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Original Article

Targeted novel surface-modified nanoparticles for interferon delivery for the treatment of hepatitis B

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The purpose of the present work was to develop hepatitis B surface antigen (HBsAg) surface-adsorbed cationic poly (D,L-lactic-co-glycolic acid) PLGA nanoparticles for interferon alpha (IFNα) delivery targeted to hepatocytes. Cationic PLGA nanoparticles loaded with IFNα were prepared using the double emulsification technique. Delipidated HBsAg was passively adsorbed on the surface of nanoparticles by using the simple dipping and drying method. Surface morphology and size distribution of nanoparticles were analyzed by scanning electron microscopy and dynamic light-scattering method, respectively. The biodistribution behavior of plain and HBsAg-coated ^{99m}Tc-tagged PLGA nanoparticles was also examined followed by intravenous injection. The results revealed that ∼75% of the radioactivity was recovered in the liver after 4 h of injection that was nearly 3-fold greater in magnitude than the plain PLGA nanoparticles. These data demonstrated that the novel formulation of nanoparticles has potential application in hepatic-targeted drug delivery.

Keywords hepatitis B surface antigen; hepatitis; liver targeting; PLGA nanoparticle

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Introduction

Hepatitis B disease is a worldwide public health problem. Hepatitis B virus (HBV) is responsible for causation of the disease and resides in the reservoir of inveterate carriers. It is estimated to be 350 million people of whom ∼105 million are expected succumbed to death owing to the chronic sequel of the infection leading to chronic liver diseases, cirrhosis, or primary hepatocellular carcinoma [1–5]. The aggrandized occurrence of hepatitis B and serious hepatotoxicity of antitubercular drugs has fueled the demand for hepatoprotectives. Hence, there is an exigent need for safe, effective, and affordable targeted hepatoprotective formulations for the treatment of hepatitis [6]. However, conventional formulations of hepatoprotective drugs have met with circumscribed success due to the poor solubility and bioavailability profiles of these drugs [7].

The liver consisting of four types of cells, namely hepatocytes (hepatic parenchymal cells), Kupffer cells, stellate cells, and endothelial cells, serves as a substantial target tissue for drug therapy, since many fatal diseases such as chronic hepatitis, enzyme deficiency, and hepatoma occur in hepatocytes [8]. Nanoparticles are easily taken up within seconds or minutes after injection by the mechanism of phagocytosis through the reticuloendothelial system (RES), including Kupffer cells of the liver and macrophages of the spleen [9]. The distribution and opsonization of nanoparticles by macrophages seem to be influenced by particle size and surface characteristics [10]. The eventuality of nanoparticles ranged in 50–250 nm to arrive at fenestrae of hepatic sinusoidal endothelium might lead to hepatic accumulation after intravenous injection. As a result, a major aspect in preparing a targeted drug carrier lies in the established procedure conditions that could control the resulting particle size with a narrow distribution.

Positively charged particles are well known to exhibit agreeable good cell adhesion properties. Furthermore, positively charged particles can bind anionic or amphiphilic substances such as protein or DNA [11]. Proteins can be exploited for targeted delivery, specifically to hepatocytes, as sinusoids of liver do not form a barrier for proteins. This strategy can be particularly appropriate for reduction of the extra hepatic side effects of nucleoside analogs in the treatment of intracellular viral infections like hepatitis. In an attempt to explore the targeting potentials of recombinant protein, hepatitis B surface antigen (HBsAg) was incorporated into a nanoparticulate system so that a multiplicity of protein molecules could be achieved over a single entity, and hence it could represent as an artificial viral vector. It was expected that bioprotein could be

directly targeted with the particulate system along with its contents to the hepatocytes and would significantly enhance the localization of encapsulated materials in those cells following receptor-mediated endocytosis [12]. Endocytosis is a process where extracellular macromolecules and particles are absorbed into an intracellular environment through engulfment [13–15]. However, prior to the evaluation of targeting potential of the developed delivery system, the immunogenic nature of the antigen should be removed.

Interferon has antiviral properties that work by inhibiting the production of viral RNA and protein. Interferon α (IFNα) is the first drug approved for chronic hepatitis B treatment. The main function of IFNα in combating hepatitis is to limit amplification and spread of viruses during infections by direct mechanisms leading to the induction of an antiviral state that either protects cells from infection or attenuates the production of progeny in already-infected cells, and indirect mechanisms leading to the activation of the adaptive immune response [16–18]. IFN formulation in poly (D,L-lactic-co-glycolic acid) PLGA micro/nanoparticles has been reported earlier [19,20], but to our knowledge no efforts have been made till now to target the IFN delivery to the liver.

Hence, the present research is an endeavor to prepare the targeted cationic nanoparticles of hepatoprotective IFNα, which enhances its bioavailability and further improves the treatment of hepatitis B and patient compliance.

Materials and Methods

Materials

PLGA with a lactide-to-glycolide ratio of 50:50 (10,000 Da), octyl glucoside, polyvinyl alcohol (30,000–70,000 Da) and cetyl trimethyl ammonium bromide (CTAB) were procured from Sigma Aldrich (St. Louis, USA), HBsAg was obtained as a gift from the Serum Institute of India (Pune, India; 2.24 mg/ml) and interferon samples were also a generous gift from the Shantha Biotech Ltd. (Hyderabad, India). Bicinchoninic acid (BCA) kit for protein estimation was purchased from Genei (Bangalore, India). All other chemicals and reagents were of analytical grade.

Preparation of interferon loaded cationic PLGA nanoparticles

Cationic PLGA nanoparticles were prepared by the double-emulsification solvent-evaporation method [21]. Briefly, 200 mg of PLGA was dissolved in 5 ml dichloromethane at room temperature. The organic phase was sonicated (20 kHz 2 kW probe sonicator) with 1 ml IFN solution with trehalose (1:2 mass ratio) for 2 min. The resulting primary emulsion was further added to a 50 ml aqueous solution of poly vinyl alcohol (PVA) and CTAB (1:3; *w*/*v*) and sonicated for up to 2 min. The emulsion was stirred continuously with a mechanical stirrer (Remi, Mumbai, India) at room temperature for 4 h for evaporation of organic solvent. Nanoparticles were isolated from bulk by centrifugation at 12,829 *g* for 20 min (Sigma; rotor 12,166), and the pellet was collected and washed three times with water. Finally, the pellet was redispersed in water, and was lyophilized.

Removal of lipids from HBsAg

Most of the lipid components of the HBsAg were removed by treatment with the non-ionic non-denaturing detergent D-octyl glucoside (OG). HBsAg dissolved in 10 mM Tris-HCl, pH 7.0, was incubated with the non-ionic non-denaturing detergent D-OG followed by centrifugation through cesium chloride linear density gradients (density 1.15–1.32 g/ml) as reported previously [22].

Adsorption of delipidated HBsAg on to IFN-loaded PLGA nanoparticles

Prepared cationic nanoparticles were incubated in a phosphate buffer saline (PBS) for 2 h. These incubated nanoparticles were then centrifuged, and the pellet taken from the antigen solution (delipidated HBsAg) was added followed by incubation at 25°C for 1 h and again incubated at 4°C for another 24 h. After that, the samples were washed three times with PBS and the pellet was taken, redispersed in PBS, and lyophilized in the same manner as mentioned.

Characterization of prepared PLGA nanoparticles

The particle size and zeta potential measurements were performed by DLS using a Malvern Nano ZS (Malvern Instruments, Warriewood, UK), which evaluates the mean diameter and size distribution profiles of nanoparticles by light scattering based on laser diffraction. The nanoparticles were immersed in an aqueous medium prior to the measurement at 25°C. The values reported are shown as the mean ± SD of at least five different batches of each nanoparticle's formulation ($n = 5$).

Morphological studies

Surface morphology of nanoparticles was characterized by scanning electron microscopy (Jeol 1640; Jeol, London, UK). For the scanning electron microscope, nanoparticles were mounted on metal stubs previously covered with double-sided adhesive, and coated with gold in a vacuum using an IB-3 ion coater (Eiko Engineering Co., Ltd, Tokyo, Japan). The coated samples were scanned at an accelerating voltage of 15 kV.

Percent incorporation efficiency of IFN in nanoparticles

The amount of protein entrapped in nanoparticles was estimated before the adsorption of delipidated HBsAg as the earlier reports [20]. Ten milligrams of the lyophilized formulations were suspended in PBS (4 ml, pH 7.4) and incubated at 37°C. After that, the nanoparticles were dried and dissolved in 1 ml of dichloromethane. The samples were centrifuged at 20,000 *g* for 15 min, and the supernatant comprising polymer solution was discarded; the precipitated protein was then extracted in NaOH (0.1 N, 1 ml). The protein content of the neutralized alkaline extraction was estimated by micro BCA kit (Pierce, Rockford, USA) and expressed as the amount of protein entrapped per mg of dry nanoparticles.

The percentage IFN incorporation efficiency was calculated by following formula:

$$\text{Incorporation efficiency}(\%) = \frac{\text{Amount of IFN released from the lysed PLGA}-\text{NPs}}{\text{Amount of IFN initially taken to prepare the NPs}} \times 100\%$$

where NPs mean nanoparticles, and for optimum parameter, the values are shown as the mean ± SD ($n = 3$).

Stability of the structure of IFN in nanoparticles

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was carried out to analyze the structural integrity of protein after encapsulation in the novel carrier. The molecular weight reference marker, HBsAg, native IFN, and extracted IFN from cationic PLGA nanoparticles were run on SDS–PAGE gel assembly (Mini Protean III cell assembly; Bio-Rad, Hercules, USA) using standard protocols [23].

Biodistribution studies

The biodistribution studies were done using female BALB/c mice (6–7 weeks old, 18–22 g), and the animals were housed in a controlled climate (23 ± 2°C; relative humidity, 60%), and photoperiod (12 h light–dark cycles). They were fed standard rodent pellets with free access to drinking water. For acclimatization with the environment, the animals were kept in the environment without any experimentation for 1 week. Different groups were designed for the study, each containing six mice. The mice were fasted overnight but had free access to water. Then, they were injected intravenously into a tail vein with therapeutic doses of ^{99m}Tc radiolabeled suspension of nanoparticles (10 mg nanoparticles/kg). Radiolabeling of nanoformulation was done as per the standard protocol.

The procedure of radiolabeling involves the following sequential steps:

(i) Identification of the best reducing agent for the particular drug.
(ii) Optimization of reaction conditions like the concentration of reducing agent, ^{99m}Tc and the drug, temperature, reaction time, stabilizers, pH value, solvent, etc. to obtain radiolabeling of >90% efficiency.
(iii) Quality control of radiopharmaceutical purity using thin layer chromatography until 24 h.
(iv) Repetition of the standardized procedure for at least three times.

In brief, radiolabeling with ^{99m}Tc is basically a chemical reaction resulting in chelation of ^{99m}Tc with the pharmaceutical (radiocomplexation) that should result in radiolabeling efficiency of >90%, and preferably 95%. The standard protocol of radiolabeling includes ^{99m}Tc solution formation in optimum conditions, ^{99m}Tc reacts with the pure drug available in solution form to give a stable radiocomplex. After the experiment animals were sacrificed by cervical dislocation at different time intervals, and different organs were removed, and were washed with normal saline.

Scintigraphic studies were performed under GE Infinia Gamma Camera equipped with Xeleris Work Station (Wisconsin, USA). And static images were acquired at 30 min, 2, and 4 h post-injection.

Fluorescence microscopy

Fluorescence microscopy was performed to confirm the uptake of surface-modified IFN-loaded nanoparticles by the liver cells. Fluorescein isothiocyanate (FITC) was used as a fluorescent marker and encapsulated along with nanoparticles. The formulation was administered intravenously to the BALB/c mice. The liver of the animals were excised, isolated, cut into small pieces washed in Ringer's solution, and dried using tissue paper. The dried tissues were embedded in paraffin, with the help of microtome, and ribbons of the section were obtained (5 μm), which were fixed on slides using egg albumin solution as a fixative. The sections were viewed under a fluorescence microscope, and photomicrographs of different areas were taken.

Results and Discussion

IFN delivery

Protein delivery has led to the development of new strategies for improving the stability of proteins encapsulated in conventional micro/nanoparticles. To target specific area we decided to prepare surface-modified PLGA nanoparticles specifically containing IFNα.

Physicochemical characteristics

It appears that the nanoparticle surface and its interactions with cell surface control the uptake and intracellular trafficking of nanoparticles and, subsequently, that of

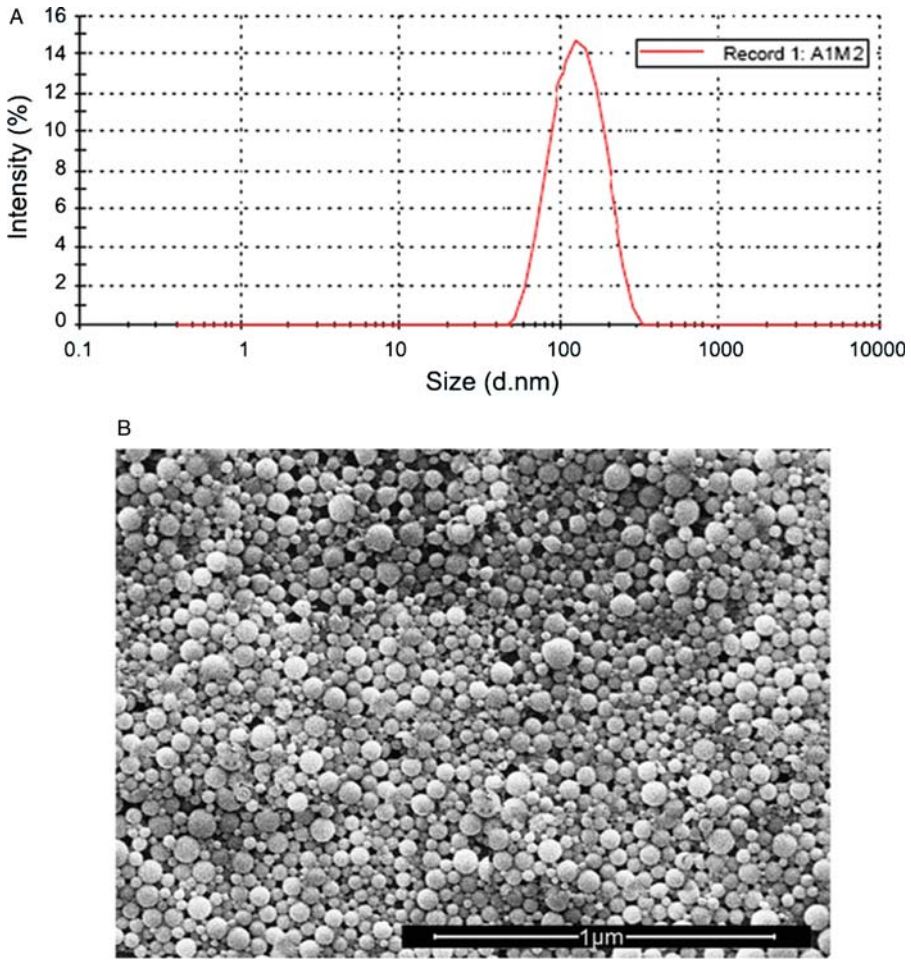

Figure 1 Surface morphology of interferon-loaded HBsAg-adsorbed PLGA nanoparticles (A) The particle size distribution of surface modifies nanoparticles. (B) Surface morphology of interferon-loaded HBsAg-adsorbed PLGA nanoparticles. It shows the morphology of all the nanoparticles that are smooth and are spherical in appearance.

encapsulated therapeutic agents. PLGA nanoparticles were distributed in a narrow particle size range, and the size of nanoparticles was found to be 174 nm as shown in **Fig. 1(A)**. The zeta potential value is an important characteristic of particles, as it can influence particle stability. In theory, more pronounced zeta potential values, being positive or negative, tend to stabilize particle suspension [24]. Bioadhesion can be promoted by a positive zeta potential value. The zeta potential result provides proof of successful cationic surface modification when a blend of PVA–CTAB (1.3%; *w*/*v*) was used. The zeta potential of the final surface-modified nanoparticles was found to be 30 mV.

Investigation done under a scanning electron microscope revealed that the nanoparticles were found to be in spherical shape with a smooth surface (free from any pores or cracks) [**Fig. 1(B)**]. The encapsulation efficiency of the protein will be enhanced due to the anionic nature of protein and the cationic nature of nanoparticles. The encapsulation efficiency of IFN was found to be 44.2%.

Structural studies carried out with HBsAg after treatment with D-OG indicate the importance of the lipid moiety for the maintenance of the native conformation. The CD spectrum of native HBsAg indicates that α-helix is the most abundant secondary structure (data not shown). Earlier, Gavilanes *et al*. reported that structural and antigenic properties of HBsAg were maintained by lipid [22]. Removal of a lipid can cause a loss of the immunogenicity of HBsAg, but still the hepatic targeting ability would be maintained on account of the small size and HBsAg epitopes present on the surface of nanoparticles.

The results of SDS–PAGE analysis suggested structural stability of IFNα in nanoparticles (**Fig. 2**). Processing the particles is important to maintain the stability and activity of the protein and prevention from aggregation during storage. Inclusion of sugars, such as trehalose, which is a well-known protein stabilizer reported by many reports [23,25], prevent aggregation and denaturation of proteins. Thus, the structural integrity of IFNα was retained in the formulation. Hence, it was concluded that the structure of

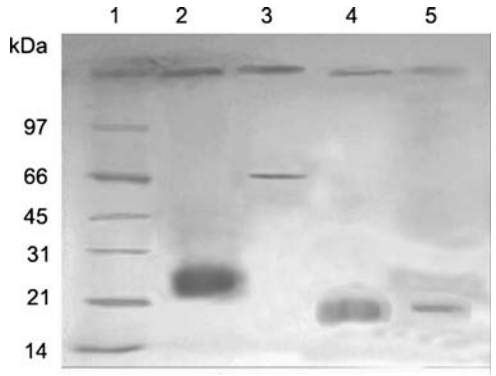

Figure 2 SDS–PAGE analysis of formulations Lane 1: protein marker; lane 2: HBsAg; lane 3: model antigen BSA; lane 4: native IFN; lane 5: IFN obtained under conditions of cationic PLGA nanoparticles containing IFN pre-treated with 0.01 M HCl for 2 h then sustained release in PBS for 24 h.

Table 1 Characterization of IFN-loaded cationic PLGA nanoparticles

Formulation code	Zeta potential (mV)	Particle size (nm)	Encapsulation efficiency (%)	IFN recovery[a]
IFN–NP	+30	174 ± 0.8	44.9 ± 1.14	91.42%

NP, nanoparticle.
[a]By micro BCA kit.

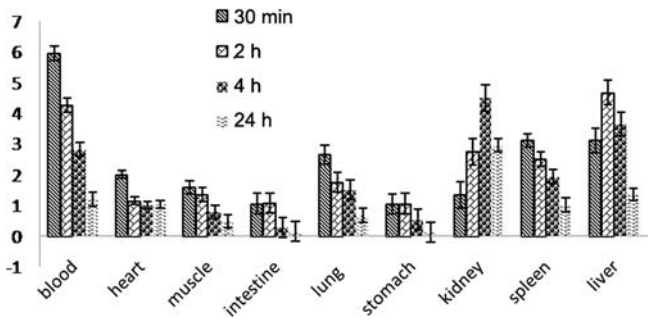

Figure 3 Organ distribution of ^{99m}Tc-radiolabeled IFN-loaded nanoparticles The time for it is 30 min, 2, 4, and 24 h post-injection. Data were expressed as the mean ± SEM.

IFN in cationic nanoparticles remained unchanged and stable. The results shown in **Table 1** exhibited that the total amount of protein recovered from PLGA nanoparticles containing trehalose and prepared to use the double-emulsion technique was 91% (as determined by micro-BCA).

The biodistribution profile of radiolabeled HBsAg-adsorbed interferon-loaded PLGA nanoparticles is shown in **Fig. 3**. The studies were conducted at intervals of 30 min, 2, 4, and 24 h of intravenous injection. IFNα-loaded cationic nanoparticles were widely and rapidly distributed into body tissues after parental

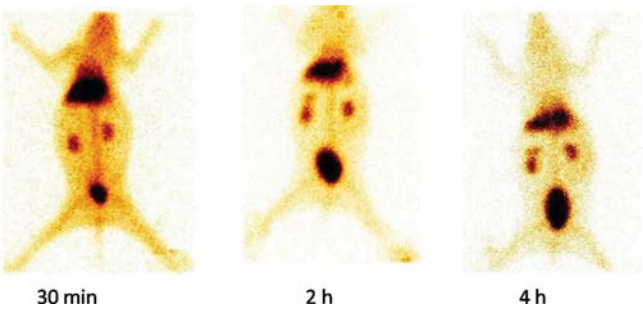

Figure 4 Gamma scintigraphic images of BALB/c mice with various time intervals

administration with the highest concentrations occurring in kidney, liver, spleen, and lung. The radioactivity (counts per gram organ) in different organs at 30 min post-injection was found to be as follows: blood (6%), liver (3%), spleen (2.6%), kidney (1.3%), lung (2.7%), muscle (1.5%), stomach (1.%), and heart (2%). The radioactivity recorded in the highly perfused organs, such as the liver and spleen, could be due to the combined activity of the circulating blood passing through organs as well as the particle uptake by cells of the RES of these organs.

It has been previously reported by other researchers that, the particle size, the polymer composition, and the surface characteristics of nanoparticles determined the particle distribution profile and stability in the physiological system [26–28]. Longer circulation of nanoparticles, with a minimum uptake by the cells of RES, is advisable for a better targeting potential of the nanoparticles. The radioactivity can mimic drug distribution before metabolism and a significant amount of radioactivity remains in the blood for a length of the period. Thus, it results in the feeding of the protein to all organs till the end, which expose a stable protein concentration for at least 24 h after a single dose. The catabolism of interferon takes place predominantly in the liver, and the waste products are primarily excreted by hepatobiliary pathways. The possibility of the reabsorption of radioactivity/peptide wastes are not ruled out. IFN α-loaded cationic PLGA nanoparticles were distributed evenly in mice as shown in the biodistribution profile in mice (**Fig. 3**), as well as the radio imaging of mice (**Fig. 4**). It is found that the blood concentration of nanoparticles remained relatively high, due to the filtration effect of the lung capillary bed that removed some large particles or their aggregates. The main route of elimination of IFNα is via renal catabolism and a negligible amount of IFNα is excreted in the urine, while hepatic metabolism and biliary excretion are minor pathways of elimination. Nanoparticles surface modified with HBsAg can stay in the circulation much longer than the uncoated counterpart resulting in considerably high blood concentration of loaded drug for a prolonged period and the targeting effect.

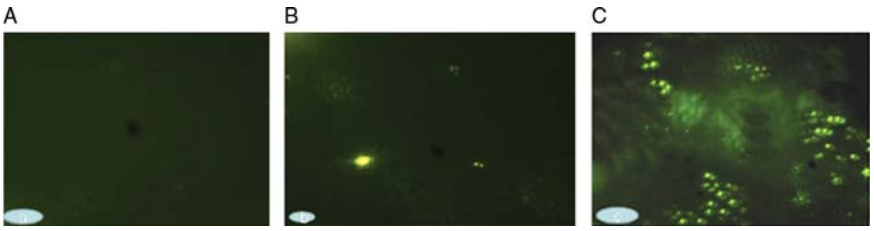

Figure 5 Fluorescent microscopy images showing the uptake of FITC–IFN loaded different nanoparticles by liver after intravenous administration (A) control; (B) plain PLGA nanoparticles; (C) targeted HBsAg-adsorbed nanoparticles.

Fluorescence microscopy was performed to observe the qualitative uptake of nanoparticles in liver. The plain IFN-loaded nanoparticles and HBsAg adsorbed IFN-loaded nanoparticles and were loaded with FITC and given intravenously to BALB/c mice. As shown in **Fig. 5**, the localized fluorescence was much higher in HBsAg-adsorbed IFN-loaded nanoparticles than in plain IFN-loaded nanoparticles. The photomicrographs clearly reveal the access of HBsAg-adsorbed interferon-loaded nanoparticles into the liver compared to that without HBsAg-adsorbed IFN-loaded nanoparticles.

Conclusion

It is concluded from the results that HBsAg-adsorbed cationic PLGA nanoparticles encapsulating interferon used in present study were successfully delivered to the hepatocytes in the liver that is the main reservoir for the HBV. The intravenous/subcutaneous administration of HBsAg-adsorbed cationic PLGA nanoparticles encapsulating interferon formulation may significantly improve patient compliance by reducing the dosing frequency from conventional doses and help in better management of hepatitis B. The biodistribution of the IFN nanoparticles demonstrate that these particles are RES evading and circulate in the blood for a considerable period of time. It may also be a potential carrier for liver-specific targeting of bioactive(s) and may improve therapeutic indices of the drug while reducing contraindicative manifestations.

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