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Research Article

Preparation of a Novel Nano-Formulation to Control Binding of a Recombinant Human Growth Hormone

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Abstract

Due to the instability of most biomolecules during the adsorption process on a hydrophobic polymer, the polymer surface can be modified to suit proteins binding mechanism. Binding of a protein to a biomolecule involves specific interaction domains that require substrate specificity. Isolating such interaction domains and incorporating them into large polypeptide chains can provide protein-binding epitopes on the surface. These exposed epitopes can recognize their partners on the protein surface and might bind with significant strength. A novel r-hGH-binding peptide named Growth Hormone Binding Peptide (GHBpep) encompasses most of the residues in the hot spot of Growth Hormone Binding Protein (GHBP) that provides most of the binding energy with Recombinant Human Growth Hormone (r-hGH). The study involves preparation and characterization of peptide-modified Poly (Lactic-co-Glycolic) Acid (PLGA) nanoparticles by covalently attaching GHBpep to the surface of these nanoparticles using conventional bioconjugation chemistry. Thus, developing a peptide-coated polymer surface may offer a viable system for controlled delivery of protein drugs. The study further explores the adsorption behavior of r-hGH onto these functionalized surfaces. The adsorption with these surfaces was studied under various microenvironmental conditions using traditional methods, such as equilibrium microdialysis, fluorescence spectroscopy and dynamic light scattering while thermodynamics of GHBpep-r-hGH interactions were studied using Isothermal Titration Calorimetry (ITC). The adsorption process was found to be affected by changes in the ionic strength, pH of the medium and length and reactivity of the cross-linker used. The energetics of GHBpep-r-hGH was found to be of high affinity type without major configurationally changes, demonstrating potential for development of controlled delivery dosage form of r-hGH.

Keywords: Poly (lactic-co-glycolic) acid; Nanoparticles; Human growth hormone; Growth hormone binding peptide; Emulsification; Adsorption

Abbreviations

r-hGH: Recombinant Human Growth Hormone; GHBpep: Growth Hormone Binding Peptide; NP: Nanoparticles; PLGA: Poly (Lactic-Co-Glycolic) Acid

Introduction

Most proteins can absorb onto hydrophobic or hydrophilic surfaces which can be useful in the design of sustained-release formulations. A wide variety of drugs can be incorporated into or adsorbed onto the nanoparticles, such as vaccines and biological macromolecules to provide a focused delivery site with minimal side effects [1-3]. Moreover, many studies have shown that proteins adsorption onto hydrophobic polymer surface is irreversible and causes loss of protein structure and subsequently, loss of its biological activity [4]. However, the surface properties of a polymer can be modulated to minimize the nonspecific adsorption by functionalizing it with ligands that possess specific binding to such proteins. Designing a polymer-based system to deliver protein drugs is a promising technique, especially for a labile protein such as Recombinant Human Growth Hormone (r-hGH).

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r-hGH is mainly used as a replacement therapy in treatment of pediatric dwarfism. The treatment requires multiple subcutaneous injections for a long-term period which will affect patient compliance. Therefore, biodegradable polymer is employed to prolong the half-life of this protein drug by encapsulating it into microspheres. Nutropin depot is a long-acting purified formulation of r-hGH that is embedded into Micronized PLAG particles. The drawback of this preparation was the uncontrolled release of r-hGH and the common injection-site reaction [5]. The interaction of protein with the polymer surface may damage its structure and affect its stability. Coating the surface of the polymeric microspheres using a peptide may protect r-hGH structure and prevent non-specific adsorption [6]. It is composed of 191 residues in a single chain from which about half of the polypeptides are in the form of α-helical structure [7].

The three-dimensional structure of the protein can change when it exists in non-physiological media which confounds the understanding of the adsorption process (Figure 1) [8]. When r-hGH adsorb on a hydrophobic surface of a polymer, it leads to a significant change in the protein structure [9,10]. Deformation of the protein structure can be alleviated by modulating the polymer surface with small biomolecules, such as peptides. Moreover, the polymer nanoparticles surface coating has been shown to control the non-specific interaction of proteins and minimize the irreversible adsorption [11,12].

The use of a peptide coating to modify the adsorption behavior of therapeutic proteins is a new field of study. Peptide-coated nanoparticles represent a promising inert carrier for therapeutic proteins [13,14]. Based on databases including peptide-protein compounds, it has been found that small peptides can interact with their partner proteins with high binding energy while causing less conformational changes in the protein [14]. Thus, we intend to functionalize the surface of polymeric nanoparticles using a specific

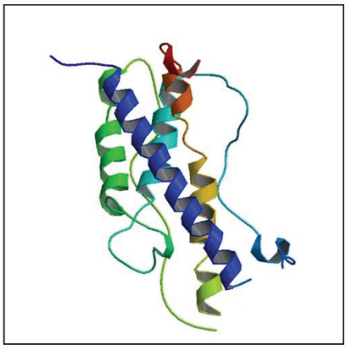

Figure 1: The crystal-structure of wild-type growth-hormone at 2.5 angstrom resolution [36].

ligand called Growth Hormone Binding Peptide (GHBpep) which interacts with r-hGH with high binding affinity.

Based on the ITC data we obtained from the interaction between r-hGH and GHBpep, we hypothesized that adsorption of r-hGH to peptide-coated hydrophobic surfaces will allow high loading amounts of r-hGH, and will reduce denaturation of r-hGH. Our project involved fabricating and modifying the surface of PLGA nanoparticles with GHBpep molecules to maximize the specific binding of r-hGH. This included preparation of PLGA nanoparticles with high amount of functional groups by embedding a stabilizer, such as Poly-Ethylene Maleic Anhydride (PEMA) and Polyethyleneimine (PEI) [11,15]. Once enhancement of the functional groups on nanoparticles surface was achieved, the study further involved preparation and characterization of peptide-modified PLGA nanoparticles by covalently attaching GHBpep on the surface of nanoparticles using various types of cross-linkers. Two monovalent linkers were compared for their efficiency in optimizing the conjugation of ligands. By increasing the ligand binding sites, the amount of r-hGH was increased. The length and chemical reactivity of spacers affected the affinity of the conjugates to r-hGH. The current research was able to explore and estimate the adsorption behavior of r-hGH to the peptide-coated colloidal system.

Materials and Methods

Materials

PLGA copolymer of a molecular weight of 48 kDa, lactide: glycolide ratio of 85:15 (PLGA 85:15), and an inherent viscosity of 0.59 dL/g, was purchased from Alkermes (Medisorb®, Wilmington, OH). r-hGH was obtained from BresaGen, Inc. (Adelaide, Australia.) Poly (ethylene-maleic anhydride) (PEMA, average Mw 100,000-500,000 g/mol) of 1:1 ratio was purchased from Sigma- Aldrich (St. Louis, MO) and hydrolyzed before use. Polyethyleneimine (PEI) (50% w/w) of high molecular weight (W=720,000 g/mol, Wn=60,000) was also purchased from Sigma-Aldrich. GHBpep (M=3296 g/mol), (Figure 2) was designed by AAPPTEC (Louisville, KY). 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide (EDC) and N-Hydroxy Succinimide (NHS) were purchased from Sigma- Aldrich Co. LLC. Disuccinimidyl Suberate (DSS) was purchased from Thermo Scientific Pierce Protein Biology Products (Rockford, IL). Cellulose Ester (CE) dialysis membranes were purchased from SpectraPor. Dichloromethane, 2-(N-morpholino) Ethanesulfonic acid (MES), sodium chloride, sodium azide and sodium hydroxide solution were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO). All dialysis membranes of different Molecular Weight Cut-Offs (MWCO) were purchased from Spectrum® Laboratories (Rancho Dominguez, CA). Ultra filtration tube (Corning®, Spin-XR UF 500) of 100 kDa MCOW) was obtained from Sigma-Aldrich. NanoPure water was purified by reverse osmosis (Barnstead Ultra filtered type I water) and used to make PEMA and buffer solutions and to wash microspheres.

Methods

Preparation and characterization of PLGA nanoparticles: PLGA nanoparticles were fabricated by using emulsification solvent-diffusion techniques which is a modified method described in the literature [11,16]. This method was a combination of different methods to produce nanoparticles of smaller particle size (<300 nm) and a higher density of functional groups. 10 mL of 4% PEMA solution (1% PEI in case of positive PLGA np) was transferred to 20 mL glass vial and placed on a magnetic stirrer at a speed of ~ 1000 Round per minute (rpm) which represented the aqueous phase. Then, 1 mL of PLGA solution (1% w/v) in organic solvent was added drop wise and gently onto the surface of the aqueous phase to form a single emulsion (W/O). The experiment was done using cold nano-pure water to prevent elevation of solvent temperature which may evaporate the organic phase during the emulsification process. Immediately after the mixing step, the emulsion was poured into 50 mL of diluted emulsifier solution (0.03% of PEMA or PEI) and kept under magnetic stirring for three hours to harden the nanoparticles and allow evaporation of the entire organic solvent. The prepared PLGA nanoparticles were collected by employing centrifugation technique using a large-volume centrifuge device (Beckman centrifuge, Model J2-21).

Characterization of nanoparticles:

Particle size: Characterization of particles size was performed by dynamic light scattering technique using Malvern Instrument's Zetasizer Nano ZS (ZEN3600). First, 50 µL of the emulsion was suspended in 950 µL of nano-pure water. The size measurement was performed immediately after 30 minutes of mixing using bath sonicator. The same samples were used to measure the zeta potential of nanoparticles. The intensity of the sample cell was detected using a detector at an angle of 173 °C illuminated by a 633 nm laser. The refractive index and viscosity of the solution were equal to that of water at 25 °C (refractive index: 1.1335, viscosity 1.03 cp). Zetasizer ZS is connected to Malvern computer program that allows computing the Polydispersity Index (PDI) and Z-average of the particles. All measurements were done in triplicate.

Surface morphology: Scanning Electron Microscopy (SEM) analysis was conducted to evaluate morphology of PLGA nanoparticles before and after lyophilization. PLGA nanoparticles were loaded onto aluminum sample stubs with double-sided carbon tape and sputter-coated with gold to capture images by scanning electron microscopy (SEM, Hitachi S-530).

Surface charge density: PLGA nanoparticles prepared using PEMA as surfactant is expected to display high density of negative charges due to carboxyl acid end groups. The number of COOH groups around PLGA nanoparticles can be quantified using conductivity measurement. The technique is based on titration of known concentration and volume of nanoparticles solution with NaOH of known volume and concentration [17]. Briefly, the particles with hydrophobic core were dispersed in aqueous solution and titrated with a known concentration of NaOH. During the titration, the conductivity was measured while the COOH groups were deprotonated by the addition of hydroxide ions. First, the conductivity

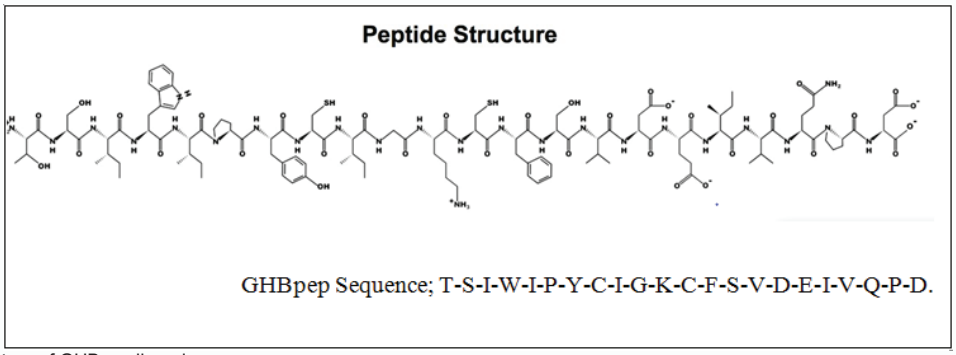

Figure 2: Chemical structure of GHBpep ligand.

decreases due to Deprotonation and neutralization of the COOH groups in solution upon addition of NaOH. Then, the conductivity increases slightly by further addition of the base reflecting the gradual Deprotonation of the carboxylate groups. Finally, the highest positive slope corresponds to end of the reprotonation reaction and presence of excess NaOH in the solution. By fitting the linear segment of the middle and last parts of the curve and extrapolating those lines, equivalent point was obtained at the intersection [17]. The volume of NaOH used is directly proportional to the density of COOH moieties as a result of the concentration of PEMA in the synthesis recipe (i.e., the total content of carboxylate groups can be maximized by applying a high amount of surfactant in the nanoparticles formulation).

The molar ratio of NaOH to carboxylate group is 1:1; therefore, the number of moles of NaOH used reflects the content of carboxylate groups. By determining the total volume and the number of moles of NaOH used, the number of moles of carboxyl acid groups can be calculated. Parameters required for this calculation includes density of the particles, particle size, weight, and the total volume of particles solution which are essential to calculate the number of nanoparticles in the solution. By obtaining the surface area of the spherical particles, the amount of COOH groups on the outer surface can be estimated accurately.

Synthesis of PLGA nanoparticles-GHBpep conjugates: The Ac-TSIWIPYCIGKCFSVDEIVQPD peptide was grafted on the surface of PLGA nanoparticles using either DSS coupling chemistry or two steps carbodiimide chemistry [18,19].

DSS coupling reaction: Six aliquots (1 ml of each) of positively charged nanoparticles suspension were prepared, to which DSS stock solution and then GHBpep solution were added as indicated in Table 1. The desired concentration of DSS molecule in each experiment was calculated based on the amount of GHBpep included. In each experiment, the reaction was allowed to proceed for 30 minutes while shaking at a speed of 200 rpm. A 50 µl volume of the quenching buffer (TRIS final concentration was 50 mM) was added and the mixture was incubated for another 15 minutes. The unreacted species were removed using dialysis by transferring the modified nanoparticles suspension to a 12kDa to 14 kDa MWCO dialysis membranes and dialyzing against 1000 ml of 25 mM sodium phosphate buffer (pH 7.4). The dialysate solution was replaced every two hours. The third dialysis was conducted overnight. On the next day, the nanoparticles suspension was collected and transferred to a glass vial and characterized for the hydrodynamic diameter and surface charges using the Zetasizer Nano ZS.

EDC coupling reaction: Five milligrams of negatively charged nanoparticles in suspension (~0.3 mg/ml in MES buffer at pH of 6.0) was mixed with 19.17 mg of EDC and 15.1 mg of NHS and incubated at room temperature for 15 minutes under gentle stirring to activate the carboxyl groups of PLGA nanoparticles. Then, the unreacted reagents were washed out by dialysis before adding the ligands using a 2,000 Da MWCO dialysis membrane. 0.5 mg of GHBpep ligands was added to covalently bind them to the activated nanoparticles. The resulted GHBpep-PLGA conjugates were dialyzed against two liters of 10 mM of phosphate buffer (pH 7.4) to get rid of the unreacted peptides [20].

Quantification of GHBpep peptide: The amount of GHBpep peptides on the surface of activated PLGA nanoparticles was quantified directly using fluorescence spectroscopy (Tecan i3 fluorescence spectrophotometer). Quartz 96- well plate black, round bottom microplate reader was used to load samples for the experiment. The excitation and emission wavelengths used were 280 nm and 348 nm, respectively. For quantification of GHBpep molecules per nanoparticles, a standard curve of GHBpep concentration in phosphate buffer (pH 7.2) *versus* emission wavelength was created. Then, emission spectrum for known volume of purified peptide-particle conjugates was obtained. The amount of ligands in the peptide-particle conjugates was calculated using the intensity of the emission maxima based on the equation from the standard curve. The amount was then divided by the total surface area of nanoparticles in the sample to calculate the amount of peptide per nanoparticles.

High-throughput equilibrium dialysis: High Throughput (HT) equilibrium dialysis was performed using a 96-well Teflon apparatus manufactured by HT Dialysis, LLC (Gales Ferry, CT) to measure the interaction between the conjugates and r-hGH [21]. The apparatus is made up of Teflon to ensure minimal non-specific binding that is constructed with vertical alignment to allow easy access to the samples [22]. The protein sample of known volume and concentration was loaded into one chamber, and the conjugates sample was loaded into the other chamber. Two chambers were separated by a semi-permeable membrane to allow passage of only the r-hGH molecules (Figure 3). The protein can diffuse freely to the other side of the membrane to interact with the conjugated GHBpep until all binding sites are saturated. At equilibrium, the concentration of protein in the particle-free chamber was quantified to estimate the binding affinity between the reactants.

Briefly, the dialysis membrane was soaked in 1:3 ethanol to nano-pure water for 30 minutes. Each sheet of cut dialysis membrane (MWCO of 100 kDa) was inserted between the Teflon block. The selected MWCO was chosen based on the molecular weight of r-hGH,

Table 1: Actual amount of linker reagents, ligands, and nanoparticles. 1) Plain PLGA nanoparticles, 2) EDC-conjugates, 3) DSS-conjugates. (Supp).

PLGA Nanoparticles characterization				GHBpep	EDC/NHS ratio		Conjugates Characterization	
Batch	Size intensity (nm)	Concentration (mg/ml)	Volume (ml)	Final Concentration (μM)	Concentration (mM)	Volume (ml)	PDI	Size Intensity (nm)
1	189.4	2.5	1	50	0	15	0.23	323
2	199.1	2.5	1	50	4/1	10	0.42	289
3	180.8	2.5	1	50	0.05*	10	0.22	218

*Only DSS was added

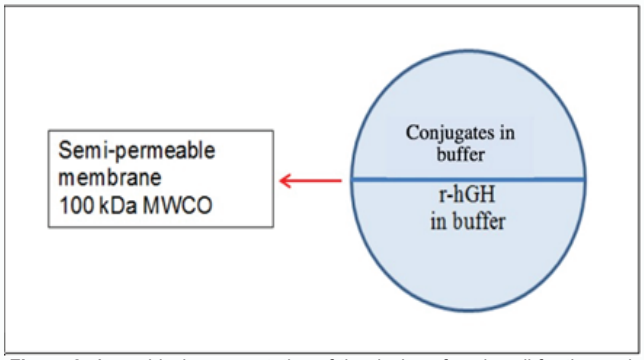

Figure 3: A graphical representation of the design of each well for the equilibrium micro dialysis.

which is 22 kDa. Moreover, the size of all conjugates was big enough to be retained by the selected membrane. Upon assembling the HT equilibrium dialysis plate, 200 μL of the conjugate's suspension was spiked on one side and 200 μL of r-hGH solution was loaded onto the other side of the membrane. The plate was shaken using plate shaker at 25 °C, and the top of the plate was covered with a parafilm. The design of the Teflon plate allowed ease of sampling from each well without disturbing the experimental set up.

Data analysis: At equilibrium, the amount of free protein (C_f) is the same in both compartments. Therefore, 100 μL was removed from the particles-free chamber, and the protein amount was estimated using fluorescence spectroscopy. The concentration of the bound protein (C_b) was calculated from the following equation;

$$C_0 = 2.C_f + C_b \qquad (4)$$

Where (C_0) is the started-protein concentration.

Isothermal titration calorimetry studies: The current studies were applied to estimate the preliminary binding affinity and driving forces of r-hGH interaction with GHBpep [28]. The energetics of binding was directly measured by ITC (Affinity ITC from TA Instruments) to determine the binding constant (K_a), enthalpy changes (ΔH), and stoichiometry number (n). Changes in Gibbs free energy (ΔG) and entropy change (ΔS) were calculated from the following relationship [28]: $\Delta G = \Delta H - T\Delta S = -RTLnK$

Where R is the gas constant and T is the absolute temperature.

Different concentrations of free GHBpep were titrated against known concentration of r-hGH. The experiment cell was filled with known concentrations of r-hGH solution. GHBpep solution was loaded into the syringe (typical concentration of 100 μM to 1000 μM) and injected in aliquots of 2 μL. The stirring rate was kept at 100 rpm. The area under each peak indicates the magnitude of heat that is generated after each addition of ligands which decreases upon each injection. The enthalpy of denaturation was calculated by integrating the area under the peak of each injection. The net thermal baseline was subtracted from the buffer thermal scan. The obtained ITC results were plotted using non-linear least squares fitting. The binding data was fitted with a single identical binding site model using TA NanoAnalyzer software (TA Instruments).

Desorption study: Desorption studies were conducted mainly at pH 7.4 (150 mM buffer) using ultrafiltration technique. The applied volume of the ultrafiltrate was mainly based on the centrifugation force and the centrifugation speed. The desorbed amount was relatively high when the surface coverage of the protein was high. Because EDC-based conjugates adsorbed a large amount of r-hGH, particularly at higher protein concentrations, more than 80% of protein was desorbed. However, the desorption amount of r-hGH from DSS-based conjugates was low at this pH condition.

Fluorescence study of r-hGH conformational transition after desorption: Due to the unstable structure of r-hGH during formulation and storage, it is susceptible to unfolding and aggregation. The structure of r-hGH is held together predominantly by forces such as hydrophobic forces, hydrogen bonding, and electrostatic interactions which are easily disrupted by any small change in the environment, such as a change in the pH, temperature, or interface [23].

r-HGH conations a single tryptophan resides that is buried inside a nonpolar environment. The fluorescence intensity is affected by the local environment, and the hydrophobic interior of r-hGH is consistent with emission maximum. When the tryptophan residue becomes surrounded by water molecules, its emission moves toward the longer wavelength which is called a red shift [24]. Adsorption of r-hGH onto a hydrophobic surface is known to decrease the fluorescence quantum yield. Upon release of r-hGH molecules, the variation in the emission maximum and quantum yield is relative to the three-dimensional structure of the protein [25].

Due to the importance of high quantum yields of the fluorescence intensity, neutral buffer solution (pH 7.2, 10 mM) was used as the medium to measure the emission spectrum of the desorbed protein. The fluorescence spectrum of the protein that is released from both approaches was plotted against the wavelength and compared to the spectra of the protein in solution.

Results

Characterization of nanoparticles

Particle size and zeta potential measurements: It was possible to obtain particle size of less than 200 nm. However, this affected the final yield and also the stability of particles. Table 2 shows the particle size results with unimodal particle size distribution (PDI~<0.2) obtained after optimizing the preparation parameters. Freeze-dried nanoparticles were characterized by a sphere shape with a very smooth surface as depicted by SEM (Figure 4) when the particles were mixed with different concentrations of cryoprotectant. The nanoparticles in the aqueous dehydration images were discrete particles without any aggregation with a diameter of less than 500 nm.

Surface density of carboxylate groups: To improve the coupling efficiency of GHBpep to the surface of PLGA nanoparticles, it was necessary to maximize the number of the activated functional groups to tether the binding peptide. It was possible to control the density of the reactive groups around PLGA nanoparticles by employing a hydrophobic surfactant with a high amount of side groups, such as PEMA. Figure 5 shows the titration of plain PLGA nanoparticles prepared without the use of surfactant, and Figure 6 describes the conductivity titration curve of surface-modified PLGA nanoparticles that are prepared with PEMA. The volume of NaOH used in the surface modified batch was higher in comparison to blank nanoparticles. This reflects the increase in the content of functional groups to provide increased binding sites for the ligands. Table 3 provides calculations for obtaining the density of COOH groups per unit surface area.

Conjugation of GHBpep peptides to PLGA nanoparticles: The results demonstrated that addition of DSS linker led to increase in particle size and the size further increased upon addition of GHBpep peptide. Figure 7 shows two types of PLGA nanoparticles, positively and negatively-charged nanoparticles. The change in particle size and surface charge occurs with each addition step. Because GHBpep molecules are negative in all types of buffer used, the final zeta potential should indicate only negative value to prove the successful coupling reaction. The increase in the particle size after addition of the linker was significant with DSS but not with EDC. However, similar increase in particle size was observed upon conjugation of GHBpep to the cationic and anionic particles. The amount of peptide attached was extremely high while using EDC chemistry for bioconjugation (Table 4). The direct binding of GHBpep ligands to the PLGA surface seemed easier compared to that to the tethered DSS linker. Amount of peptide bound to the polymer surface was much lower in absence of cross-linker (plain PLGA nanoparticles). This indicates that most of the attached peptide on the surface can be achieved only by covalent binding rather than by physical binding.

Interaction between r-hGH and GHBpep: The ITC studies were performed to evaluate the forces involved in the binding affinity of r-hGH molecules to the GHBpep ligands. The results demonstrated that the reaction between GHBpep and r-hGH was exothermic. With addition of each injection of ligand to the protein solution, the magnitude of heat decreased gradually until a constant enthalpy was obtained. This indicated the saturation of all binding sites and the heat generated after the saturation was the heat of dilution. The ITC results in Figure 8 consist of upper and lower panels. The upper panel

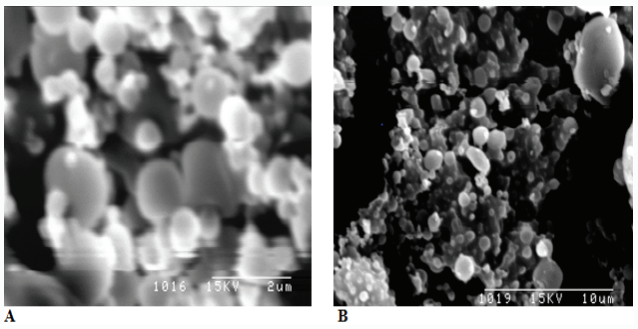

Figure 4: Morphology of nanoparticles determined by SEM. A-B after lyophilization using 5% sucrose as cryoprotectant.

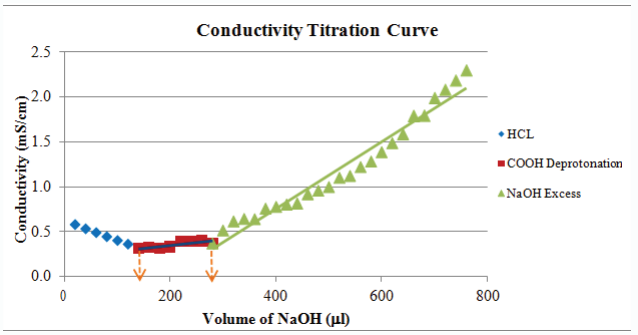

Figure 5: Estimation of the density of the carboxyl acid groups on plain PLGA nanoparticles using conductivity titration method.

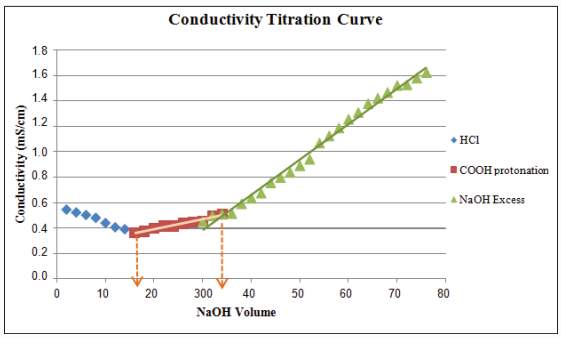

Figure 6: Estimation of the density of the carboxyl acid groups on PLGA nanoparticles with high amount of PEMA on the surface using conductivity titration method.

Table 2: The physical properties of PLGA nanoparticles prepared under different experimental conditions.

Batch	PLGA Polymer	Organic solvent composition	Surfactant (w/v)	Concentration of PLGA (mg/ml)	Stirring speed (rpm)	PSD (nm)	PDI	ZP (mV)
5	50:50:00	Acetone: EtOH (85:15)	PEMA 0.8%	10	1000	187 (15.8) *	0.202	-42.3
9	85:15:00	Acetone: EtOH (85:15)	PEI 1%	10	1200	264 (8.9) *	0.255	39.2

*mean (SD)

Table 3: Carboxylic acid group's density on designated batches estimated by conductivity titration method.

Batch Number	Particles size (nm)	Concentration (mg/ml)	Mole of NaOH Used	No. of COOH/particle	No. of COOH/cm²
1	125	5	2.2×10^{-6}	3.63×10^{5}	7.4×10^{14}
4	230	5	1.78×10^{-6}	1.83×10^{6}	1.12×10^{15}
7	360	10	1.4×10^{-6}	2.76×10^{6}	1.014×10^{15}

Table 4: Surface coverage of GHBpep on PLGA nanoparticles. 1) EDC-conjugates, 2) DSS-conjugates, 3) Plain PLGA nanoparticles.

Modified-PLGA Nanoparticles			Amount of Peptide Used	Surface Coverage (Γ)			% Recovery
Batch	Intensity PSD (nm)	PDI	µg/mL†	SA (m²)	µg/mL	mg/m²	%
1	384 ± 21	0.23	50	0.972	11.27	115.9	22.5
2	817 ± 15	0.54	50	0.573	2.301	40.2	4.6
3	426 ± 45	0.19	50	0.497	1.17	14.06	2.3

†Final concentration

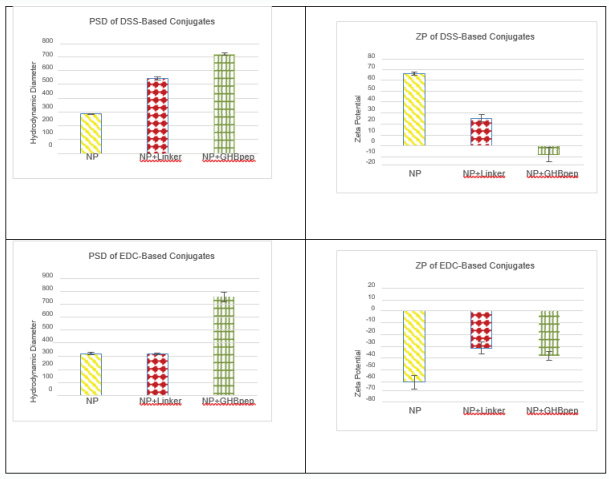

Figure 7: Particle size and zeta potential comparison of different linker-based conjugates (pH 7.2, 10 mM). A) Represents the size and zeta potential characterization of positively-charged PLGA np. B) Represents the size and zeta potential characterization of negatively-charged PLGA np.

demonstrates the raw data of bonding enthalpy after each injection. The area under each peak represents the amount of heat released upon binding of ligands to the protein. All points were separated by specific time intervals and were corrected for the heat of dilution. The lower panel corresponds to the binding isotherm of the whole binding process after integration using a suitable model of interaction.

Preparation of protein solution and particles suspension: Protein stock solution was prepared fresh just before the adsorption experiment. The lyophilized protein was reconstituted to a concentration of 1 mg/ml using nano-pure water. The obtained protein solution was dialyzed against an appropriate buffer, 1000 times more that the volume of the sample using 3500 Da MWCO regenerated cellulose dialysis membrane. The dialysis was continued for two hours with changing the buffer three times to completely remove the sugar content (lactose and trehalose) in the protein solution. Then, the protein solution was filtered using 0.22 µm to remove any protein aggregate.

Modified PLGA nanoparticles were prepared at a concentration of 1 mg/ml. Before running the adsorption study, the protein solution and particles suspension were equilibrated using the designated buffer solution using dialysis method. Both solutions were dialyzed for two hours, then the protein concentration was determined in a Shimadzu UV-1601 Spectrophotometer using the molar absorptivity of r-hGH at 277 nm (A277 nm=0.93 at 1 cm, 0.100%). The working concentrations of the protein were adjusted in the range of 0.002 mg/ml to 0.066 mg/ml (i.e. 0.09 µM to 3 µM). The particle concentration was used without further dilution for all adsorption studies.

Adsorption study using equilibrium dialysis: The binding isotherms of r-hGH molecules on different batches of conjugates are demonstrated in Figures 9 and 10. They present the surface coverage of r-hGH onto conjugates in different media in comparison to adsorption behavior of r-hGH onto plain nanoparticles. The percent adsorption of r-hGH was normalized to the surface coverage, and plotted against the initial concentration of the protein. The pH values were selected to vary the charge status on the r-hGH molecules and the conjugates in order to study different types of interactions [26].

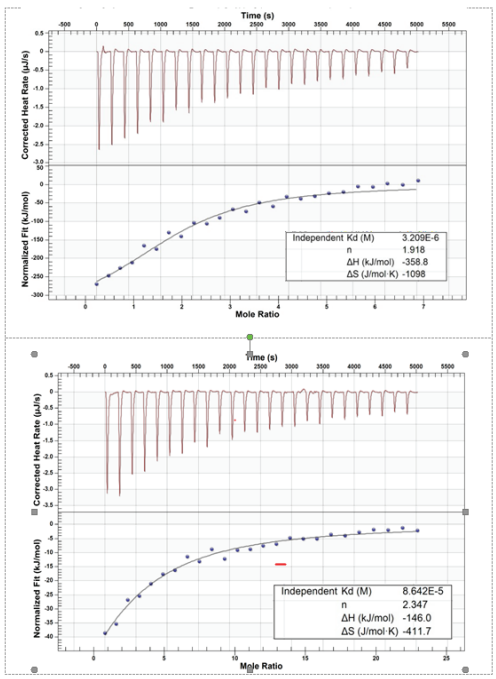

Figure 8: ITC data of GHBpep (0.5 mM) and r-hGH (5 µM) (0.5 mM) (upper), and r- hGH (15 µM) (lower).

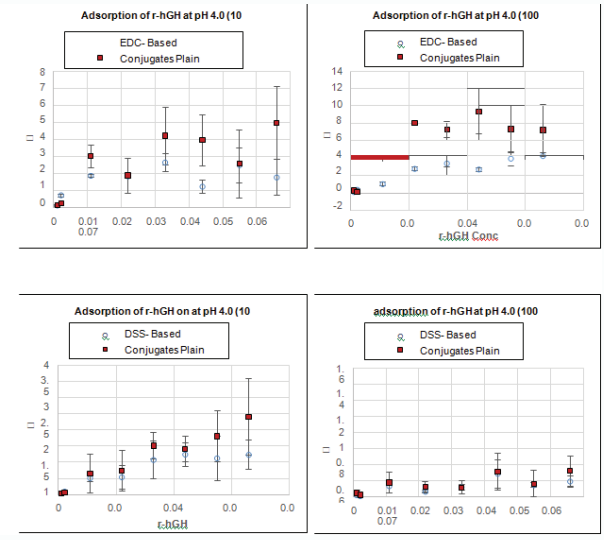

Figure 9: Surface coverage of r-hGH on conjugates (circle) and unmodified particles (squares) at pH 4.0 of different ionic strength. The upper graphs are for EDC conjugates and the lower graphs are for DSS conjugates.

At pH 4.0, higher surface coverage was noted for EDC based conjugates compared to DSS based conjugates while the surface coverage was higher for plain nanoparticles compared to both the conjugates irrespective of the ionic strength of the media. For EDC based conjugates, the surface coverage was higher at higher ionic strength while that was higher at lower ionic strength for DSS based conjugates. In case of DSS based conjugates, the amount of r-hGH adsorbed was lower than the full surface coverage on the EDC-based conjugates.

At pH 5.3 and 7.2, higher surface coverage of protein onto EDC based conjugates compared to DSS based conjugates was observed in

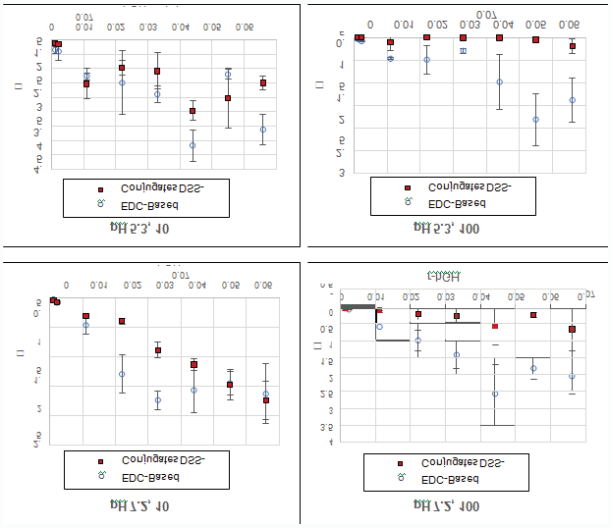

Figure 10: Surface coverage of r-hGH on EDC-based conjugates (closed circle) and DSS-based conjugates (closed square). The upper graphs demonstrate the adsorption at pH 7.2 of different ionic strength. The lower graphs demonstrate the adsorption at pH 5.3 of different ionic strength.

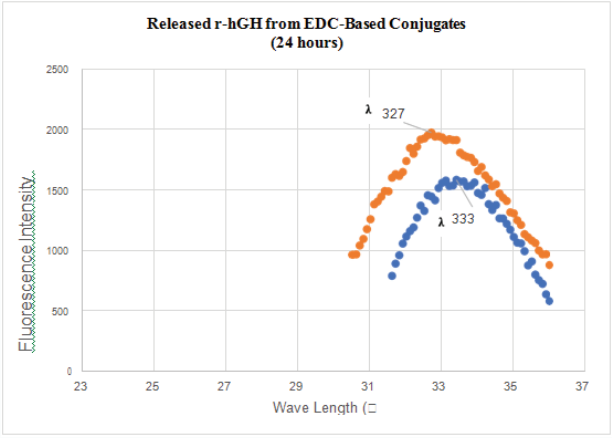

Figure 11: The fluorescence spectra of released r-hGH from the EDC-based conjugates and in solution. The measurement was taken after 24 hours release time-frame.

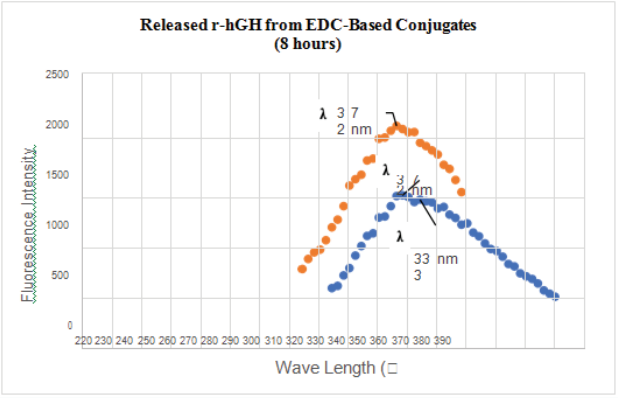

Figure 12: The fluorescence spectra of released r-hGH from the EDC-based conjugates and in solution. The measurement was taken after 8 hours release time-frame.

both 10 mM and 100 mM ionic strength buffer media. Both conjugates adsorbed a significant amount of protein in low ionic strength media of pH 5.3.

Discussion

Preparation and Characterization of nanoparticles

Multiple methods can be applied to prepare PLGA nanoparticles [27], and emulsification solvent- diffusion is most common one. The single oil in water emulsion (O/W) was prepared and the nano-droplets were formed by adding the organic phase drop wise to the aqueous phase under a high speed of stirring (~1000 rpm). Slow addition of the organic phase into the aqueous phase under a very high speed of constant stirring, resulted in extreme size-reduction of the nano-droplets. Use of mixture of suitable organic solvents causes interfacial turbulence between the solvent molecules due to the spontaneous diffusion of the organic phase [16]. One of these organic solvents is a highly water-miscible (ethanol 95% v/v) that allows its quick diffusion from the mixture. This phenomenon causes a reduction in the particle size as a result of decrease in interfacial tension between the emulsion phases [12].

Effect of surfactant concentration on the nanoparticle's properties

A satisfactory yield of nanoparticles as a function of polymer concentration was obtained even at low concentration of PLGA. This was certainly because the viscosity of the solution resulted in good dispersibility of PLGA in the aqueous phase [28]. Using PEMA as a surfactant stabilizes the organic droplets and coats the particles by embedding more carboxyl acid groups leading to high repulsive forces among nanoparticles. However, the final concentration of PEMA in the emulsion at the time of nanoparticles preparation could affect their size as well as the zeta potential. Kwon et al. [29] found the higher concentration of stabilizers used, the smaller particle size obtained. When the applied concentration of the cationic Didodecyl dimethyl Ammonium Bromide (DMAB) surfactant was 2%, the mean particle size reduced to 76 nm. With a higher concentration of DMAB molecules (4%), a slight reduction in the particle size was observed. DMAB surfactant causes a considerable decrease in the surface tension [29]. Therefore, using a higher concentration of surfactant will decrease the size of nanoparticles and improve the zeta potential. However, applying a low concentration of surfactant minimizes the density of functional groups and provides unstable nanoparticles system. The nanoparticles exhibited a larger mean particle size upon reconstitution after lyophilization measured by Zetasizer ZS. The reason might be the swelling of the particles in aqueous dispersion [12].

PLGA nanoparticle-GHBpep conjugation

Crosslinking GHBpep peptides to PLGA nanoparticles using DSS linker: PLGA nanoparticles with amino groups on surface were obtained by applying PEI as a stabilizer. The coupling reactions using DSS monovalent linker comprised of one additional step. The GHBpep molecule has a lysine (K) residue at position number 11, and it is available as an anchor for DSS reagent. In each aliquot, the DSS linkers and GHBpep molecules were added to the nanoparticle's suspensions simultaneously, while maintaining the total volume of the solution to 1 ml. The NHS ester groups react selectively and efficiently with the primary amino groups on molecules, PLGA nanoparticles and GHBpep molecules, because they only attack the amino groups [30]. The molar ratio of the linker and biomolecules were optimized

by applying an excess amount of DSS molecules. The concentration of 10 mM of DSS was the optimum ratio (1:400) to minimize the growth of mean particle size. Moreover, the different molar ratio had an impact on the zeta potential of the intermediate and final products of the conjugation reaction. The NHS moieties of DSS decreased the charge density on particles which rendered the surface unstable. When employing a high concentration of DSS linker, the observed amount of bound ligands was high compared to the low ratio of the linker to GHBpep. Zeta potential value of the intermediate product and the final conjugates and the change in particle charges reflected the density of activated groups and attached GHBpep molecules onto the surface, respectively. For example, the zeta potential of the DSS linker-based conjugates switched from positive (60 mV) to negative value (-17 mV) due to the amount of GHBpep on the outmost surface (Figure 7).

Crosslinking GHBpep peptides to PLGA nanoparticles using carbodiimide chemistry

EDC is not an efficient reagent in bioconjugation when it is used alone because of the production of Oacylisourea ester, an unstable intermediate which undergoes fast hydrolysis to regenerate carboxyl acid group. The bioconjugation reaction using EDC chemistry was performed according to the two-step mechanism. The COOH groups of the nanoparticles were first activated with EDC/NHS reagents in MES buffer (pH=6.0) before reacting with the GHBpep ligands. The GHBpep ligands were added in the second step after purifying the COOH activated particles from any unreacted species. The lifetime of NHS-esters on the COOH groups determines the coupling efficiency. 15 minutes and two-hours of activation and coupling reactions, respectively; resulted in the highest amount of ligands on nanoparticles. The mixture of EDC and NHS reagents was maintained at a ratio of 4:1 based on a study in the literature [31]. For EDC, 6 molecules are equivalent to 6 COOH groups; however, 1.5 molecules of NHS reagent are equivalent to COOH groups. The final volume was kept at 1 mL using MES buffer. As a zero-length linker, EDC/NHS linkers were not part of the final conjugates. The amino groups of GHBpep molecules attached directly to the activated carboxylate groups of nanoparticles to form amide bonds. Therefore, these types of linkers are not aimed to provide some form of handles to attach ligands.

The increase in particles size after addition of ligand molecules corresponds to the presence of intermolecular Crosslinking. The structure of the GHBpep ligands contains many hydrophobic moieties which may adsorb on the hydrophobic surface of the particles through hydrophobic forces. Accumulation of the ligands on the surface through the non-specific binding may attract other GHBpep molecules especially upon breaking the disulfide bridge which may lead to exposure of their hydrophobic parts. Another factor for susceptibility of aggregation is the presence of neutral NHS esters that lead to a neutral surface of particles and diminish the electrostatic forces. The tendency of adjacent particles to agglomerate will be higher in this case, and aggregation may happen even before the addition of GHBpep ligands to the reaction medium.

Quantification of GHBpep peptide on nanoparticles surface

Highly sensitive quantification of the attached peptides on the nanoformulation was achieved without structural modification or tagging. Fluorescence spectroscopy is a robust technique to quantify the concentration of biomolecules containing fluorophore moieties. GHBpep molecules contain aromatic amino acids that emit fluorescence upon excitation. Unlike traditional proteins quantification method, such as BCA protein assay, fluorescence spectroscopy allows direct and fast analysis of intact biomolecules [26]. However, peptide quantification with this technique can be difficult due to the signal-to-noise ratio. Therefore, quantification with fluorescence spectroscopy requires the use of standards of GHBpep molecules during each analysis.

There are many factors that influence the surface coverage of GHBpep on the PLGA nanoparticles. The size and biomolecule concentration have been evaluated to determine the maximum loading condition. Increase in the concentration of the binding peptide in the conjugation reaction resulted in a corresponding increase in the amount of bound GHBpep molecules on the nanoparticles (Table 4). The increase in the fluorescence intensity with the highest GHBpep concentration may be indicative of the unsaturation of the binding sites in PLGA nanoparticles which could allow further peptide attachment.

Interaction between r-hGH- GHBpep peptide

The interaction of GHBpep ligands with r-hGH molecules was found to be exothermic under pH 7.2 condition. Hydrogen bonding and vander Waals' interaction, which are a consequence of the hydrophobic forces, can be contributing factors of the negative values of ΔH and ΔS [32]. They play a major role in the stability of protein association complexes. We found that thermodynamic parameter, ΔG, is mainly of negative sign. The large negative effect of ΔH and ΔS indicated that the type of interaction is due to non-classical hydrophobic binding [33]. Moreover, the magnitude of enthalpy is much higher than that of ΔS, which indicated the association between the binding peptides and r-hGH molecules was enthalpy driven. Observed value of $\Delta G<0$ is indicative of spontaneous association between the biomolecules. The large net value of ΔG is due to the tremendous negative value of ΔH as a result of extreme ligands polarization. The change in free energy (ΔG) for the binding was found to be approximately similar for different concentration ratios studied. Unfortunately, we couldn't perform the ITC experiment on the peptide-modified PLGA np because of the instability issues. The degradation profile of the chosen PLGA was less than three months. The presence of monomers in the particle suspension, which occurred immediately after reconstituting PLGA np with aqueous solutions, interfered with the r-hGH binding to the conjugates and produced data that could not be interpreted correctly (not shown).

Study of r-hGH adsorption using equilibrium dialysis

The magnitude of surface coverage of GHBpep played an important role on the dominant type of interaction. Proteins are more prone to structural change at low surface coverage where the adsorption is primarily controlled by protein-sorbent interactions. However, at high surface coverage, the interaction is dominant mainly by the adsorbed protein molecules [34]. The stability of the native conformation of globular proteins can be manipulated by changing the microenvironment condition, such as pH and ionic strength of buffer solution. At the isoelectric point of r-hGH, the protein molecules exist relatively in a compact structure where the maximum protein adsorption occurs on hydrophobic surfaces. The length of the linker acts as a barrier for r-hGH to adsorb on the hydrophobic surface of the nanoparticles. Theoretically, the hydrophobic arm of DSS linker may interact with r-hGH. This type of binding is considered non-specific binding of r-hGH molecules to the conjugates. Therefore, the

adsorption mechanism was conducted at different pH value to vary the charge on the protein.

At all pH conditions, the ligands possess overall negative charges which may play a crucial role in either reducing or enhancing their interaction with the r-hGH molecules. Furthermore, at low ionic strength, complete surface coverage was seen only with the plain nanoparticles. The electrostatic repulsive forces between r-hGH and GHBpep molecules exerted some effects on the amount of protein on the surface. This holds true for the firm hydrophobic surface of plain nanoparticles that might cause protein denaturation and attract more protein on the denatured parts. Studies were done in our lab to investigate the structural change upon adsorption on plain nanoparticles using Circular Dichroism spectroscopy (CD) [26]. Based on the CD spectra scans at pH 7.2, the secondary structure remained almost stable at all the full surface coverage conditions. However, the CD data showed a loss in the α-helix structure at low surface coverage. This is consistent with other finding of rhGH adsorption behavior onto different hydrophobic sorbent obtained by using Total Internal Reflection Fluorescence (TIRF) [23].

In low ionic strength, EDC-based conjugates adsorbed low amount of r-hGH which is relatively similar to that of the plain nanoparticles. Overall, the content of the GHBpep ligand on the conjugates contributed favorably to control the amount of reversibly adsorbed protein. The adsorption of r-hGH on the conjugates was different at pH 5.3 and 7.2. The amount of r-hGH was high at low ionic strength of pH 5.3. This explained the role of the binding peptide in improving the amount of adsorbed protein. It was noticed that EDC-based conjugates contained the largest amount of GHBpep. Therefore, upon interaction of r-hGH with these conjugates, GHBpep provided a controlled binding mechanism.

Figures 11 and 12 show the fluorescence intensity of released r-hGH from EDC-based conjugates after 8 and 24 hours, respectively. The intensity of r-hGH in solution is used as a reference irrespective to the protein concentration. It is well known that r-hGH in solution revealed an emission maximum at wavelength of 327 nm [35]. Desorption of protein is a slow process consists of many steps including relaxation and detachment of the protein followed by transportation of the detached protein into the bulk solution. After eight hours of protein desorption process, the slightly bound protein detaches quickly from the hydrophobic surface with almost no structural change. The slight conformational change could be due to the specific interaction of r-hGH molecules with the GHBpep ligands. Then, the slowly released molecules undergo some extent of structural change due to the strong adsorption to the surface. Modifying the three-dimensional design allows polar molecules accessibility and formation of hydrogen bonds with the hydrophobic interior. This process was indicative by a change in the emission maximum.

There is a low chance for the tightly bound protein molecules to be released back into the solution. The structural change in the protein was significant when the protein adsorbed on a low surface load of ligands, which is the case with DSS-based conjugates. r-hGH molecules interacted not only with the covalently-attached ligands but also with the hydrophobic part of nanoparticles surface.

This caused extreme alterations in structural orientation and a corresponding shift in the emission maximum.

Conclusion

It was possible to conjugate GHBpep ligands to modified surface of nanoparticles using two different types of linkers, EDC and DDS. Overall, the adsorption was favorable with EDC linker- based conjugates with respect to amount adsorbed and structural stability of r-hGH under pH 7.2 and 5.3. If desorption studies are performed on these conjugates at physiological pH, these can prove to be critical path for development of sustained delivery of r-hGH. Use of other linkers can also be explored to achieve enhanced loading efficiency of biomolecules, GHBpep and r-hGH.

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