Original Research Article

Stability of Insulin on Polycaprolactone Nanoparticles as a Function of Surface Properties

Abstract:

The purpose of this research was to evaluate the structural stability of insulin upon interaction with polycaprolactone (PCL) nanoparticles using a combination of fluorescence spectroscopy and high pressure liquid chromatography (HPLC). The size and morphology of the nanoparticles were characterized using dynamic light scattering (DLS) and scanning electron microscopy (SEM). Fluorescence emission data revealed that insulin is most stable with multilayer adsorption at pH close to its isoelectric point. The obtained particles size ranged from 130-140 nm+22 (SD). The loading amount of insulin onto the PCL nanoparticles was low at pH 7.4 and relatively high at pH 5.3. Adsorption phenomenon of protein onto hydrophobic nanoparticles provide a promising noninvasive carrier system for insulin.

Key words:
Protein adsorption, Insulin, PCL nanoparticles, Fluorescence spectra, dynamic light scattering,

1. Introduction:

In the last decade, the advancements in biotechnology have paved the way for the development of new formulations that employed for the delivery of therapeutic proteins (Bajracharya et al., 2019). Some of these approaches utilized biodegradable polymers in order to prolong the stability and increase the biological half-life of labile proteins. As such, controlled drug delivery of insulin using

polymeric nanoparticles has been an interesting delivery option to overcome many challenges associated with macromolecules formulation (Duncan et al., 2019). Insulin is a fragile molecule that is subjected to physical and chemical instability. Denaturation of such protein results in loss of the therapeutic effect as well as eliciting the structure-related toxicity and immunogenicity. Moreover, extreme agitation of this labile protein by emulsification or nanoprecipitation leads to a major loss in protein activity (Castellanos et al., 2002; Schellekens et al., 2008).

Diabetes is a worldwide health problem that impacts the patient's quality of life. The number of diabetic patients is increasing every year and it is expected to grow to 693 million by 2045 (Cho et al., 2018). Insulin is an important treatment for Type 1 diabetic patients which is usually administered by the subcutaneous route. Due to the short half-life of insulin, repeated injections are needed to maintain the therapeutic activity. Repeated and frequent daily subcutaneous injections throughout lifetime could affect patient compliance. Thus, sustained drug delivery systems have been implied to overcome many challenges associated with adverse effects of conventional dosage forms leading to improve therapeutic outcomes.

Insulin is one of the most available proteins in nature which represents a key role in the human body. Like most proteins, insulin usually interacts with solid materials that might cause reversible or irreversible modification in the structure based on the nature of the material's surface (Johnson et al., 1983). Due to the nature of proteins structure (Figure 1), they adhere to polymer surfaces with different mechanisms which makes controlling their drug delivery a challenging aspect. Buijs J et al have studied the adsorption behavior of r-hGH with different lipophilic and hydrophilic surfaces that cause significant structural change (Buijs et al., 1998). However, limited studies have been conducted to characterize insulin adsorption onto extreme hydrophobic polymers with respect to

structural changes assessment using fluorescence spectra. It is a crucial to utilize biodegradable polymers to control and optimize the reversible interaction of insulin aiming in providing a sustained drug delivery system.

Polymeric nanoparticles are a versatile approach for controlled release systems because of the possibility to tune their properties including biocompatibility, biodegradability and subcellular size (Panyam and Labhasetwar 2003; Chavanpatil et al., 2006). The impact of nanoparticles on therapeutic protein delivery is a critical issue that is appealing increasing attention from scientists. Their sizes range between 1–100 nanometers in diameter which are preferable for use in nanomedicine due to their ability to traverse micro-capillaries. (Singh and Lillard 2009). The major obstacle in developing protein-based nanoparticles as a drug delivery system is controlling the release rate and minimizing the possible protein denaturation associated with the formulation condition (Sung et al., 1998; Klose et al., 2008).

Polycaprolactone (PCL) is a semi-crystalline synthetic polyester that has been studied over several decades as a drug carrier, including microparticles, nanoparticles, films and nanofibers (Valmikinathan et al. 2009; Woodruff et al., 2010). Due to its biocompatibility, biodegradability and lack of toxicity, it has been used extensively to produce several drug delivery systems for controlled release of a variety of drugs (Chang et al., 2005; Dang et al., 2013; Jiang et al., 2020). PCL is characterized by high hydrophobicity making it a good candidate to adsorb biomolecules such as proteins to improve its cytocompatibility. Moreover, PCL has good permeability to proteins due to its slow degradation profile that does not cause an acidic environment in contrary to polylactic glycolic acid (PLGA) which negatively affect protein structure (Jameela et al., 1996).

Insulin exhibited a strong binding to hydrophobic surfaces at pH closes to its isoelectric point which can be applied to be adsorbed onto nanoparticles with high quantity. The release of adsorbed insulin from the PCL nanoparticles is attributed to a change in the net charge as a function of the pH. In the present study, we aim to characterize the adsorption mechanism and the release kinetics of insulin-loaded PCL nanoparticles. The fluorescence spectroscopy was used to explore any change in protein structure and subsequent insulin release in media of various pH conditions. We also utilized the dynamic light scattering (DLS) technique to determine the change in particle size as a result of insulin adsorption.

2. Materials and Methods

2.1 Materials

Insulin was purchased from Shanghai Hengyuan Biotechnology Co., Ltd. (Shanghai, People's Republic of China). PCL (Mw = 45,000 Da) was obtained from Shenzhen ESUN, China. Dichloromethane (DCM) and all buffer ingredients (acetic acid, sodium chloride, hydrochloric acid and sodium hydroxide) were obtained from Sigma Aldrich. All chemicals were of analytical grade and used without any further purification. Deionized water was used throughout the study. Tools and other materials were obtained from Fisher Scientific Co. (Fairlawn, NJ)

2.2. Methods

2.2.1 Preparation of Buffers

To investigate the effect of pH on the adsorption behavior of insulin acetate and phosphate buffers, pH 5.3 and pH 7.4, respectively were prepared. Deionized water was used to prepare the

buffer at 10 mM ionic strength. 1 N of sodium hydrochloride and hydrochloric acid were used to adjust the pH of each condition. The ionic strength was adjusted using sodium hydroxide.

2.2.2 Preparation of polycaprolactone nanoparticles

PCL nanoparticles were obtained by nanoprecipitation method that depends on the diffusion of the organic solvent from the oil phase into the aqueous phase. This leads to precipitation of small colloidal particles of the polymer. Briefly, PCL was first dissolved in DCM at a concentration of 10 mg/ml in a thermostatic water bath at 40 °C. The oil phase was continuously and slowly added to aqueous phase under rapid magnetic stirring (>1200 rpm). The stirring was continued for 3 – 4 hours to allow for solvent extraction and hardening of nanoparticles. The obtained nanoparticles were collected by centrifugation at 14,000 rpm at 25◦C for 10 min. The PCL nanoparticles were redispersed in water, lyophilized and stored at -80 °C.

2.2.3 Physicochemical characterization of nanoparticles

The properties of nanoparticles were characterized for their particle size and zeta potential by DLS Malvern Instrument's Zetasizer, Nano ZS ZS (Malvern Instrument, UK). The intensity of autocorrelation was measured at a scattering angle (θ) of 173^{o} (λ_{ex} 633 nm). Clean square polystyrene cuvettes were filled with ~1.2 mL of solution with gentle stirring on a vortex stirrer and measured at 25 ^{0}C.

$$D = \frac{kT}{3\pi\eta d_p}$$

(**D**=The diffusion coefficients, **k**=Boltzmann constant, **T**=temperature, **η**= viscosity, **dp**, hydrodynamic diameter)

2.2.4 Scanning electron microscopy

Scanning Electron Microscopic (SEM) analysis was employed using Hitachi S-4500 SEM machine in order to observe the shape and surface morphology of the produced particles. A very small amount of the sample was dropped on a carbon coated copper grid and the extra solution was removed using a blotting paper. The sample on the grid were allowed to dry by putting it under a mercury lamp for 5 min.

2.2.5 Preparation of Protein Solution and Nanoparticle Suspension

Insulin was first dissolved in 0.1M HCl and then dialyzed with the PCL nanoparticle suspension (each in a separate dialysis bag) against an appropriate buffer using 3500 MWCO cellulose membrane to equilibrate the solutions condition. After dialysis, the protein solution was filtered using a syringe filter (pore size of 0.22 μm) to exclude any aggregates. The insulin concentration was obtained using the specific absorptivity of tyrosine (Tyr) at 276 nm (A276 nm = 0.1362 $cm^{-1}.M^{-1}$) by Shimadzu UV-1601 Spectrophotometer. Working concentrations of protein were adjusted in the range of 0.01 to 0.25 mg/ml to obtain protein coated nanoparticles at low and complete surface coverage. (for conformational change studies). These concentrations were adjusted using the dialysate for each of the studies. The nanoparticles concentration was maintained at 1 mg/ml for all studies using the dialysate.

2.2.6 Adsorption study

Equilibrium dialysis is an accurate classical way to evaluate the binding of one protein to another. This method is easy to perform and can produces results even at low affinity interactions that are difficult to measure with other methods (Schuhmacher et al 2004). High throughput (HT) dialysis apparatus is 96 well Teflon plate arranged in 8 rows and 12 columns (Waters et al., 2008). Each well is separated to two vertical chamber to eliminate any air-pocket. The vertical alignment of the wells facilities transfeing the samples solutions to a corresponding well in the micro-plate for spectroscopy analysis.

Knowing concentration of insulin was placed in the two chambers that are separated by a semipermeable regenerated cellules membrane (100 kDa MWCO). The nanoparticles suspension was loaded in one chamber and left on a shaker to allow equilibration at 25 ^{0}C for 4 hours. Insulin molecules can freely diffuse between the two sides through the membrane and interact with the nanoparticles. 100 ul was withdrawn from the nanoparticles-free chamber and the protein concentration was quantified spectrophotometrically using HPLC.

2.2.7 Calculation of bound insulin

HPLC with diode array detection is a simple and reliable technique for the determination of insulin amount. Chromatographic analysis was carried out on an HPLC system equipped with Shimadzu accessories. A good separation peak was achieved on a C_{18} column with a mobile phase consisting of acetonitrile and 0.1% TFA aqueous solution. The ratio was initially at 30:70 (v/v), which was linearly changed to 40:60 (v/v) over 5 min. Eluent was pumped at a flow rate of 1 mL/min with injection volume 20 uL and detection wavelength of 276 nm. All experiments occurred at room temperature and the total area of peak was used to quantify the insulin. Calibration curve was linear

within the concentration range of 0.01 – 1 mg/ml. Relative standard deviations of insulin for intra-day and inter-day variability were less than 6.3 and 8.5%, respectively.

The amount of insulin adsorption onto the nanoparticles was determined according to the following equation.

$$C_b = C_0 - 2C_f$$

Where C_b is the concentration of bound insulin, C_0 is the initial concentration of insulin and C_f is the free insulin concentration in both chambers.

2.2.8 Study the Structural change of insulin

Conformational change was studied using fluorescence spectroscopy technique. A Tecan i3 fluorescence spectrophotometer was applied to measure the fluorescence spectra using 96 well black quartz plate. The excitation wavelength was at 280 nm (for INS) with scanning speed at 250 nm /min, and slits was set at 5 nm. Fluorescence emission data was collected from 305 to 400 nm for the free INS and that adsorbed on PCL nanoparticles. % quenching was obtained from the peak height and blank solution was subtracted from each sample measurements. The temperature of the sample was maintained at 25°C in all experiments.

3. Results

3.1. Particle size and zeta potential measurement

Nanoparticles with size range of 130-150 nm and zeta potential of –15 mV were obtained (described in Table 1). From the SEM micrographs (Fig. 6), it is shown that the nanoparticles

prepared by nanoprecipitation are generally with uniform particle size distribution and spherical in shape.

Moreover, the isoelectric point of crystalline insulin was found to be between pH 5.3 and pH 5.35. Size characterization of insulin in solution regarding to different pH at variable time intervals shows the stability of insulin in these conditions as described in figure 1. Size analysis of protein adsorption onto PCL at pH 7.4 and pH 5.3 are shown in Figs. 4 and 5, respectively. Size change of particles (Dh) was plotted against increasing concentration of insulin (mg/ml). Results of protein adsorption indicate the increase particle size with the rise of protein concentration in the solution. At pH 7.4, the increase in size at time zero was in the range of 5 nm. After three hours, the size change was stable at lower concentrations. However, at higher concentrations there was a dramatic increase in the particle size in the order of ~20 nm. At pH 5.3, the particle size increase was in the order of ~30 nm at the higher concentration at time zero. The size was increased in the order of ~120 nm where the protein is at its maximum adsorption condition. At full surface coverage, the ratio of insulin to PCL nanoparticles was found to be 1: 0.21 and 1:0.17 at pH 7.2 and pH 5.3 respectively. Surface coverage of insulin according to the total surface area (SA) of nanoparticles (mg/m^2) and the ratio of protein concentration to nanoparticles concentration were also described in Table 2.

3.2. Fluorescence Spectroscopy Analysis

The change in the fluorescence emission spectra at pH 5.3 for free insulin relative to the adsorbed one is shown in Figure 7 and 8. pH conditions had no effects on the wavelength of maximum emission of insulin in solution. Insulin structure comprises interior hydrophobic residues composed of four tyrosine (Tyr) and three phenylalanine (Phe). The structure contains two polypeptide chains (A and B) that are linked by two disulfide bonds and another disulfide bond

located within the A chain (figure 1). The emission spectra originate mainly from the Tyr residues (Casamayou-Boucau, Y. and Ryder, A., 2020).

Figure (7) shows the emission spectra at pH 5.3 of insulin in solution as well as of the adsorbed one on PCL nanoparticles at time zero. Any change in the insulin structure due to adsorption is reflected in the peak height of the emission spectra. Irrespective to insulin concentration, the emission maxima of the protein in solution were similar at pH 5.3, and 7.4. Table 2 presents the shifts in the emission maximum and the changes in intensity associated with adsorption of insulin onto PCL nanoparticles. All adsorption isotherm exhibited a red shift in the emission spectra except with high surface coverage at pH 5.3 where the shift went toward a lower wavelength (blue shift). Also, quenching of fluorescence emission was significant specially at low surface coverage.

4. DISCUSSION

4.1. Particle size characterization

DLS records the intensity of the scattered light at a fixed angle depending on the Brownian motion of the particles. The time-dependent fluctuation is important because it reflects the movement of the particles and relates it to the size of particles according to the Stokes-Einstein relationship (Goldburg 1999). These measurements were conducted in order to determine the change in particles size upon protein adsorption as a function of pH at different time limit (time zero and three hours later). The results showed a dependency of insulin binding on the pH of the solution as well as the hydrophobicity of the polymer.

The pH conditions govern the ionization extent of the surface functional groups. Based on the charge density on the protein and nanoparticles surface, the electrical double layers overlap leading to protein-particle attraction or repulsion. Insulin acquires positive net charges below the isoelectric

point and negative net charges above it. High pH medium maximizes the negative charge density of the protein. Thus, adsorption behavior was conducted at different pH conditions (5.3 and 7.4). Therefore, the charge status on protein is varied while on nanoparticles surface remain negative under all pH.

PLC contains carbonyl groups with a pKa of 3.85 where the magnitude of nanoparticles charge decreases at more acidic pH according to the ionization extent of the polymer functional groups. The zeta potential is expected to be the lowest in the magnitude of negative charge at pH 5.3 (acetate buffer). This demonstrates the dominant hydrophobicity of nanoparticles at pH close to the pKa of PCL nanoparticles. The shielding effect of exciting ions can attribute to a decrease in the zeta potential magnitude. Therefore, increasing the polymer concentration resulting in a reducing the shielding effect and subsequently improving the zeta potential value.

At pH 7.4 and 5.3, the polydispersity index (PDI) was low for the free nanoparticles in solution, confirming that the obtained PCL nanoparticles are generally monodispersed. However, a slight increase in the PDI was seen at pH 7.4 with the inclusion of insulin in the mixture. This could be attributed to the adsorbed protein on the particles surface and possible coalescence between the adjunct particles resulting in wide particles size distribution (Nanda et al., 2006). Both the protein molecules and particles possess negative charges which indicates presence of electrostatic repulsions between the molecules. Upon adsorption of insulin onto PCL nanoparticles due to the hydrophobic forces, the negative charges will decrease in magnitude. This explains the slight increase in particles size indicating a possibility of insulin adsorption in a monolayer.

At pH 5.3, insulin surface coverage onto PCL nanoparticles follows the order of low to high protein concentration which corroborates the increased in PDI value at higher protein concentration.

At higher protein concentration, large amount of protein molecules transfers toward the polymer hydrophobic surface (Larsericsdotter et al., 2005). Although the hydrodynamic diameter of insulin is barely 5 nm while that of PCL nanoparticles is about 130-140 nm, it is unlikely the increased particle size was solely due to protein adsorption. The dramatic increase in the particles size at the IEP at high protein concentrations is explained by higher surface coverage due to strong hydrophobic interaction. This process at this pH attribute to the exposed hydrophobic moieties of the protein on the polymer surface resulting in particles aggregation. It has been known that rearrangement of protein structure upon adsorption accounts to exposure of the hydrophobic residues and attracting other protein molecules. This phenomenon is confirmed when the particle size continues to increase after three hours of mixing.

Insulin is at its most stable condition at the IEP due to relatively equal distribution of positive and negative making a compact structure as a result of an intramolecular interaction. Absence of net charge between the adsorbed protein and hence, greater structure stability may have produced multilayers adsorption due to closer protein packing on the surface in comparison where the protein possesses a net negative charge (Demanèche et al., 2009; Rabe et al., 2011). The slight increase in particle size at low protein concertation even after three hours of adsorption can be attributed to less structural rearrangement within the protein molecules as a result of burying the hydrophobic region internally to become energetically stable (Pinholt et al., 2011).

4.2. Fluorescence spectroscopy analysis

Fluorescence spectroscopy technique is very suitable to study the conformational change of the insulin molecule's structure. The quantum of Tyr is higher making it responsible for the main fluorescence properties of insulin. Figure (1) depicts the crystal structure of insulin with a focusing

on the Tyr residues. Binding of the protein to the hydrophobic surface of the polymer causes a movement in the amino acids toward the water molecules due to the quenching effect. Subsequently, their emission is shifted to higher wavelength which is called a red shift. Adsorption of protein as multilayers cause burring of the hydrophobic residues and their emission is shifted to lower wavelength (blue shift) (Yang et al., 2015). In the current study, PCL nanoparticles was considered a quenching agent and adsorbed protein molecules at different magnitude according to the protein concentration. Interaction of insulin with PCL nanoparticles shows the lowest quenching effect at pH 7.4. whereas the quenching effect was high at pH 5.3 specially at low concentration. This fact can be illustrated by the change in the amino acids environment due to exposing to a hydrogen-bonding groups indicating a conformational change in the protein structure. It is indicated that at higher quenching in the fluorescence intensity reflects a higher adsorption force of protein on the PCL nanoparticles.

5. Conclusion

Interaction of insulin with PCL nanoparticles surface have been investigated. The adsorption mechanism of insulin with the surfaces was found to be governed by the pH of the medium and the concentration of the protein in solution. At low surface coverage, insulin is strongly bound to the hydrophobic forces which is accompanied by large structural change specially at low pH. The extent of conformational changes is less if insulin is adsorbed as multilayers onto the PCL surfaces. A low affinity is shown for insulin binding at neutral pH with low packing density and apparently less conformational change. Despite of the high PDI after the adsorption mechanism, one of these approaches can be considered for potential intravenous formulation with maximum loading amount depending on the stability of the protein structure.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table 1: Preliminary measurement of particle size and zeta potential at different pH condition.

polymer/Insulin	Size Average (nm) ±SD	PDI	Zeta Potential (mV) ±SD	
			PH 7.4	PH 5.3
PCL	140±10.3	0.086	-17±4.1	-11±SD4.5
Insulin	4±0.4	0.084	-10±4	0.0

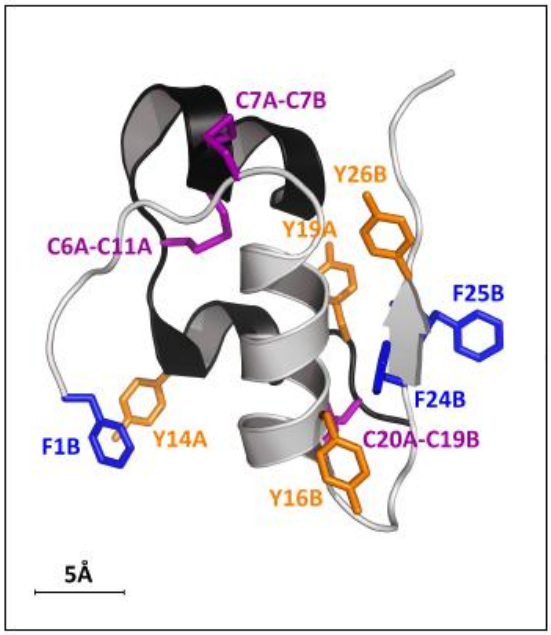

Figure 1: The three-dimensional structure of insulin monomer with A chain consists of two a-helices (black color), and β-chains contains only one α-helix (gray color). The residues are shown with tyrosine (Y), phenylalanine (F), and disulfide bridges (C). *This image is reproduced with permission from the following citation: "Quantitative analysis of weakly-bound insulin oligomers in solution using polarized multidimensional fluorescence spectroscopy". Y. Casamayou-Boucau and A.G. Ryder. Analytica Chimica Acta, 1138, 18-29, (2020).* ***DOI:*** *10.1016/j.aca.2020.09.007.*

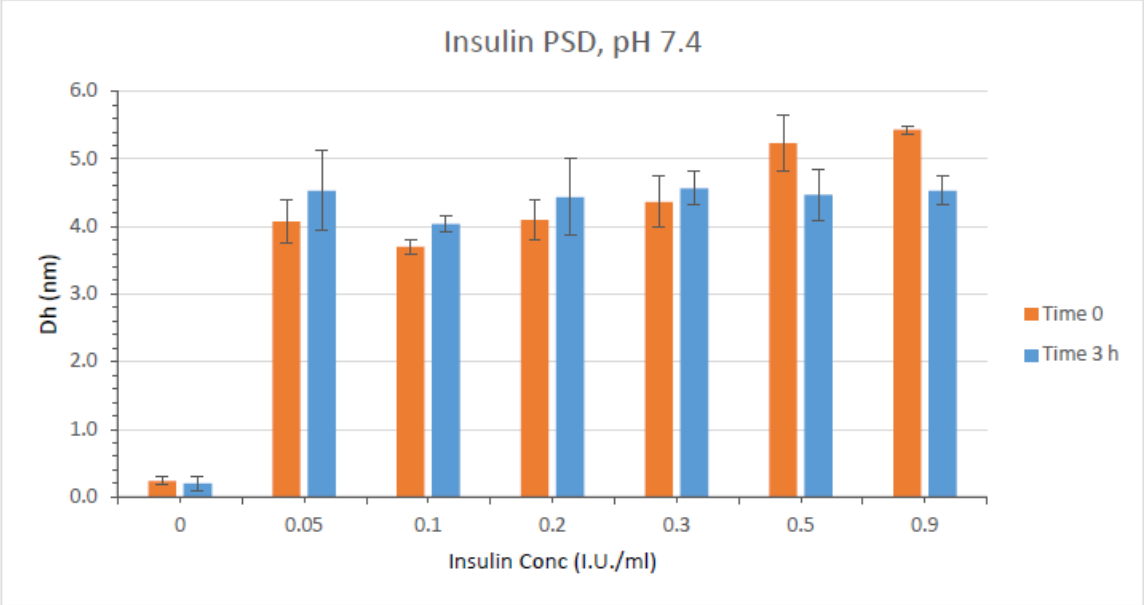

Figure 2: Hydrodynamic diameter measurements, Dh (nm), of different concentrations of insulin at pH 7.4.

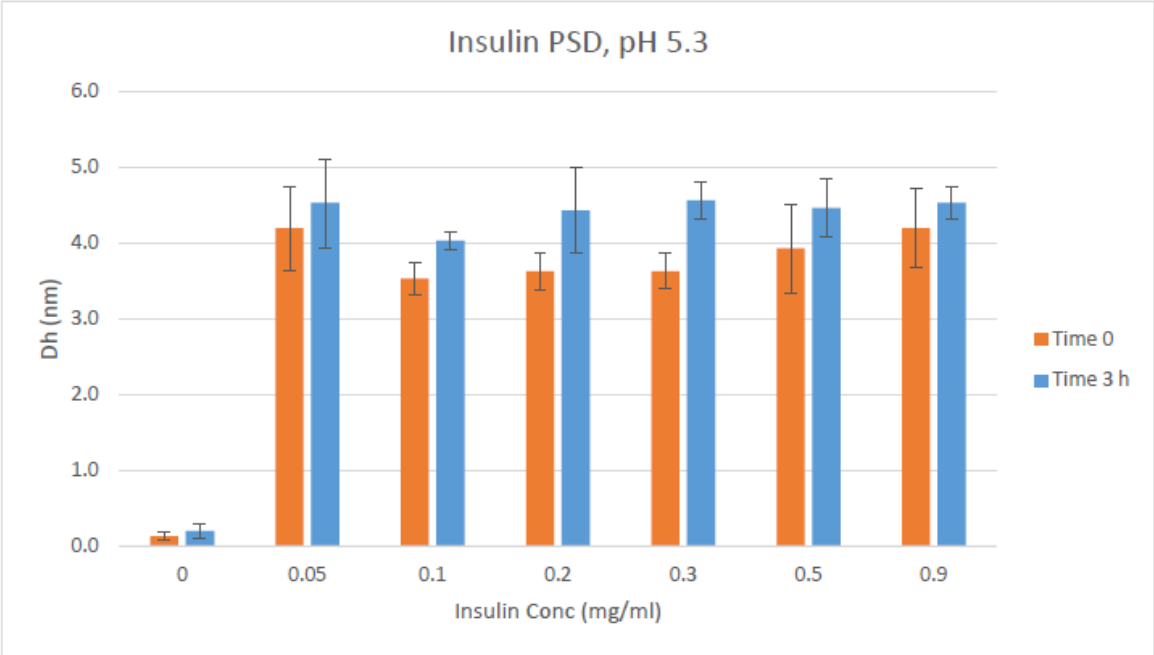

Figure 3: Hydrodynamic diameter measurements, Dh (nm), of different concentrations of insulin at pH 5.3.

Table 2: Summary of fluorescence emission maxima of insulin adsorbed onto PCL nanoparticles after three hours. The data shows the shifts in the emission maximum and the changes in intensity associated with protein adsorption onto nanoparticles.

pH	Ratio of INS to PCL	$\mu g/m^2$	Shit in λMax (nm)	%Quenching
5.3	1:0.05	1.08	5 (red shift)	45%
	1:0.1	2.73	3 (red shift)	31%
	1:0.2	2.81	-2 (blue shift)	23%
7.4	1:0.05	0.17	2 (red shift)	21%
	1:0.1	0.19	2 (red shift)	19%
	1:0.2	0.23	2 (red shift)	16%

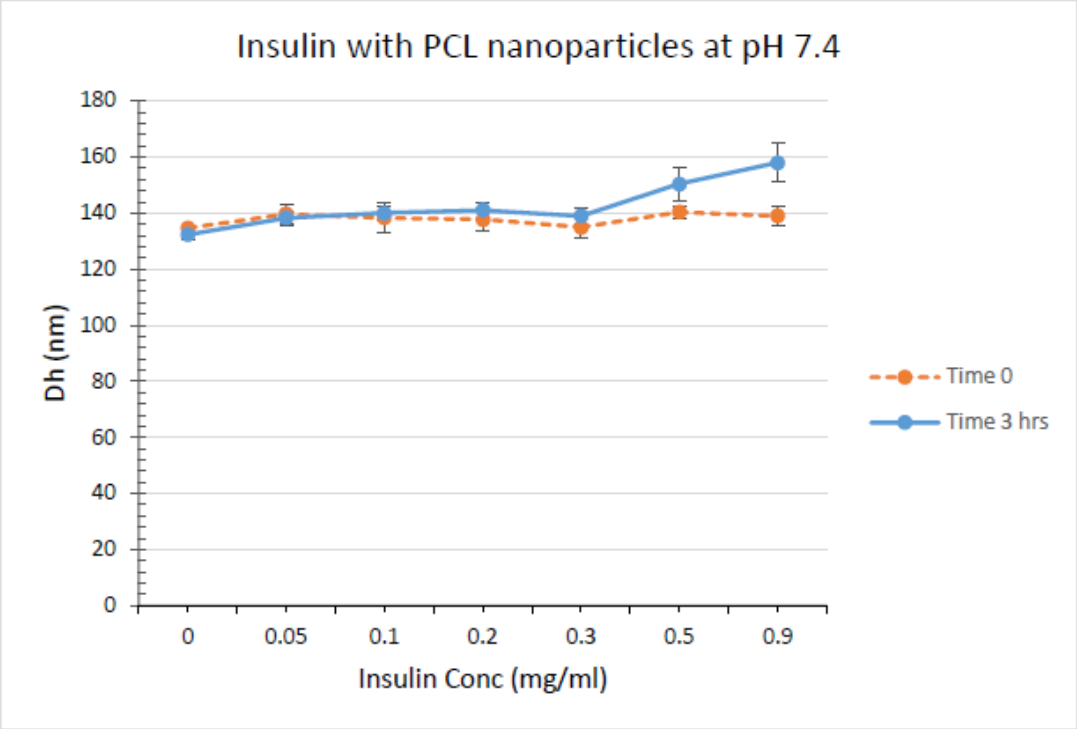

Figure 4: Measured hydrodynamicdiameter (Dh) of insulin adsorbed on PCL nanoparticles at pH 7.4 at two different time intervals.

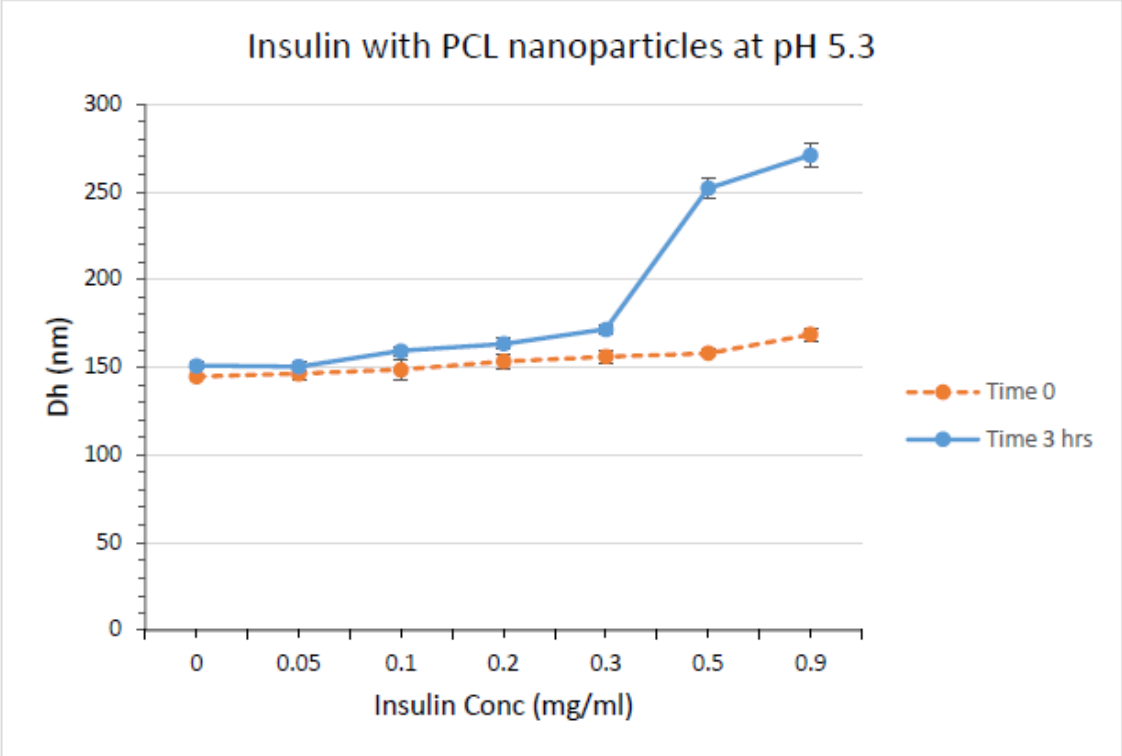

Figure 5: Measured hydrodynamicdiameter (Dh) of insulin adsorbed on PCL nanoparticles at pH 5.3 at two different time intervals.

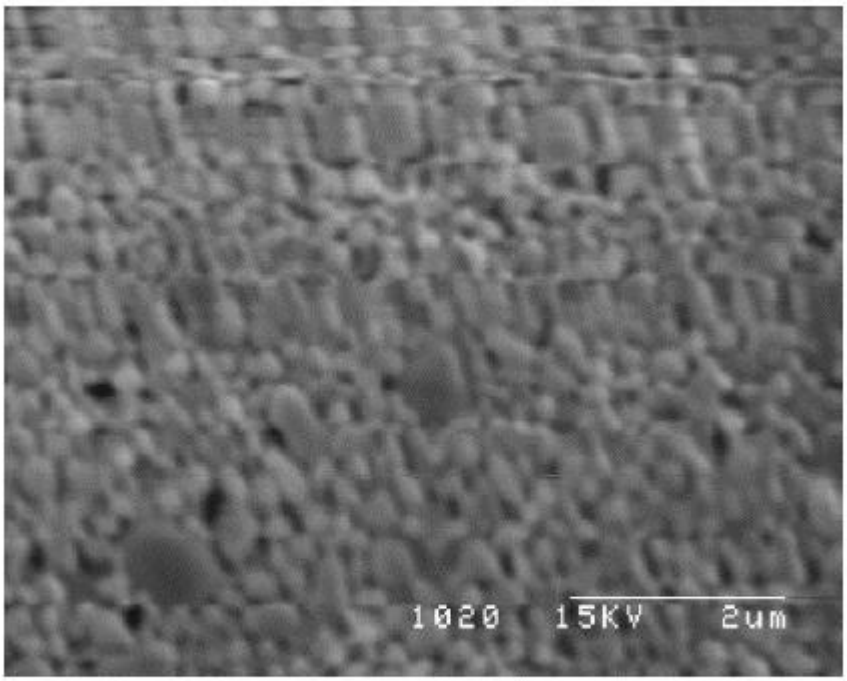

Figure 6: The SEM micrograph of PCL nanoparticles.

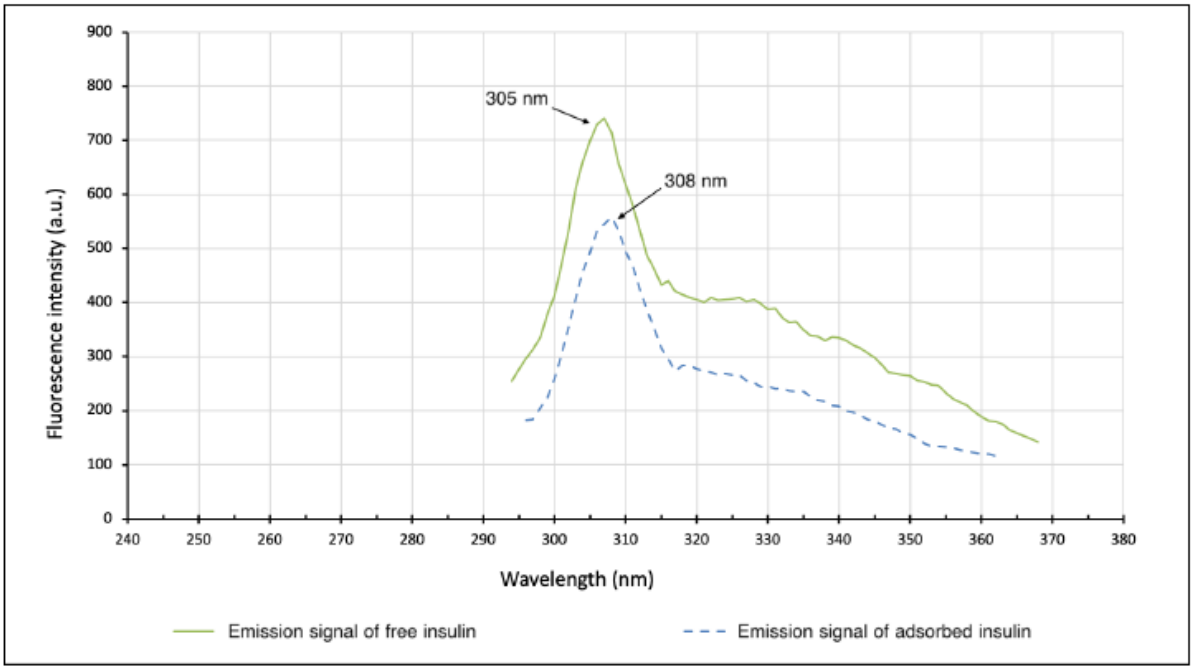

Figure 7: The emission spectra of insulin in solution (solid line) and the adsorbed one on PCL nanoparticles (dashed line). The emission spectra of the protein in solution are similar at pH 5.3 and 7.4. A change in insulin structure due to adsorption is reflected in the fluorescence emission spectra.

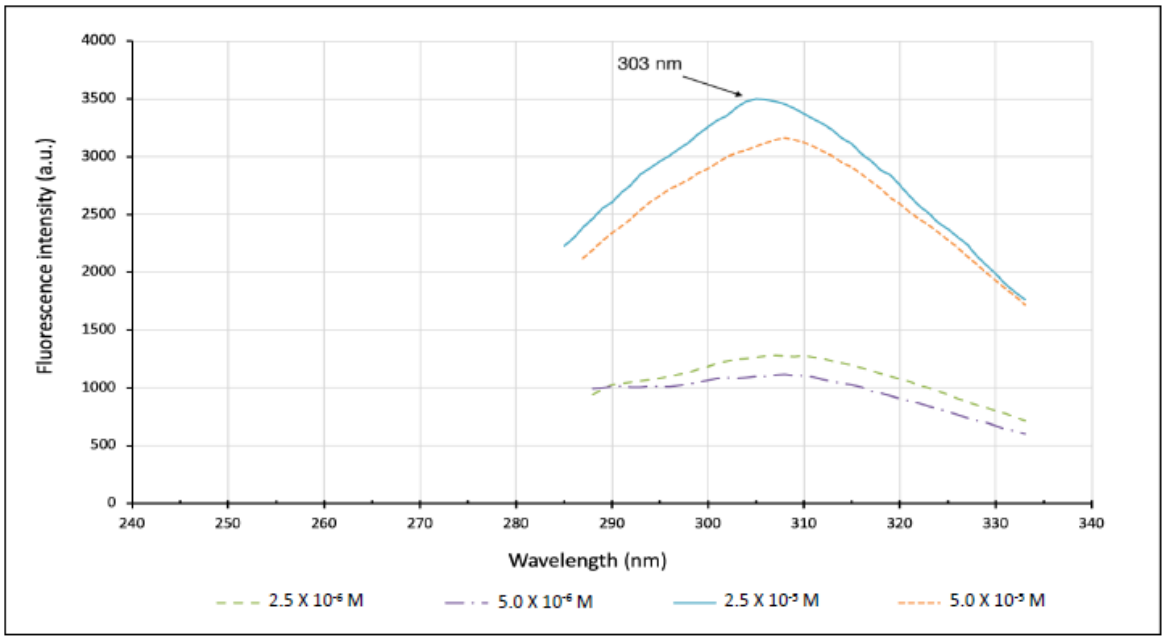

Figure 8: The emission spectra of adsorbed insulin at four different concentrations. Adsorption at high concentration caused blue shift in the emission maximum.