application.

**Keywords:** Drug delivery, electrospaying, insulin, microparticles, polycaprolactone.

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## 1.0 INTRODUCTION

Electrospaying has been a simple method to produce the drug/protein-loaded microparticles (MPs) for the application in the pharmaceutical biotechnology with a lot of advantages such as easy preparation, high possibility in obtaining the mono-sized particles, high potential for scalability, cost-effectiveness, and the ability to maintains the integrity and biological activity of protein and drug due to its encapsulation of drug/protein [1-3]. Besides, electrospaying is a simple method to fabricate the drug/protein-loaded microparticles with high encapsulation efficiency and loading capacity [2, 3]. In the electrospaying method, the high voltage is applied between the steel needle and the collectors which connected to the grounded electrode during electrospaying. As consequence, the electric field force was formed and accumulated the positive charged solution issued from the needle ejected towards the collector. The solid

polymeric MPs can be formed on the aluminum foil due to the Coulomb fission and the evaporation of solvent when the droplets are sprayed to the collector. Therefore, this method is feasible to create the drug/protein-loaded particles with the reproducible release profile of protein or drug [4]. The release mechanism of drug/protein from MPs usually contains two main removal processes: i) the initial burst is released from the particle surface; ii) the second stage was the more constant release rate by the erosion polymer matrix and the diffusion of drug or protein. The erosion of the polymer matrix in the biological environment depends on the degradation of the polymer chains [5, 6]. Therefore, various kinds of polymers ranging from natural polymers (e.g., chitosan, gelatin, elastin and pullulan) to synthetic polymers (e.g., polycaprolactone (PCL), poly(vinyl alcohol) (PVA), poly (lactic acid) (PLA), and poly (lactic-co-glycolic acid) (PLGA)) have been exploited for drug carrier applications using the electrospaying method [3, 7-9]. In

particular, PCL which is a biodegradable polymer and able to be degraded by random scission of ester bond, has been applied to long-term degradation and drug release [10, 11].

The morphology and the size of electrospayed particles ranging from nanometers to micrometers were effectively adjusted by varying the process parameters including conductivity of polymer solution, polymer concentration, solvent evaporation rate, applied voltage, flow rate and collecting distance [12, 13]. When the morphology and size of MPs are tuned, the degradation of polymer matrix as well as their erosion can be controlled, thereby the release of drugs can be designed [4]. The drug/protein delivery systems have been developed to enhance the biological activity and their effectiveness. Several methods such as a double emulsion or emulsification-solvent evaporation, spray drying, precipitation, and electrospaying have been significantly exploited to fabricate the drug/protein-loaded particles [6]. Moreover, it gave advantages such as an optimized dose of drug/protein administration, increasing safety and reducing side effects by controlling the release of drug/protein [9, 14, 15]. In previous studies, the influences of polymer concentration and electrospaying parameters on the drug/protein-loaded PCL MPs morphology including Paclitaxel or Taxol [8, 16],  $\beta$ -Oestradiol [7], Bovine serum albumin [17] were studied. In stomach, insulin couldn't have preserved the structure and the biological activity because of the low pH and enzymatic degradation. Therefore, it is necessary to be encapsulated in PCL MPs [14, 18].

Herein, the influences of electrospay processing parameters on the PCL MPs morphology, size and size distribution were systematically investigated. When the entanglements formed enough in the droplet, the electrospayed MPs were formulated. Moreover, the PCL MPs morphology as well as their size were impacted on the degradation rate and decomposition of PCL. The larger PCL MPs area expose to PBS solution, the faster penetration rate of aqueous solution into the PCL MPs, resulting in the degradation of particles [12, 19, 20]. In this study, we focused on the preparation of insulin-loaded PCL microparticles by the electrospay method and evaluate the degradation as well as the release profile of insulin from PCL MPs by gel permeation chromatography (GPC) and high performance liquid chromatography (HPLC). The structural analysis, materials characterization and the cytotoxicity of insulin-loaded PCL MPs were carefully analyzed using scanning electrode microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) analysis and cell viability.

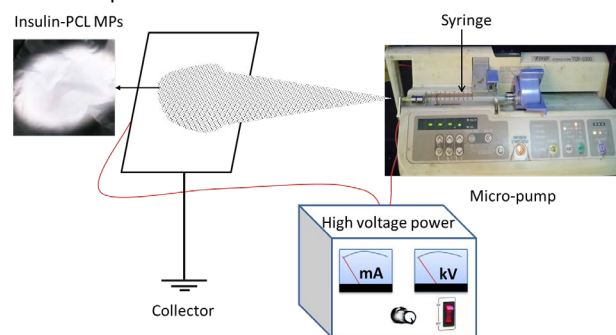
## 2.0 METHODOLOGY

### Materials

Insulin (human recombinant,  $M_w = 5,800$  Da) was obtained from SAFC Biosciences, US. Polycaprolactone (PCL) ( $M_n = 45,000$  Da), trifluoroacetic acid (TFA), (HPLC grade,  $\geq 99.0\%$  purity), tween 20 and Phosphate buffered saline (PBS, tablet form) were supplied by Sigma-Aldrich Pte. Ltd, Singapore. Dichloromethane (DCM) and acetonitrile (ACN) (HPLC grade, 99.9% purity) were purchased from Fisher Scientific, UK. Water used in this study was double-distilled and deionized.

### Fabrication of Insulin-loaded PCL Microparticles (Insulin-PCL MPs) by Electrospaying

Firstly, a transparent PCL solution (9 wt%) in DCM was prepared. After that, insulin was mixed into the solution for 1 hour slightly stirring. The 20In-PCL sample and 25In-PCL sample have an amount of insulin in PCL matrix is 20 wt% and 25 wt%, respectively. The suspension of insulin and PCL solution was transferred to a syringe with the flat needle (stainless, 20G) and then placed on a syringe micro-pump (model Top-5300 from Japan). The controllable micro-pump was used to adjust the flow rate of PCL solution at 0.7 ml/h. And some processing parameters were set up including the working distance of 22.5 cm, the applied voltage of 18 kV at room temperature (Figure 1). Finally, the insulin-PCL MPs were dried at ambient temperature for 2 days to remove residual solvents. The collected insulin-PCL MPs were powder and stored at 0 °C.



**Figure 1** Schematic fabrication of insulin-loaded particles by electrospaying

### Characterization of PCL MPs

**Scanning Electrode Microscopy (SEM):** The surface morphology and size of insulin-loaded PCL microparticles were characterized using scanning electrode microscopy (SEM, S-4800 Hitachi, Japan). A sputter coater was used to coat the insulin-PCL MPs with platinum for 30 seconds before SEM analysis. All samples were fixed with a double-sided conductive tape on a metallic stud.

**Differential Scanning Calorimetry (DSC):** The compatibility of insulin and PCL in the electrospayed microparticles was evaluated by observing the melting peaks of samples. The DSC machine (NETZSCH DSC 204F1 Phoenix, Germany) was used to determine the thermal properties of these samples. In each test, 3 mg PCL MPs were placed in a standard aluminum pan, then heated from 25 °C to 250 °C at the heating rate of 5 °C/min. All experiments were carried out under nitrogen environment.

### Encapsulation Efficiency of Insulin from The Electrospayed PCL Microparticles

Before testing the encapsulation efficiency (EE), the calibration curve of insulin standard in PBS was established to determine accurately insulin amount. Insulin solution stock (IS) was obtained 1000 ppm by mixing 2 mg original insulin in 2 mL PBS (pH 7.4), stored at 5-8 °C. IS was diluted to solutions following 200 ppm, 150 ppm, 125 ppm, 100 ppm, 75 ppm, 50 ppm, 25 ppm, 12.5 ppm, 10 ppm, 5 ppm. Each sample was conducted 3 repeated times.

Insulin solutions were investigated by High performance liquid chromatography system (HPLC, 1200 Agilent, US) with degasser, binary pump, DAD. 60% solvent A (deionized water solution +0.1% v/v TFA) and 40% solvent B (acetonitrile solution +0.1% v/v TFA) were applied as mobile phase at 1.2 ml/ min. The C18 reversed phase column (RP C18, 100 x 3.6 mm, 3  $\mu$ m, pore size 130  $\text{\AA}$ ) was used. The temperature of the column was set at 30°C. Chromatograms were recorded at 206 nm (UV detector). Elution mode: isocratic. Sample injection: 20  $\mu$ l.

The insulin-PCL MPs were dissolved in DCM solvent with 5 mg/mL (w/v) for 3 min to create a suspension. Next, 2 ml PBS were added in this suspension and further stirred gently in 5 min (pH 7.4). The mixture was separated 2 phases: the aqueous solution of insulin and PBS was in the bottom of the mixture; the lipophilic solution of PCL and DCM was on top of the mixture. Then, the mixture was dried at 42 °C for 5-6 min in order to evaporate completely DCM solvent and finally the PCL plastic was formed on top of the mixture. The insulin solution was collected with content 500 ppm (on theory) and repaired for HPLC analysis. These independent samples were conducted triplicate.

### Degradation of PCL MPs In-vitro Test

The electrosprayed PCL MPs were dispersed in PBS 10 mM solution containing 0,2% Tween 20 (pH 7.4) with the MPs concentration of 10 mg/ 6 mL, and then incubated in the water bath at 37 °C. At interval times of 7 to 70 days, PCL MPs samples were collected by a freeze-drying machine. Each sample was conducted triplicate and stored at 0°C before testing its properties. The number average molecule weight ( $M_n$ ) of all samples had been determined using the gel permeation chromatography (PL-GPC 50, Agilent 1100 Series, US) with Resipore column ( $M_n = 200 - 200,000$  g/mol), polystyrene was used as a standard and chloroform as a solvent. Fourier Transform Infrared Spectroscopy (Bruker Tensor 37, Germany) was used to determine the structure of the initial PCL MPs and the degraded PCL MPs.

### Insulin Release Study In-vitro Test

The insulin-loaded microparticles were weighed approximately 10 mg of 25In-PCL MPs and 12.5 mg of 20In-PCL MPs into a vial (4 ml, Germany), each sample had 2.5 mg insulin in PCL MPs. Then, 3 ml PBS (stabilized with 0.02% w/v Tween 20, pH 7.4) was added in these vials. The samples were eroded at 37°C in a water bath (WNB14, Memmert, Germany). At intervals time, 1.5 ml supernatant was withdrawn, and the exactly similar volume of fresh PBS solution was added in these vials. The supernatant was analyzed by HPLC with the same mobile phase and method which was used to calculate the encapsulation efficiency of insulin.

### Microparticles Cytotoxicity

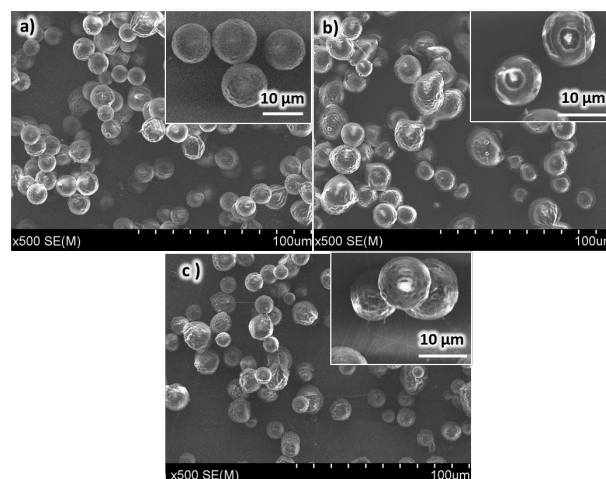
Any cell lines can be used to evaluate the cytotoxicity of PCL MPs, so that the A549 cells were chosen. The A549 cells were incubated with PCL MPs before cell viability was assessed by MTT assay. The 96-well plate was used to seed A549 cells with  $1 \times 10^5$  cells/well density. Cell culture propagation and maintenance were conducted as following: The cell line A549 (ATCC), passage number between 27 and 56, was used in this

work. The cell line was grown and maintained in T75 tissue culture flasks, with 1640 Roswell Park Memorial Institute medium containing 10% (v/v) of Fetal bovine solution (FBS). The cells were fed every day and trypsinized once reached about 80% cell confluence. Next, cells were incubated with different concentrations of the PCL MPs were dispersed in Hank's balance salt solution (HBSS) at concentrations from 50 – 2000  $\mu$ g/mL for 4 hours, 5%  $\text{CO}_2$  at 37 °C. After the incubation time, HBSS was used to wash cells 2 times and 0.5 mg/ml MTT in HBSS was further incubated in the dark for 4 hours. After withdrawing supernatant from the wells, the Dimethyl sulfoxide (DMSO) was used to dissolve the formed formazan crystals. Finally, the samples were analyzed by an Infinite M200Pro plate reader (Tecan, Germany) with the absorbance at 550 nm. The cells treated only with HBSS were applied as a negative control (designed as 100% cell viability), while the cells incubated in HBSS medium with 1% Triton<sup>TM</sup> X-100 were applied as the positive control (0% cell viability). Each sample was conducted three independent experiments. The percentage of cell viability was determined by comparing with negative and positive controls.

## 3.0 RESULTS AND DISCUSSION

### Preparation The Insulin-loaded PCL MPs by Electrospinning

The morphology of insulin-loaded PCL MPs fabricated with different insulin contents is presented in Figure 2. As can be seen, the insulin concentration affects the morphology of PCL MPs since the 25In-PCL MPs and 20In-PCL MPs were generated heterogeneous morphology. Due to suspending in PCL solution, insulin was not effectively incorporated with PCL matrix, therefore insulin-PCL MPs could not form the spherical shapes when insulin concentration was high as 25 wt%. It should be noted that the main purpose of this work is to fabricate insulin-loaded PCL MPs with the highest insulin content. However, the Insulin-PCL MPs could not form in spherical shape when the content of insulin was over 25 wt%. Therefore, the 20In-PCL MPs and 25In-PCL MPs were used to the release in-vitro test.



**Figure 2** SEM images of insulin-loaded PCL microparticles with various insulin contents in PCL: (a) 0 wt% insulin, (b) 20 wt% insulin and (c) 25 wt% insulin

### Encapsulation Efficiency

It should be noted that the calibration of insulin was needed before the encapsulation efficiency of insulin-PCL MPs was investigated. Correspondingly, a wavelength of 206 nm was selected to acquire minimal tailing of a symmetrical peak by UV detection. The accurate determinations of insulin concentration were obtained to determine accurately peak area ratio. The total analysis time was 10 min for insulin standard and extracted insulin, while the retention time of insulin is 3.05 min.

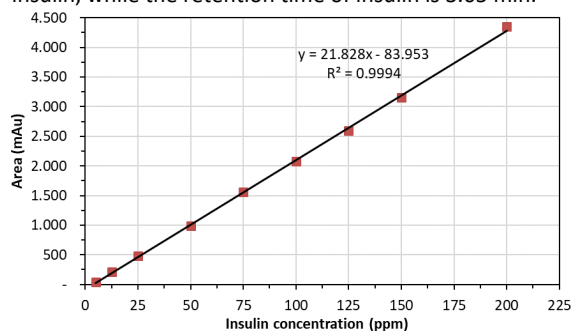


Figure 3 The calibration curve of insulin solution in PBS (n=3)

The calibration curve of insulin solution in PBS is presented in Figure 3. As can be seen, the calibration curve of insulin standard demonstrated a linearity in the range of 5–200 ppm. The correlation coefficient value ( $R^2$ ) is 0.9994 and interception  $y = 21.828x - 83.953$ , where  $x$  is a concentration of insulin.

The encapsulation efficiency (EE) of 20In-PCL MPs was 90% and EE of 25In-PCL MPs was 80%. With higher insulin concentration (25 wt%), the 25In-PCL MPs exhibited lower EE than 20In-PCL MPs. It was assumed that insulin dispersed ineffectively inside the PCL MPs and gathered on the MPs' surfaces, therefore the drug diffused significantly to PBS solution and dissolved into water. With another drug, their amount in PCL matrix below 16 wt%, the drug-loaded PCL MPs has EE = 78% of Taxol, EE = 89% of  $\beta$ -Oestradiol and EE = 30% of BSA [16, 17, 21]. This research indicated that the electrosprayed PCL MPs could encapsulate insulin effectively.

### DSC Analysis

DSC thermograms of pure insulin, PCL MPs, and insulin-loaded PCL MP are depicted in Figure 4. As seen, the melting peak of PCL is at around 60°C while the peak of insulin is in a range of 210–220 °C. When insulin was encapsulated into the MPs, the melting peak of insulin-loaded MPs is the combination of these melting peaks from insulin and MPs, which implies that insulin is in the crystalline phase in the polymer matrix [22]. In other words, insulin was not incorporated in the PCL matrix, resulting in the heterogeneous morphology of insulin-PCL MPs as shown in Figure 2.

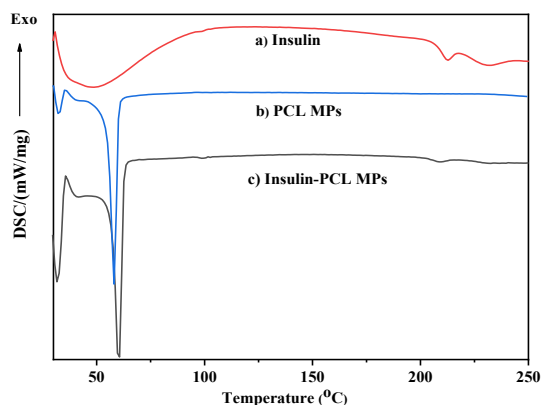


Figure 4 DSC thermogram of (a) insulin (b) PCL MPs and (c) In-PCL MPs

### Degradation In-vitro Test

The electrosprayed PCL MPs were degraded when they contacted with PBS solution in the in-vitro test. The ester bonding of PCL chain can be hydrolyzed in aqueous solution, consequently, the  $M_n$  of polymer was reduced [10, 11].

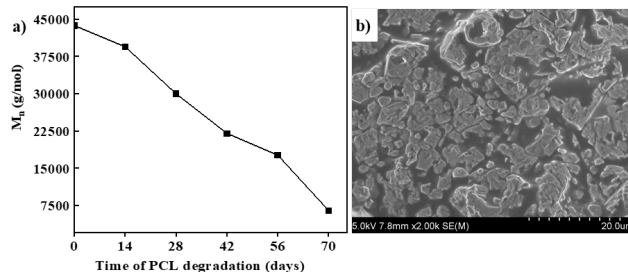
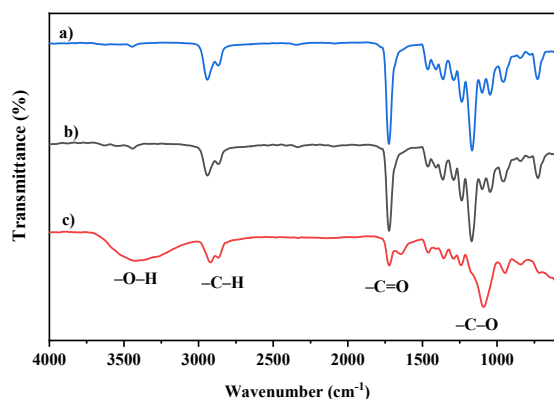


Figure 5 (a) the degradation of PCL MPs in-vitro test by GPC analysis and (b) SEM images of PCL MPs after 14 days-degradation

The degradation of PCL MPs in-vitro test is presented in Figure 5. As seen,  $M_n$  of PCL decreased 9.65% ( $M_n = 39442$  g/mol) after 2 weeks, and it continuously decreased around 50% at 6 weeks. The result can be explained that the PCL MPs were just eroded their surfaces in the early degradation stage, then the PBS solution would diffuse rapidly into the PCL MPs through the holes, which causes the decay of MPs (Figure 5b). However, the initial degradation of PCL would execute slowly owing to the highly crystalline structure. Therefore, the  $M_n$  of PCL was 17588 g/mol after 8-weeks of degradation and reduced quickly to 6432 g/mol (over 80%) at 10 weeks of the in-vitro degradation test. At the end of the in-vitro degradation test (after 10 weeks), the  $M_n$  of polymer was enough low to be excreted from body through kidney. The FTIR analysis was used to investigating the structure of these samples.



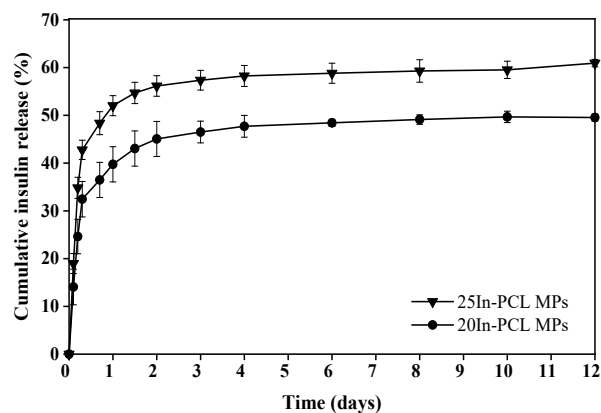


**Figure 6** FTIR spectra of (a) raw PCL pellet (b), the electro sprayed PCL MPs and (c) PCL MPs after 70 days' degradation in in-vitro test

As shown in Figure 6, the absorption band at 1723 and 1722  $\text{cm}^{-1}$  is characterized to  $-\text{C}=\text{O}$  stretching vibrations of ester carbonyl group, the characteristic peaks at 3447 to 3422  $\text{cm}^{-1}$  are assigned to  $-\text{O}-\text{H}$  group. The FTIR spectra of raw PCL pellet and the electro sprayed PCL MPs are observed to be similar, indicating that the structure of PCL is conserved after electro spraying. Besides, the lower intensity of  $-\text{C}=\text{O}$  peak implies the decrease of ester carbonyl group in PCL, as a result of hydrolysis of ester bonding in aqueous solution. PCL MPs were degraded significantly after 70 days in the in-vitro test, corresponding to the lowest intensity of peak  $-\text{C}=\text{O}$ . In addition, the electro sprayed PCL MPs after 70 days of degradation in the in-vitro test have the broad peak between 3026 and 3645  $\text{cm}^{-1}$ , which can be assigned to  $-\text{O}-\text{H}$  groups. The result can be explained by the scission of PCL backbone to alcohol and acid carboxylic [11].

#### Insulin Release In-Vitro Test

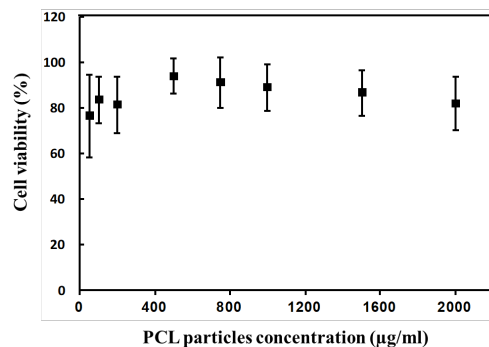
The insulin release profiles from the electro sprayed PCL MPs with different insulin contents were obtained by HPLC method as shown in Figure 7. As seen, a rapidly initial release period of PCL MPs was determined in both samples during first 24 hours, corresponding to 52.03% drug release from 25% insulin- PCL MPs (triangle line) and 39.75% drug release from 20% insulin- PCL MPs (circle line). When exposed to PBS medium, insulin which is encapsulated in surfaces and the internal matrix of both MPs, was rapidly released into the aqueous solution without the resistance from an outside shell of MPs. The diffusion of insulin from the surface of PCL MPs in the PBS solution caused the burst release of insulin. The obtained results well agree with the drug release behaviors of the electro sprayed drug-loaded microparticles, from previous reports [7, 8, 16]. After the initial release of insulin, the release rate became slower and more stable for 2 to 7 days. In conclusion, drug release from PCL MPs happened in two continuous stages, the initial burst release from the microparticles surface and the constant rate which related to the degradation or erosion of polymer and the diffusion of drug and protein [5-8]. Moreover, the release of insulin was not obviously changed after 6 days (Figure 7). This is because the release of insulin at this stage only depends on the degradation of PCL matrix and the erosion of external microparticles.



**Figure 7** The in-vitro release of insulin from PCL MPs, triplicate samples were conducted for each released point (n=3), 25In-PCL MPs and 20In-PCL MPs

#### Cytotoxicity of PCL Microparticles

Using MTT assay to evaluate the cytotoxicity of PCL microparticles with the human A549 cell line in in-vitro test. Figure 8 shows the cell viability after 4 hours of incubation with PCL microparticles with various concentrations from 50 to 2000  $\mu\text{g}/\text{ml}$ . At 50  $\mu\text{g}/\text{ml}$  of MPs concentration in HBSS, the percentage of viable cells was lower than 80% and the standard deviation broadened from 59% to 95% cell viability. When increasing the concentration of PCL MPs from 100 to 2000  $\mu\text{g}/\text{ml}$ , the viability of the cells was higher than 80%, corresponding to 83% of 200  $\mu\text{g}/\text{ml}$  PCL MPs and 97% of 500  $\mu\text{g}/\text{ml}$  MPs, respectively. These results indicate that the PCL MPs are good biocompatible with cells. Therefore, the electro sprayed PCL microparticles are a potential biomaterial for drug/protein delivery applications.



**Figure 8** Cell viability of the electro sprayed PCL microparticles

## 4.0 CONCLUSION

The insulin-loaded PCL MPs were fabricated by electro spraying with the spherical shape. The physical and chemical characteristics of the insulin-PCL MPs were investigated using SEM, IR, DSC, GPC and HPLC. The insulin-PCL MPs conserved the physicochemical properties of insulin and exhibited high encapsulation efficiency (over 75%). The insulin-PCL MPs reduced the initial burst release and prolonged the insulin release for 7 days. The A549 cell viability test indicated that the electro sprayed PCL MPs were biocompatible to these cells. In spite of some restrictions, the electro sprayed insulin-PCL MPs

are still potential for controlled release in long term treatment such as diabetes, hormone disorders.

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