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Uptake and transport of insulin across intestinal membrane model using trimethyl chitosan coated insulin niosomes

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ABSTRACT

This study reports the development of a highly stable niosomal nanostructure based on Span 60/cholesterol (CH)/N-trimethyl chitosan (TMC) system and its potential application for oral delivery of insulin. Insulin loaded niosomes were prepared by reversed-phase evaporation and TMC coating was performed by incubation of niosomal suspensions with TMC solution. The efficiency of nanoparticulate delivery system in enhancement of insulin permeation was evaluated by Caco-2 cell monolayer as intestinal membrane models. The prepared niosomes were characterized for entrapment efficiency (EE), particle size, zeta potential and stability. The particles were between 100 and 180 nm in diameter, and they were stable for over 60 days at 4 °C. Insulin permeability through Caco-2 cell monolayer was enhanced 4-fold by niosomal nanoparticles, compared with insulin alone. Further work is demanded to optimize this formulation with the object of maximizing its potential to facilitate oral delivery of insulin.

1. Introduction

Proteins and peptides are an important class of drugs or treatment of various medical disorders. Insulin is a peptide drug which is administered parenterally and two or three injections are required for the better control of diabetes, thereby reducing the long term complications of hyperglycaemia. However, repeated injections can cause lipodystrophy and damage to tissues over time. In addition, subcutaneously injected insulin goes into general circulation causing peripheral hyperinsulinemia, which is associated with peripheral hypertension, hypoglycemia, atherosclerosis development, cancer, and other harmful metabolic side effects [1]. Therefore, alternative routes for non-invasive, safe yet effective delivery of insulin have been proposed [2–4]. The oral route for insulin administration not only improves the overall well-being of diabetic patients who routinely receive insulin by the subcutaneous route, but also takes insulin directly to the liver through portal circulation, which causes rapid hepatic insulinization, and no peripheral hyperinsulinemia resembling what occurs in a non-diabetic individual [5]. However, oral delivery system suffers from weaknesses such as enzymatic degradation and limited capabilities to cross biological barriers. Therefore, development of an efficient delivery vehicle for oral administration of protein/peptide drugs presents an interesting challenge [6–8]. So far, different approaches such as chemical modification, administration with absorption enhancers, mucoadhesive polymers and protease inhibitors as well as design of functional delivery systems have been adopted for improving bioavailability of orally administered insulin [6,9–11]. Nanostructures are among the functional systems designed to the delivery of the carried insulin to the absorption sites, facilitate its permeation across biological membrane, and consequently to achieve potential pharmacological effects. Control of particle size, surface properties and release of active pharmaceutical ingredients are the main objectives in development of nanostructures to attain site-specific action of a drug at the therapeutically optimal rate and dose regimen [12]. Nano-sized drug carrier systems, such as niosomes, are new excellent candidates for delivery of different types of therapeutic agents. Niosomes are non-ionic surfactant vesicles, presenting similar structure to liposomes. Therefore, they can be used as a carrier for hydrophilic and lipophilic drugs. The surfactants used in preparation of niosomes are assumed to be chemically stable, compared to phospholipids vesicles in liposomes. Niosomes are biocompatible, biodegradable, non-immunogenic, non-toxic and capable of site specific delivery of proteins [13]. In addition, the simplicity of large scale production and storage, greater stability, healthy and low-cost ingredients have made niosomes an interesting alternative to other micro- and nano-encapsulation technologies.

Chitosan, a cationic natural polysaccharide obtained from chitin deacetylation, is also considered a safe, biocompatible and biodegradable

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material. It is a sustained release agent in oral drug formulation and can be used in numerous areas of biopharmaceutical industry to enhance intestinal absorption and permeability, thanks to its mucoadhesive property. The mucoadhesion of chitosan can be explained by the ionic interaction between its positively charged amino groups and negatively charged functional groups in epithelial cell surface. It has also been shown to enhance the solubility of nanoparticles in aqueous solutions [14–16]. However, chitosan remains a major challenge as it is insoluble at physiological pH value. In fact, as an absorption enhancer, it is soluble and active only in acidic medium, wherein chitosan amino groups are positively charged [17]. In neutral and alkaline environments, these amino groups lose their positive charge and the polymer consequently become insoluble. To address this weakness, a partially quaternized chitosan derivative, known as N-trimethyl chitosan chloride (TMC), was synthesized and evaluated at different degrees of substitution as a safe and efficient permeation enhancer in vitro [18–21]. TMC shows mucoadhesive characteristic and enhanced absorption effects even at neutral pH due to its permanent cationic charge and good water solubility over a wide pH range [17,18]. Therefore, niosomal nanocarriers coated with TMC can be a viable candidate for oral delivery of proteins and peptide drugs as they are able to overcome some of the inherent limitations related to these molecules [22–26].

Several studies have highlighted the potential use of TMC in drug delivery [19,20,27]. However, there are not yet published work on formulation and optimization of insulin loaded niosomes coated by TMC-nanostructure in order to enhance insulin permeation through the intestinal membrane. This work has mainly focused on evaluating the ability of the TMC-coated niosomal nanocarriers to open tight junctions and facilitate insulin transport across Caco-2 cell monolayer as intestinal membrane models.

2. Materials and methods

2.1. Materials

Insulin was obtained from Exir Pharmaceutical (Lorestan, Iran); Sorbitan monostearate (Span 60) and cholesterol were purchased from Sigma Chemical Co. (Germany); Diethyl ether and acetic acid were from Merck Chemical Co. (Germany); HBSS (Hanks Balanced Salt Solution), Dulbecco's modified of Eagle's medium (DMEM), fetal calf serum (FCS), trypsin/EDTA solution (0.2% of EDTA and 0.25% of trypsin in PBS (phosphate buffered saline, 0.01 M pH 7.2)) were from Gibco-BRL Life Technologies (Grand Island, NY). Enzyme-linked immunosorbent assay (ELISA) plates (96 wells) and other plastic wares were obtained from Nunc (MAX-ISORP, Roskilde, Denmark). Transwell (12-well plates) cell culture chambers inserted with 3.0 μm pore size were purchased from Corning Life Sciences (Massachusetts, USA). Chitosan with low molecular weight was obtained from Primex (Iceland). N-methyl pyrrolidone (NMP), iodomethane, triethylamine, sodium hydroxide, sodium chloride and acetone were purchased from Merck (Darmstadt, Germany). All other chemicals were of the highest grade commercially available. Ultrapure water (Millipore, Bedford, MA) was also used in reactions.

2.2. Methods

2.2.1. Synthesis of TMC polymer

TMC with substitution degree of 37.5% was synthesized using minor modifications of previously described protocol [28,29]. In brief, low molecular weight chitosan (12 g) was dispersed in a basic solution of methyl pyrrolidone at room temperature for 12 h with constant stirring. Subsequently, 15% (w/v) aqueous solution of sodium hydroxide (8 ml) and sodium iodide (3 g) were added and then the resulting mixture further stirred for 15 min at 60 °C. Finally, iodomethane (8 ml) was added in about 3-h intervals to the resulting mixture and stirred for a further 24 h at 60 °C. The product was precipitated with acetone and then separated by centrifugation. The obtained polymer was dissolved in 10% NaCl aqueous solution in order to exchange the iodide. The final solution was dialyzed against distilled water for 3 days and then lyophilized. The quaternized derivatives were characterized by ^{1}H NMR and D_2O was used as solvent for ^{1}H NMR spectroscopy. Degree of substitution and degree of quaternization were also determined from integrated data obtained from ^{1}H NMR spectra.

2.2.2. Preparation of insulin loaded niosomes

Niosomes were prepared by reversed-phase evaporation method. Briefly, a mixture of Span 60 and cholesterol (CH) (1:1, 2:1, 3:1, 4:1 and 5:1 molar ratio) was dissolved in 10 ml of diethyl ether, in a flask. Subsequently, 4 mg insulin solution in 3 ml phosphate buffer (PB, pH 7.4, 0.01 M) was added to the flask and mixed for 1 min. The mixture was then emulsified using 10, 15 and 20 min sonication in a water bath at 10 °C. The emulsion was rotary-evaporated at 40 °C with a rotating speed of 50 rpm for about 15 min to remove the organic solvent. The suspension finally underwent high-pressure extrusion, while passing four filters of different sizes (1, 0.4, 0.2, 0.1 μm). The suspension was passed from these filters for five times. Free insulin was removed by two step ultra-centrifuge (25,000 ×g, 4 °C, and 10 min) and washed with PB. TMC-coating was carried out by incubation of the niosomal suspensions with the TMC solution (1 mg/ml) at 37 °C for at least 1 h. Free TMC was finally removed from the suspension by two step ultra-centrifuge (25,000 ×g, 4 °C, 10 min) and then washed with PB [30,31].

2.2.3. Characterization of nanoparticle

Vesicles were characterized before and after extrusion. The particle size and zeta potential value of niosomes were measured by using a submicron particle analyzer, Zetasizer (Malvern instruments Ltd., Worcestershire, UK). Morphological examination of the niosomal nanoparticles was studied by negative stain transmission electron microscopy (TEM) following a standard procedure. For this purpose, lyophilized nanoparticles were re-suspended in freshly prepared distilled water and related size and shapes were investigated by TEM using a CEM 902A transmission electron microscope (Zeiss, Oberkochen, Germany) [31,32]. To determine the amount of insulin entrapped into the nanoparticles, the niosome sample was separated from unentrapped protein by ultra-centrifugation. In the next step, entrapment efficiency of insulin was (indirectly) determined by the difference between initial amount of insulin in niosomal suspensions and the unentrapped amount of free insulin found in the supernatant after centrifugation.

This value determined by ELISA, indicating the percentage of encapsulated insulin with respect to the total amount used for the nanoparticle preparation. To perform ELISA, microtiter plates were coated with 100 μl of two samples containing total amount of insulin and the supernatant after centrifugation. The plates were then incubated overnight at 37 °C. The following day, the plates were blocked with blocking buffer of PBS containing 3% skim milk for 30 min at 37 °C. Following the blockade of the non-specific binding sites, the wells were treated with anti-insulin mAb (monoclonal antibody) (1 μg/well), and incubated at 37 °C for 1 h. The residual binding was detected with anti-mouse HRP (horseradish peroxidase) conjugated antibody and compared with the standard curve. The amount of insulin was determined from a calibration curve based on the known concentrations of insulin [33–36].

2.2.4. The storage stability of nanoparticle

The biological stability and activity of the nanoparticles were determined using noncompetitive indirect ELISA. For this purpose, the plates were coated with 100 μl of the stored and freshly prepared insulin loaded niosome (1 μg/well) in PBS and incubated overnight at 37 °C. Following the blockade of the non-specific binding sites, the wells were treated with the anti-insulin specific antibody at a concentration of 1 μg/well. The residual binding was determined by adding the peroxidase-labeled anti-mouse IgG secondary antibody and expressed as a percentage of the optical density value given by a control [34,37].

Table 1
Effect of Sur/CH ratio on size, zeta potential, PDI and EE% of insulin-loaded niosomes.

Sur/CH	Without TMC				With TMC			
	Size (nm)	Zeta potential (mV)	PDI	EE%	Size (nm)	Zeta potential (mV)	PDI	EE%
1:1 (50% CH)	186.706 ± 10.130	−33.341 ± 1.251	0.495 ± 0.140	78.948 ± 2.071	240.350 ± 9.384	21.002 ± 0.322	0.571 ± 0.004	80.215 ± 1.221
2:1 (33% CH)	238.140 ± 8.331	−42.000 ± 1.800	0.611 ± 0.004	71.180 ± 2.840	269.121 ± 9.032	6.710 ± 0.111	0.628 ± 0.003	70.514 ± 3.841
3:1 (25% CH)	688.572 ± 15.140	−40.401 ± 0.754	0.869 ± 0.021	62.192 ± 3.100	734.383 ± 8.151	14.634 ± 0.262	0.691 ± 0.003	63.535 ± 2.803
4:1 (20% CH)	394.410 ± 10.090	−41.500 ± 0.920	0.631 ± 0.011	71.323 ± 2.780	439.010 ± 8.572	12.712 ± 0.224	0.554 ± 0.001	71.656 ± 2.112
5:1 (17% CH)	244.612 ± 5.881	−35.474 ± 0.392	0.661 ± 0.010	78.422 ± 3.000	298.343 ± 8.330	19.701 ± 0.251	0.592 ± 0.003	77.422 ± 3.910

2.2.5. Cell viability

Biocompatibility of nanoparticles was also evaluated via cell viability measurements [31,32]. About 5×10^3 Caco-2 cells were seeded in a complete growth medium in 96-well plates 24 h before treatment. Cells were incubated with coated and un-coated nanoparticles containing insulin and compared with cell exposed to PBS and 1% (v/v) Triton X-100 in PBS as the positive and negative controls, respectively. Cells were then incubated for 2 days at 37 °C in a humidified atmosphere of 5% CO_2. Methylene blue in 0.01 M-borate buffer (pH 8.5) was added to each well. After 30 min, excess dye was removed by two times washing with 0.01 M-borate buffer (pH 8.5). To elute the dye, 100 μl of 1:1 (v/v) ethanol and 0.1 M-HCl were added to each well. The plates were then gently shaken and the absorbance was read at 650 nm using an ELISA reader [9].

2.2.6. Experimental design studies

Design expert software version 8.0.7.1 was used for optimizing and mathematical modeling. The effect of three and five leveled numerical variables (duration of sonication and surfactant (Sur)/CH) on physicochemical properties of insulin loaded niosomes was evaluated using hexagonal response surface methodology (RSM). In this study, Sur to CH ratio and sonication time were independent factors, whereas, zeta potential, polydispersity index (PDI), and EE% were dependent variables [38–41]. According to the software, 10 runs were designed to obtain the best fitted models.

2.2.7. In vitro release of insulin from Span 60/CH/TMC vesicles

The optimized and experimentally designed nanoparticles, Span 60/CH/TMC vesicles, were used for in vitro release study of insulin at 37 °C in PB (pH = 6.8), known as simulated intestinal fluid (SIF), under gentle stirring for 12 h. Appropriate amount of lyophilized insulin loaded nanoparticles, equal to 2.00 mg of insulin, providing sink condition was placed into 8.00 ml of PB, and the dispersion was incubated at 37 °C under gentle shaking. In specific time interval, 0.2 ml of supernatant release medium was collected and replaced by freshly prepared buffer. The individual samples were centrifuged at 25,000 rpm for 10 min and the insulin concentration was measured by previously described ELISA [7,42]. All the experiments were performed in triplicate.

2.2.8. Transepithelial electrical resistance measurements and insulin transport study

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin and streptomycin. The cells were grown under standard conditions until 60–70% confluency. The cells from passages 20–40 were used for all of the experiments. Caco-2 cells were seeded into 12-well polycarbonate transwell plates at a density of 2×10^4 cells/cm^2 and grown for 21 days in order to form a confluent monolayer. The integrity of the cellular barrier was assessed using transepithelial electrical resistance (TEER). Samples of media were collected from the basal compartment of each transwell plate and replaced with fresh HBSS at 0 (before sample addition), 15, 30, 45, 60, 90, 120, 180 and 240 min. The insulin concentrations of these samples were determined using ELISA, and the final presented values were adjusted to reflect the change in volume at each successive sampling point. TEER measurements were taken at 0 h (before sample addition), at 0.5, 1, 1.5 and 2 h [7,9].

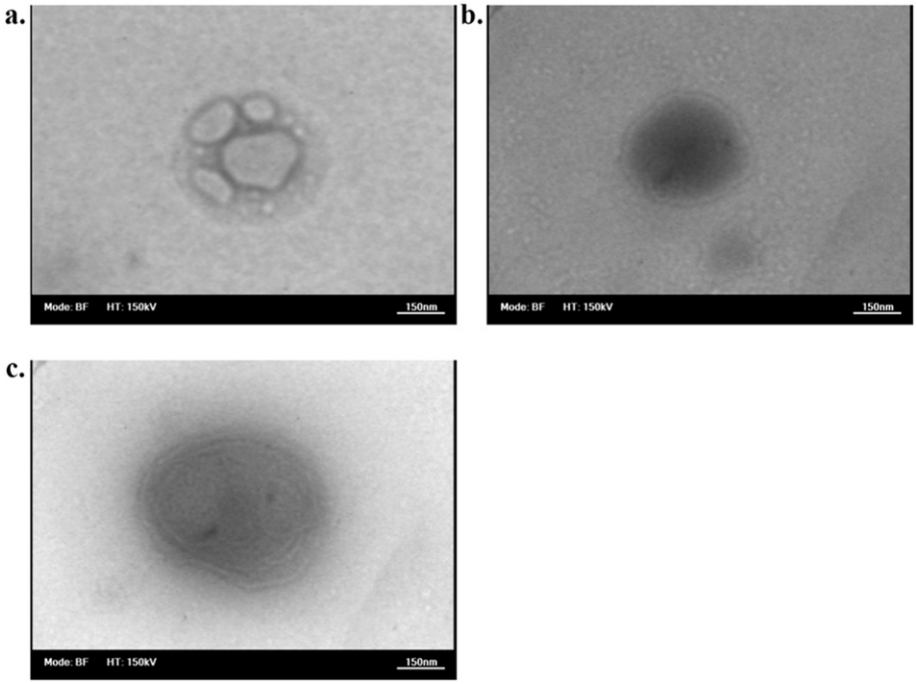

Fig. 1. Transmission electron photomicrographs of niosomes: (a) blank (niosome alone); (b) insulin loaded niosomes; (c) insulin-loaded niosomes coated with TMC.

Table 2
Experimental results for hexagonal RSM designed experiments to determine the effect of ratio of Sur to CH and duration of sonication on zeta potential, PDI, and EE% of insulin-loaded niosomes, (n3).

Run	Factor		Responses		
	Sur/CH	Time (min)	Zeta (mV)	PDI	EE%
1	1:1	15	−32.100 ± 0.780	0.551 ± 0.003	75.063 ± 1.640
2	2:1	10	−42.000 ± 1.800	0.611 ± 0.004	71.180 ± 2.840
3	2:1	20	−41.100 ± 2.190	0.663 ± 0.004	65.847 ± 2.120
4	3:1	15	−38.700 ± 2.600	0.697 ± 0.005	59.535 ± 2.020
5	3:1	15	−39.100 ± 1.440	0.701 ± 0.004	60.615 ± 1.980
6	3:1	15	−38.100 ± 2.120	0.718 ± 0.017	60.723 ± 2.130
7	3:1	15	−38.500 ± 0.780	0.710 ± 0.004	58.988 ± 1.980
8	4:1	20	−39.700 ± 2.040	0.705 ± 0.004	66.790 ± 1.340
9	4:1	10	−41.500 ± 0.920	0.631 ± 0.011	71.323 ± 2.780
10	5:1	15	−31.200 ± 1.110	0.623 ± 0.004	73.023 ± 3.010

2.2.9. Enzymatic stability study of insulin by trypsin

Trypsin was first dissolved in 10 mM Tris buffer (pH 7.4), and the concentration was adjusted to 3000 BAEE IU/ml. One hundred microliters of the solution was then added to 900 μl of insulin solution and nanoparticle suspensions containing 500 μg/ml of insulin. The initial concentrations of insulin and trypsin in the sample solution (1 ml) were 450 μg/ml and 300 BAEE IU/ml, respectively. Samples were incubated for 1 h at room temperature and the enzymatic reaction was stopped by the addition of 1 ml of acetonitrile/purified water mixture (3/1, v/v) containing 0.1% trifluoroacetic acid. The insulin content was determined by ELISA as described above.

2.2.10. Statistical analysis

The data were reported as mean ± SD ($n = 3$) and statistical analysis of the data was carried out using one way ANOVA followed by least square difference (LSD) test at a level of significant of $p < 0.05$.

3. Results and discussion

3.1. Characterization of nanoparticle

The efficiency of vesicle entrapment relies on vesicle stability, which itself is highly dependent on the type and amount of Sur forming the bilayers, as well as the amount of CH and drug load.

CH is one of the common additives in preparation of stable niosomes. It makes the membrane more arranged and eliminates the gel to lipid phase transition of niosome systems. Therefore, it has the capacity for effective prevention of drug leakage from niosomes [43,44].

Here, niosomal nanoparticles were synthesized and the effect of CH, added within the lipid composition, on insulin EE was assessed by changing the Sur/CH molar ratio from 1:1 to 5:1 (Table 1). A significant increase was observed in insulin EE% when 1:1 molar ratio of Sur/CH was incorporated into niosomes prepared from Span 60 ($p < 0.05$).

The decrease in CH then followed by a decrease in insulin EE%, except for lower amounts of CH (4:1 and 5:1) [45]. The formation of a TMC layer on the surface of the niosomes was confirmed by comparing the PDI, zeta potential and also the particle size of niosomes before and after TMC coating (Table 1).

The PDI value is an estimate of width distribution, representing the size heterogeneity. It was between 0.495 and 0.869. There was a slight difference between the PDI values measured for the prepared niosomes and changes in the CH content and TMC-coating. Z-average sizes of the niosomes with various Sur to CH molar ratios, in the presence and absence of TMC, were in the range of 186–734 nm. The lowest value was for 1:1 molar ratio of Sur to CH and Z-average size of coated niosomes slightly increased, compared with uncoated niosomes.

By increasing the zeta potential, the charged particles repel one another and they become more stable against aggregation. As shown in Table 1, zeta potential of uncoated nanoparticles, in various molar ratio of Sur to CH, varied from −33.341 to −42.000 mV.

TMC-coated niosomes, on the other hand, showed positive zeta potential values ranging between 6.710 and 21.002 mV. The zeta potential value reached its maximum level, at 21.002 mV, while the molar ratio of Sur to CH was 1:1. However, it reduced in other molar ratios of Sur to CH.

Fig. 1 shows transmission electron micrographs of the blank niosomes, insulin loaded niosomes, and insulin-loaded niosomes coated with TMC. These samples prepared with 1:1 Sur/CH ratio and 11 min sonication, based on experimental design studies. TEM morphology analysis confirmed the formation of almost spherical shape structures for both coated and uncoated nanostructure. Loosely held TMC layer was also visualized in TEM image of insulin-loaded niosomes coated with TMC. TEM results further confirmed encapsulation of insulin into niosomal nanostructure; and no changes were observed in the morphology of TMC coated niosomes as after drug loading. These findings

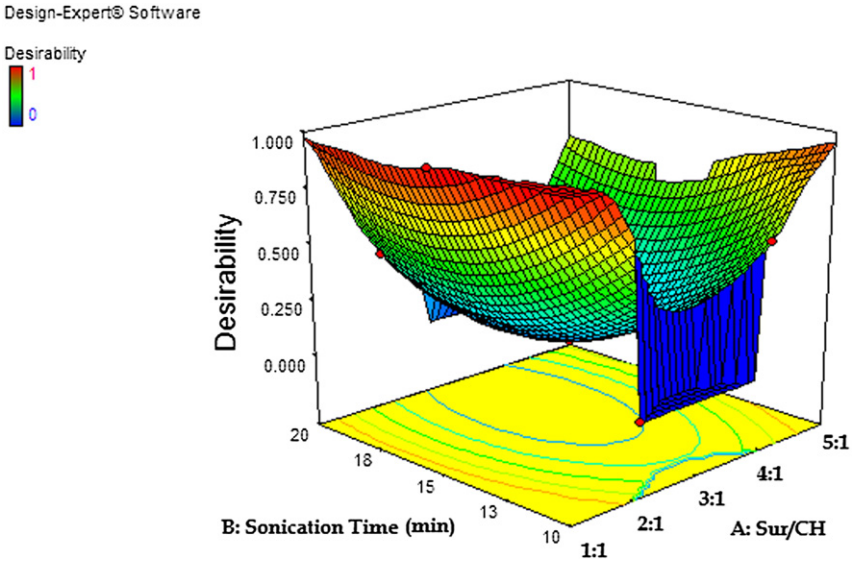

Fig. 2. 3D plot of desirability vs. ratio of Sur to CH and sonication time.

Table 3
Observed responses, predicted responses and error for validating of the model.

Factor		Zeta potential			PDI			EE%		
Sur/CH	Time of sonication	Observed responses	Predicted response	Error %	Observed responses	Predicted response	Error %	Observed responses	Predicted response	Error %
A = 1:1	B = 11	33.74 ± 1.041	35.142	4	0.538 ± 0.074	0.515	4	78.948 ± 2.072	79.373	0.5

were in well agreement with the results of dynamic light scattering (DLS) analysis (Table 1).

3.2. Experimental design studies

Experiments were performed based on the ratio of Sur to CH and sonication time; and responses including zeta potential, PDI, and EE% were added to the software as shown in Table 2.

Factors and responses were evaluated and optimum ratio and model were then calculated by the software. Following models were suggested for each response:

Eq. (1) is the model fitted to zeta potential:

$$\text{zeta} = -63.825 - 10.11667(\text{Sur/CH}) + 5.19B + 1.7375(\text{Sur/CH})^2 - 0.1685\,\text{time}^2. \quad (1)$$

Eq. (2) is the model fitted to PDI:

$$\text{PDI} = +0.0745 + 0.19642(\text{Sur/CH}) + 0.03525\,\text{time} - 0.029875(\text{Sur/CH})^2 - 0.000965\,\text{time}^2. \quad (2)$$

Eq. (3) is the model fitted to EE%:

$$\text{EE\%} = +147.491 - 21.36613(\text{Sur/CH}) - 6.85367\,\text{time} + 3.51944(\text{Sur/CH})^2 + 0.21201\,\text{time}^2. \quad (3)$$

All the models have significant *p*-value and insignificant lack of fit, indicating that the suggested models are proper. Reasonable agreement between adjusted R-squared and predicted R-squared proved the statistically significant selected modes, which could be used to navigate the design space.

By obtaining the optimum ratio of Sur to CH, outcome goals were set to each response. Zeta potential values determine the physical stability of nanosuspension. Minimum PDI and maximum EE% were needed while Zeta potential was at the minimum range of 30 mV. Generally, stability of nanosuspension is higher at high zeta potential values due to the great electrostatic repulsion force between them [46,47]. The optimum point with 1 desirability was suggested by hexagonal RSM in 1:1 ratio of Sur to CH at 11 min sonication. Fig. 2 shows the graph of desirability versus ratio of Sur to CH and sonication time. The software predicted the point with low error (less than 5%) from the experimental data.

Table 3 compares the experimental and predicted responses.

3.3. In vitro release study

Drug release rate is an important parameter in the development of drug delivery systems and must be carefully analyzed. In vitro studies in physiological conditions conducted to have an idea of in vivo performance of the proposed system [11,48]. Fig. 3 illustrates in vitro release of insulin from optimized insulin-loaded niosomal nanoparticles coated with TMC in SIF medium.

Biphasic kinetic release of insulin, including primary relatively rapid drug release, and an equilibrium state or a slower release phase, was observed in our formulation. The insulin release rate in our formulation was significantly slow, compared to insulin alone (Fig. 3). After 5 h, the released drug reached its maximum level, while it remained constant for other times. By 5 h, about 63.42% of insulin released from free solution, whereas, only 12% released from niosome system. These findings suggest that niosomes have the capability of sustainable and controlled release of insulin, and of course, support other studies [48], concluding that niosomes play an important role in development of controlled release formulations. This kind of release profile has also been observed for another sort of insulin-loaded niosome developed by Pardakhty et al. [25].

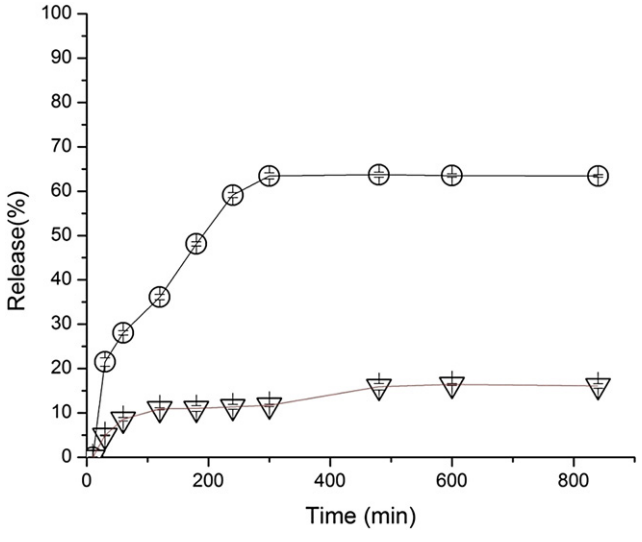

Fig. 3. Release of insulin from TMC-coated niosomes in SIF at 37 °C (mean of ± SD, n = 3). (▽) Insulin-loaded niosomes coated with TMC, (○) insulin alone.

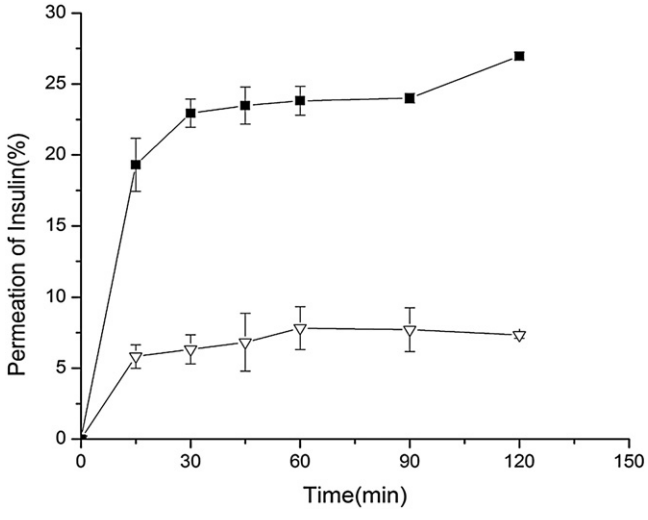

Fig. 4. Transport of insulin across Caco-2 cell monolayer. Data (means ± SD; n = 2) are shown as percentage of the total dose of insulin permeated: (■) TMC-coated niosomal nanoparticles, (▽) insulin alone. Insulin permeation enhanced by using insulin-loaded niosomal nanostructure, $p < 0.05$.

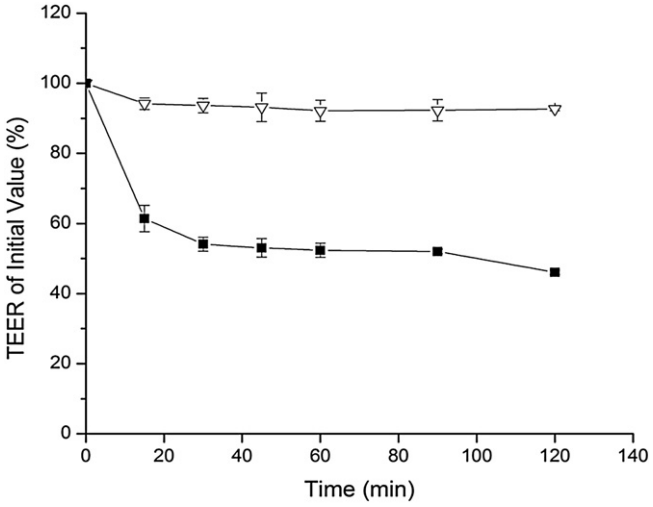

Fig. 5. The effect of insulin formulations on TEER values of Caco-2 cell monolayer: (■) TMC-coated niosomal nanoparticles, (▽) insulin alone.

3.4. Effect of niosomal nanoparticles on insulin permeation across intestinal cell model

Caco-2 human colon adenocarcinoma cell lines are regarded as more representative of the small intestine. When they are grown on semipermeable filters, they spontaneously differentiate in culture to form confluent polarized, columnar cell monolayers. Therefore, they have widely been selected as an in vitro model for evaluation of intestinal drug absorption [9].

To evaluate the ability of the niosomal nanoparticles to facilitate paracellular transport of insulin across a cell monolayer, Caco-2 cells were grown in transwell plates over 21 days and then treated with insulin and insulin-loaded niosomal nanoparticles coated with TMC.

Fig. 4 shows permeation of insulin through the intestinal model, which is presented as cumulative transport over time. The largest amount of insulin transport was facilitated by the niosomal nanoparticles. Most of insulin transport occurred in the first 15 min, with little additional transport in 90 min, except for the TMC-coated niosomal nanoparticles which still continued to improve insulin transport until 120 min. Compared to insulin alone, insulin permeation through Caco-2 cells was significantly increased by niosomal nanoparticles, representing 4-fold enhancement of insulin permeation coefficient.

Several studies have highlighted the potential use of chitosan as a stabilizing constituent of vesicular structure like liposomes. Chitosan-coated vesicular delivery system shares mucoadhesive characteristic allowing delay in intestinal transit time in order to increase absorption of protein drug [14,17]. However, modifications have been introduced to chitosan to enhance its intestinal mucoadhesion and permeation. It has been shown that drug transport across the rat intestinal mucosa can be positively affected by addition of thiol groups to chitosan and also thiolated polymers in combination with reduced glutathione (GSH) [14]. Modified chitosan derivatives, such as TMC have also been evaluated in vivo for absorption enhancing properties [14]. However, there is no obvious report regarding the formulation and optimization of TMC-coated niosomal nanostructure and its effect on enhancing intestinal drug permeability.

As shown in Fig. 4, the obtained results were aligned with what the scholars observed after oral administration of chitosan-coated liposomes in mice, where they were shown to have the capability of reducing tryptic digestion on insulin and improving enteral absorption of insulin [49].

3.5. TEER evaluation

TEER measurements for intestinal membrane models can be used to predict their paracellular permeability. Since the tight junctions are open, the TEER of Caco-2 cell monolayers is significantly reduced due to the passing of ions via their paracellular route [7,9]. Fig. 5 compares TEER measurements across Caco-2 cells incubated with insulin loaded TMC-coated niosomal nanoparticles or insulin alone for 120 min. Since insulin is not known to affect the tight junctions and consequently the TEER, it was used as control for making comparisons [9]. TEER of Caco-2 cell monolayer incubated with free insulin showed slight change, compared with the loaded insulin which showed a stronger effect on reducing the TEER, $p < 0.05$ (Fig. 5).

After 120 min incubation, the initial TEER value in Caco-2 cell monolayers incubated with niosomal nanoparticles decreased by 47%. As shown in Fig. 5, nanoparticles caused increased TEER reductions, compared with insulin control, ($p < 0.05$). However the decrease in TEER values may be explained by decreased cell viability after long period of incubation.

It has been shown that the permeation enhancement ability of chitosan and chitosan derivatives, such as TMC, is influenced by pH environment [14]. For example, unlike unmodified chitosan hydrochloride or 12.3% quaternized trimethyl chitosan, a trimethyl derivative with 61.2% quaternization was shown able to decrease TEER of Caco-2 cells and increase mannitol permeability at pH 7.4 [14]. The possible explanation for this difference is existence of positive charges on these compounds, mediating interactions with tight junction proteins, such as

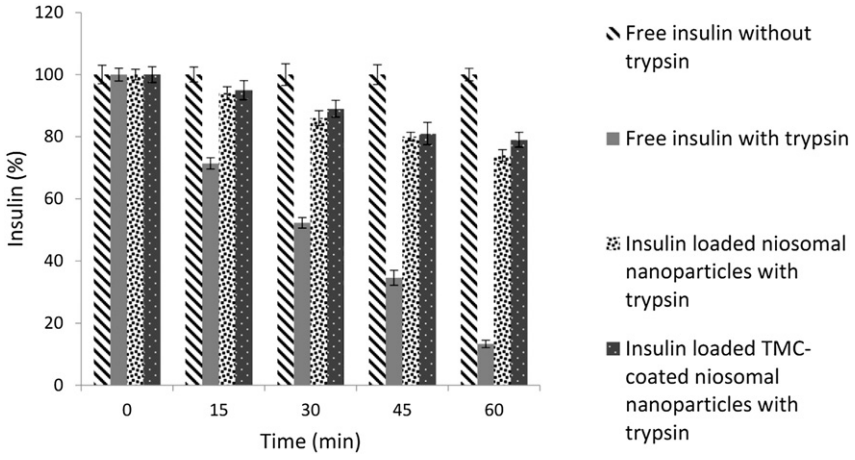

Fig. 6. Enzymatic degradation of insulin by trypsin. Each value represents the mean ± SD of three experiments. The initial concentrations of insulin and trypsin were 450 μg/ml and 300 BAEE IU/ml, respectively.

occludin and zonula occludens protein-1 (ZO-1), and also redistribution of F-actin cytoskeleton. In fact, electrostatic interactions with these proteins cause reversible opening of the junctions. During the interactions, changes in F-actin cytoskeleton and cell localization of ZO-1 and occludin modify cell morphology, thereby allowing opening the junctions.

This study came to the conclusion that the prepared nanoparticles were capable of transiently and reversibly opening the tight junctions between Caco-2 cells.

3.6. Insulin protection from trypsin degradation

As illustrated in Fig. 6, under the experimental conditions, about 86.7 ± 2.5% of free insulin control solution was degraded within 60 min. Compared to free insulin solution, both insulin loaded niosomal nanoparticles showed an excellent protective effect on insulin digestion ($p < 0.05$). Although, coated nanoparticles was more effective than uncoated nanoparticles, it seemed that TMC coating of nanoparticles could not significantly increase insulin stability and protect it from trypsin digestion ($p > 0.05$).

3.7. Stability of the system

The stability of encapsulated insulin is critical for effective oral delivery of insulin. The stability of the stored and freshly prepared insulin loaded niosomes was assessed every 10 day for over 60 days at 4 °C by noncompetitive indirect ELISA. The system maintained its function and there was no distinctive difference between the stored and freshly prepared samples due to high zeta potential and great electrostatic repulsion force between vesicles. The stored insulin-loaded niosomal nanoparticles kept 78% of its residual activity after storage at 4 °C for over 60 days (Fig. 7).

3.8. Biocompatibility of the system

Methylene blue was used to estimate the number of live cells present in the culture medium. The dye methylene blue can be taken up by dead or severely damaged cells, but not by living cells. The intensity of color is inversely proportional to the number of viable cells, and therefore a measure of biocompatibility. The percentage of cell viability was in reference to full cell viability of untreated cells incubated in PBS buffer. No significant decrease in cell viability was observed during the incubation of the niosomal nanoparticles with Caco-2 cells. The cell viability remained between 90 and 93% after 2 days and there was no significant difference between treated and untreated cells (Fig. 8). Coating of the nanoparticles with an additional biocompatible layer, such as TMC, did not cause any significant difference in cell viability. Cells

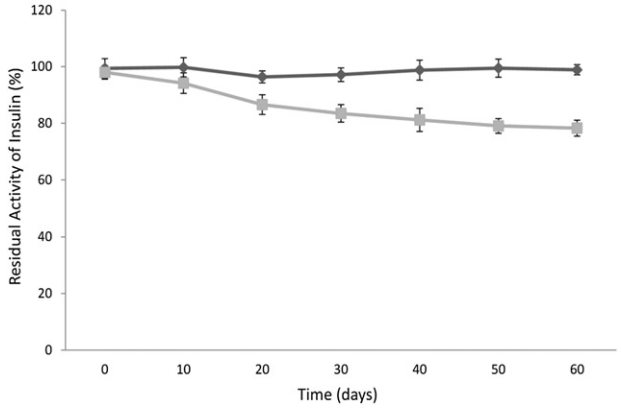

Fig. 7. Stability measurements of the stored and freshly prepared insulin loaded niosome every 10 days for over 60 days at 4 °C by ELISA. Residual reactivity of the stored (■) and freshly (♦) prepared insulin loaded niosomes.

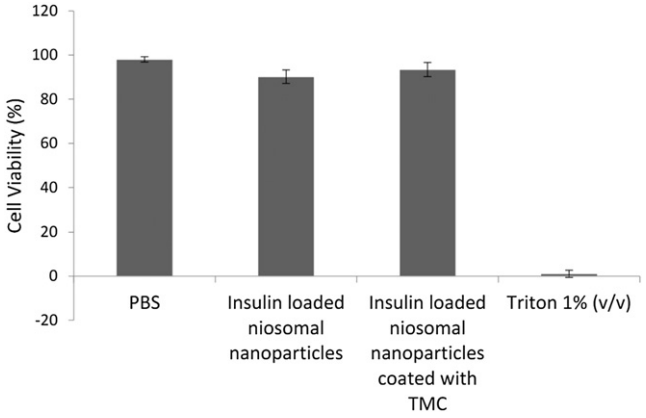

Fig. 8. Cell viability of Caco-2 cell lines after 2 days, expressed as the mean ± SD, n = 3. Caco-2 cells were incubated with PBS, insulin loaded niosomal nanoparticles, insulin loaded niosomal nanoparticles coated with TMC and Triton 1% (v/v). Both nanoparticle samples are not statistically different from the cells exposed to PBS, $p < 0.05$.

incubated with 1% Triton, showed 98%–100% decrease in cell viability due to toxicity. The proposed nanostructure was biocompatible to the Caco-2 cells, indicating no toxicity to the intestinal membrane models.

4. Conclusion

Niosomes have a great potential for oral drug delivery purposes. In current report, insulin loaded niosomal nanostructure coated by TMC was successfully designed by reversed-phase evaporation technique. The prepared nanoparticles were characterized by Zetasizer and TEM, and their biocompatibility was evaluated via cell viability measurements. The prepared niosomal formulation represented good function for opening the tight cell junctions and was able to facilitate insulin transport across Caco-2 cell monolayer as intestinal membrane models.

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