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RESEARCH ARTICLE

GALACTOSYLATED POLY (D, L-LACTIC-CO-GLYCOLIC ACID) NANOPARTICLES FOR LIVER TARGETED DELIVERY OF ACYCLOVIR

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ABSTRACT

Introduction: The present study discusses d-galactose (Gal) –acyclovir- poly (D, L-lactic-co-glycolic acid) nanoparticles (Gal-PLGA-NPs) using galactose as an asialoglycoprotein receptor (ASGPR) ligand for hepatic targeting.

Methods and Materials: The PLGA nanoparticles (PLGA-NPs) were prepared by double emulsification method and galactose was conjugated to the free amine group of amine ended PLGA. Galactosylation of poly (D, L-lactic-co-glycolic acid) was confirmed by FTIR study and zeta potential measurements. The Gal-PLGA-NPs obtained were characterized for their morphology, particle size, polydispersity index and zeta potential.

Results and Discussion: The spherical nanoparticles prepared with Gal-PLGA were in the 198.1 nm size range exhibited a negative electrical charge (-8.5 mV), with 84.1% acyclovir entrapment efficiency and showed lower extent of *in vitro* drug release (40% over 48 h). The Gal-PLGA nanoparticles were remarkably targeted to the liver, and keep at a high level during the experiment. The accumulation in the liver was 36.71±0.68% at 24 h after administration. The Gal-PLGA nanoparticles were remarkably targeted to the liver, and keep at a high level during the experiment. The accumulation in the liver was 36.71±0.68% at 24 h after administration.

Conclusion: These results suggest that Gal-PLGA-NPs are safe and potentially promising for hepatocyte-selective targeting.

KEYWORDS: Galactosylation, Galactosylated-PLGA-Nanoparticles, Liver-Targeting, asialoglycoprotein, Acyclovir.

INTRODUCTION:

About one-third of the world's populations have been infected with the hepatitis B virus. Transmission of hepatitis B virus results from exposure to infectious blood or body fluids containing blood [1]. The development of an efficient targeted drug delivery system into cells is an important subject for the advancement of drug carriers. Active targeting has been achieved by many investigators to gain the high selectivity to a specific organ and to enhance the internalization of drug-loaded carriers into target cells [2]. Receptor mediated, drug targeting is a promising approach to active targeted drug delivery. Receptor systems cannot only bind specific ligand, but can also internalize them within endosomes. Once a ligand binds the receptor, the ligand–receptor complex is rapidly internalized, and the receptor recycles back to the surface. Various ligands such as folic acid, galactose and asialoglycoproteins have been introduced into drug carriers to enhance the intracellular localization into target cells [3].

Asialoglycoprotein receptors (ASGP-R), also known as the hepatic lectin represents a promising target for hepatocyte-specific delivery. ASGP-R is predominantly present in large numbers on the sinusoidal cell membrane of hepatocyte and internalizes sugars such as galactose or lactose and glycoproteins with terminal galactose or N-acetylgalactosamine by endocytosis [4]. Ligands such as galactose and galactosylated or lactosylated residues, including galactosylated cholesterol, galactosylated lipid , glycolipids and galactosylated polymers have been explored for selective targeting to be liver. Galactose (Gal) is a monosaccharide. It actively takes up by asialoglycoprotein receptors (ASGP-R) which are present exclusively on hepatic parenchymal cells and rapidly phosphorylated within liver cells and can be irreversibly removed from the portal circulation [5]. So, if exogenous galactose is used as a ligand for hepatic delivery, it mimics like endogenous particles and specifically targeted to the liver and internalized by the mechanism of receptor mediated endocytosis. In the last half a decade, galactose has been used as a ligand for the transport of different delivery systems in the liver [6].

Acyclovir shown anti-viral activity through phosphorylation by thymidine kinase (TK) and inhibition of viral DNA synthesis by competitive inhibition with guanosine triphosphate and chain termination after incorporation of acyclovir triphosphate into DNA [7]. Poly

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(D, L-lactic-co-glycolic acid) (PLGA) is a widely used polymer for fabricating NPs because of biocompatibility, long-standing track record in biomedical applications and well-documented utility for sustained drug release compared to the conventional devices up to days, weeks or months, and ease of parenteral administration via injection. Macromolecular drugs such as proteins, peptides, genes, vaccines, antigens and human growth factors, are successfully incorporated into PLGA or PLGA-based nano/microparticles [8]. PLGA is one of the most successfully used biodegradable polymers for the development of nanomedicines because it undergoes hydrolysis in the body to produce the biodegradable metabolite monomers, lactic acid and glycolic acid. They are effectively processed by the body, resulting in minimal systemic toxicity [9]. The objective of this study was to prepare/synthesize and characterize novel galactosylated-nanoparticle as hepatic targeting and controlled drug release carriers for acyclovir. It is expected that dissociation of nanoaprticles by hydrolysis of its ester linkage in the presence of water into two monomer lactic acid & glycolic acid.

MATERIALS:

Acyclovir was received as a gift sample from CIPLA Pharmaceuticals Ltd. Mumbai, India. Poly (lactic-co-glycolic acid) with 50:50 monomers and mol. wt. 17000 obtained from Sigma USA, dichloromethane (>99.5% pure, DCM) were procured from Spectrochem, Mumbai, India. Sephadex G-50 (dry bead diameter 50–150 μm, bed volume 9–11 ml/g), 1-Ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), Fluorescein isothiocyanate and D-Galactose were purchased from sigma, USA. Approval to use experimental animals taken up by Institutional Animal Ethical Committee, ADINA Institute of Pharmaceutical Sciences, Sagar, M. P. India. All other reagents were of analytical grade and used as such without further modification.

METHOD:

STRATEGY FOR GALACTOSE CONJUGATION:

Preparation of Gal-PLGA conjugation was done in two steps. In the first step, PLGA (100mg) was added to 5ml of distilled water and placed in an ice bath. Further ethylene diamine (EDA) (80μl) was added in it then aqueous solution of 1-Ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) 250 mg/2 ml, was added and adjusted to pH 5 using 1N HCl. The solution for slowly stirred for overnight, then dialyzed against distilled water for 24 hours using dialysis membrane (12KDW). D-galactose (12 mg) was dissolved in a sufficient quantity of phosphate buffer (pH 4). This solution was slowly added to the solution and stirred for two days. The resulting formulation was extensively dialyzed using dialysis membrane (12KDW) and lyophilized [10].

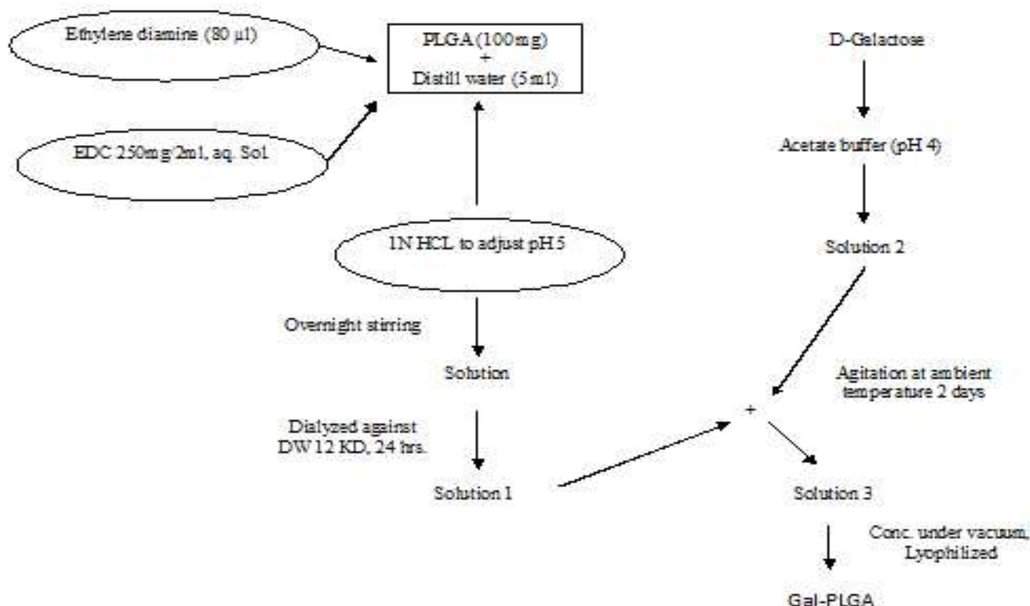

Figure 1: Strategy of galactose conjugation.

PREPARATION OF DRUG LOADED Gal-PLGA-NPs:

The nanoparticles were prepared by using double emulsification method as reported by Tewes et al. (2007) with slight modification [11]. Gal-PLGA was dissolved in a mixture of DCM and acetone (3:1 v/v) then the drug was dissolved in 1.5% polyvinyl alcohol (PVA) solution (0. 5ml). This solution was emulsified by sonication for 30 sec in an ice bath and w/o emulsion was formed. Again, 1.5% PVA solution (4 ml) was added and sonicated for 60 sec in an ice bath. This resulting w/o/w emulsion was diluted with 20 ml

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of 0.15% PVA solution and slow speed stirring was continued for 6 hr to facilitate evaporation of the solvent. The NPs so formed were collected, washed and freeze dried.

CHARACTERIZATION OF Gal-PLGA-NPs

PARTICLE SIZE, POLYDISPERSITY INDEX AND ZETA POTENTIAL:

The average particle size and polydispersity index of the NPs were determined by photon correlation spectroscopy using a Zetasizer (DTS Ver. 4.10, Malvern Instruments, England). The particle size distributions are represented by the average size (diameter). Hashida et al. (1998) [12] reported that most of the fenestrate of the liver sinusoid is usually smaller than 200 nm. Additionally drug carriers with a diameter larger than 200 nm readily scavenged non-specifically by monocytes and the reticuloendothelial system [13]. The zeta potential of a particle is the overall charge that the particle acquires in a particular medium and can be measured on a Zetasizer Nano instrument (DTS Ver. 4.10, Malvern Instruments, England). The magnitude of the measured zeta potential is an indication of the repulsive force that is present and can be used to predict the long-term stability of the product.

Table 1: Particle size, PDI, % drug entrapment and zeta potential of nanoparticulate formulation.

Sr. No.	Formulation Code	Average Particle Size (nm)	PDI	% Entrapment efficiency	Zeta potential (mv)
1	PLGA-NPs	163±1.4	0.102	80.5±1.5%	-20.03±1.3
2	GAL-PLGA-NPs	188.1± 1.2	0.107	84.1 ± 0.3	-8.5±1.5

TRANSMISSION ELECTRON MICROSCOPE:

Transmission electron microscope (TEM) was used as a visualizing aid for particle morphology. The sample (10 µl) was placed on the grids and allowed to stand at room temperature for 90 sec. Excess fluid was removed by touching the edge with filter paper. All samples were examined under a transmission electron microscope (Philips Morgagni 268, Eindhoven, Netherlands) at an acceleration voltage of 100 KV, and photomicrographs were taken at suitable magnification.

Figure 2: TEM of Gal-PLGA-NPs.

DRUG ENCAPSULATION AND *IN VITRO* RELEASE STUDY:

The acyclovir-loaded Gal-PLGA nanoparticles were separated from suspension in eppendorff tube and ultra-centrifuged at 30,000 rpm for 30 minimums. The amount of free acyclovir in the supernatant was measured by UV-Vis spectrophotometer (Shimadzu 1800, Japan) at 251 nm. The encapsulation efficiency (EE) and the loading capacity (LC) of the nanoparticles for acyclovir were calculated by eqs. (1) and (2) respectively.

EE (%) = (X-Y)/X × 100% -- (1)

LC (%) = (X-Y)/Z × 100% --------------------------------------- (2)

Where X is the total amount of the acyclovir added, Y was the free amount of the acyclovir in the supernatant, and Z is the weight of the nanoparticles.

The *in vitro* release profiles of the acyclovir from the nanoparticles were evaluated in PBS (pH= 7.4) using the following procedures. Two milliliters of the nanoparticle suspension were transferred to a dialysis membrane bag with a molecular cutoff of 12 kD, and the bag was immersed into 40 ml of PBS. It was incubated at 37°C with the magnetic stirrer at a constant speed of 100 rpm. At appropriate intervals, 5 ml of the release medium were removed and replaced by 5 ml fresh medium. The amount of the Acyclovir in the release medium was evaluated by UV-Spectrophotometer (Shimadzu 1800, Japan).

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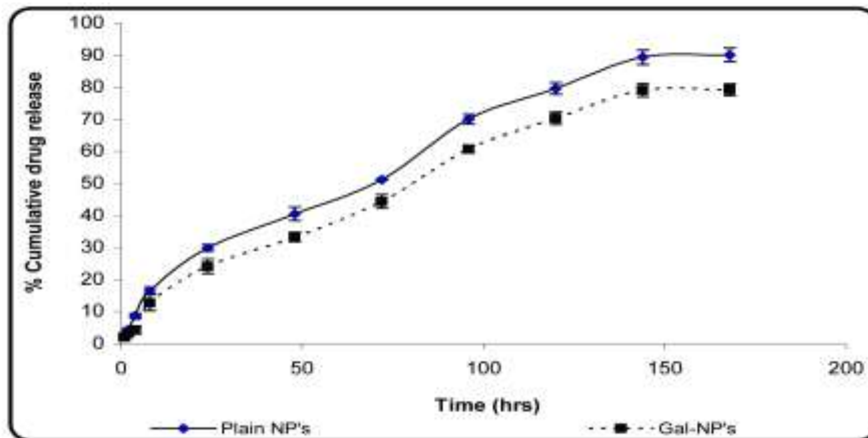

Figure 3: % Cumulative drug release profile from different nanoparticles formulation in PBS (pH 7.4).

STABILITY STUDIES OF Gal-PLGA-NPs:

The purpose of stability testing is to provide evidence that how the quality of a formulation varies with time under the influence of a variety of environmental factors such as temperature, humidity and light. In most of the stability studies, the major emphasis has been directed towards the accelerated stability studies, but the stability studies of aged products have been of greater pharmaceutical significance [14]. For this purpose, the samples were taken in borosilicate glass vials and sealed, and the vials were stored in-room temperature (28±2°C), refrigerator (4±1°C), and relative humidity 75% over a period of time i.e. 10, 20, 30, 45 and 60 days. Samples were evaluated effects of storage on their residual drug content as well as on particle size of nanoparticles.

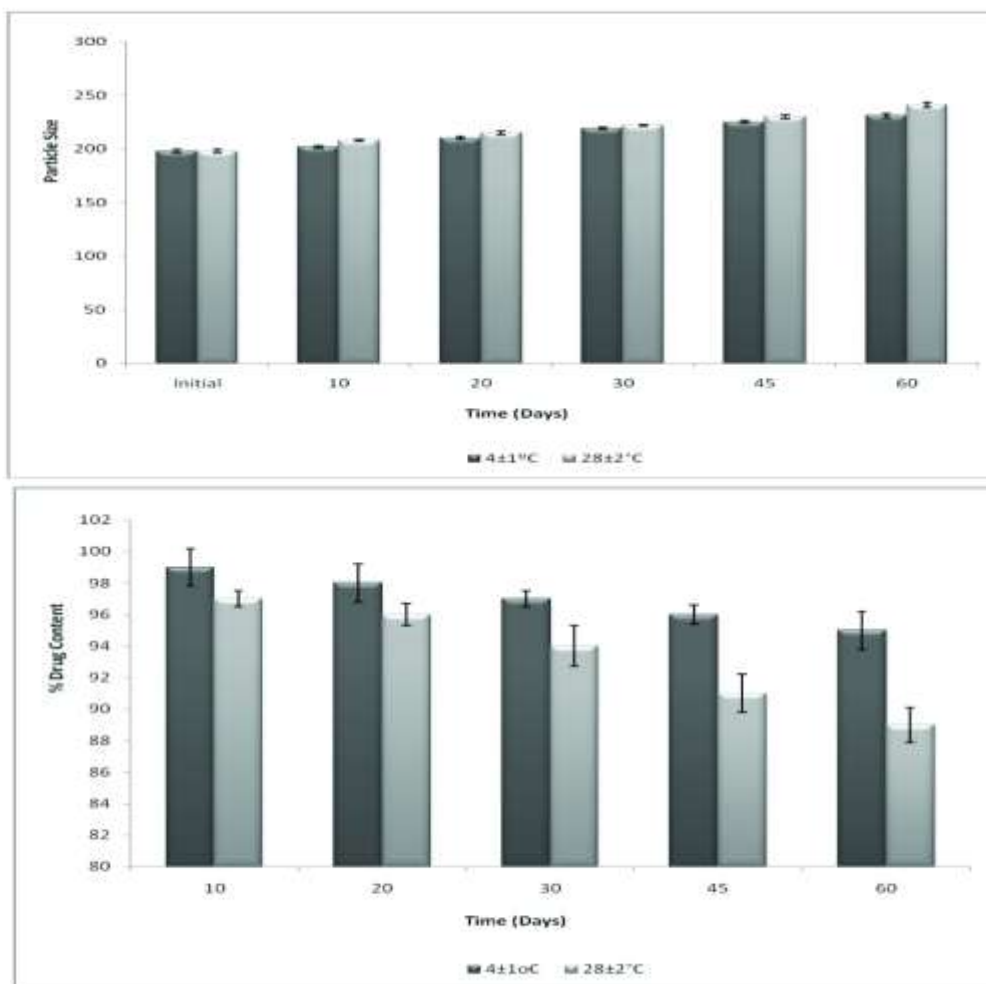

Figure 4: (A) Effect of storage temperature on particle size of Gal-PLGA-NPs (B) Effects of storage temperature on % residual drug content of Gal-PLGA-NPs.

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IN VIVO ORGANS DISTRIBUTION STUDY:

Biodistribution studies were performed with some modification in procedures described by Tu et al. (2004) [15] using albino mice of with average body weight 20-25 g were used for the study. The animals were maintained under standard laboratory conditions (14 h: 10 h dark/light cycle, a temperature of 22 ± 2° C and 50–70% humidity). Pelleted feed and water were provided at one's a day. The institutional Animal Ethics Committee of ADINA Institute of Pharmaceutical Sciences, Sagar, India, approved the experimental protocol for study.

The mice were fasted overnight before administration of formulations. Phosphate buffer solution, plain drug solution, PLGA-NPs and Gal-PLGA-NPs formulations were injected to mice of different groups by tail vein. The various formulations were administered with the dose of drug equivalent to 64 mg/kg body weight through the tail vein. Acyclovir concentration was determined by HPLC method developed by Bhrami et al. (2005) [16]. The mixture of methanol-phosphate buffer (0.05 M, pH 2.3, 5:95v/v) containing sodium dodecyl sulfate (200 mg/l) and triethylamine (2ml/l) was used as mobile phase at flow rate of 2ml/min. For the estimation of drug in various organs (liver, lung and spleen), it was required to prepare the standard curve of drug in the extracts of tissue homogenates of these organs.

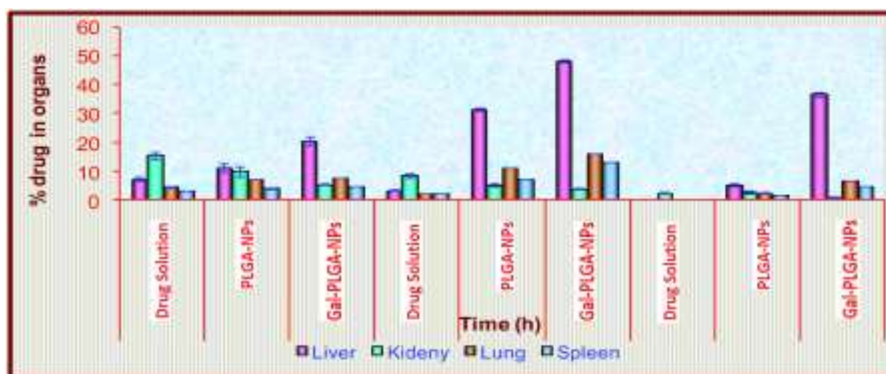

Figure 5: Biodistribution of drug solution, PLGA-NPs and Gal-PLGA-NPs in various organs of mice.

RESULT AND DISCUSSION:

The Gal-PLGA conjugation was prepared by two steps. The first step consisted of introduction of amine terminal to the PLGA via conjugation of ethylene diamine. The galactose conjugation with PLGA was estimated by FT-IR spectrophotometer. In the second step, CHO-group of open chain form of galactose was conjugated to the free amine group of amine ended PLGA under ambient temperature with agitation for two days without the use of high temperature, organic solvents, surfactants and other special experimental technology. The Fig. 5 presents FT-IR spectra of the PLGA nanoparticles (without adding galactose) and the Gal-PLGA nanoparticles. The conjugation of galactose with PLGA polymer was proven by FTIR. Fig. 5A show that the EDA conjugated PLGA one peak at 1690.2 cm^{-1} was found which shows the bending of free N-H bond, peak 1755.5 cm^{-1} represents C=O stretch, peak 1496.0 cm^{-1} represents N-H bending and peak at 1334.9 cm^{-1} represent C-N stretch. In the Gal-PLGA conjugates, the peak 1632.2 cm^{-1} represents C=N stretch of galactose ethylene diamine-PLGA conjugation, peak 1755.5 cm^{-1} represents C=O stretch, peak 1496.0 cm^{-1} represents N-H bending and peak at 1334.9 cm^{-1} represents C-N stretch shown in Fig. 5B.

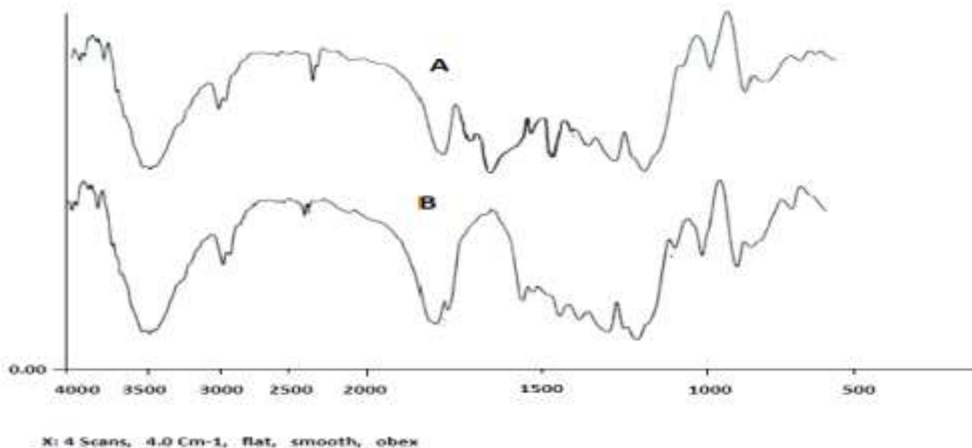

Figure 6: FT-IR spectra of (A) PLGA-NPs and (B) Gal-PLGA-NPs

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PLGA nanoparticles are usually prepared by double emulsification method. In this study, we prepared the Gal-PLGA-NPs composed of PLGA, EDA and EDC, using the emulsification process, in which the targeting ligand galactose was conjugated to the amine terminal of PLGA. The particle size, encapsulation efficiency and the zeta potential of the nanoparticles are described in Table 1. Thus, the nanoparticles with larger size would be produced. Further increase in the concentration of PVA from 2% w/v to 3% w/v increases the particle size, because the energy required for the formation of NPs decreases. Therefore, the amount of surfactant plays an important role in the emulsification process and in the protection of the droplets, because it can avoid the coalescence of globules. The size of the nanoparticles increased, and the zeta potential decreased as the Gal-PLGA concentration increased.

It was previously reported that PLGA interacts with EDA to form a PLGA-EDA semi-interpenetrating network through interaction between a sugar and the base. Thus, the presence of EDA-Gal could enlarge the size and reduce the zeta potential of the nanoparticles. The increase in the particle size, and the reduction of the zeta potential observed for the nanoparticles, was additional evidence for the existence of EDA-Gal molecules in Gal-PLGA-NPs. In this work, the nanoparticles used in the following experiments were prepared with the Gal-PLGA to ensure a sufficient liver-targeting ability. The morphology of the Gal-PLGA nanoparticles was observed by TEM study nanoparticles were well dispersed as individual nanoparticles with a spherical shape.

To investigate the feasibility of using Gal-PLGA-NPs as a drug carrier, Acyclovir as a model drug was encapsulated into the nanoparticles. Measured by UV-Vis spectrophotometer, the encapsulation efficiency was 84 ± 0.3%. The release profiles of the acyclovir from the Gal-PLGA nanoparticles in PBS are showed in Table 2 and Figure 6. *In vitro* release studies of PLGA-NPs and Gal-PLGA-NPs showed a % cumulative drug release was 90.0±2.1% and 79.1±1.8% respectively after 168 hours in PBS (pH 7.4). A significant decline in the % cumulative release rate of acyclovir from Gal-PLGA-NPs was observed in comparison to PLGA-NPs in PBS (pH 7.4). The results indicated that coupling of galactose to PLGA slows down the release of drug from the nanoparticles and thereby imparts a sustained-release nature. The different release property of the Gal-PLGA- NPs at different pH value would be beneficial for application in the body. Lower amounts of acyclovir are released in the blood (pH 7.4) during transport to the target site, and most of the active drug could be released after reaching the lesion site, as a result of a pH decrease in the environment or in the endosomes (pH 5-5.5) after entering the cells via receptor-mediated endocytosis. Therefore, the side-effects of the drug could be reduced, and the therapeutic efficacy could be enhanced greatly.

Stability of a drug in a dosage form at different environmental conditions is important, because it determines the expiry date of that particular formulation. Gal-PLGA-NPs formulation was subjected to stability studies. The formulation was stored at 4±1°C and 28±2°C. Change in the particle size and residual drug content after the time interval of 10, 20, 30, 45 and 60 days were determined. The average particle size of the nanoparticles was found to increase on storage, which may be due to aggregation of particles. This effect was encountered lower in the case of formulation stored at 4±1°C. This signifies that storage temperature can regulate aggregation and hence ideal storage temperature for nanoparticles is 4°C. By keeping the initial drug content 100%, the determination of percentage residual drug in nanoparticles showed that 4-5% of drug was lost from the formulation within 60 days(Figure 4 A & B).

The in vivo targeting ability of the Gal-PLGA-NPs for the liver was evaluated by fluorescence microscopy and biodistribution studies. Fluorescence microscopy is an optical microscope that can be effectively used to visualize the biodistribution of carriers in vivo. The animals were sacrificed and acyclovir concentration was determined by HPLC method . In Figure 5 shows the distribution of the nanoparticles in each organ at 2, 8 and 24 hours after intravenous injection. The animals treated with Gal-PLGA-NPs showed significantly higher accumulation in the liver than in other tissues. The accumulation in the liver was 48.3% at 8 hours after injection.

As previously reported, several factors, such as particle size, polymer composition, molecular weight and surface characteristic of nanoparticles determine the particle distribution in the body. The PLGA-NPs and Gal-PLGA-NPs nanoparticles had different particle sizes and zeta potentials (Table 1). The particle composition was the main reason for the distinct difference in body distribution. It has already been shown that there are receptors for Gal on the cellular membrane of rat hepatocytes. We believe that Gal plays a key role in the different distribution of the two kinds of nanoparticles. Nanoparticles modified with Gal could be recognized by the galctose receptors on hepatocytes and were transferred into hepatocytes via receptor mediated endocytosis. This enhanced their ability to target to the liver and enabled the longevity of the Gal-PLGA-NPs in the liver.

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CONCLUSION:

This work report that Gal-PLGA nanoparticles-NPs can be used as the carrier of acyclovir for hepatocyte targeting in effective management of Hepatitis B. Liver-targeted nanoparticles (Gal-PLGA) composed of Gal and PLGA could be prepared conveniently by the double emulsification process. Gal-PLGA-NPs are nearly spherical of 150–200 nm in diameter with homogeneous structure and smooth surfaces. This study drug loaded Gal-PLGA-NPs were tested for storage stability to provide, evidence on how the quality of a formulation varies with time under the influence of temperature. Data obtained from stability tests indicated that nanoparticles formulations stored at 4°C were more stable than those stored at room temperature. A significant decline in the % drug release of acyclovir from Gal-PLGA-NPs was observed in comparison to PLGA-NPs in PBS (pH 7.4). The results indicated that coupling of galactose slows down the release of drug from the NPs and thereby imparts a sustained release nature. When Acyclovir given by i.v. route, blood circulation distributed it to all the organs, it goes into the kidney and retained there, in higher amount and become the cause of renal toxicity. However, when DNPs were given, because of their size (less than 200 nm) these were taken up by passive targeting and when Gal-PLGA-NPs were given, they were selectively taken up by ASGP-R, exclusively present on liver parenchymal cells, because of conjugation with galactose, these receptors taken up Gal-PLGA-NPs by receptor-mediated endocytosis and retain the drug in the liver.

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REFERENCES:

1. Barker LF, Shulman NR, Murray R, Hirschman RJ, Ratner F, Diefenbach WC, et al. Transmission of serum hepatitis. JAMA. 1996; 276: 841-844.
2. Cho CS, Cho KY, Park IJ, Kim SH, Sasagawa T, Uchiyama M, et al. Receptor-mediated delivery of all transretinoic acid to hepatocyte using poly(L-lactic acid) nanoparticles coated with galactose-carrying polystyrene. J Control Rel. 2001; 77: 7-15.
3. Stella S, Arpicco MT, Peracchia D, Desmaele J, Hoebeke M, Renoir J, et al. Design of folic acid-conjugated nanoparticles for drug targeting. J Pharm Sci. 2000; 89:1452-1464.
4. Wu J, Nantz MH, Zern MA. Targeting hepatocytes for drug and gene delivery: emerging novel approaches and applications. Front Biosci. 2002; 7:717- 725.
5. Kim TH, Park IK, Nah JW, Choi YJ, Cho CS. Galactosylated chitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier. Biomat. 2004; 25:3783-3792.
6. Tygstrup N, Winkler K, Lund E, Engell H. A clinical method for determination of plasma galactose in tolerance tests. Scand J Clin Lab Invest. 1954; 643-48.
7. Giannavola C, Bucolo C, Maltese A, Paolino D, Vandelli MA, Puglisi V, et al. Influence of preparation conditions on acyclovir-loaded poly-d,l-lactic acid nanospheres and effect of PEG coating on ocular drug bioavailability. Pharm Res. 2003; 20:584-590.
8. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly (D,L-lactide-co-glycolide) and its derivatives. J Control Rel. 2008; 125:193-209.
9. Wu XS. Synthesis and properties of biodegradable lactic/glycolic acid polymers. In: Wise et al., ed. Encyclopedic handbook of biomaterials and bioengineering. New York: Marcel Dekker, 1995:1015-1054.
10. Akamatsu K, Yamasaki K, Nishikawa M, Takakura Y, Hashida M. Development of a hepatocyte specific prostaglandin E1 polymeric prodrug and its potential for preventing carbon tetrachloride induced fulminant hepatitis in mice. J Pharmaco Exp Ther. 1999; 290:1242-1249.
11. Song CX, Labhasetwar V, Murphy H, Qu X, Humphrey WR, Shebuski RJ. Formulation and characterization of biodegradable nanoparticles for intravascular local drug delivery. J Control Rel. 1997; 43:197-212.
12. Hashida M, Takemura S, Nishikawa M, Takakura Y. Targeted delivery of plasmid DNA complexed with galactosylated poly (L-lysine). J Control Rel. 1998; 53:301-310.
13. Litzinger DC, Buiting AMJ, Rooijen NV, Huag L. Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly (ethylene glycol) -containing liposomes. Biochim Biophys Acta. 1994; 1190: 99-107.
14. Kauper P, Rossi N, Laue C, Schmitt F, Lagopoulos L, Juillerat L, et al. Chitosan-based nanoparticles for medical applications : Stability in physiological environments. Eur Cel Mat. 2007; 13:3-4.

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15. Tu J, Zhong S, Li P. Studies on acyclovir–dextran conjugate, synthesis and pharmacokinetics. Drug Dev Ind Pharm. 2004; 30: 959-965.
16. Bahrami G, Mirzaeei S, Kiani A. Determination of acyclovir in human serum by high-performance liquid chromatography using liquid – liquid extraction and its application in pharmacokinetic studies. Life Sci. 2005; 816:327-31.

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