Original Research Article

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

Abstract:

The purpose of this research was to formulate insulin-loaded polycaprolactone (PCL) nanoparticles and evaluate structural stability of the protein using fluorescence spectroscopy. 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 (IEP). The obtained particle 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. The adsorption phenomenon of protein onto hydrophobic nanoparticles provides 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 are employed for the delivery of therapeutic proteins [1]. 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 [2]. Insulin is a sensitive 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 [3, 4].

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 [5]. 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 improved 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 [6]. Due to the nature of protein 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 [7]. 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 crucial to utilize biodegradable polymers to control and optimize the reversible interaction of insulin aiming in providing a sustained drug delivery system.

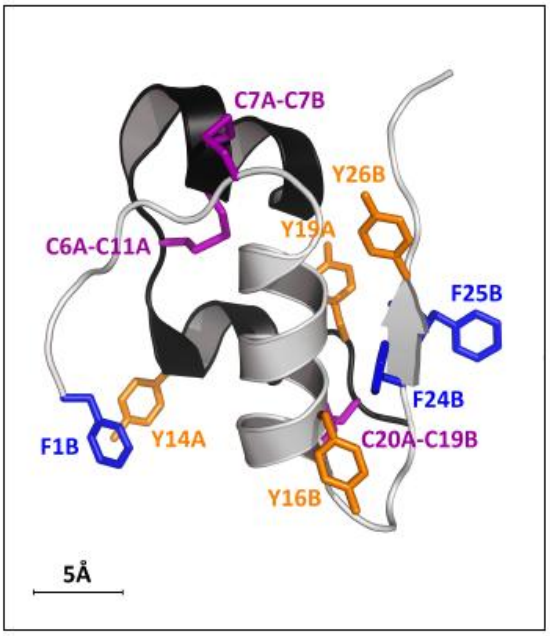

Figure 1: The three-dimensional structure of insulin monomer with A chain consists of two a-helices (black color), and β-chains contains only one α-helix (gray color). The residues are shown with tyrosine (Y), phenylalanine (F), and disulfide bridges (C). *This image is reproduced with permission from 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.*

Polymeric nanoparticles are a versatile approach for controlled release systems because of the possibility to tune their properties including biocompatibility, biodegradability and subcellular size [8, 9]. The impact of nanoparticles on therapeutic protein delivery is a critical issue that is appealing to 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 [10]. 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 [11, 12].

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 [13,

14]. 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 [15-17]. Previous studies have evaluated the loading of insulin into PCL-based nanoparticles [18, 19]. However, fabrication of polymeric nanoparticles necessitates using organic solvent which can influence protein folding and cause denaturation. Therefore, association of insulin with nanoparticles is promising because it will keep its intact structure and provide a sustained-release profile. Moreover, there is limited literature on the adsorption behavior of insulin onto PCL nanoparticles with respect to structural changes at different buffer media. PCL possesses high hydrophobicity making it a good candidate to adsorb therapeutic proteins to improve its permeability. Moreover, PCL has good compatibility with proteins due to its slow degradation profile that does not cause an acidic environment in contrast to poly lactic-glycolic acid (PLGA) which negatively affects protein structure [20].

Insulin exhibits a strong binding to hydrophobic surfaces at pH close 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. Dialysis membranes of different molecular weight cut-offs (MWCO) was purchased from Spectrum® Laboratories (Rancho Dominguez). Tools and other materials were obtained from Fisher Scientific Co. (Fairlawn, NJ)

2.2. Methods

2.2.1 Preparation of Buffers

The IEP of insulin is 5.3 which is considered the basis to investigate the effect of pH variation on its adsorption behavior [21]. 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 hydroxide and hydrochloric acid were used to adjust the pH of each condition. The ionic strength was adjusted using sodium chloride.

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 minutes. 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 using Malvern Instruments Zetasizer Nano ZS (ZEN3600) based on photon correlation spectroscopy. 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 °C. The diffusion coefficient (D) is related to the hydrodynamic diameter (Dh) by Stoke-Einstein relationship

$$\mathbf{D} = \frac{\mathbf{kT}}{\mathbf{3\pi\eta D_h}} \qquad (1)$$

(**D**=The diffusion coefficients, **k**=Boltzmann constant, **T**=temperature, **η**= viscosity, D_h, 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 was 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 transferred to the dialysis bag. PCL nanoparticle suspension was also transferred to another dialysis bag. Both the insulin solution and nanoparticles suspension was dialyzed against an appropriate buffer 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 ($A_{276\ nm} = 0.1362\ cm^{-1}.M^{-1}$) 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. 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 produce results even at low affinity interactions that are difficult to measure with other methods [22]. High throughput (HT) dialysis apparatus is a 96-well Teflon plate arranged in 8 rows and 12 columns [23]. Each well is separated into two vertical chambers to eliminate any air-pocket. The vertical alignment of the wells facilitates transfeing the sample solutions to a corresponding well in the micro-plate for spectroscopy analysis.

Known concentration of insulin was placed in the two chambers that are separated by a semipermeable regenerated cellulose membrane (100 kDa MWCO). The nanoparticles suspension was loaded in one chamber and the plate was left on an electric orbital shaker to allow equilibration at 25 ^{o}C for 3 hours. Insulin molecules can freely diffuse between the two sides through the membrane and interact with the nanoparticles. Then, 100 μl was withdrawn from the nanoparticles-

free chamber and the protein concentration was quantified spectrophotometrically using HPLC. Moreover, another 100 µl was withdrawn from the chamber that contains PCL/insulin complex for evaluating the particle size change as a function of different insulin concentration.

2.2.7 Calculation of bound insulin

HPLC with diode array detection is a simple and reliable technique for the determination of insulin amount [24]. 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 µl and detection wavelength set at 214 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.

$$C_b = C_0 - 2C_f \qquad (2)$$

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 a 96-well

black quartz plate (round bottom). The excitation wavelength was at 280 nm with scanning speed at 250 nm/min, and slits were set at 5 nm. Fluorescence emission data was collected from 305 to 400 nm for the free insulin and that adsorbed on PCL nanoparticles. % quenching was obtained from the peak height and blank solution was subtracted from each sample measurement. The temperature of the sample was maintained at 25 °C in all experiments.

3. Results

3.1. Particle size and zeta potential measurements

Size characterization of insulin at various buffer media proves the stability of protein in these buffer media as described in figure 2 and 3. Nanoparticles with size range of 130-150 nm and zeta potential of –15 mV were obtained (as described in Table 1). From the SEM micrographs (Figure 4), it is shown that the nanoparticles prepared by nanoprecipitation are generally with uniform particle size distribution and spherical in shape.

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

polymer/Insulin	Size Average (nm) ±SD		PDI	Zeta Potential (mV) ±SD	
	pH 7.4	pH 5.3		pH 7.4	pH 5.3
PCL	140±10.3	138±10	0.186	-17±4.1	-11±4.5
Insulin	4±0.4	4±0.2	0.084	-10±4	0.0
PCL/insulin	147±4.3	142±4.1	0.301	-19.3±3.2	-11+2.4

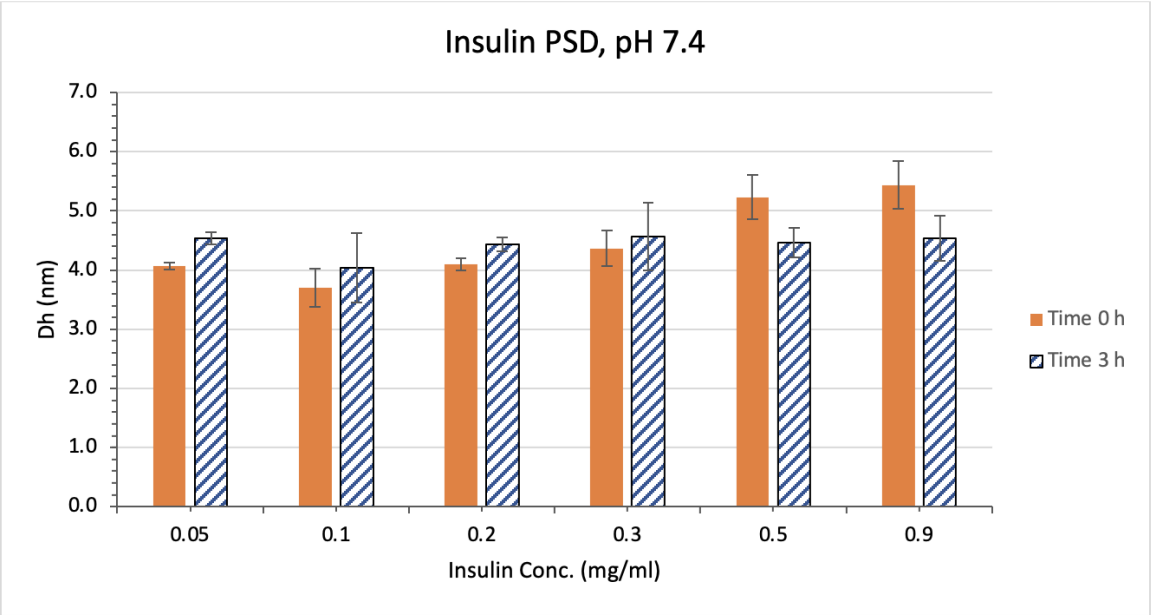

Figure 2: Hydrodynamic diameter measurements, Dh (nm), of different insulin concentrations at pH 7.4.

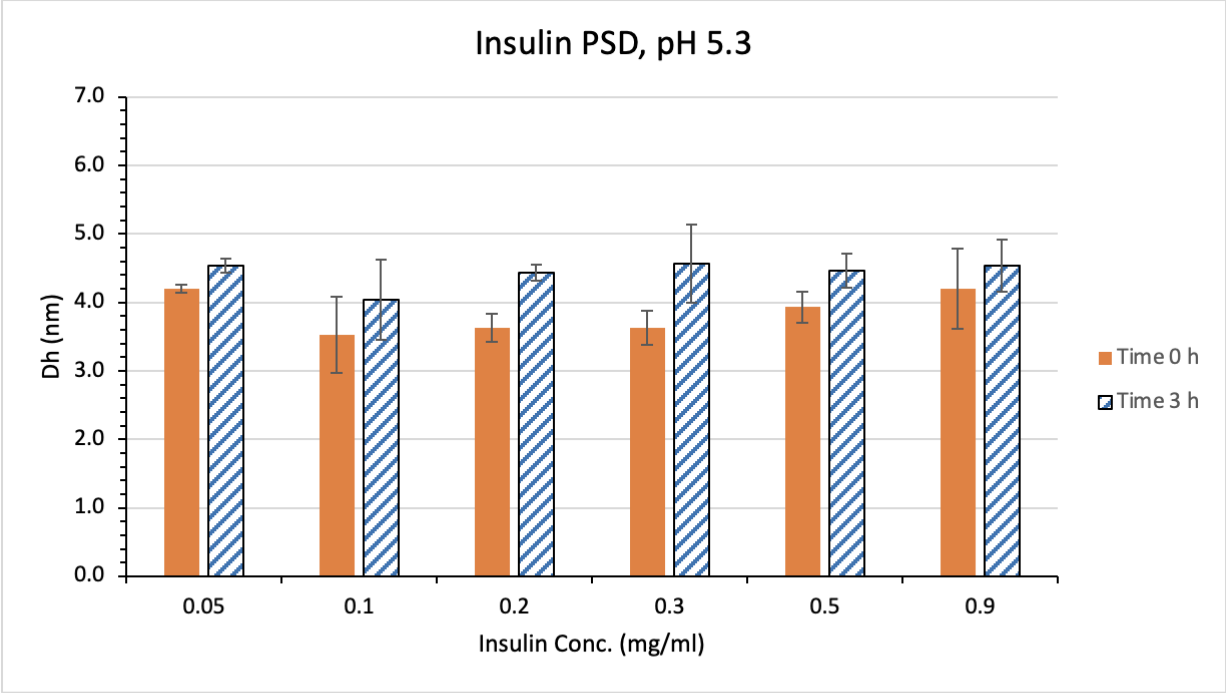

Figure 3: Hydrodynamic diameter measurements, Dh (nm), of different insulin concentrations at pH 5.3.

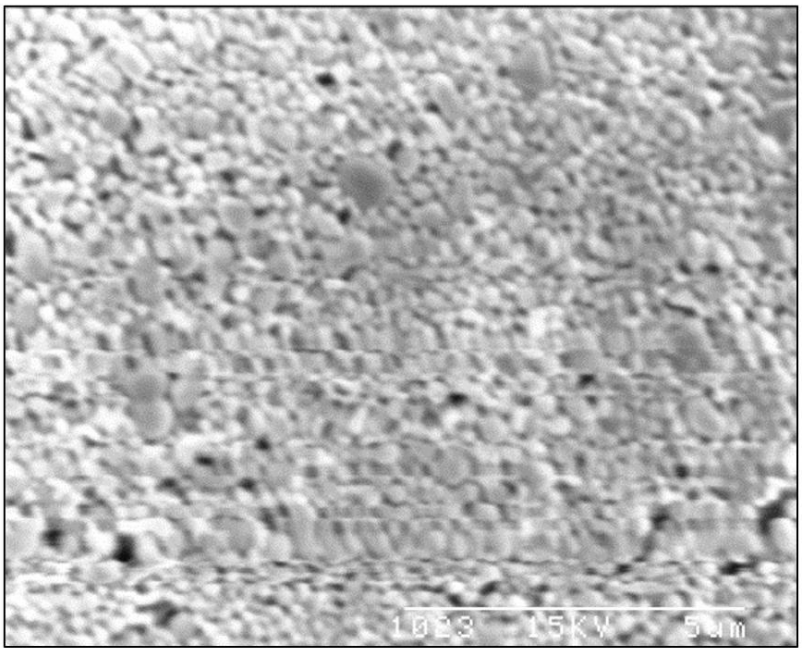

Figure 4: The SEM micrograph of PCL nanoparticles.

Size analysis of protein adsorption onto PCL at pH 7.4 and pH 5.3 are shown in figures 5 and 6, respectively. Size change of particles (Dh) was plotted against increasing concentration of insulin (mg/ml). Results of protein adsorption indicate the increased 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 low protein concentrations. However, at high protein 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.

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

pH	PCL/insulin	mg/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%

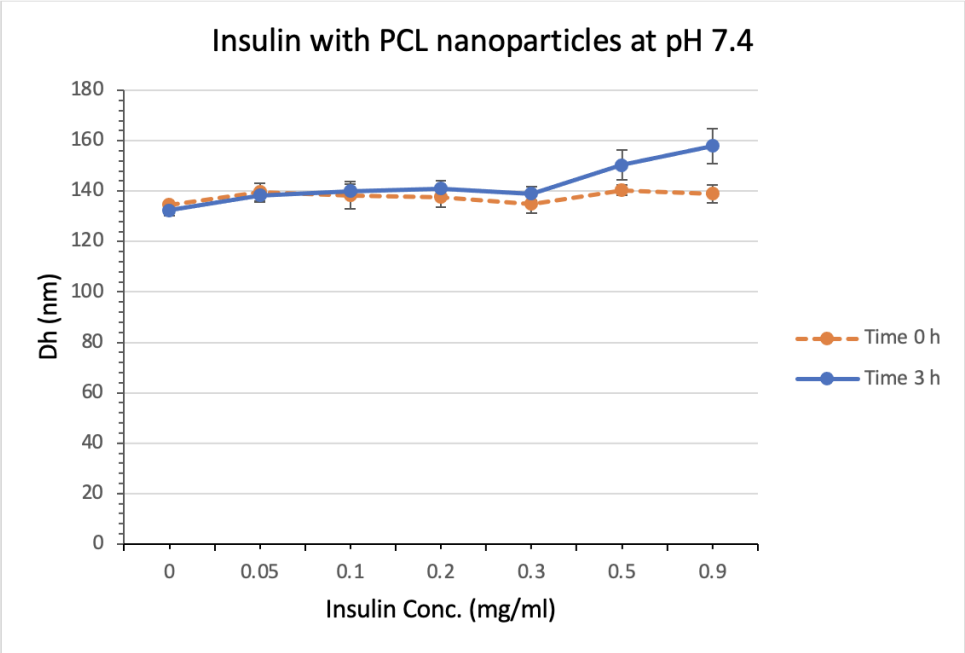

Figure 5: Measured hydrodynamic diameter (Dh) of adsorbed insulin at pH 7.4 and two different time intervals.

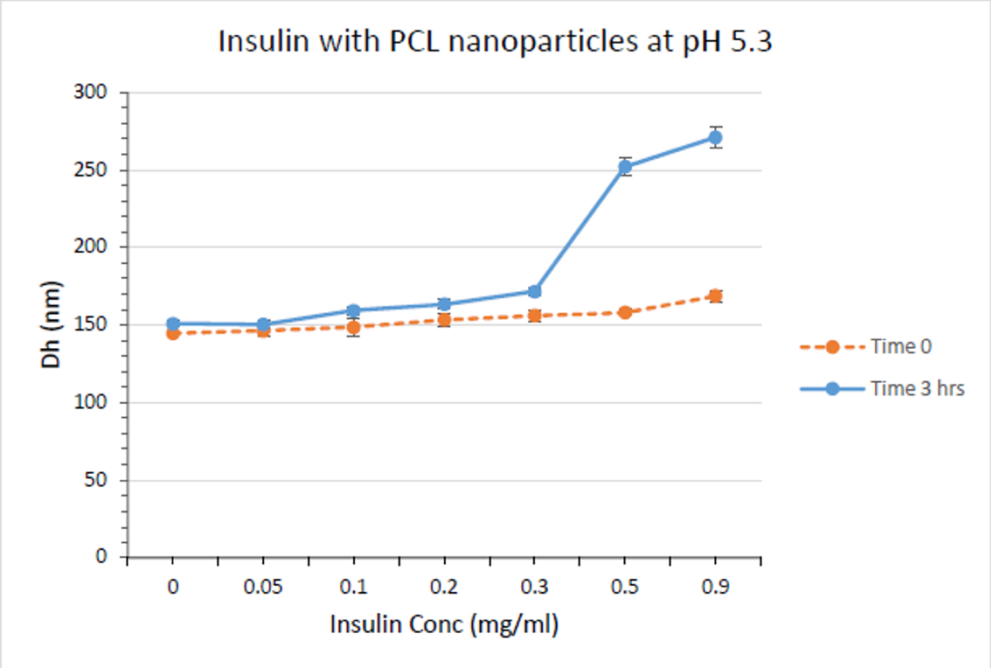

Figure 6: Measured hydrodynamic diameter (Dh) of adsorbed insulin at pH 5.3 and two different time intervals.

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 [25].

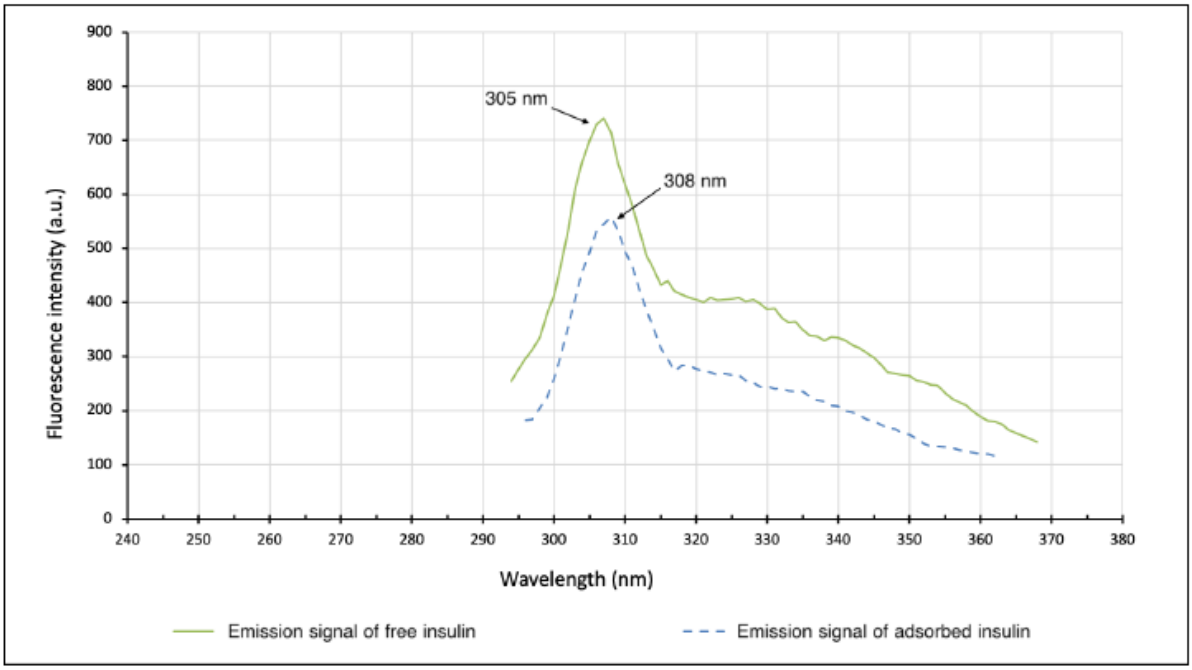

Figure 7: The emission spectra of insulin in solution (solid line) and the adsorbed one on PCL nanoparticles (dashed line). The emission spectra of the protein in solution are similar at pH 5.3 and 7.4. A change in insulin structure due to adsorption is reflected in the fluorescence emission spectra.

Figure (7) shows the emission spectra at pH 5.3 of the 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 isotherms 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 at 303 nm in figure 8). Also, quenching of fluorescence emission was significant especially at low surface coverage.

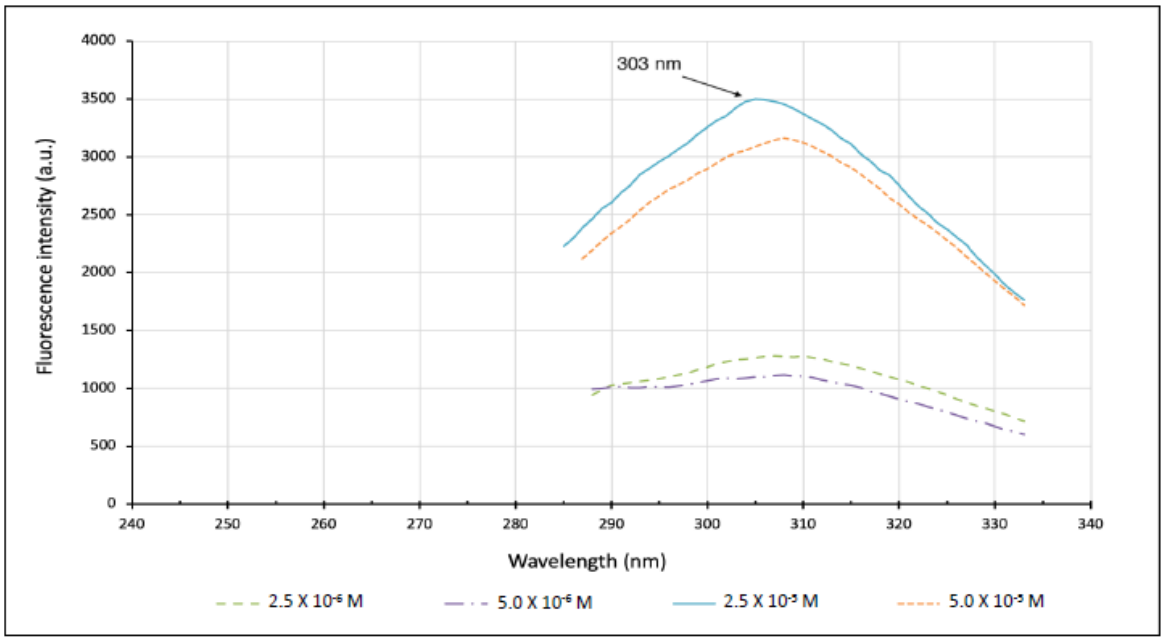

Figure 8: The emission spectra of adsorbed insulin at four different concentrations. Adsorption at high concentration caused blue shift in the emission maximum.

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 [26]. These measurements were conducted in order to determine the change in particle size upon protein adsorption as a function of pH at different time limits (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 of the media governs 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 IEP 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 the surface remains negative under all pH.

PLC contains carbonyl groups with a pKa of 3.85 where the magnitude of nanoparticle 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 contribute to the decrease in the zeta potential magnitude. Therefore, increasing the polymer concentration results 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 nanoparticle surface and possible coalescence between the adjunct particles resulting in wide particle size distribution [27]. 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 particle 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 increase in PDI value at higher protein concentration. At higher protein concentration, a large amount of protein molecules transfers toward the polymer hydrophobic surface [28]. 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 particle size at the IEP at high protein concentrations is explained by higher surface coverage due to strong hydrophobic interaction. This process at this pH is attributed to the exposed hydrophobic moieties of the protein on the polymer surface resulting in particle 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 (data not shown).

Insulin is at its most stable condition at the IEP due to relatively equal distribution of positive and negative charges, making a compact structure because of intramolecular interactions. 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 [29, 30]. The slight increase in particle size at low protein concentration 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 [31].

4.2. Fluorescence spectroscopy analysis

Fluorescence spectroscopy is a very suitable technique to study the conformational change of the insulin structure. The quantum of Tyr is higher making it a responsible residue for the main fluorescence properties of insulin. Figure (1) depicts the crystal structure of insulin with a focus 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 causes burring of the hydrophobic residues and their emission is shifted to lower wavelength (blue shift) [32]. In the current study, PCL nanoparticles was considered a quenching agent and adsorbed protein molecules at different magnitudes according to the protein concentration. Interaction of insulin with PCL nanoparticles at pH 7.4 shows the lowest quenching effect, 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 exposure to hydrogen-bonding groups indicating a conformational change in the protein structure. It is indicated that higher quenching in the fluorescence intensity reflects a higher adsorption force of protein on the PCL nanoparticles.

4.3. Binding isotherm

The surface properties of protein and nanoparticles are the keypoint in determining the amount of adsorbed protein. Figure 9 clearly shows a high amount of adsorbed protein where the hydrophobic

interactions are dominant. Interaction of insulin at the liquid/solid interface in the presence of the electrostatic forces seems a slow process and is accelerated when high concentration of protein is used.

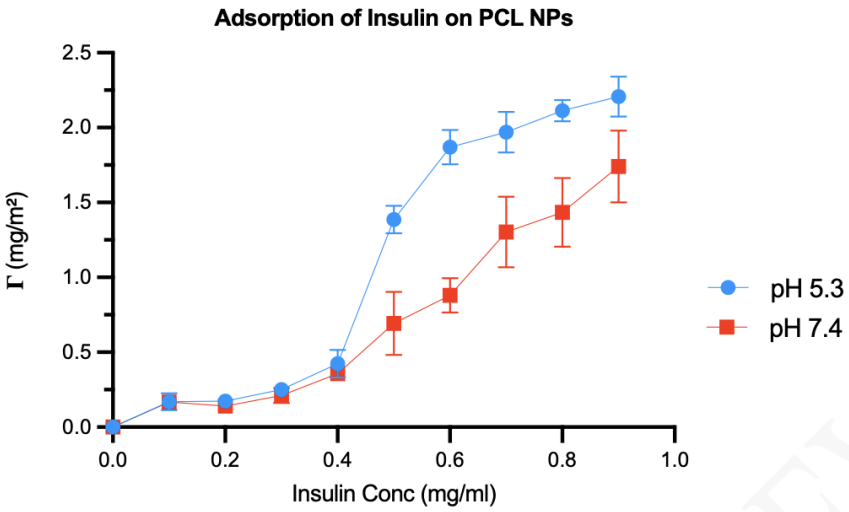

Figure 9: Surface coverage of different concentrations of insulin after adsorption on PCL nanoparticles at pH 5.3 (circle) and pH 7.4 (square).

Adsorption of insulin on the nanoparticles as multilayers attracts other molecules in the solution to bind to the surface and exceeds the full surface coverage quantity. This adsorption mechanism protects the compact structure of insulin (figure 8). Therefore, the insulin/PCL interaction at the high surface coverage is controlled mainly by the adsorbed protein molecules [33]. Even with the presence of repulsive forces at neutral pH, insulin molecules bind to the nanoparticles through the specific binding with the hydrophobic residues on both sides.

4.4. Desorption study

Protein adsorption starts at a fast rate then the process slows down until it reaches a plateau where the desorption mechanism begins. Protein detachment is a slow process that includes several steps followed by transportation into the bulk of solution [34]. Strongly bound protein detaches very slowly due to the repulsive forces that eliminates the hydrophobic interactions. The slightly bound protein detaches rapidly from the surface with almost no structural change.

Figure 10 shows that a significant amount of insulin was detached specially at the plateau values. At pH 7.4, the amount of protein molecules that remain on the substrate are much less. When introducing low concentration of protein in solution, few amounts of insulin transfer to the surface for adsorption. This explains why at low surface coverage it is unlikely for a single protein to be desorbed from the surface after adsorption. A molecule with a higher affinity to the surface needs to be incorporated in the solution to displace the preadsorbed proteins.

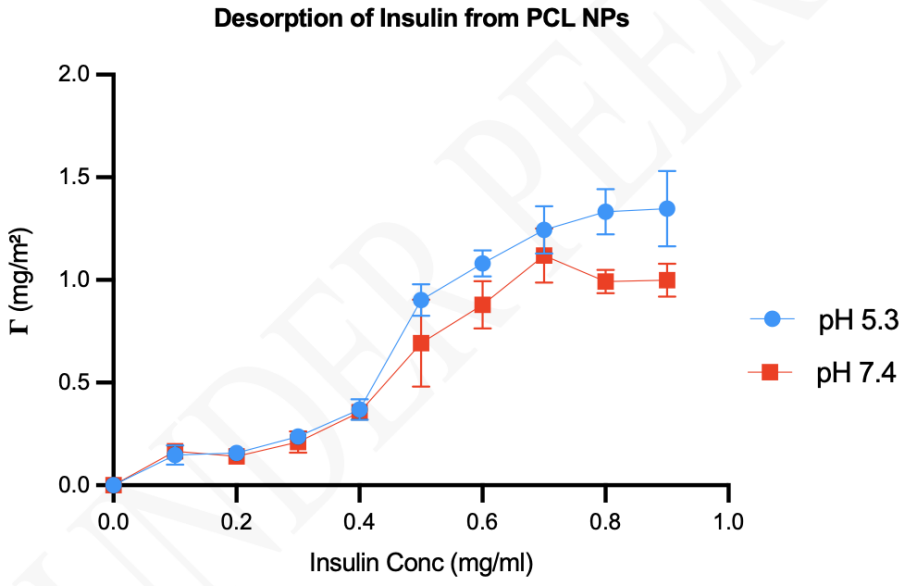

Figure 10: Surface coverage of different concentrations of insulin after desorption from PCL nanoparticles at pH 5.3 (circle) and pH 7.4 (square).

5. Conclusion

Association of insulin with PCL nanoparticles 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 by hydrophobic forces which is accompanied by large structural change especially 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. Minimal or no desorption was seen at low surface coverage upon dilution of the adsorbed insulin. Establishing reversibility is required for all the adsorption processes by evaluating differences in the structure of adsorbed and desorbed proteins. Despite the high PDI after the adsorption mechanism, one of these approaches can be considered for potential intravenous formulations 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.

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