Enhanced Effects of Metformin Loaded Chitoson Nanoparticles in L6 Myotubes: In vitro

Chinnaiyan Santhosh Kumar[1*], Deivasigamani Karthikeyan[1], Venkata Radha Gadela[2]

[1]*Department of Pharmaceutics, Srikrupa Institute of Pharmaceutical Sciences, Vill: Velikatta, Mdl: Kondapak, RD: Siddipet, Dist: Medak-502277, Telungana state, India.*

[2]*Department of Pharmaceutics, GITAM University, Rushikonda, Visakapatnam-530045, Andhra Pradesh, India*

*Corresponding author: Chinnaiyan Santhosh Kumar, Department of Pharmaceutical Biotechnology, Srikrupa Institute of pharmaceutical sciences, India, Tel: +91-8008928923; Email:skredmind7@gmail.com

ABSTRACT

In this present study aimed to formulate and evaluate metformin loaded chitosan (CSM) nanoparticles (NPs) with sustained action for NIDDM (Non-Insulin Dependent Diabetes Mellitus), These nanoparticles have been developed by ionic gelation technique and were subjected to various studies including PCS (Photon correlation spectroscopy), SEM (scanning electron microscopy) and surface charge analysis. The in vitro haemolysis assay, SDS-PAGE test of stability and glucose uptake studies were performed with nanoparticles. The particle size of the nano-formulations 541.2 nm and exert spherical morphology with high stability. Moreover, formulated nanoparticles point up that, there was no interaction between drug and polymer. The CSM4 formulation showed better in vitro prolonged release characteristics. In addition, the CSM nanoparticles were reasonably stable in the presence of excess bovine serum albumin. The percentage of hemolysis induced by metformin and placebo CSNPs were less than 5% and CSMNPs are highly hemocompatible. The glucose uptake significantly augmented in skeletal muscle cell line. Above results recommended nanoparticle had prolonged release, reduced dose frequency, better patient compliance and targeted action on skeletal muscles.

Key words: *Chitosan, Metformin. Ionic gelation, Nanoparticle, L6 skeleton muscle*

INTRODUCTION

Diabetes mellitus (DM) is pandemic in the contemporary world and one of the major determinants of morbidity and mortality. Diabetes is a major health problem in the modernized society [1, 2]. The WHO also estimates that 80 per cent of diabetes deaths occur in low and middle-income nations and projected that such deaths will twofold between 2016 and 2030. It has been further

48

approximated that the global burden of type-2 diabetes is expected to augment to 438 million by 2030 from 285 million people in 2010. Similarly, for India this increase is estimated 51 million people in 2010, 69.2 million people living with diabetes (8.7%) as per the 2015 data and undiagnosed in more than 36 million people and may rises to 87 million in 2030 [3, 4] This surprising increase is primarily rooted in genetic level and modern life style related factors like obesity, physical inactivity, aging less, nutrition and stress [5]. The disruption of insulin secretion important factor to accretion of blood glucose level and other complications like neuropathy, blindness, renal dysfunction, organ damages, heart disease and related mortality is 2–4 times more in DM. The two categories of diabetes mellitus are type I (insulin-dependent) and type II (non-insulin dependent). Type II diabetes is a progressive and complex disease that is difficult to manage effectively in the long-term, which often requires multi medication strategy in order to achieve better glycemic control [6,7].

Metformin (1,1-dimethyl biguanide) is an oral anti-hyperglycemic drug, which its action by improving the peripheral sensitivity to insulin by increasing glucose uptake in skeletal cells and by inhibiting intestinal glucose uptake as well as hepatic glucose production [8]. Metformin is highly soluble in water, and its absolute bioavailability is 50 to 60%. The absorption site for metformin is the proximal part of the small intestine where the GIT absorption is complete after 6 h [9]. To increase the bioavailability of metformin, several approaches in controlled release dosage forms have been reported.

Anti-diabetic drug containing nano-formulation may enhance the therapeutic efficacy of the drug and also releases in a predetermined controlled approach for a long-lasting action. The biodegradable polymers have major advantages since they do not require any elimination after application [10]. Chitosan is one of the most important candidates for biomedical materials because of its biocompatibility and its relatively stronger mechanical properties. Chitosan is a copolymer of β (1-4)-glucosamine and N-acetyl-D-glucosamine prepared from partial deacetylation of chitin from crustacean shells. Protonation of amine groups on chitosan glucosamine monomers is facilitated at slightly acidic pH, below chitosan pKa of ~ 6.5; thus conferring cationic nature allowing chitosan to interaction with anionic components such as cell membrane, macromolecules and nucleic materials. Nanoparticles, due to their nanoscale and comparatively larger surface area, may interact with biological systems in a more efficient manner produce better therapeutic action [11, 12].

The present study was carried out to formulate and evaluate a stable nanoparticle using biodegradable polymer such as chitosan, to deliver metformin at a controlled rate for a long lasting action at targeted site.

MATERIALS AND METHODS

Materials

Metformin was donated by Micro labs limited (Hosur, India). Chitosan was donated by Central Marine Fisheries Research Institute Kochi, India. Sodium tripolyphosphate (TPP), glacial acetic acid, sodium hydroxide and all other chemicals

49

_____

were analytical grade purchased from Sigma–Aldrich (Bangalore, India) and ultrapure water was used throughout this study.

Preparation of placebo and Metformin loaded chitosan nanoparticles

The anti-diabetic drug metformin containing chitosan nanoparticles was formulated by *ionic gelation* method. Chitosan was solubilised in an aqueous solution of acetic acid (0.2 mg/mL) to form a 0.5 mg/mL chitosan solution. The polymeric solution was stirred overnight at 25ºC using a magnetic stirrer. The final pH of the chitosan solution was adjusted to 4.7-4.8 using 20 % w/v aqueous sodium hydroxide solutions and then passed through a syringe filter pore size (0.45μm, Millipore, USA). TPP was dissolved using ultrapure water at a concentration of 0.5 mg/mL and also filtered through a micron syringe filter (pore size 0.22 μm, Millipore, USA). The 10 mL of chitosan solution in a 100 mL beaker was preheated in a water bath at 60 °C for 10 min, the flask was then placed on the mechanical stirrer stirring at 700 rpm, in which the ambient temperature was controlled at 2-4°C, temperature fluctuations and flow of cold air were avoided as much as possible, and 3.0 mL TPP solution was quickly added to the chitosan solution using a plastic Pasteur pipette. The reaction was carried out for 10 min and the resulting suspension was subjected to further analysis. Nanoparticles were recovered by centrifugation at 10000 rpm for 40 min. The Metformin containing nanoparticles was prepared similarly 2.5mg/ml drug pre-incubated with chitosan solution [13, 14].

Drug Entrapment efficiency

The drug entrapment efficiency of formulated nanoparticles was estimated by separating the nanoparticles by ultracentrifugation at 10000 rpm for 30 min. The sum of free metformin in the supernatant was calculated by UV spectrophotometer at 232nm. The drug loading efficiency in the prepared nanoparticles was calculated by the following formula.

Entrapment efficiency (%) = Tp-Tf/Tpx100

Where, Tp = Total amount drug, Tf = free drug

Nanoparticle characterizations

The hydrodynamic diameter of nanoparticles was calculated by DLS high performance particle seizer (Beckman counter, Delsa™ Nano). The size confirmation and surface morphology analysis of NPs were done using SEM (JEOL-JSM-6490-LA Scanning Electron Microscope). Surface charge analysis carried zeta potential measurements using Zetasizer (Beckman counter, Delsa™ Nano). The FT-IR spectrum of materials was examined using KBr pellet with a resolution of 4 cm^{-1} and 100 scans per sample on Perklin Elmer Spectrum RXI FT-IR spectrophotometer.

In vitro drug release

In vitro drug release studies of a metformin loaded chitosan nanoparticle was performed in triplicate by dialysis method. The formulated nano preparation (10 ml) was taken in dialysis tubing (MW cut-off = 10 kDa from Hi media) and

50

allowed for dialysis by immersing it into a reservoir containing 100 ml of phosphate buffer pH 6.8 and continuously stirred 50 rpm at 37°C. The amount of drug release was calculated by sampling the receptor media (5ml) at predetermined time intervals and 5 ml of fresh buffer was replaced. The amount of metformin released in the buffer was calculated by a UV spectrophotometer at 232nm (ELICO SL-244 spectrophotometer).

In vitro Haemolysis assay

Fresh blood was collected from human volunteers into acid citrate dextrose (ACD) contained in tubes. The different concentrations of samples were prepared in 0.9% w/v saline. The 1% v/v Triton-X 100 was kept as positive and 0.9% w/v saline and negative control. 0.1 mL of the samples was mixed with 1 mL of blood and kept at 37°C for 5 h at 40 rpm. After incubation, plasma was recovered by centrifugation at 4500 rpm for 15 minutes and optical density at 450 nm was measured. % Haemolysis was calculated as:

$$\text{Haemolysis (\%)} = \text{Plasma Hb content in test} \,/\, \text{Total Hb content} \;\times 100$$

SDS-PAGE test of nanoparticle stability

The *in vitro* stability of metformin nanoparticles was examined by the SDS-PAGE. The stability of formulate nanoparticle was confirmed by calculated the molecular mass changes in each preparation following reaction with or without bovine serum albumin (BSA). The placebo and metformin nanoparticle (1 mg/ ml) was dissolved in reaction buffer (10 mM Tris−HCl (pH 7.5), 150 mM NaCl and 1 mM EDTA) with 20 mg/ml BSA. After incubation time of 6 h, each sample was loaded onto a vertical slab gel consisting of a 5% stacking gel 10% separating gel. All gels were run at a constant voltage mode of 120 V in a Tris /glycine/ SDS buffer. BSA run on the SDS-PAGE gel was stained with Coomassie Brilliant Blue (0.1 w/v) in 10% of acetic acid in a solution methanol: water (1:1 v/v).

In vitro glucose uptake assay

Glucose uptake assay of metformin loaded chitosan nanoparticle was determined by using L6 myotube cells. In brief, the 24 h old cell cultures with 70-80% confluence in 60mm petri plates were allowed to differentiate by keeping in Dulbecco's Modified Eagles Medium (DMEM) with 2% Fetal bovine serum (FBS) for 4-6 days. The degree of differentiation was established by monitoring multi-nucleation of cells. The separated cells were serum starved over night and at the period of analysis cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) and incubated with KRP buffer with 0.1% BSA for 30 min at 37°C. The differentiated cells were treated with different safe concentrations of standard and test drugs for 30 min at 37°C along with negative controls. 2 D-glucose solution was added concurrently to each petridish and incubated at 37°C for 30 min. After incubation, the uptake of the glucose was ended by aspiration of solutions from wells and wash down three times with ice-cold KRP buffer. Cells were lysed with 0.1M NaOH and an aliquot of lysates were used to calculate the cell-associated glucose. The amount of glucose in cell lysates was measured using glucose assay kit.

RESULT

Preparation of nanoparticles

51

Metformin loaded chitosan Nanoparticles was successfully formulated by ionic gelation method. The pre-incubation of drug with polymer was conceded to facilitate electrostatic interaction between the amphoteric chitosan polymer and cationic metformin. In a while the drug was entrapped within the nanoparticle system by cross-linking chitosan polymer using TPP (Tri polyphosphate). The CSM nanoparticles were formulated using different concentration (CSM1: 0.5, CSM2: 0.7, CSM3: 0.9, CSM4: 1.1, CSM5: 1.3 mg/ml) of chitosan polymer. However, based on the drug entrapment efficiency, among the five different concentrations, 1.1 mg/ml showed the 70.17 % entrapment efficiency. The other formulation produced low amount of drug entrapment which causes high amount drug wastage during the formulation. The hydrophilicity of drug cause complexity in achieving high entrapment as it can easily move towards to the aqueous phase outside. The results elicited on (Figure 1).
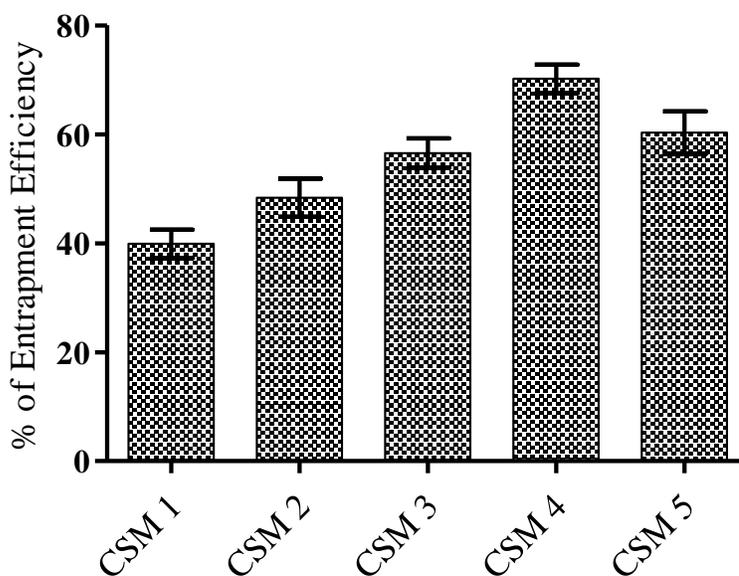

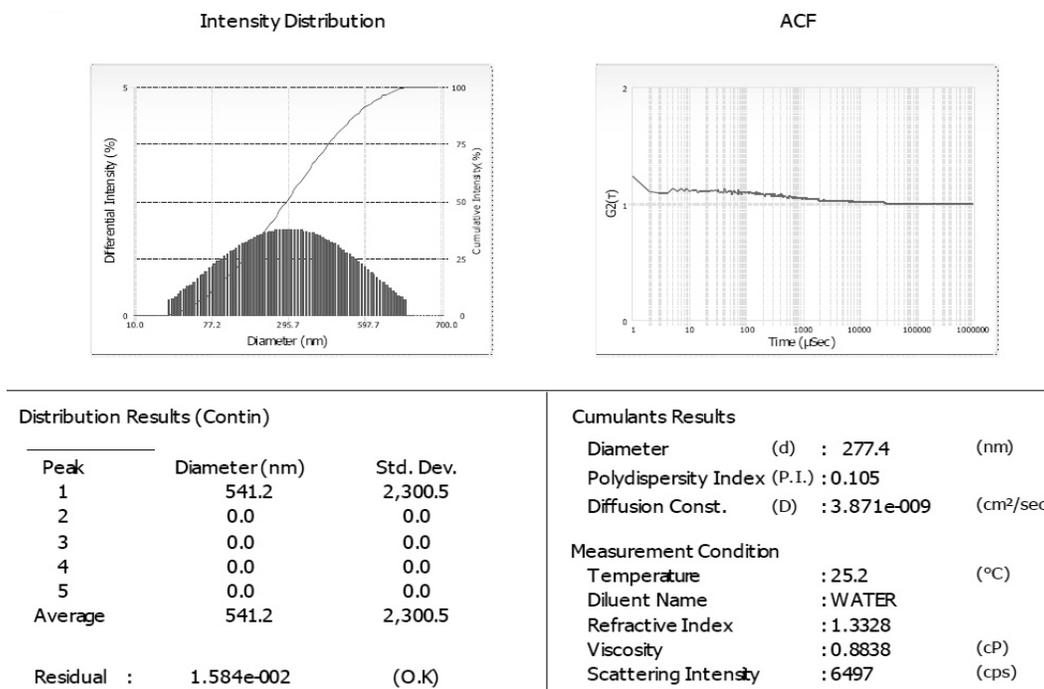**Figure 1.** Entrapment Efficiency of CSM Nanoparticles.

Figure 2: Particle size distribution of CSM4

Particle Size Analysis

The particle size formulated nanoparticle was found to be in the nanometer range of 270-643nm (Table 1). The features of the CSM particles prepared with different concentrations of chitosan were studied, when the concentration of chitosan solution increase its influences increasing in particle size of the nanoparticle. The optimized formulation CSM 4 showed the mean particle size 541 nm and broad unimodal particle size distribution with PDI value 0.105. (Figure 2).

Table 1: Particle size distribution and Zeta potential of CSM nanoparticle.

Formulation code	Mean particle diameters (nm)	Polydispersity Index (PDI)	Zeta potential (mV)
CSM 1	270 ± 15	0.26 ± 0.03	29.09 ± 2.1
CSM 2	347 ± 27	0.22 ± 0.01	33.04 ± 4.2
CSM 3	476 ± 12	0.18 ± 0.04	37.21 ± 1.5
CSM 4	541 ± 05	0.10 ± 0.06	42.12 ± 2.5
CSM 5	643 ± 20	0.24 ± 0.05	35.63 ± 3.4
Note: The values were expressed in Mean±SD n=3			

53

Zeta potential

As results displayed in (Table 1), the formulated NPs exhibit positive zeta potential (from +29 to +42mV). The results indicate of NPs surface charge, as well as nano system stability. Regarding the CSM 4 formulations consisting of CS/TPP/MET 1.1/0.5/2.5 (w/v), which have higher CS content, registered increased entrapment efficiency (70.17%) and zeta potential (+42.12 mV) compared to the other formulation (Figure 3), this means that the previous Nanoparticles are more reticulated than the others and it is very likely that this reticulation allowed them to imprisonment of more metformin. On the other hand, the NPs made up of CS 0.5, 0.7, 0.9 (w/v), showed loading capacity of 39.88%, 48.34%, and 56.52%, respectively, but smaller size (270 - 476 nm) than the NPs made up of CS 1.1 (w/v).
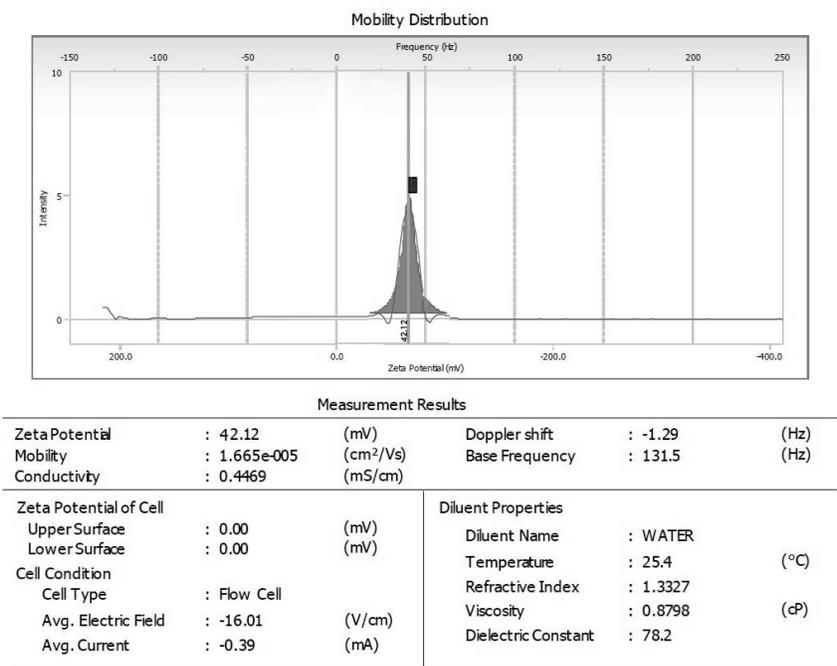

Figure 3: Zeta potential of CSM4

Morphological characterizations of CSM nanoparticles

The CSM4 nanoparticles have smooth spherical shaped appearance. (Figure 4). the peripheral appearance of formulated NPs based on saturation of polymer solution, produced smooth and high yield of NPs. The un-dissolved polymer solution generate irregular and rod shaped particles. In this preparation the polymer was fully saturated and leading to the smooth and spherical configuration, independently distributed homogeneous particles and has no evidence of collapsed particles.
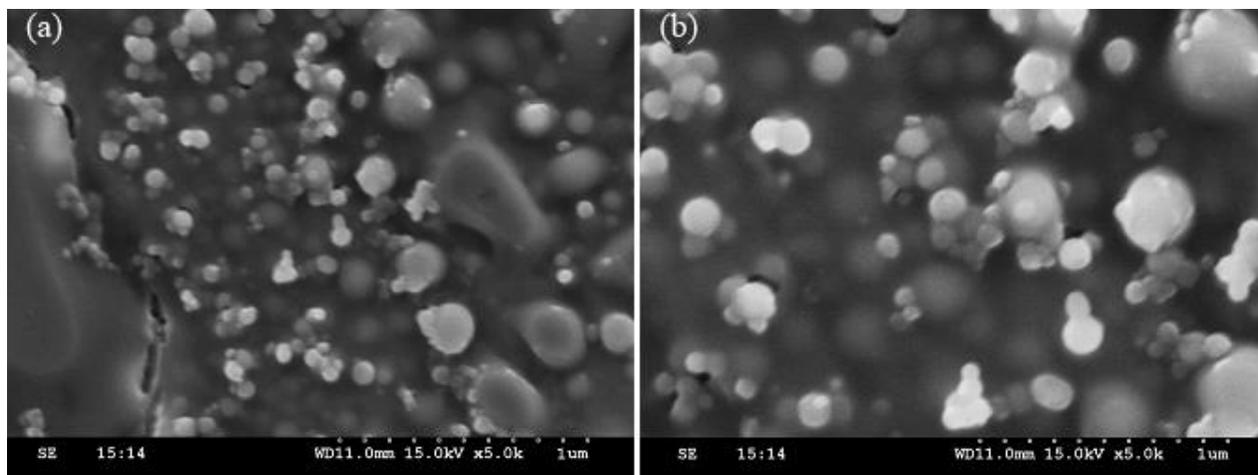
Figure 4: Scanning electron microscopy photograph of CSM 4 nanoparticles. a) X10,000 Magnification, b) X 20,000 Magnification

FTIR Compatibility study

FTIR spectral data (Figure 5) were used to confirm the chemical stability of metformin in polymeric nanoparticle. FTIR of the pure metformin showed the peak at 3372 cm^{-1}(NH stretching of NH$_2$), 3297 cm^{-1} (NH stretching), 3173 cm^{-1} (CH stretching), 1628 cm^{-1} (C-N stretching), 1566 cm^{-1} (C=N stretching), 936 cm^{-1} (C-H deformation). The finger print characteristic vibration bands of chitosan polymer appears at 3400-3650 cm^{-1} (OH stretching), 3200-3400 cm^{-1}(NH stretching of NH$_2$), 2920 cm^{-1}(CH stretching), 1126 cm^{-1}(C-O stretching).
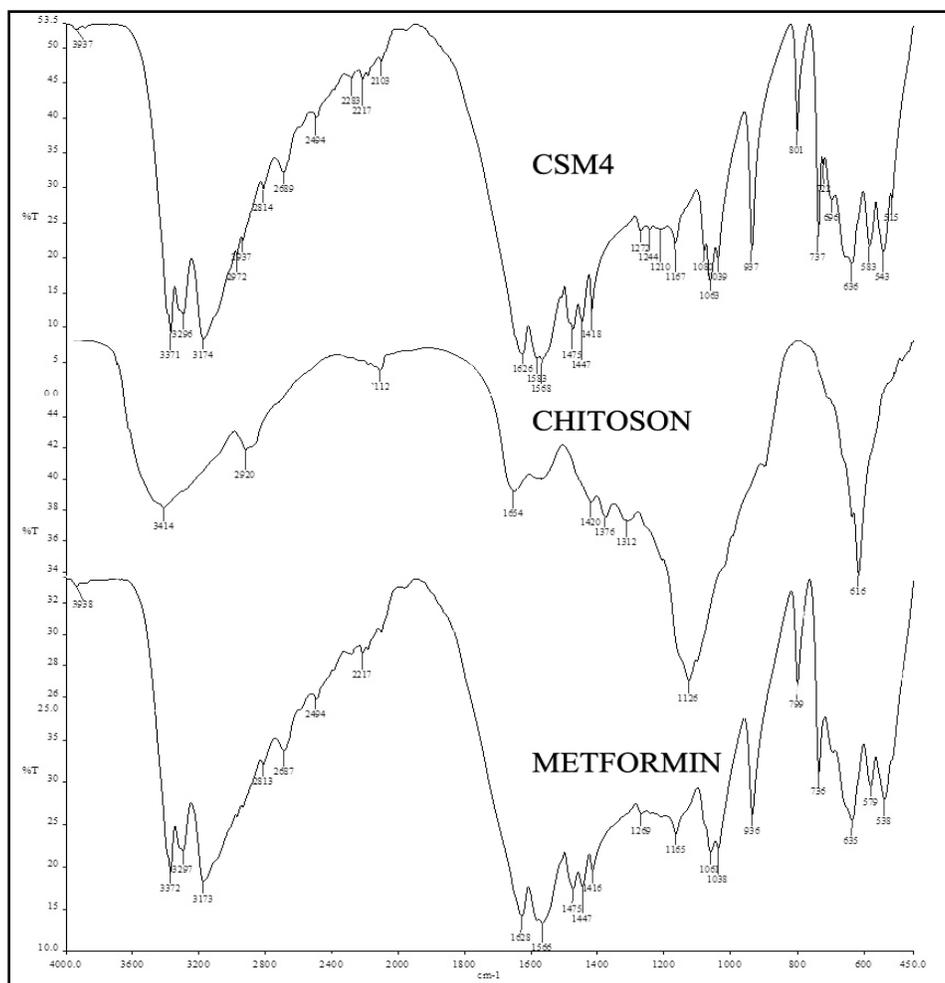
Figure 5: FT- IR showing Metformin, Chitosan and optimized formulation CSM 4.

In vitro release study

To assess the potential of metformin loaded chitosan nanoparticles as nano-sized drug carriers, release tests were performed in phosphate buffer (pH 6.8, 37°C). The *in vitro* drug release pattern of initial burst release of surface adsorbed drug was observed followed by slow and sustained release of entrapped drug from the CSMNPs (Figure 6). The initial burst effect on the release of metformin may be due to the loosely associated metformin on the surface of chitosan nanoparticles. The burst release is clinically significant to achieve initial high drug concentrations in the target tissue. The slow release of the drug is controlled by the speed of the degradation of chitosan polymer. During the drug release study the reservoir condition was maintained by regularly replacing the dialysis medium. With 67% initial release of CSM4 after 6 h and further zero order release profit was observed up to 24 hours. The two phase drug release behaviour might be responsible that the initial rapid drug release is due to the release drugs weakly networked with hydrophobic moiety of nanoparticles, and the following steady release is due

56

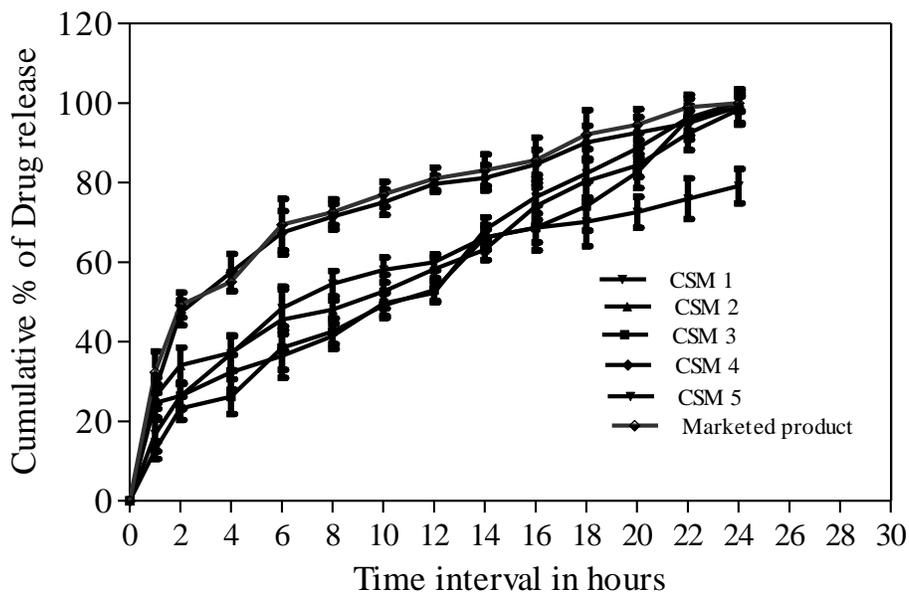to the release of drug robustly interacted with hydrophobic cores of nanoparticles [21].

Figure 6: *In vitro* drug release profile of CSM nanoparticle in PBS $_p$H 6.8

Hemolysis assay

The percentage of hemolysis induced by metformin, bare CS NPs and CS-metformin NPs were less than 5% (critical safe hemolytic ratio for bio-materials according to ISO/TR 7406) (Figure 7). This is most likely due to that the nanoparticles caused negligible damage to erythrocyte membrane, and moreover, the negatively charged free hemoglobins were readily adsorbed by the positively charged nanoparticles, causing the hemolysis was simar to negative control PBS (Figure 8). The results suggested that the CSM NPs are hemocompatible and safe for *in vivo* administration[22,23].
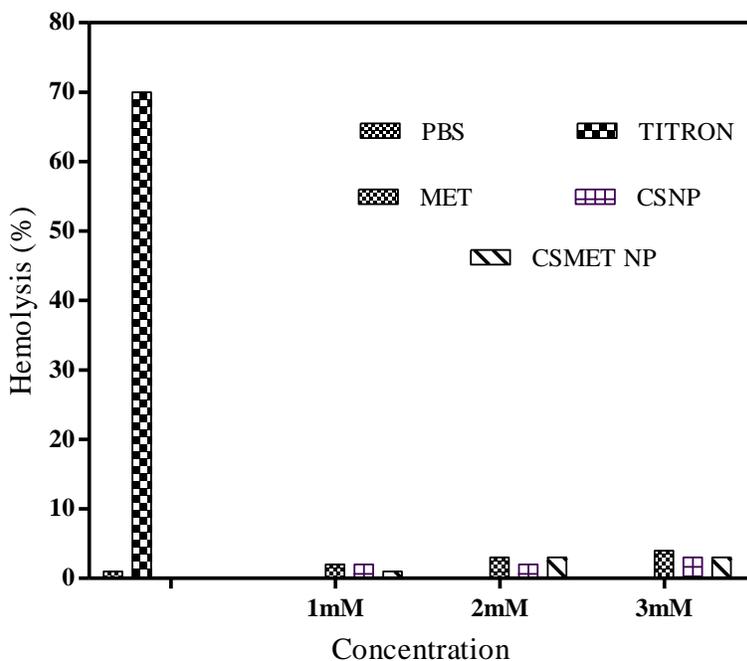
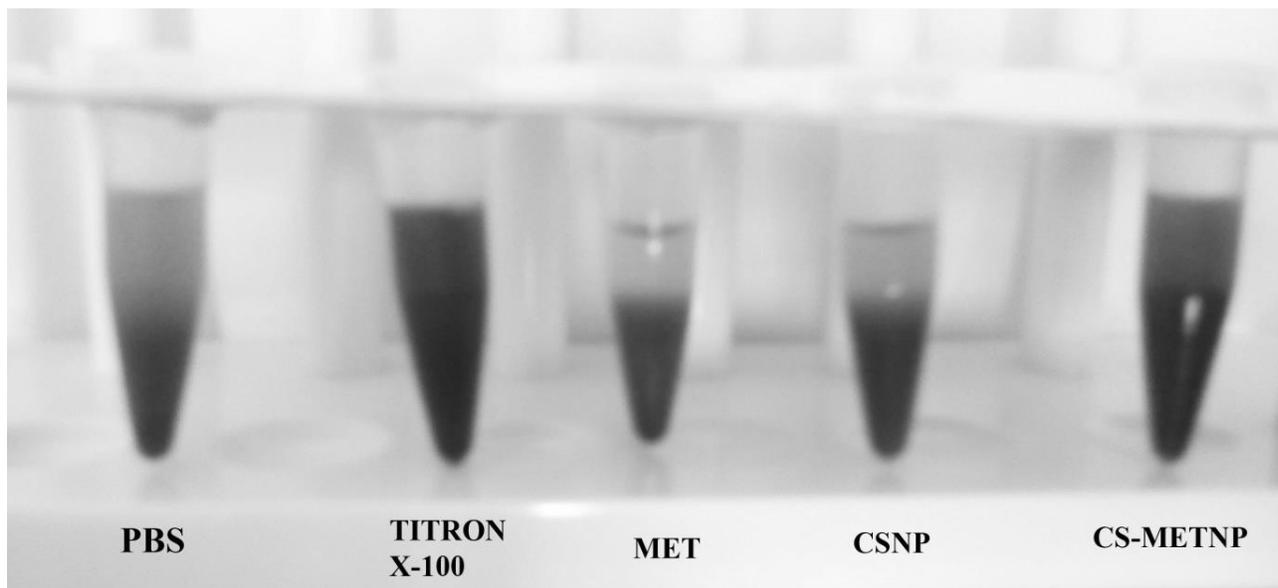**Figure 7:** Graph showing % hemolysis versus concentration of samples.

Figure 8: Hemocompatibility of 3mM metformin, bare CSNPs and CSM4.

In vitro stability study of nanoparticles

In vitro stability tests of nano-sized drug carriers in aqueous media do not provide a reasonable estimate of the *in vivo*

58

stability, because such tests are carried out primarily in a buffer solution without blood components, particularly proteins [24]. After administration of nano-sized drug, various proteins spontaneously interact with nano drug carriers in the blood stream and even though our nano-sized formulation are stable in such a buffer, non-specific protein binding to nano-sized drug may cause destabilization and dissociation of drug carriers in the blood stream [25, 26]. Based on the *in vivo* conditions in which nanoparticles must exist, the stability of nano sized formulation should be examined in the presence of blood protein. Thus, we examined the stability of CSM nanoparticles in the presence of bovine serum albumin, which is the most abundant plasma protein in the blood, using SDS-PAGE. The CS and CSM nanoparticles were very stable in the running buffer. Following incubation of the CS and CSM nanoparticles with excess BSA (20 mg/ml) for 6 h, CS and CSM nanoparticles continued to maintain their nanoparticles structure, and stability. The (Figure 9), showed the control band of BSA at SDS-PAGE.
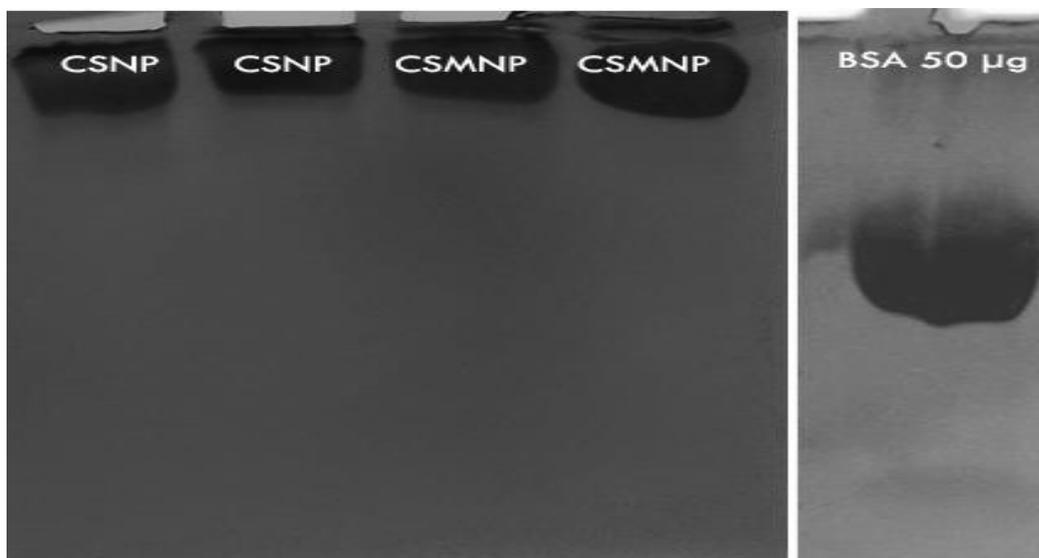

Figure 9: *In vitro* stability test of CS and CSM4 an SDS-PAGE test.

Glucose Uptake Study

The prepared CSM nanoparticle was assayed the uptake of 2D Glucose (2DG) in cultured skeletal muscle cells. L6 myotubes are distinguished in a 96-well microplate were stimulated with CSM nanoparticle for 4h, and then incubated with 2DG for 30min before the uptake of 2DG was determined. The L6 myotubes stimulated by insulin showed an increase in 2DG uptake (Table 2). These results projected that 2DG uptake is increased by standard insulin and should be reflect in the amount of 2DG that was transported by glucose transporters (GLUTs) and phosphorylated. The CSM nanoparticle dose dependently increased 2DG uptake into L6 myotubes in the absence of insulin [27].

Table 2*: In vitro* glucose uptake studies in L-6 myotoube cell line.

Name of the formulation	Test concentration (µg/ml)	% glucose uptake over control
CSM4	100	115.0±3.05*
CSM4	50	53.09±4.78*
Insulin	1 IU/ml	131.50 ± 17.62
Note: Data are expressed as mean±S.E.M. (n=6) and analyzed by one way ANOVA * Represents statistically significant as compared to standard (p < 0.05).		

DISCUSSION

The chitosan metformin nano particles was prepared with different concentrations of chitosan solution were studied. The results indicated when increasing the concentration of chitosan which increase particle size and found that the formation of nanoparticles was only feasible for at particular concentrations of chitosan polymer and TPP [15]. This piece of evidence was also confirmed in our study that in order to evade the any micro-particles configuration, the concentration of chitosan solution and TPP needed to be below 1.5 mg/mL and 1.0 mg/mL, respectively. In these concentration ranges, chitosan solution had mild effect on the monodispersity of the nanoparticles, since their PDI values were all below 0.05. It is known that under acidic nature, there is an electrostatic repulsion between chitosan molecules due to the protonated amino groups of chitosan polymers; in the meantime, there also exist interchain hydrogen bonding exchanges between chitosan molecules. Hence, in this concentration range, as chitosan concentration increases (0.5-1.3mg/ml), chitosan molecules approach each other with a limit, leading to a limited increase in intermolecular cross-linking, thus larger but still nanoscale particles are formed [16]. Above this concentration, microparticles are easily produced possibly due to the stronger hydrogen bonding interactions leading to plenty of chitosan molecules concerned in the cross-linking of a single particle. The arrangement of micro-particles usually leads to a flocculent precipitate due to the electrostatic repulsion between particles are not adequate to uphold the stability of these large particles [17].

The low concentration of chitosan may form stable nanoparticles even at a low mass ratio of chitosan to TPP, while higher concentration of chitosan could only form stable nanoparticles at a higher mass ratio of chitosan to TPP. For illustration, the fixed concentration of TPP at 0.5 mg/mL, a chitosan concentration of 0.5 mg/mL could form stable nanoparticles at a mass ratio of 3.3:1, while a chitosan concentration of 1.0 mg/mL would form aggregates at this mass ratio [18].

The positive charge of zeta potential from +29 to +42 mV of the particle surface charge is crucial for the interaction with the negatively charged mucosa, increasing the residence time of the nano delivery system at the absorption site of skeleton muscle. As a consequence, the association of metformin with the positively charged CS/TPP NPs is favoured, increasing the zeta potential. The optimised formulation CSM 4 showed higher entrapment efficiency (70.17%) and zeta potential (+42.12 mV).

This spherical arrangement suggested that the application of the Stokes–Einstein equation (relating the hydrodynamic and thermodynamic view on the diffusion of microspheres) could be reasonable to estimate the particle size of CSM nanoparticle [19]. In addition, this nano formulation showed brilliant topography, representing the coating of polymer in the external layer. The cross linking agent TPP stabilized the nano-sized configuration and prevented CSM NPs from coagulation.

The drug and polymer interaction study between was carried out to evaluated the compatibility. The characteristic band peak at 1626 and 1583 cm^{-1} subsequent to the stretching vibration of C=N and peaks at 937, 801 and 737 cm^{-1} typical for N-H wagging in metformin indicated the presence of drug within the NPs. Furthermore, the decreased intensity of metformin peaks at 3371 and 3296 cm^{-1} suggested the possibility of hydrogen bonding between the metformin and chitosan in the NPs [20]. Comparing the FTIR spectra of pure drug, polymer, and formulation we confirmed that good chemical stability drug and polymer because of no significant interaction of formulation ingredients.

These SDS-PAGE data indicated that CSM nanoparticles maintained their stability with or without excess proteins, which will enhance *in vivo* bio-distribution of nano-sized drug carriers [21].

This result suggests that the increased 2DG uptake induced by CSM was dependent on its high affinity for the nuclear peroxisome proliferator- activated receptor ɣ (PPAR ɣ) which promotes Glucose transporter type (GLUT4) translocation to the cell membrane. Importantly, skeletal muscle is a major mass peripheral tissue, accounting for ~40% of the total body mass and >30% of energy expenditure. Hence, skeletal muscle has a supreme role in energy balance and is the primary tissue for insulin-stimulated glucose uptake, disposal, and storage (showing fourfold the glycogen content of liver) [28, 29]. Therefore, skeletal muscle glucose uptake must be considered an important therapeutic target tissue in the battle against NIDDM.

Conclusion

Metformin loaded chitosan NPs were successfully prepared by ionic gelation method. The prepared NPs were analyzed using particle size distribution, zeta potential and SEM for its size and shape. The FT-IR analysis confirmed the incorporation of the drug within the nano carrier. The optimized nano-formulation CSM4 showed burst drug release followed by slow and sustained release of the drug at pH 6.8 and hence may increase the drug retention time in blood circulation. Also, the CSM nanoparticles showed a reasonable stability in the presence of excess bovine serum albumin, indicating the *in vivo* stability of CSM nanoparticles in the blood stream. The CSM nanoparticles did not induce any haemolysis and thus proved to be

haemocompatible. The CSM 4 NPs dose dependently increased glucose uptake in L6 skeleton muscle. Hence by the application

of this nanoformulation, bioavailability of metformin can be increased for efficient treatment of type 2 diabetes mellitus.

Acknowledgements

REFERENCES

[1]   Wild, S., Roglic, S., Green, A., et al. Global prevalence of diabetes estimates for the year 2000 and projections for 2030. *Diabetes Care* **2004.** 27: 1047–1053.
[2]   Horenstein, RB., Shuldiner, AR., Genetics of diabetes. *RevEndocr Metab Disord* **2004.** 5: 25–36.
[3]   Zimmet, P., Alberti, KG., Shaw, J., Global and societal implications of the diabetes epidemic. *Nature,* **2001.** 414: 782–787.
[4]   King, H., Aubert, RE., Herman, WH., Global burden of diabetes 1995–2025: prevalence, numerical estimates and projections. *Diabetes Care*. **1998.** 21: 1414–1431.
[5]   Mohan, V., Sandeep, S., Deepa, R., et al. Epidemiology of type 2 diabetes: Indian scenario. Indian *J Med Res* **2007.** 125: 217–230.
[6]   Akiyama, S., Katsumata, S., Suzuki, K., et al. Hypoglycemic and hypolipidemic effects of hesperidin and cyclodextrin-clathrated hesperetin in Goto-Kakizaki rats with type 2 diabetes. *Biosci Biotechnol Biochem*. **2009**. 73: 2779–2782.
[7]   Brahmachari, G., Bio-flavonoids with promising antidiabetic potentials: a critical survey. Challenge **2011.** 661: 187–210.
[8]   Green, J., Feinglos, M., New combination treatments in the management of diabetes: focus on sitagliptin metformin. *Vasc Health Risk Manag* **2008.** 4:743–751.
[9]   Graham, GG., Punt, J., Arora, M., et al. Clinical pharmacokinetics of metformin. Clinical Pharmacokinetics **2011.** 5: 81– 98.
[10] Mi, FL., Shyu, SS., Lee, ST., et al. Kinetic study of chitosan–tripolyphosphate complex reaction and acid-resistive properties of the chitosan–tripolyphosphate gel beads prepared by in-liquid curing method. *J Polymer Sci Part B*: Polymer Physics **1999.** 37: 1551–1564.
[11] Kalotia, M., Bohidar, HB., Kinetics of coacervation transition versus nanoparticle formation in chitosan–sodium tripolyphosphate solutions. Colloids and Surfaces B: *Biointerfaces* **2010.** 81: 165–173.
[12] Chen, RH., Tsaih, ML., Effect of temperature on the intrinsic viscosity and conformation of chitosans in dilute HCl solution. *Int J Biol Macromolecules* **1998.** 81: 135–141.
[13] Cho, MC., Heuzey, A., Begin, PJ., Viscoelastic properties of chitosan solutions: effect of concentration and ionic strength. *J Food Engineering* **2006.** 74: 500–515.
[14] Amid, M., Romeijn, SG., Borchard, G., et al, Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system, *J Control Release* **2006.** 111: 107–116.
[15] Sattarahmadya, N., Helib, H., Faramarzi, F., Nickel oxide nanotubes-carbon microparticles/ Nafion nanocomposite for the electro-oxidation and sensitive detection of metformin. *Talanta* **2010.** 82: 1126 –1135.
[16] Jayakumar, R., Prabaharan, M., Nair, S., et al. Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications. *Progress in Materials Sci,* **2010.** 55: 675-709.
[17] Wei, Yanb., Zushun, Xub., Hong, Nia., Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. *Colloids and Surfaces B: Biointerfaces*, 2012. 90; 21-27.
[18] Al-Qad, A., Grenha, D., Carrion Recio, B., et al. microencapsulated chitosan nanoparticles for pulmonary protein delivery: In vivo evaluation of insulin loaded formulations. *J Control Release,* **2011.** 4:1-8.
[19] Berger, J., Reist, M., Mayer, JM., et al. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications, *Eur J Pharm Biopharm*, **2004.** 57: 19–34.
[20] Snima, KS., Jayakumar, R., Unnikrishnan, AG., et al. O-Carboxymethyl chitosan nanoparticles for metformin delivery to pancreatic cancer cells. *Carbohydrate Polymers* **2012.** 89: 1003-1007.
[21] Costa, P., Lobo, J., Modeling and comparison of dissolution profiles. *Euro J Pharm Sci* **2001.** 13: 123–133.
[22] Barshtein, G., Ben-Ami, R., yedgar, S., Role of red blood cell flow behavior in hemodynamics and hemostasis. *Expert Rev Cardiovascular Ther.* 2007. 15: 743–752.

[23] Oberdorster, E., Oberdorster, J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect,* **2005.** 113: 823–839.

[24] Jin Hee, Na., Seung-Young, Lee., Sangmin, Lee., et al. Effect of the stability and deformability of self-assembled glycol chitosan nanoparticles on tumor-targeting efficiency. *J Control Release,* **2012.** 163: 2-9.

[25] Anitha, A., Maya, S., Deepa, N., et al. Efficient water soluble O-carboxymethyl chitosan nanocarrier for the delivery of curcumin to cancer cells. *Carbohydrate Polymers*, **2011.** 83: 452–461.

[26] Lavertu, M., Methot, S., Tran-Khanh, N., et al. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation, *Biomaterials,* **2006.** 27: 4815–4824.

[27] Patil, SA., Kuchekar, BS., Chabukswar, AR., et al. Formulation and evaluation of extended-release solid dispersion of metformin hydrochloride. *J of Young Pharmacists*, **2010.** 12: 121–129.

[28] Csaba, N., Koping-Hoggard, M., Alonso, MJ., Ionically crosslinked chitosan/ tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery, *Int J Pharmaceutics* **2009.** 382: 205–214.

[29] Myriam, Jean., Mohamad, Alameh., Diogo, De Jesus., et al. Chitosan-based therapeutic nanoparticles for combination gene therapy and gene silencing of in vitro cell lines relevant to type 2 diabetes. *Eur J Pharmaceutical Sci,* **2012.** 45: 138-149.