

Liver targeted functionalized chitosan nanoparticles for the delivery of tenofovir against hepatitis-B

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Abstract: The liver targeting and controlled release nanoparticles based on chitosan derivative was prepared: firstly, novel lactose modified chitosan (LC) was synthesized; its chemical structure was characterized by ^1H NMR and FTIR spectroscopy. Then, tenofovir disoproxil fumarate (TDF) an antiviral drug was chosen as model drug and encapsulated within nanoparticles by ionic gelation of LC with tripolyphosphate (TDF-LC-NP) (F2, F3). The TDF-loaded chitosan nanoparticles (TDF-NP) were prepared as a control (F1). The NP's were characterized for the morphologies (TEM), particle size, zeta potential, entrapment efficiency, *in vitro* drug release and *in vivo* liver targeting studies. Formulations F1 and F3 were in the size range of 226.4 and 176.5 nm with a PDI of 0.26 and 0.1 and exhibited a positive electrical charge (+28.4 and +11.7 mV). In the optimal aqueous conditions, the % EE of TDF-LC-NP was 39.3% (F2). With 50% (v/v) isopropyl alcohol (IPA)/0.2% acetic acid solution as alternative solvent, % EE increased to 65.9% (F3). The *in vitro* drug release behavior from nanoparticles displayed zero order kinetics with initial burst release and consequently sustained release and the studies showed that the nanoparticles could prolong the drug release (up to 48 h) compared with TDF solution (F0) (2 h). The tissue distribution of TDF-LC-NP, and TDF-NP were carried out and the results showed that the distribution of TDF-LC-NP to liver was higher than that of TDF-NP. According to these results, the TDF-LC-NP have the potential to be used as drug delivery system with hepatic targeting and controlled release properties.

Key words: Lactose modified chitosan, liver targeting, tenofovir disoproxil fumarate, nanoparticles.

Introduction

Although effective vaccines are available, chronic hepatitis B virus (HBV) infection is still a serious global health problem. Hepatitis B caused by hepatitis B virus (HBV) is an infectious inflammatory illness of the liver, and is also considered as a causative factor of the development of hepatocellular carcinoma.^{1,2}

According to the WHO, worldwide around 2 billion people are infected with the hepatitis B virus and 600,000 die each year due to its consequences. And in India approximately 80 million people harbor the hepatitis B virus, which results in around 2,50,000 deaths annually due to complications from the disease such as liver cirrhosis and hepatocellular carcinoma.^{3,4}

The development of an efficient targeted drug delivery system into cells is an important subject for the advancement of drug carriers. Recently polymeric nanoparticles have been widely studied as drug carriers. These nanoparticle drug delivery systems can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged. They can be used to provide drug targeting, to improve oral bioavailability and to sustain drug in target tissue. Polymeric nanoparticles play an important role in controlling drug release and delivering drug to the desirable action site.^{5,6}

As the chronic hepatitis occurs in hepatocytes of the liver, they are the important target tissue for drug therapy. At present, some drugs can reach the anticipative curative effect, but drugs were difficult to be transferred to the target site specifically and accurately by the drug delivery system, which caused toxicity, side-effect, a low bioavailability and limited clinic application. An effective approach to overcome this critical issue is the development of targeted drug delivery systems that release the drugs at the desired site of action. This could increase patient compliance and therapeutic efficacy of pharmaceutical agents through improved pharmacokinetics and distribution. There are a lot of advantages in targeting delivery of drugs, which include the accumulation of drug in the action site, increase in therapeutic efficacy, reduction of therapeutic dose and toxicity, etc. It is well known that hepatocytes can recognize the asialoglycoprotein receptor (ASGP-R) among the liver-associated cell surface receptors and that ASGPR is present in several human hepatoma cell lines. Several studies indicated that the delivery system of galactose receptor-mediated endocytosis would be useful for drug targeting to hepatocyte and hepatoma cells.⁶

Among polymeric nanoparticles, chitosan-based nanoparticles as drug delivery systems have received much attention. It is a macromolecule with reactive functional groups, high adsorption capacity and biodegradability. The biocompatible and highly charged chitosan can be chemically modified to allow it bearing specific liver targeting function. Considerable effort has been made to exploit lactose-modified chitosan as a liver-specific drug carrier.⁷

Among the antiviral drugs, tenofovir, an acyclic phosphonate nucleotide analogue, has shown good results in the treatment of HBV infection in human beings. Tenofovir [9-(R)-(2-phosphonomethoxypropyl) adenine, PMPA] was approved by the U.S FDA on AUG 11, 2008 for the treatment of chronic hepatitis B.⁸

Tenofovir diphosphate selectively inhibits the reverse transcriptase- DNA polymerase of HBV through competition with the natural substrate deoxyadenosine 5'-triphosphate for incorporation into DNA during HBV transcription. As tenofovir lacks a 3'-hydroxyl group, incorporation in DNA prevents further DNA chain elongation, and causes termination of viral DNA growth.^{1,9}

Tenofovir contains a phosphate group and is negatively charged in NaOH solution. It can interact with chitosan through electrostatic forces. Chitosan nanoparticles could offer a controlled release drug delivery system for tenofovir.¹⁰

Experimental

Materials

Chitosan, lactose and pentasodiumtripolyphosphate were obtained from Sigma-Aldrich. Tenofovir disoproxil fumarate was provided by Micro labs, Bangalore. Sodium cyanoborohydride was obtained from Avra synthesis private limited, Hyderabad. The mice weighing 18-22g were provided by Chalapathi Institute of Pharmaceutical Sciences, Guntur. Acetic acid, isopropyl alcohol, methanol and other reagents and solvents were of analytical grade.

Synthesis and purification of Lactosylated Chitosan (LC)

1.5 g chitosan and 3.0g lactose were dissolved in 20 ml of a 1:2 mixture of methanol and 3% (v/v) acetic acid (pH 4.5), into which 0.1g sodium cyanoborohydride was added. The solution was stirred at room temperature for 24 hr. The resulting product was exhaustively dialyzed against triple distilled water and then freeze-dried for 48 h. The chemical structure of LC was analyzed using a Fourier transform infrared (FT-IR) and ¹H nuclear magnetic resonance (¹H NMR) spectroscopy.^{11,12}

Preparation of TDF loaded nanoparticles

Chitosan (2mg/ml) and lactose modified chitosan (LC) (2mg/ml) were dissolved in 10 ml of acetic acid aqueous solution (1% and 0.2% v/v) and TPP (1.2mg/ml) was dissolved in purified water. The TPP solution was added to the polymer solution during magnetic stirring (500 rpm) at room temperature, spontaneously forming an opalescent suspension.¹³

For the preparation of TDF loaded NPs, TDF was dissolved in 2 ml of 0.5 M NaOH. Then the drug solution was dropped into the polymer solution during magnetic stirring, followed by the addition of the TPP

solution. The pH of the mixture was adjusted (5-6.5) under continuous stirring for 10 minutes at room temperature.

Table No.1: Preparative variables of TDF -NP'S

Parameters	Formulation Code		
	F1	F2	F3
Chitosan concentration (mg/ml)	2	-	-
LC concentration (mg/ml)	-	2	2
Isopropyl alcohol (% v/v)	-	-	50
Drug (TDF) in mg	20	20	20
TPP concentration (mg/ml)	1.2	1.2	1.2

LC can be dissolved in a mixture of 0.2% acetic acid aqueous solution and isopropyl alcohol (IPA). Therefore, beside distilled water used alone, the solvent mixture was made of 1:1 ratio of IPA and 0.2% acetic acid solution. NPs were recovered by centrifugation at 20,000 rpm and 20°C for 60 min and freeze dried.¹⁰

Physicochemical characterization

Particle size distribution, poly dispersity index and zeta potential of the TDF loaded nanoparticles (F1, F3 and F5) were measured in distilled water at 25°C using zetasizer (Malvern instruments, UK).

The transmission electron microscopy (TEM) was used to assess the morphology of nanoparticles. To obtain the specimens, drops of nanoparticle suspension were placed on a copper grid with a carbon support film and air dried. The NPs were viewed under a Scanning Transmission Electron Microscope at 80 kV accelerating voltage.

To determine the encapsulation efficiency (EE), the TDF loaded nanoparticles were separated from the aqueous suspension medium by ultracentrifugation with 20,000 rpm at 4°C for 30 min. The content of TDF was calculated from the difference between the total amount of drug added in the NP preparation and the amount of free drug in the supernatants. The amount of unencapsulated drug was measured by UV spectrophotometer at a wavelength of 260 nm. The drug % EE and %LC was calculated as follows:

$$\%EE = \frac{\text{Total amount of TDF} - \text{Free TDF}}{\text{Total amount of TDF}} \times 100$$

$$\%LC = \frac{\text{Total amount of TDF} - \text{Free TDF}}{\text{Total nanoparticle weight}} \times 100$$

In vitro drug release assay

Lyophilized nanoparticles were suspended in pH 7.4 phosphate buffer and placed in the donor compartment separated by dialysis membrane with a molecular weight cut-off of 4–8 k Da. The other compartment was filled with 30 ml of pH 7.4 phosphate buffer to determine the amount of TDF diffused through the diffusion membrane. The entire phosphate buffer pH 7.4 was kept at 37°C with continuous magnetic stirring. At time intervals 5 ml of buffer was removed from donor compartment and 5 ml of fresh buffer was added into the system. The amount of TDF was evaluated by UV – Spectrophotometer at 260 nm.

In vivo liver distribution study

The amount of the micelles distributed in liver was estimated by using HPLC. In brief, TDF loaded chitosan and LC nanoparticle suspensions prepared by dispersing required amount of nanoparticles in PBS (pH = 7.4 buffer) were injected (0.2 ml) into the tail veins of mice of two groups (each group containing six mice) at a dose of 20 mg/kg, respectively. The mice were sacrificed after 2 hrs and liver was excised. With the exception of blood, after carefully washing with PBS and wiping with filter paper, tissue was weighed and dispersed in PBS. The mixture was homogenized and the lysates of the tissue was centrifuged at 10,000 rpm for 10 minutes at 4°C. 20µl of supernatant was injected into the HPLC system for analysis. Consequently, the amount of drug accumulated in the liver (% Biodistribution) was estimated.

Results

Synthesis and characterization of LC

Lactose is a disaccharide consisting of galactose and glucose. The amino groups of chitosan were combined with the aldehyde groups derived from the glucose moiety of lactose to form the Schiff bases, which are then reduced into secondary amino groups by sodium cyanoborohydride. In this reaction, the glucose moiety was changed into another structure while the structure of galactose remained unchanged.¹¹

Figure 1 shows the FT-IR spectra of chitosan (CS) and lactosylated chitosan (LC). CS has characteristic amide absorptions at 1649 and 1567 cm^{-1} . The very weak amide I band and the remarkably strong amide II band indicates the presence of abundant free amino groups in the CS molecule. In LC the amide bands II and I shift to 1646 cm^{-1} and 1545 cm^{-1} , respectively, indicating the new amide bond formation.

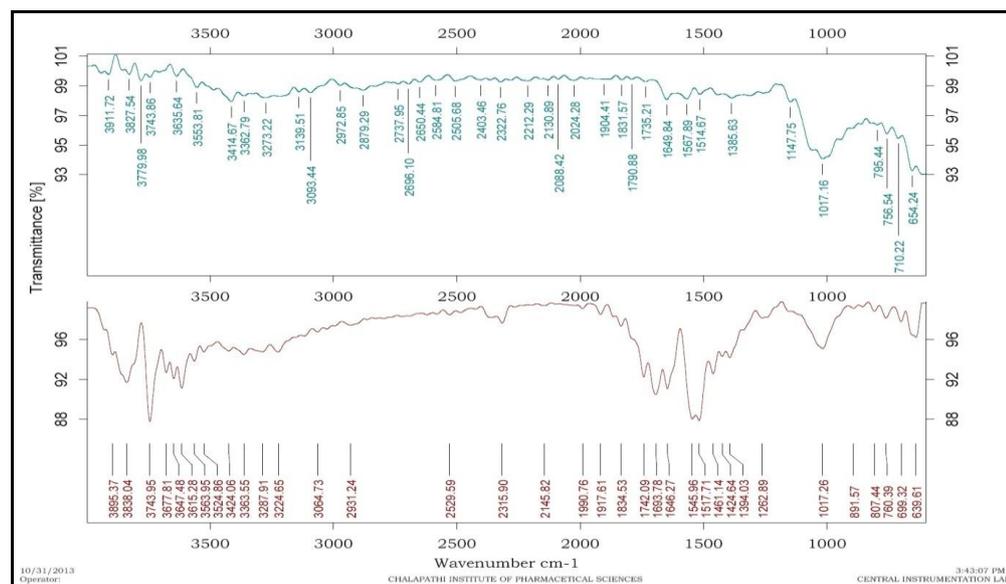


Figure 1: FTIR- spectrum of Chitosan and Lactosylated chitosan

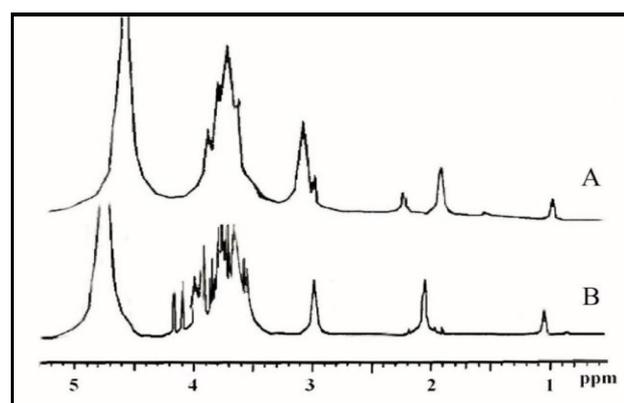


Figure 2: ^1H NMR spectrum of A) chitosan and B) LC

The ^1H NMR spectrum (Fig 2) of CS displayed typical peaks including the peaks of protons on the carbon of CS (at 3.00–4.00 ppm). The ^1H NMR results showed that the number of hydrogen's in LC at δ 3.5–4.0 was higher than that in CS, implying that lactose was introduced on to the CS.

Characterization of drug-loaded nanoparticles

Mean particle size of chitosan nanoparticles and surface characteristics of nanoparticles are of prime importance. So the surface morphology of chitosan nanoparticles were analyzed by transmission electron microscopy (TEM) shown in fig.3 and the particles were found to be spherical. The mean particle size of nanoparticle formulations were 226.4 nm and 176.5 nm for F1 and F3 respectively (fig. 4 and table 2) with a PDI of 0.264 and 0.1.

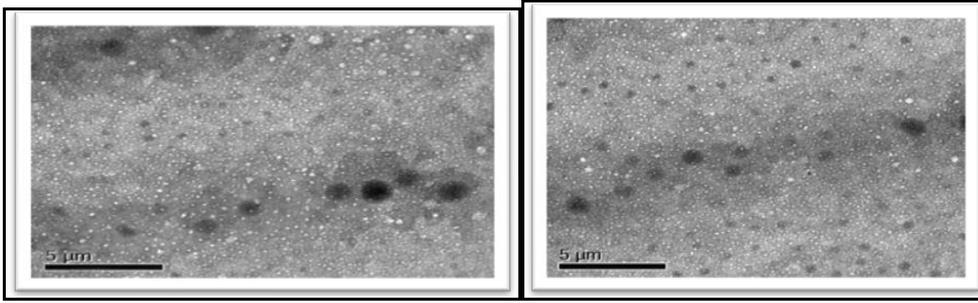


Figure 3: TEM images of formulations F1 and F3

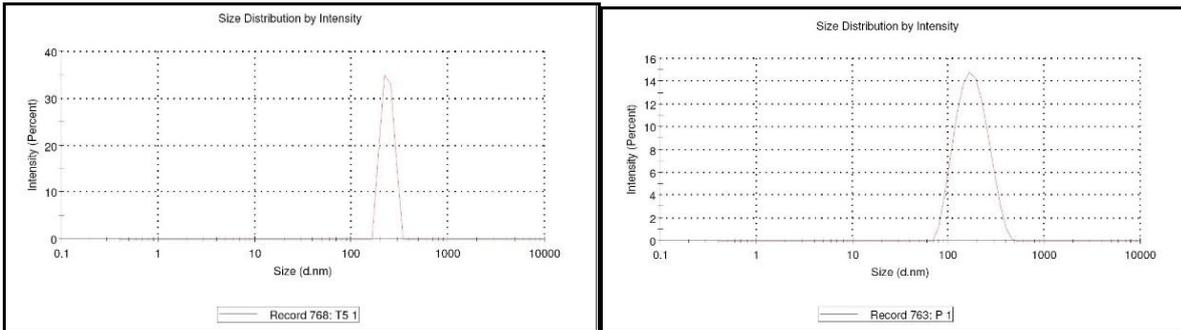


Figure 4: The size distribution of formulations F1 and F3

Table No.2: Zeta potential and size distribution study of NPformulations

Formulation code	Average particle size (nm)	% Area of peak indicating volume of particle formulation	Poly dispersity index(PDI)	Zeta potential (mv)
F1	226.4	245.6	0.264	28.4
F3	176.5	185.4	0.100	11.7

Nanoparticles were all positively charged which is a typical characteristic of chitosan particles. Zeta potential measurement indicated positive zeta potential value of +28.4 for F1 and +11.7 for F3 (table 2). The results indicated that zeta potential decreased for modified chitosan nanoparticle formulation (F3) compared to chitosan nanoparticles (F1).

Entrapment efficiency and loading capacity

The results of entrapment efficiency and loading capacity of prepared nanoparticles were shown in figure 5. The formulation F3 (used IPA in the formulation) showed higher entrapment efficiency than the other formulations.

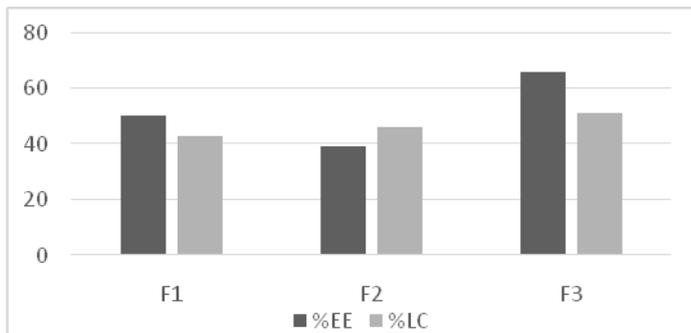


Figure 5: % Entrapment Efficiency and Loading capacity of nanoparticle formulations

Tenofovir could not dissolve in isopropyl alcohol (IPA), and the addition of IPA reduced the amount of required water. As a result, the solubility of tenofovir in IPA was lower than that in water alone. Therefore, the drug could not diffuse out in massive amounts during the nanoparticle preparation, leading to a higher EE%.

In vitro drug release

The *in vitro* release study was carried out using dialysis membrane. The release profiles of TDF from nanoparticles were shown in fig 6. The results showed that the release of drug from the nanoparticles extended to 48 hrs. The *in vitro* release kinetics of the formulations were fitted to various standard release equation viz., zero order, first order, higuchi model and korsmeyer-peppas equation. The model that best fits the release data was selected based on the highest coefficient of determination value (r) of various models. The results indicated that the release of the drug from nanoparticles followed zero order kinetics. When the release data was analyzed using the korsmeyerpeppas equation, the n values indicated that the mechanism of drug release from the nanoparticles was non-fickian

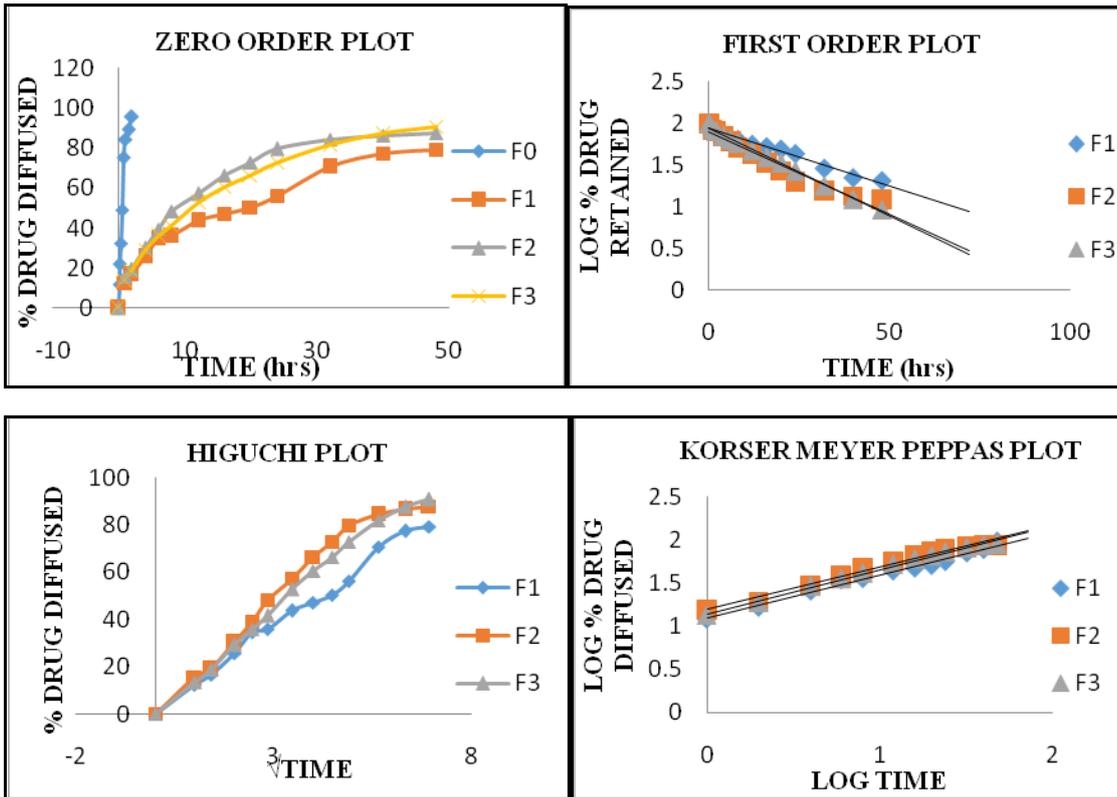


Figure 6: In vitro drug release data of TDF loaded nanoparticles

In-vivo liver targeting study

Fig. 7 represents the TDF % in the liver after two hours of i.v. administration of chitosan and lactose modified chitosan (LC) nanoparticles. The result shows that the LC nanoparticles (TDF-LC-NP) were accumulated more in the liver compared to chitosan nanoparticles (TDF-NP), which demonstrates that LC nanoparticles could enhance targeting effect and reduce side effects.

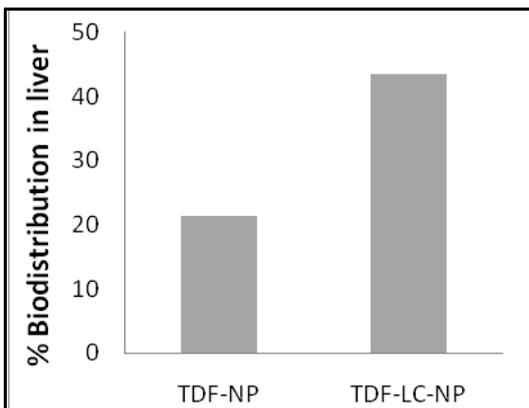


Figure 7: % Liver distribution of F3 nanoparticles after two hours of injection



Figure 8: Dissected mice and excised liver

Discussion

The present study showed that the liver targeted drug delivery nanoparticles composed of lactosylated chitosan could be prepared conveniently by the ionic gelation process. The nanoparticles were prepared without using any organic solvents and surfactants, which are more suitable for pharmaceutical applications. The % EE of tenofovir could be improved significantly by using isopropyl alcohol as a solvent of chitosan. The average diameter, % EE and polydispersity index (PDI) was found to be good for the formulation F3 compared with other formulations. Hence formulation F3 was selected for *in vivo* liver targeting study and the results indicated that these nanoparticles accumulated more in the mice liver compared to chitosan nanoparticles (control).

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