

# Subcutaneously Delivered Nano-Insulin Drug as Label Free Nanotheranostic

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## Research Article

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# Abstract

In this study, it has aimed to understand the relationship between purified insulin and insulin receptor, also nanoinsulin and insulin receptors. The insulin receptor has separated from rat liver using a cryogel column material that is photosensitively insulin cross-linked in the Fast Protein Liquid Chromatography (FPLC) system based on the affinity between insulin and insulin receptor. In the second step, an isolated insulin receptor has used to synthesize insulin receptor cross-linked cryogels for purifying insulin from rats. Subcutaneously delivered nano-insulin drug has prepared from the purified insulin using **Amino Acid (monomer) Decorated and Light Underpinning Conjugation Approach (ANADOLUCA)** method. Lastly, Reflectometric Interference Spectroscopy (RIfS) study has performed to understand the interaction between purified insulin receptor and purified insulin, commercial insulin analog, and nano insulin. These studies have demonstrated that nano-insulin drugs can be effectively used as a theranostic platform to monitor affinity and blocking interactions of nanoprotein drug and its receptor.

## 1. Introduction

Insulin is an influential hormone that regulates the uptaken and storing of carbohydrates, fatty acids, amino acids in the form of protein, fat, and glycogen. Its main mechanism is carbohydrate and fat in the body. Localization of insulin receptor (IR) is important for starting the insüline activity at the cellular level. IR is the tyrosine kinase receptor family into plasma membranes. Mature peptide in pancreas  $\beta$  cells has been synthesized and IR starts the process after this organization [1–3].

High or low-affinity binding sites could be indicated according to kinetic and equilibrium binding studies between insulin and its receptor, IR. High-affinity interactions between insulin and IR depends on the dimeric structure of IR. Two receptor binding sites of insulin provide a bivalent interaction with the IR [4]. On the other hand, synthesized insulin-conjugated GQDs could be used as a specific marker for IRs in 3T3-L1 adipocytes [5].

Nanomaterials that their size and shape are defined nanomaterials functions are very important for new applications such as treatment, detection [6, 7]. They are used for diagnosis and treatment at nanomedicine with a specific route of administration like oral, intravascular, pulmonary, or subcutaneously [8]. There are several studies about insulin detection with nanoparticles. especially metal nanoparticles are very useful detection insulin. Also, Au nano particles have been used for biorecognition. The administration way is determined according to the most safe and effective delivery for nano drugs [9–11]. Subcutaneous injection has been broadly preferred as a delivery way of drug formulations, especially for diabetes treatment because, subcutaneous injection is a great way to bypass low oral bioavailability [12, 13]. Over the years, many studies have been done to replace subcutaneous injection due to a bit painful way, to oral route for insulin. However, oral administration causes protein degradation and decrease the effectiveness of insulin preperats. Therefore, subcutaneous injection still remains a priority way for the treatment of diabetes, and new drug formulation as a nano-drug carrier is being delivered by subcutaneously [14].

Several types of nanodevices or strategies have been developed as drug delivery systems so far. Nano-drug structure, in general, protects drug degradation, provides increased drug absorption, and alter drug tissue distribution [15, 16]. One of the advantages of using nano-drug particles is the basic chemical structure because they don't include any additives or excipients [17]. Liposomes, proteins and antibodies, micelles, etc. are widely used as nano-drug carriers and the potential of those developed nano-drug carriers are investigated both in patients and animals. Types of clinically well-understood nano-drug carrier materials are liposomes, polymers, micelles, proteins, and antibodies. These nano-drug formulations have been investigated and modified both in animal models and in patients [18]. Polymer-based nanoparticle delivery strategies, polymer therapeutics, lipid-based nano-drug delivery systems are common techniques among nano-drug carrier formulations [19]. Some features such as good stability in storage and in vivo, non-antigenicity, non-toxicity, biodegradability, and metabolizability make protein-based nanoparticles especially useful materials among several drug carrier systems [20, 21]. Nano albumin particles have great properties for drug delivery. They include a significant amount of drug into the matrix of nanoalbumin matrix, since albumin molecule has different drug binding sides [22].

Nano-albumin drug carriers are very well for lipophilic drugs applications. Therefore, albumin-based nanoparticles provide transportation of hydrophobic drugs without toxic solvent in the body [23]. For the drug formulation process, micro and nanoencapsulation techniques are attractive. These techniques have some advantages such as site-specific drug delivery, controlled release, minimizing side effects, and protecting sensitive drugs [24]. Transport of monomers, free radicals, surfactants, and inhibitors in a heterogeneous system between organic and aqueous phases is carried out by microemulsion polymerization technique [25]. Particle nucleation, adsorption, and desorption of radicals and monomer partitioning between the phases provided the control of the polymerization rate. Stability of particles affected by the pH of the medium and type of the surfactant [26]. AmiNoAcid (monomer) Decorated and Light Underpinning Conjugation Approach (ANADOLUCA) provides a new effective perspective in complex heterogeneous processes, microemulsion polymerization, using photosensitive cross-linking strategies. According to the ANADOLUCA method, nanostructured proteins are synthesized through photo-electron transfer using ruthenium-based amino acid monomers in microemulsion polymerization media [27–30].

Since supermacroporous cryogels have excellent heterogenous-open porous systems, there is a considerable interest in this type of cryogel [31]. Cryogel preparation is carried out at subzero. During the polymerization which occurs in the thawed solution, freezed solvent crystals result in forming interconnected large macropores. Water and water-soluble substances are often selected in cryogelation reactions. In addition, water crystal molecules generate pores and flow channels randomly in macroporous and sponge-like polymeric structure [32].

The aim of this study is to investigate the interaction of subcutaneously deliverable nano-insulin drugs and insulin receptors. For this purpose, the insulin receptor from rat liver was purified using Fast Protein Liquid Chromatography (FPLC), and then purified insulin receptor was used to separate insulin from rat serum. After purification steps, subcutaneously nano-insulin drugs as diagnostics were prepared from the

purified insulin using the ANADOLUCA method. We monitored affinity assays of the nano-insulin drugs by chromatography, Reflectometric Interference Spectroscopy (RIfS) detection, and sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Western Blot analysis.

## 2. Materials And Methods

Detemir was purchased from Novo Nordisk (Copenhagen, Denmark). Hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EDMA), N,N,N,N-tetramethylene diamine (TEMED), ammonium persulfate (APS) and N-N' methylene bis acrylamide (MBAAm) were supplied from Fluka A.G. (Buchs, Switzerland). HEMA and EDMA were distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4°C until use. Polyvinyl alcohol (PVA) was bought from Sigma. Ruthenium based amino acid monomer, bis (2-2'-bipyridyl) - methacryloyl tyrosine - methacryloyl tyrosine ruthenium(II), (MATyr-Ru(bipyridyl)<sub>2</sub>-MATyr), was synthesized according to patented ANADOLUCA procedure [27].

A piece of the dried cryogels was placed on a Scanning Electron Microscopy (SEM) (FEI Quanta 200 FEG, Oregon USA). All chromatographic separations were performed by AKTA-FPLC (Amersham Bioscience Uppsala, Sweden) system equipped with a UV detection system. M-925 mixer, P-920 pump, UPC-900 monitor INV-907 injection valve, and Frac920 fraction collector were the system equipments. Both isolation analyses were performed using a linear gradient at a flow rate of 1.0 mLmin<sup>-1</sup>. The average particle size and size distribution of nano insulin were determined by Zeta Sizer (Malvern Instruments, Model 3000 HSA, England) and an FEI 120kV transmission electron microscope (TEM). Fluorescence feature of nano-insulin was determined by Varian Cary Eclipse, Fluorescence Spectrometer. The RIfS sensors (Analytik Jena, BIAffinity, Jena, Germany) were used for real-time detection of isolated insulin from rats by attaching to the RIfS system. In SDS-PAGE and Western Blot analysis, insulin detemir and nano insulin detemir were used to determine the molecular weight and purity of purified insulin.

### 2.1. Animal Study

Ten male 12-week-old Wistar rats, weighing approximately 300 g, were individually housed in a temperature and -humidity-controlled room under a 12 h light/dark regimen. The food and water were provided ad libitum. At the end of the study, intraperitoneal injection of thiopental was applied for anesthesia of the rats and the rats were euthanized by total blood collection. The local ethics committee for animal care and use approved the study protocol.

### 2.2. Blood and Tissue sampling

Blood samples were collected with a cardiac puncture. Na-EDTA was used for coagulation of intracardiac blood samples and intracardiac blood samples were centrifuged at 1500 x g for 10 min. The serum samples were used for insulin purification and the liver tissue was stored at -70°C and used for insulin receptor isolation.

### 2.3. Isolation of Insulin Receptor from Rat Liver Tissue

The insulin receptor was isolated from rat liver according to the study of Cuatrecasas [33] with some modifications. Briefly, the liver tissue was homogenized with Janke & Kunkel, Ultra-Turrax T25 in ice-cold 0.25 M sucrose, and the homogenate was centrifuged at 600 x g for 10 min.

## 2.4. Preparations of Insulin Receptor and Insulin Cross-Linked Cryogels

For the synthesis of the insulin receptor, cross-linked cryogel was prepared using the ANADOLUCA method based on photosensitively cross-linking and conjugation approach. On the other hand, the main monomer was HEMA due to its mechanical strength, blood compatibility, inertness, and stability. HEMA based cryogel was synthesized via free-radical cryopolymerization technique. The monomer (HEMA, 300  $\mu$ L) and the crosslinker (MBAAm, 56.6 mg) were dissolved in 4.74 mL of deionized water. Any dissolved oxygen was eliminated with degassing the mixture under vacuum for 5 min. The total monomer concentration was 7.1 % (w/v) in cryogel solution. Then, 100  $\mu$ L of ruthenium-based amino acid monomer, MATyr-Ru(bipy)<sub>2</sub>-MATyr, and 25 mg insulin receptor together were prepared and added into the cryogel mixture. The free-radical polymerization process was applied for the preparation of cryogel using TEMED and APS as initiator solution pairs. After the addition of APS (4 mg, 1 % (w/v) of the total monomers) the cryogel mixture was cooled in an ice bath for 12–13 min. After the addition of TEMED (5  $\mu$ L, 1 % (w/v) of the total monomers), the mixture was stirred for 3 min. The cryogel mixture was poured into a plastic syringe (5 mL, id. 0.8 cm, bottom closed outlet). The polymerization solution was frozen at – 12°C for 24 h and thawed to room temperature. 200 mL of deionized water was used to wash the cryogel. The cryogel was stored in 0.02 % sodium azide solution was used to store at 4°C until use.

The surface morphology of cryogels was investigated by SEM. Firstly, the cryogels were dried at room temperature. A piece of the dried cryogels was stuck on the SEM sample holder and coated with gold for 2 min before analysis. Then, the sample was placed in SEM.

## 2.5. Purification of Insulin and Insulin Receptor via Fast Protein Liquid Chromatography

Insulin receptor cross-linked cryogel was used for the purification of insulin from rats. In this process, A buffer (0.1 M ammonium acetate, pH: 6.0) and B buffer (1 M ammonium acetate, pH: 6.3) were used as FPLC mobile phases. 100 % of mobile phase A was passed through the column for 4 min for the starting period. Then, linear-gradient started with 0 % B and reached 100% B in 8 min. 100 % B was passed for 6 min, finally 100 % A was passed for the last 12 min through the column. For the purification of the insulin receptor, insulin cross-linked cryogel was used as a column material in the FPLC system. FPLC mobile phases were buffer A (0.1 M ammonium acetate, pH: 6.0) and buffer B (1 M ammonium acetate, pH: 6.3). After 1 min starting period with 100 % of mobile phase A, a linear gradient started with 0 % and reached 100 % B in 8 min, continued with 100 % B for 6 min, and finished with 100 % A for the last 26 min. Chromatographic separation took place at room temperature and all buffers were filtered before use.

## 2.6. Synthesis of Nano-insulin Drug as Labelfree Theranostic

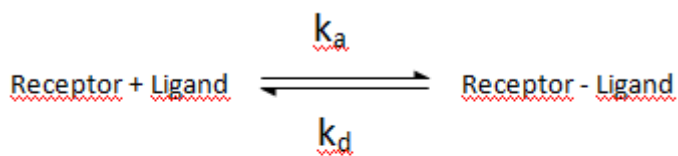
Nano-insulin drug was synthesized using the microemulsion polymerization process. Microemulsion media was obtained by dispersing 0.5 g PVA in 45 mL of deionized water. On the other hand, 2000 ppm 3 mL of isolated insulin (from rats) interacted with 25  $\mu$ L of ruthenium based MAT having monomer hapten. Then, this hapten was added to 25 mL of microemulsion media. 20 mL of the prepared initiator solution, with dissolving 0.02 g APS in 45 mL of deionized water, was added into the mixture, finally. Copolymerization was carried out for 48 h under nitrogen atmosphere at room temperature in daylight. Nano-insulin particles that occurred at the end of the reaction were removed from the reaction media by centrifugation at 6000 rpm for 20 min. After the centrifugation process, the occurred nanoparticles were washed with deionized water to remove unreacted substances.

## 2.7. Characterization of Nano-Insulin as Labelfree Nanotheranostic

500  $\mu$ L of nano insulin solution was diluted to 3 mL with deionized water to measure the fluorescence feature of nano insulin. Then, it was excited at 310 nm to measure the fluorescent emission.

## 2.8. Monitoring of Reflectometric Interference Spectroscopy Sensor Performance

The activation of the RIfS sensor surface was functionalized using the carbodiimide method with 70  $\mu$ L, 1:1 NHS/EDC mixture in deionized water [28, 29, 34]. And for RIfS measurements under continuous flow was used degassed and filtered buffer (pH: 7.4, 10 mM phosphate buffer containing 0.5 % SDS) at a flow rate of 5  $\mu$ Lmin<sup>-1</sup>. Immediately, 200  $\mu$ L of the purified insulin receptor in sodium acetate was injected and the reactive groups which were still remaining on the sensor surface were deactivated with ethanolamine. The flow rate was adjusted to 5  $\mu$ Lmin<sup>-1</sup> all the time. The interactions between purified insulin receptor and purified insulin, nano-insulin theranostic and commercial analog were determined and the volumes of 200  $\mu$ L of all samples, purified insulin, nano-insulin theranostic and commercial analog, were used to observe association properties. Running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA), pH 7.4, was used for dissociation and 50  $\mu$ L of Glycine and 50  $\mu$ L of HCl solutions were used for the regeneration of the surface.



$$\text{at equilibrium} = \frac{k_d}{k_a} = K_D$$

Purified insulin (5.8 kDa) and nanopurified insulin (15 kDa), insulin detemir (6 kDa), and nanoinsulin detemir (16 kDa), as well as SDS biomarker, were prepared in a mixture containing 1 % SDS and 5 % mercaptoethanol. Then, they were boiled for 5 min. 10  $\mu\text{g}$  protein was loaded to SDS-PAGE gel which includes 12 % w/w running gel, 4 % w/w ratio of stacking gel. The prepared gel was run under constant 80  $\text{Vgel}^{-1}$  voltage using running buffer (192 mM glycine, 25 mM Tris and 10 % SDS). The pH value of the running buffer was 8.3. When the coomassie blue marker reached 1 cm away from the bottom, it generally pointed out that the running time was 4–5 h. Then, the proteins were stained for 5 h using 0.25 % coomassie brilliant blue and destained using the mixture of 7 % acetic acid, 10 % methanol, and 83 % water. Proteins were transferred to a polyvinylidene fluoride filter (PVDF; Immobilon-P, Millipore) from the SDS gel [35]. ProteoQwest colorimetric kit included TMB substrate (Sigma) was used for western blotting process, with Anti-Insulin antibody or Detemir specific antibody and secondary antimouse antibody (Sigma, A5225). For the determination of molecular mass, Kaleidoscope prestained standards (Bio-Rad) were used as a marker.

### 3. Results And Discussion

#### 3.1. Characterization of Cryogels

The equilibrium swelling ratios of the insulin and insulin receptor linked cryogels were found to be 795.48 and 832.28 %, respectively. The equilibrium swelling ratio test was also applied for PHEMA cryogel and determined as 756.05 %.

SEM micrographs of the synthesized insulin receptor and insulin cross-linked cryogels were given in Fig. 1. As seen in Figure, both cryogels have a randomly interconnected macroporous sponge-like structure and the size of the pores are in the range of 200–300  $\mu\text{m}$ . Fast and effective separation of biological molecules can be provided by interconnected supermacroporous character.

#### 3.2. Characterization of Nano-Insulin Theranostic Particles

Figure 2A showed the size distribution of nano-insulin particles. According to data, the average particle size and the fluorescent intensity of synthesized nano-insulin theranostic particles were found to be 74.64 nm and 598 intensity (a.u.) at 610 nm (Fig. 2B), respectively. This showed that nano theranostic insulin

particles have a strong fluorescence feature that could be used in different studies such as imaging or labeling.

TEM photographs (Fig. 2C) also supported that nano insulin particles have an average particle size of about 70–80 nm.

### 3.3. Purification of Insulin and Insulin Receptor

Insulin receptor cross-linked cryogel was used in the FPLC system (Fig. 3A) as a column material for the purification of insulin from rats. For this chromatographic process, a gradient elution was used at a flow rate of  $1.0 \text{ mLmin}^{-1}$ . During the 0–8 min interval, the solution A was passed through the column for the separation of other species except insulin in the rat's blood. So, the peak at 2nd min indicated the other species in rat's blood rather than insulin. Then, the buffer solution was changed and buffer B was passed through the column for 6 min. In this interval, the peak at 11th min indicated the insulin peak. The separated insulin was collected at the collector and then, lyophilized for the binding studies in RIFs analysis. Insulin cross-linked cryogel was used as a column material for the purification of insulin receptors from the sample that had been prepared as explained in the previous section. For this separation, gradient elution was used at a flow rate of  $1.0 \text{ mLmin}^{-1}$ . Figure 3B showed the chromatogram for this separation process. According to this chromatogram, the peak at 2nd min indicated the other species rather than the insulin receptor and the peak at 13th min showed the insulin receptor. The separated insulin receptor was collected and lyophilized for the binding studies for RIFs analysis.

### 3.4. RIFs Study

The activation of the sensor surface achieved using 70  $\mu\text{L}$  of NHS/EDC (each of 0.1 M). Then, the purified insulin receptor was immobilized on the sensor surface. During the immobilization routine process of purified insulin receptor, reference sensor surface for determination of nonspecific binding effects of the purified insulin for the following measurements was generated [34]. Therefore, only flow channel 1 was linked with the purified insulin receptor. The results of the RIFs study about the interaction of the insulin receptor and purified insulin, nano-insulin theranostic, and commercial insulin were given in Fig. 4. According to obtained data from binding curves, the  $K_D$  values of purified insulin, nano-insulin theranostic, and commercial insulin were found to be  $1,10 \times 10^{-4}$ ,  $1,12 \times 10^{-4}$  and  $3,01 \times 10^{-3}$  M, respectively.  $K_D$  values indicated that nano-insulin and purified insulin had a near affinity for the insulin receptor. Actually, nano-insulin theranostic had one receptor binding site and this  $K_D$  value indicated that this binding site was sufficient for efficient binding.

### 3.5. Western Blot Analysis

Western Blot (Fig. 5) was used for determination of the purity of purified insulin and nano insulin from purified, insulin detemir and nanoinsulin detemir eluted from FPLC analysis. Western blot analysis for anti-insulin antibody and detemir specific antibody showed purified insulin (5.8 kDa) and insulin detemir molecules (6 kDa) in Fig. 5 as Lane 1 and Lane 2. Lane 3 and 4 indicated nano insulin from purified (15



kDa) and nano-insulin from insulin detemir (16 kDa), respectively. Furthermore, the purity of nanopurified insulin after desorption of the synthesized cryogel was observed as one band at Lane 3. Only one band observed in Lane 4 indicated the purity of nanoinsulin detemir purified by FPLC system from the blood sample. Western Blot analysis showed that purified insulin and insulin detemir proteins had been separated in high purity from the blood sample in this study.

## 4. Conclusions

There is a huge attention to developing novel insulin analog for diabetes mellitus treatment. In general, the interaction between insulin and insulin receptor is used for trying new approaches. The interaction is very crucial for treatment. For that reason, we investigated the binding interaction of insulin to the insulin receptor. As seen from the swelling results, both insulin receptor and insulin cross-linked cryogel have hydrophilic character as HEMA cryogel. The linkage of the insulin receptor and insulin to the cryogel did not change the hydrophilic character and the swelling degree of the cryogel.

Label-free detection using the RfS system is used for monitoring the interaction of purified insulin receptor and purified insulin, nano insulin, and commercial insulin in real-time. Thin silica layer's surface modification by binding could be detected using this technique. The shift of the interference spectrum shows the binding curves [36, 37].

In our study, we offered a new perspective to the investigations using nano insulin approach. Nano-sized insulin theranostic was synthesized using the ANADOLUCA concept and the binding interactions with the insulin receptor was determined using RfS analysis. Besides this, we investigated the binding interactions between purified insulin receptor and purified insulin and commercial insulin. Actually, the interactions in separation and purification steps and modifications during the synthesis of nano-insulin decreased the affinity of nano insulin compared to commercial insulin. But, in the reusability aspect of nanostructures still causes advantages such as usage of many times without deformation in their protein structures and decorated easily with other drug and labeling materials and separated easily in the reaction media. Besides these, they show resistance in harsh conditions such as acidity, alkalinity or high temperatures of the reaction media. Also, in the nanoprotein synthesis process, the modifications in insulin structure may cause the less affinity to insulin receptor. On the other hand, the advantage of using nano-insulin in diabetes treatment could be that the usage of many times without any deformation in the nanostructure of insulin and any deficiency in affinity to insulin receptors. Western blot analysis and RfS studies demonstrated that nano-insulin drugs can be used for insulin receptor–nanoinsulin affinity interactions, which are great advances within nano-bio platforms for theranostics and drug discovery.

## 5. Declarations

-Ethical Approval: Not applicable

-Consent to Participate: Not applicable

-Consent to Publish: Not applicable

-Authors Contributions:

Rıdvan Say ensured coordination and wrote the main manuscript text

Almila Şenat performed purification steps and all lab processes

Özlem Biçen Ünlüer performed nano-insulin and cryogel synthesis

Fahrettin Akyüz performed in vivo processes and pre-purification steps

Arzu Ersöz investigated insulin-insulin receptor interactions ve characterizations

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-Competing Interests: The authors declare no conflicts of interest to this work

-Availability of data and materials: Not applicable

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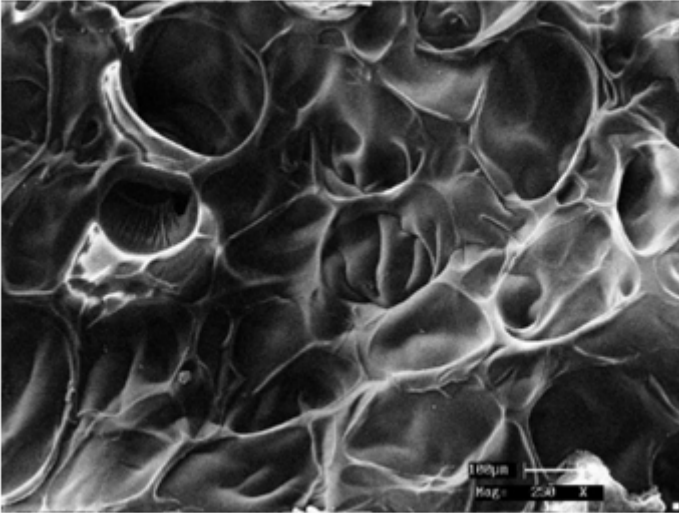
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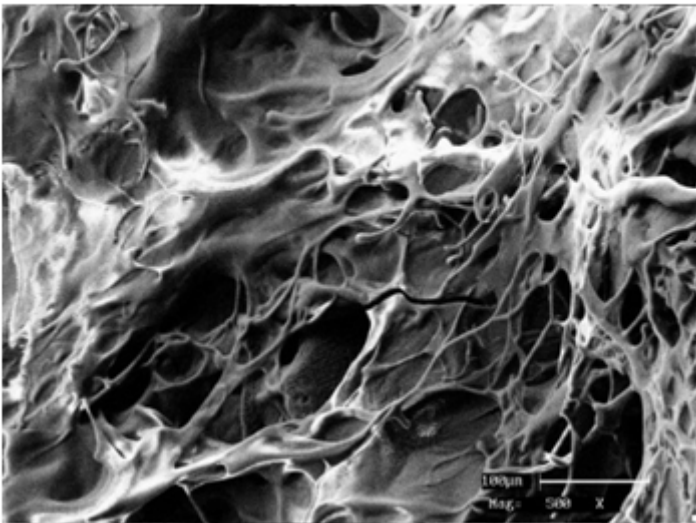
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## Figures

**A**

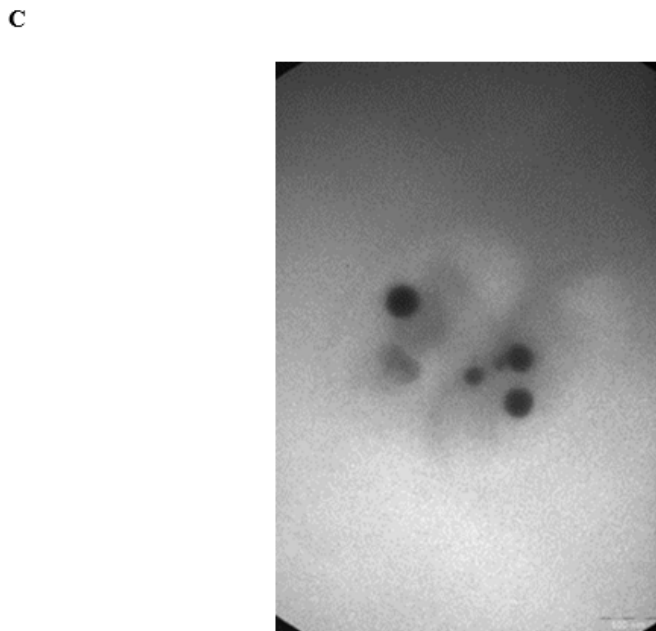
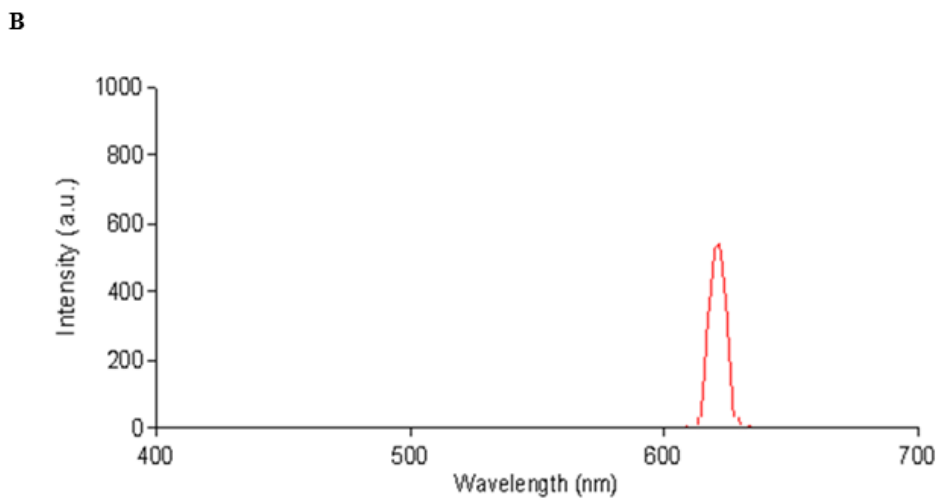
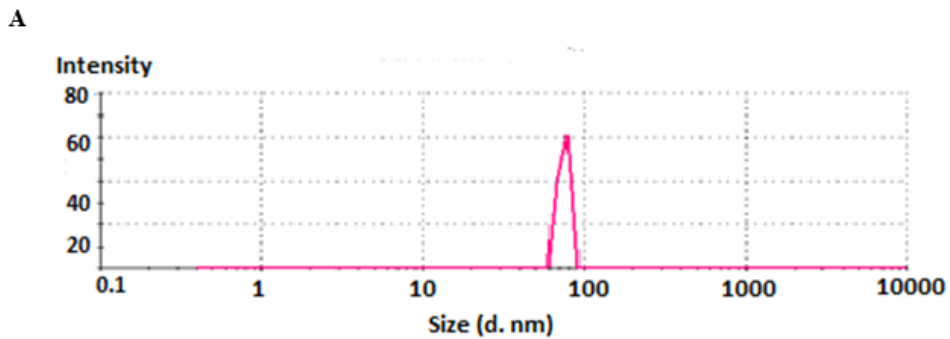


**B**



**Figure 1**

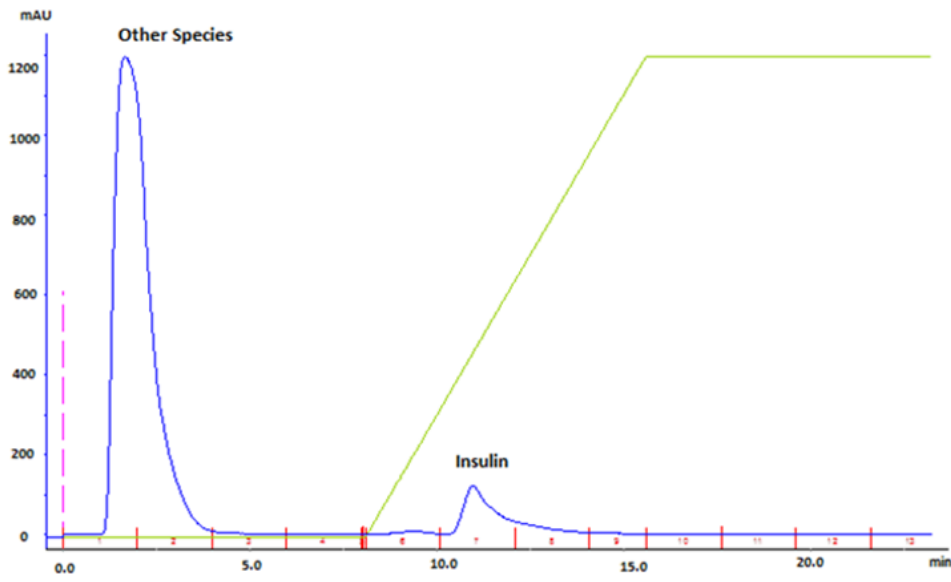
SEM micrograph of A. insulin cross-linked cryogel, B. insulin receptor cross-linked cryogel



**Figure 2**

A. Size distribution of nano insulin particles. Distribution result (Mean/area) = 74.64 d nm /100.0 % Zeta potential = -32 mV, Conductivity: 0.0258 mScm<sup>-1</sup>, B. Fluorescence spectrum of nano insulin particles (Fluorescence intensity is 598 a.u. at 610 nm), C. TEM images of nano insulin particles (Nano insulin particles are about 70 - 80 nm)

A



B

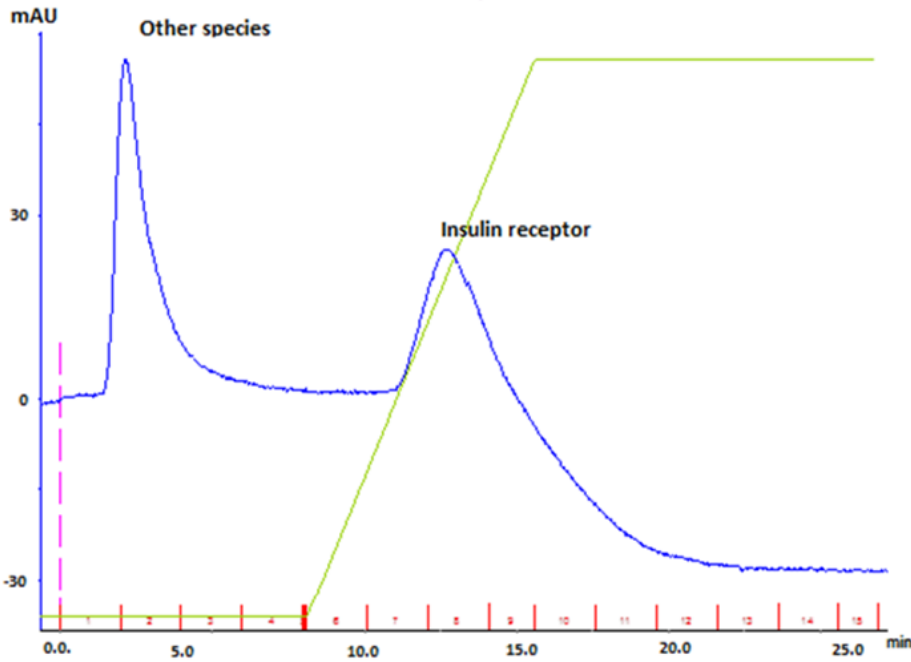
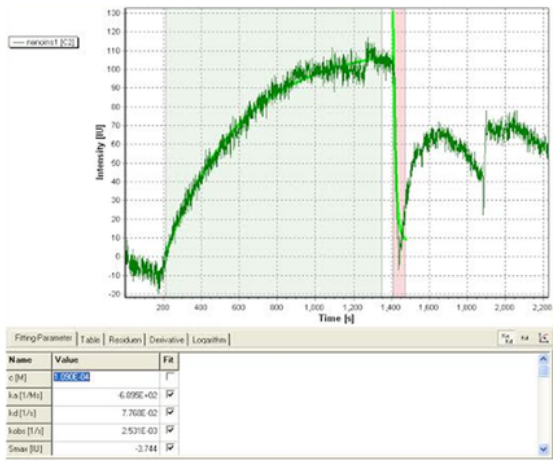


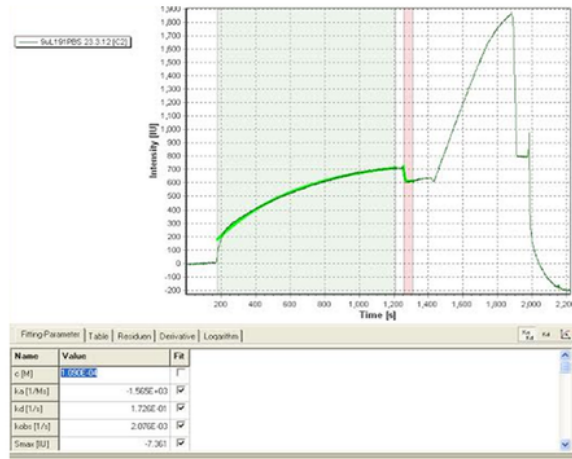
Figure 3

Chromatographic separation of A. insulin from rats via FPLC, B. insulin receptor from rat liver tissues via FPLC

A



B



C

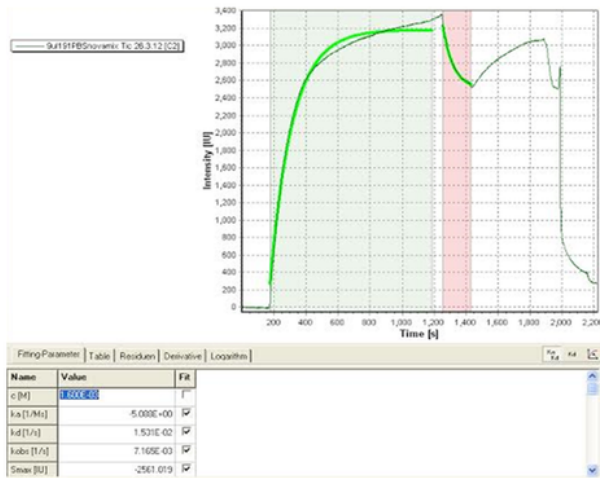
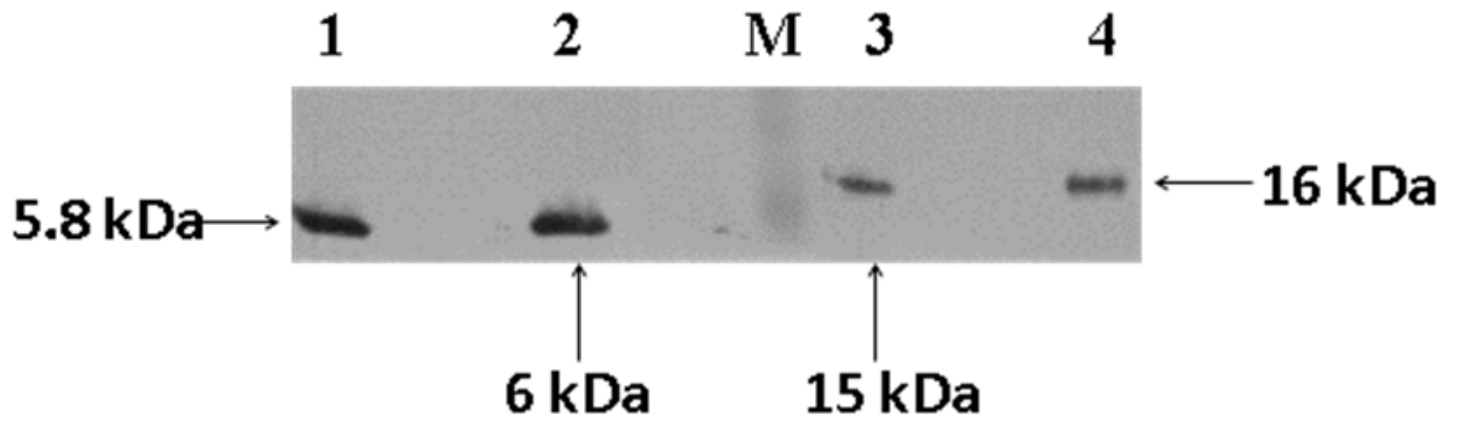


Figure 4

RlfS Spectrum of A. nanoinsulin for insulin receptor immobilized on the sensor, B. commercial insulin analog for insulin receptor immobilized on the sensor, C. purified insulin for insulin receptor immobilized on the sensor



**Figure 5**

Western blot analysis: purified insulin and nanopurified insulin, insulin detemir, and nanoinsulin detemir. Protein bands were detected with 10 % SDS-PAGE and blotted to PVDF membranes. The membranes were probed with an anti-Insulin antibody or an anti-Detemir specific antibody. Lane M is kaleidoscope prestained standards (Bio-Rad). Lane 1, purified insulin; lane 2, insulin detemir; lane 3, nanopurified insulin; lane 4, nanoinsulin detemir.