DEVELOPMENT AND CHARACTERIZATION OF INSULIN-LOADED LIPOSOME-CHITOSAN NANOPARTICLE (LCS-NP) COMPLEX AND INVESTIGATION OF TRANSPORT PROPERTIES THROUGH PANCREATIC BETA TC CELL LINE

İNSÜLİN YÜKLÜ LİPOZOM-KİTOSAN NANOPARTİKÜL (LCS-NP) KOMPLEKSİNİN GELİŞTİRİLMESİ VE KARAKTERİZASYONU VE PANKREATİK BETA TC HÜCRE HATTINDAN GEÇİŞ ÖZELLİKLERİNİN ARAŞTIRILMASI

Short title: Liposome-Chitosan Nanoparticle (LCS-NP) Complex Containing Insulin: Development and Characterization

Kısa başlık: İnsülin İçeren Lipozom-Kitosan Nanopartikül (LCS-NP) Kompleksi: Geliştirme ve Karakterizasyon

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ABSTRACT

Objectives: In recent years, studies on oral use have been rapidly underway due to the restrictive aspects of parenteral administration of indispensable peptide-structured insulin in the rapidly increasing worldwide diabetes treatment. The aim of the study was to examine the development of novel insulin-loaded liposome chitosan nanoparticle (LCS-NP) complex, characterization and efficacy on pancreatic cells responsible for insulin release.

Materials and Methods: Blank liposomes and insulin loaded CS-NPs were prepared by dry film hydration and ionotropic gelation methods respectively. LCS-NP complex was prepared by mixing liposome/nanoparticle 2:1(w/w) ratio. Cytotoxic effects of the various concentrations of insulin and formulation components on the pancreatic cell line were determined by MTT assay and quantities to be used in the formulation were determined. Particle size, zeta potential, encapsulation efficiency, *in vitro* release profile and release kinetics and transport property of the prepared complex were investigated.

Results: The newly developed insulin-loaded LCS-NP complex had a particle size of 2.85 ± 0.035 μm and zeta potential of 8.11 ± 1.025 mV. Encapsulation yield was found to be 48 ± 1.1%. *In vitro* insulin release from the complex was 80.9 ± 2.71%. Insulin transport from β TC cells was 30.50%. Permeability coefficients (log k) were calculated as -1.280 ± 0.070 for insulin solution and -1.020 ± 0.062 for insulin loaded complex.

Conclusion: In conclusion, this study suggests that insulin could be successfully loaded into the newly developed LCS-NP complex, and it is thought that this complex carries an effective formulation potential for long-term efficacy in the treatment of diabetes.

Keywords: Insulin, Diabetes, Chitosan nanoparticle, Liposome-chitosan nanoparticle complex

ÖZ

Amaç: Son yıllarda dünya çapında hızla artan diyabet tedavisinde vazgeçilmez peptit yapılı insülinin parenteral uygulamalarının kısıtlayıcı yönleri nedeniyle oral kullanıma yönelik çalışmalar hızla sürmektedir. Çalışmada amaç, insülin yüklü yeni geliştirilen lipozom-kitozan nanopartikül (LCS-NP) kompleklerinin geliştirilmesi, karakterize edilmesi ve insülin salımından sorumlu pankreatik hücreler üzerinde etkinliğinin incelenmesidir.

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Gereç ve Yöntemler: Boş lipozomlar film hidrasyon metodu ile, insülin yüklü kitozan nanopartiküller ise iyonik jelasyon metodu ile hazırlanmış ve fosfolipit/nanopartikül 2:1 oranında karıştırılması ile kompleksler hazırlanmıştır. İnsülinin çeşitli konsantrasyonları ve formülasyon bileşenlerinin pankreatik hücre hattı üzerinde sitotoksik etkileri MTT testi ile belirlenmiş ve formülasyonda kullanılacak miktarları belirlenmiştir. Hazırlanan kompleksin karakterizasyonu (partikül büyüklüğü, zeta potansiyeli, enkapsülasyon etkinliği), *in vitro* salım profili ve salım kinetiği ve pankreatik hücreler üzerindeki geçiş özellikleri incelenmiştir.

Bulgular: Yeni geliştirilen insülin yüklü LCS-NP kompleks, 2,85±0.035 µm partikül büyüklüğüne ve 8.11±1.025 mV zeta potansiyele sahiptir. Enkapsülasyon verimi, % 48±1.1 olarak bulunmuştur. Komplekslerden *in vitro* insülin salımı % 80,9±2.71 bulunmuştur. β TC hücre hattından insulin geçişi %30,50 olarak elde edilmiştir. Permeabilite katsayıları (log k) insülin çözeltisi için -1.280±0.070, insülin yüklü kompleksler için -1.020±0.062 olarak hesaplanmıştır.

Sonuç: Sonuç olarak bu çalışma ile yeni geliştirilen LCS-NP kompleksine insülinin başarılı bir şekilde yüklenebildiği, bu kompleks yapının uzun süreli salım ile diyabet tedavisinde kullanılabilecek etkili formülasyon potansiyeli taşıdığı düşünülmektedir.

Anahtar kelimeler: İnsülin, Diyabet, Kitozan nanopartikülü, Lipozom-kitozan nanopartikül kompleksi

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INTRODUCTION

Diabetes mellitus is a metabolic disorder that results in hyperglycemia associated with abnormalities in carbohydrate, fat and protein metabolism and insulin deficiency due to beta cell loss.[1,2] Insulin, a peptide hormone produced by pancreatic β-cells, is used for the treatment of diabetes by regulating glucose concentration in blood. Although insulin therapy is the oldest and most effective in diabetes, some limitations has occurred. Insulin is commonly used via parenteral route which provides immediate action. However, there are many disadvantages of parenteral route including pain discomfort and hypoglycemic episodes associated with multi dose injections which cause poor patient compliance.[3] Administration of therapeutic peptide drugs such as insulin via the oral route, especially the gastrointestinal tract, represents one of the greatest challenges. Colloidal drug carriers have been developed for controlled drug release and represent an exciting approach to increase the uptake and transport of orally administered peptide drugs such as insulin.[4] Besides, these systems have many advantages including decrease in multi dose injections, improved patient compliance, decrease in drug plasma level fluctuation in blood and total drug usage, increase bioavailability of some drugs and minimize drug toxicity.[5] Liposomes and polymeric nanoparticles are suitable colloidal carriers for insulin delivery and many investigations were performed for administration routes e.g. parenteral, ocular, nasal, pulmonary, transdermal, oral and buccal. Liposomes are spherical vesicules consisting of cholesterol and nontoxic phospholipids that are biodegradable, biocompatible and non immonogenic colloidal carriers. They increase peptide stability with protecting bioactive agents from digestion in the stomach and show significant levels of absorption in the gastrointestinal tract.[6] Polymeric nanoparticles are solid colloidal nanocarriers that provide controlled release of peptides depending on surface modifications by biodegradable polymers. Chitosan, a hydrophilic natural polymer, has been used in protein and peptide encapsulated nanoparticle formulation for its unique characteristics including biocompatibility, biodegradability and mucoadhesivity.[7,8]

Cell culture is a laboratory process based on survival of cells under controlled conditions while preserving their viability and shows *in vivo-in vitro* characteristics. The major advantages of using cell culture are the consistency and reproducibility of results that can be obtained from using a batch of cells and high *in vivo* correlation.[9]

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(Yücel Ç. Development and investigation of efficiency of embryonic stem cell and insulin-loaded liposome, nanoparticle and cochleate formulations. Ph. D. Thesis, Gazi University, Institute of Health Sciences, Ankara 2015: 2-3.)

In the present study, insulin was encapsulated in liposome-chitosan nanoparticle complex (LCS-NP) and *in vitro* characterization studies were performed on complexes including particle size, zeta potential, surface morphology, release and transport studies through pancreatic beta TC (β TC). Furthermore, *in vitro* cytotoxicities of insulin solution with different concentrations and formulation components on β TC cells were evaluated.

EXPERIMENTAL

Materials

Insulin (Humulin R), (Humulin® R 100 IU/mL), Chitosan hydrochloride (CS) (Protasan CL 110 and pentasodium tripolyphospate, (TPP) were purchased from Lilly (USA), FMC Biopolymers (Norway) and Kimetsan (Turkey) respectively. Diasteroylphoshatidylcholine, (DSPC) and cholesterol (Chol) were supplied by Sigma (St. Louis, USA). All other chemicals used were analytical grade. β TC cell lines were obtained from the American Type Culture Collection (CRL-11506). Cell culture flasks surface area 25 cm^2 and 75 cm^2 and cell culture plates 6 well were purchased from Corning®. Fetal Bovine Serum, Tripsin-EDTA solution, DMSO for cell culture, penicillin-streptomycin solution and MTT (3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide) were purchased from Sigma (St. Louis, USA). Cedex Smart Slides and Trypan Blue solution were purchased from Roche (Switzerland). Insulin mouse ELISA kit was obtained from Sunredbio. DMEM was purchased from Capricorn Scientific, Germany.

Analytical method and calibration

Drug content in formulations was measured using UV-spectrophotometry (Shimadzu-UV 1800). Initially absorbance of insulin was determined at respective wavelength. Stock solution of insulin (100 µg/mL) was diluted from insulin market preparation with distilled water. Working standard solutions were prepared by diluting the stock solution in the concentration range of 0.5-100 µg/mL for calibration curves. Spectrophotometric determination was carried out at 756 nm. The method was found to be linear (r^2 = 0.9992) and reproducible.

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Preparation of Chitosan Nanoparticles (CS NPs)

Blank and insulin-loaded CS NPs were prepared according to the ionotropic gelation process as described by Aktaş et al.[10] The ratio of CS/TPP was established as 1:1 according to the preliminary studies. Blank nanoparticles were obtained upon the addition of a tripolyphosphate (TPP) aqueous solution (0.4 mg/mL) to a CS solution (1.75 mg/mL) stirred at room temperature on magnetic stirrer for an hour. The same protocol was applied to obtain insulin-loaded CS NPs. Amount of initial insulin amount was determined according to the preliminary drug loading studies.

Preparation of Liposomes (LPs)

Blank liposomes were prepared by dry film hydration method according to Bangham et al.[11] DSPC and cholesterol were added to the round-bottomed flask in 6:4 molar ratio and dissolved in chloroform. The organic solvent was then evaporated on a rotary evaporator (Heidolph) at ~45°C. The resulting thin film was then hydrated with 10 mL water for 30 min and vortexed.

Preparation of the Complexes

Blank and insulin loaded LCS-NP complexes were formed by the combination of liposome/nanoparticle at 2:1 ratio (w/w) followed by lyophilisation (Labconco) in the presence of 5.0 % mannitol for 24 h.[12,13] Finally, the lyophilised powders were reconstituted with distilled water for the their physicochemical characterization and encapsulation efficiency determination.

Characterization of the LCS-NP Complexes

The surface morphologies of the blank and insulin-loaded complexes were determined by inverted microscope (Zeiss Primo Star, Germany). Particle size and zeta potential were determined by Malvern Zeta Sizer. The encapsulation efficiency (EE) of insulin was determined after centrifugation at 14000 rpm, for 25 min of the LCS-NP complex dispersions. The amount of free insulin in the supernatant was measured by Lowry method at 756 nm.

$$EE(\%) = \frac{\text{theoretical total amount of insulin} - \text{free insulin}}{\text{theoretical total amount of insulin}} \times 100$$

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In Vitro Release Studies

LCS-NP complex were placed in a tube and 2 mL of pH 7.4 phosphate buffer was added as release medium. The tubes were immersed in a water bath (37±0.5 ºC) with a horizontal shaker. At various time intervals, samples of 1.5 mL were withdrawn and replaced with equal volume of fresh medium. The samples were centrifuged at 14000 rpm, for 25 min. Insulin in the supernatant was measured by Lowry method as described above.[14]

Cell Culture Studies

β TC cells were grown in a medium composed of DMEM containing 25 mM glucose, 5 mM glutamine supplemented with 15% fetal bovine serum and %1 penicilin-streptomycin in an incubator at 37ºC under 5% CO_2 atmosphere. The medium was replaced with fresh DMEM every 48 h. The presence of a confluent monolayer was controlled with a microscope.

Cytotoxicity assay

The effect of insulin solution and formulation components on cell viability was investigated using MTT (3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide) test.[15] β TC cells were seeded (20.000 cells/well) in 96-well culture plates and kept at 37ºC for 24 h for cell adhering.[16] Then, the cells were treated with various insulin concentrations (100-6.25 µg/mL) for 24 hours. The color intensity corresponding to cellular activity was measured at 570 nm with a multi-well ELISA reader.

Transport experiments

β TC cells were seeded at $4x10^4$ cells/well on polycarbonate membranes (Corning® 6 Well Transwell® Inserts) with a pore size of 0.4 µm.[17] Insulin, blank and insulin-loaded complexes were dissolved in medium for donor compartment. Ninety-five percent % O_2 and 5% CO_2 were delivered to the system at 37ºC to maintain cell viability. Samples were collected from the receptor compartment after 30, 60, 120, 180, 240, 360, 480, 720 and 1440 minutes. Insulin content of samples was analyzed by Lowry method and ELISA kit and apparent permeability (Papp) value was calculated using following equation.[18]

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$Papp = (dQ / dt) \times (1 / A \times C_0)$

dQ/dt refers to the permeability rate, A (cm^2) is the membrane diffusion area, and C_0 (mg/mL) is the initial concentration of insulin in the donor compartment.

RESULTS

In vitro studies

The surface morphologies of the LCS-NP complexes were shown in Figure 1. Mean particle size and zeta potential of the LCS-NP complexes were shown in Table 1.

Figure 1. The surface morphologies of the LCS-NP complexes: a) Blank LCS-NP complexes, b) Insulin loaded LCS-NP complexes

Table 1. Characterization parameters of the LCS-NP complexes (n=3).

Extent of the in vitro insulin release from LCS-NP complexes for 24 h was found 80.9 %. Insulin release was found to be compatible RRSBW kinetics and correlation coefficient was found 0.8995 as shown in Figure 2.

Figure 2. In vitro release profiles of insulin from LCS-NP complexes at pH 7.4 phosphate buffer (error bars represent standard deviations, n =3).

Cell culture studies

The MTT assay was used to determine the toxic effects on β TC cells. The effect of insulin solution, blank and insulin loaded LCS-NP complexes on cell viability were investigated for 24 h. The wells containing only the medium were accepted as a positive control with a cell viability of 100%. The viability of cells as percentages is given on Figure 3.

Figure 3. Cytotoxicity of various concentrations of insulin solutions (a) blank and insulin loaded LCS-NP complexes (b).

Transport experiments of insulin from the solution and LCS-NP complex through β TC cells were evaluated. Cumulative amount of transported insulin from solution and LCS-NP complex at the end of the 24 hours were found 37.46 % and 30.50 % respectively. Results were given on Figure 4. Papp (log k) values were calculated for insulin solution and LCS-NP complex (Table 2).

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Figure 4. Cumulative amount of insulin from solution and LCS-NP complex transported through β TC cells (error bars represent standard deviations, n = 3). **Table 2.** Papp (log k) values was calculated from β TC cell transport study results (n=3)

DISCUSSION

Diabetes is a disease with an increasing prevalence in the world wide that is occurs as hyperglycemia due to a relative deficiency of the production of insulin by the pancreatic beta-cells.[19] Insulin is a hydrophilic molecule, used in the treatment of diabetes mellitus that is commonly administered as multiple daily subcutaneous injections. Alternative routes for administration to improve patient compliance for insulin therapy have been investigated by designing drug delivery systems. Furthermore, various studies have been made on insulin loaded drug delivery systems such as liposomes and polymeric micro/nanoparticles to improve bioavailability and provide long-term stability.[20,21]

The aim of the present study was to combine the CS-NPs with similarity of the liposomes to the biological membranes to obtain a good alternative to insulin treatment. We developed and characterized insulin-encapsulated LCS-NP complexes to improve drug entrapment efficiency and evaluate their sustained efficiencies on pancreatic β TC cell line.

In this study, blank liposomes and insulin loaded CS-NPs were prepared by dry film hydration method[11] and ionotropic gelation process as described by Aktaş et al.[10] respectively. For the preparation of LCS-NP complex, liposome/nanoparticle at 2:1 ratio (w/w) was used according to the method described by Carvolho et al.[21] In characterization studies, the mean particle size and zeta potential of blank and insulin loaded LCS-NP complexes were found 3.25±0.083 µm, 10.395±2.411 mV, 2.85±0.035 µm and 8.115 ±1.025 mV respectively. Encapsulating efficiency of insulin loaded LCS-NP complex was found 48±0.1 % which is reported to be quite high.[22] In literature, Diebold et al. developed and studied LCS-NPs, a class of colloidal system that combines liposomes and CS-NPs as a potential ocular drug delivery system which had mean size of from 407.8±9.6 to 755.3±30.0 nm and zeta potential of from +14.7±0.4 to +5.8±1.3 mV.

In another study, microspheres containing lipid/chitosan nanoparticles complexes were prepared for pulmonary delivery of therapeutic proteins. Lipid and CS-NPs

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complexes had a particle size of approximately 2 μm and zeta potential of 2 mV.[23] Cui et al. prepared nanoparticles with biodegradable polymers such as poly(lactic acid) (PLA), and poly(D,L-lactide–co-glycolide acid) (PLGA), loaded with insulin–phospholipid complex for oral delivery. Spherical particles of 200 nm mean diameter and a narrow size distribution were obtained.[4] In another study, bioadhesive polysaccharide chitosan nanoparticles containing insulin were prepared by ionotropic gelation and particle size distribution and zeta potential were determined. The ability of CS-NPs to enhance intestinal absorption of insulin and increase the relative pharmacological bioavailability of insulin was investigated. They concluded that CS-NPs had shown an excellent capacity for the association of insulin. CS-NPs loading insulin showed a positive charge and rapid release kinetics in vitro. Also to obtain a positive zeta potential is important for interaction with the negatively charged cell membrane. Therefore, our findings from characterization appeared to be sufficient.[8,24]

In vitro release experiment was performed in pH 7.4 phosphate buffer. The medium for LCS-NP complex was selected as pH 7.4 phosphate buffer because the pH of DMEM (cell culture medium) was measured as 7.37 pH. The kinetic releases of insulin were found to be with RRSBW (r^2=0.8995) for insulin solution and LCS-NP complex. In this RRSBW kinetic, steeper initial slope followed by a flattened tail in final part was obtained.[25]

For cytotoxicity studies, we used MTT test that is the most commonly used. The effects of insulin solution and formulation components on β TC cell viability were investigated for 24 h. According to MTT test results, insulin did not cause any cellular toxicity with the used dose as a 33 μg/mL was decided as the encapsulated concentration in preparing CS-NPs. Additionally, blank and insulin-loaded LCS-NP complexes were also not found to be toxic to cells even at the highest concentrations as 75%. In literature, nanosystems were designed consisting chitosan nanoparticles and liposomes for ocular delivery. Diebold et al. hypothesized that combination of chitosan nanoparticles and liposomes will protect the peptide (fluorescein isothiocyanate (FITC)-conjugated albumin (BSA) from harsh environmental conditions while providing its sustained release. They have shown that these complexes interacted with mucus layer and transported the conjunctival cells. Furthermore toxicity of the LCS-NP complexes in conjunctival cells was found very low.[26] Also, Carvalho et al. developed nanosystems by same method. They

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encapsulated insulin and (FITC)-conjugated BSA in LCS-NP complexes then evaluated these activities in conjunctival cells by *in vitro* cell culture studies. They also investigated activities of complexes application via oral route. They have found that these complexes were well suited for controlled release with great stability in biological fluids and were provided an significant reduction in plasma glucose levels. They also found that the toxicity of LCS-NP complexes in conjunctival cells was found very low.[21]

The transport studies were performed for insulin solution and insulin-loaded LCS-NP complex through pancreatic β TC cells and permeability cofficients were calculated (Table 2).

Permeability coefficients (log k) were also calculated from solution and LCS-NP complex and the lower value was -1.280 cm/h for insulin solution, the penetration value found for the insulin loaded LCS-NP complex was -1.020 cm/h.

CONCLUSION

To conclude, although further experiments are warranted, all these data point out LCS-NP as potentially useful candidates for insulin delivery. The membrane structure of LCS-NP complex, due to the similarities of liposomes in the cell membrane structures, complex is able to penetrate more into the cells and get endositozed easier by the cells.

TABLES/FIGURES/IMAGES

a) b)

Figure 1. The surface morphologies of the LCS-NP complexes: a) Blank LCS-NP complex X40, b) Insulin loaded LCS-NP complex X40.

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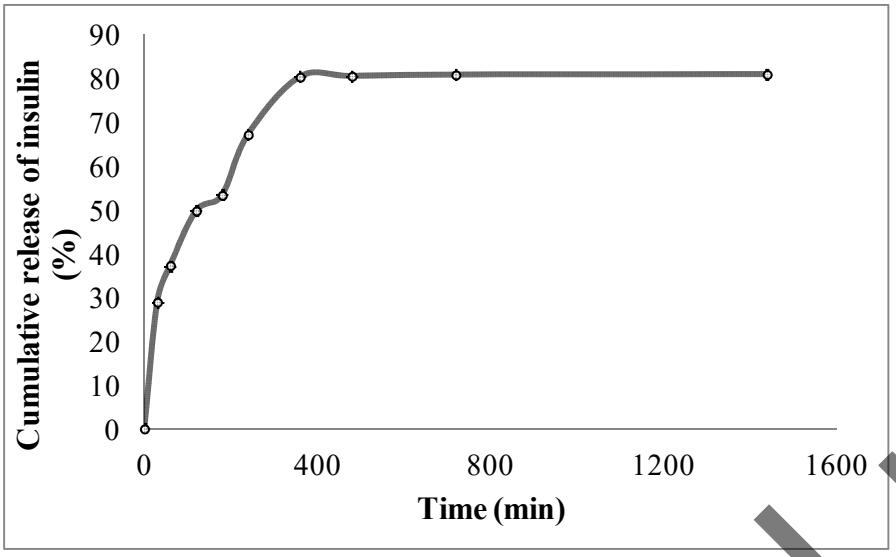
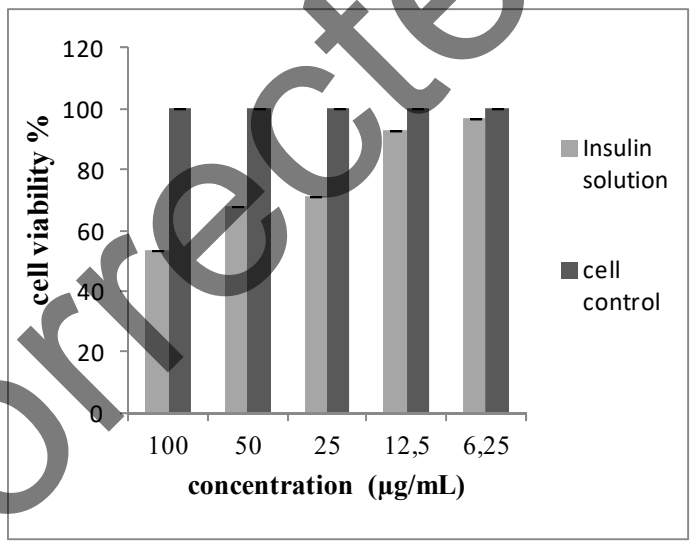


Figure 2. In vitro release profiles of insulin from LCS-NP complexes at pH 7.4 phosphate buffer (error bars represent standard deviations, n =3).



a)

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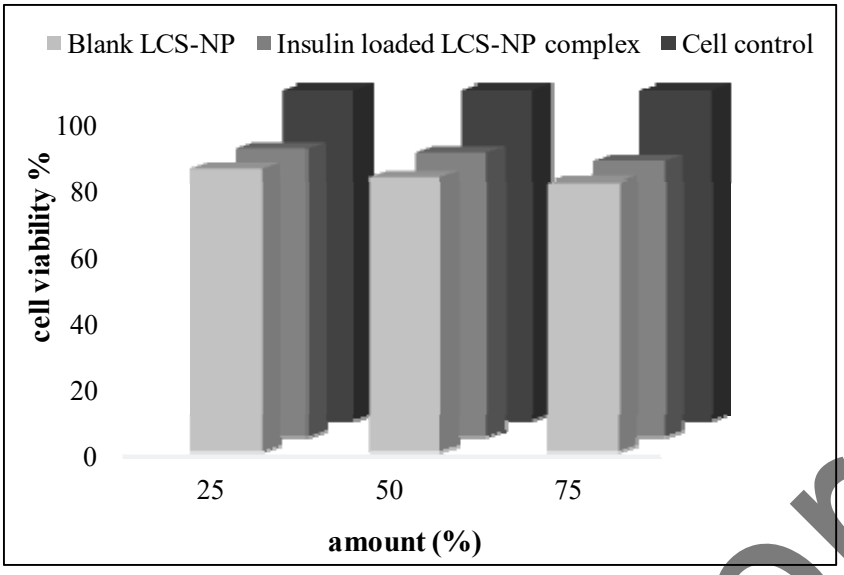

b)

Figure 3. Cytotoxicity of various concentrations of insulin solutions (a) blank and insulin loaded LCS-NP complexes (b).

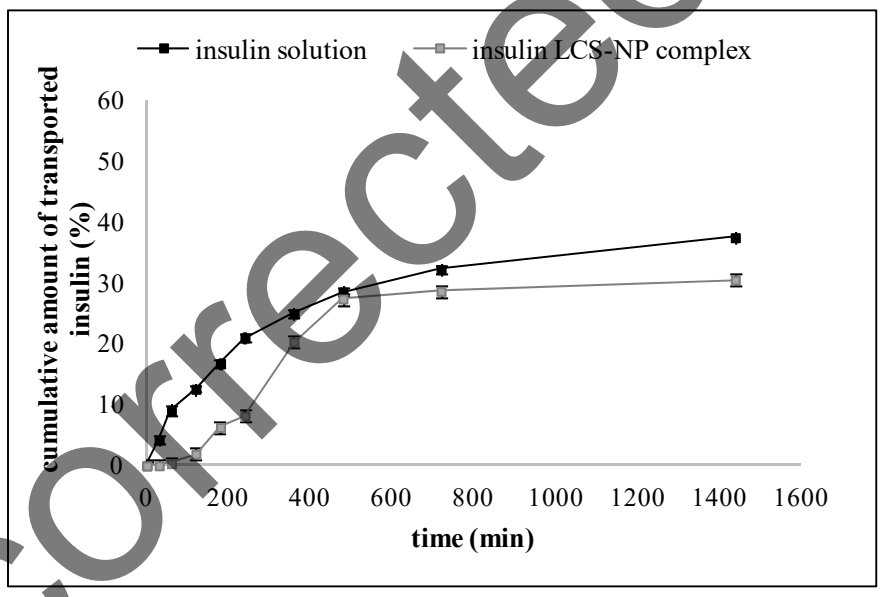

Figure 4. Cumulative amount of insulin from solution and LCS-NP complex transported through β TC cells (error bars represent standard deviations, n = 3).

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Table 1. Characterization parameters of the LCS-NP complexes (n=3).

FORMULATIONS	PARTICLE SIZE±SD (µm)	ZETA POTENTIAL±SD (mV)	ENCAPSULATION EFFICIENCY±SD (%)
Blank complex	3.25±0.083	10.40±2.411	----
Insulin loaded complex	2.85±0.035	8.11±1.025	48±1.1

Table 2. Papp (log k) values was calculated from β TC cell transport study results (n=3)

Samples	Log k values
Insulin solution	-1.280±0.070
Insulin LCS-NP complex	-1.020±0.062

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