Development of Biodegradable Nanoparticles for Liver-Specific Ribavirin Delivery

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ABSTRACT: Ribavirin is an antiviral drug used for the treatment of chronic hepatitis C. However, ribavirin induces severe side effects such as hemolytic anemia. In this study, we prepared biodegradable nanoparticles as ribavirin carriers to modulate the pharmacokinetics of the drug. The nanoparticles encapsulating ribavirin monophosphate (RMP) were prepared from the blend of poly(D,L-lactic acid) homopolymer and arabinogalactan (AG)–poly(L-lysine) conjugate by using the solvent diffusion method in the presence of iron (III). RMP was efficiently and stably embedded in the nanoparticles and gradually released for 37 days in phosphate-buffered saline at 37°C. The coating of AG on the nanoparticles surfaces was verified by measuring the zeta potentials and performing an aggregation test of the nanoparticles using galactose-binding lectin. Moreover, the nanoparticles were efficiently internalized in cultured HepG2 cells. Ribavirin was drastically accumulated to the liver of mice after intravenous administration of the RMP-loaded nanoparticles, after which the ribavirin content gradually decreased for at least 7 days. Our results indicated successful development of nanoparticles with dual functions, targeting to the liver and sustained release of ribavirin, and suggested that the present strategy could help to advance the clinical application of ribavirin as a therapeutic agent for chronic hepatitis C. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:4005–4011, 2014

Keywords: polymeric drug carrier; nanoparticles; controlled release; site-specific delivery; hepatocytes; polymer biodegradation; encapsulation

INTRODUCTION

Chronic hepatitis C results from infection of hepatocytes with hepatitis C virus (HCV) and is the major risk factor for the development of cirrhosis and hepatocellular carcinoma. At present, the combination of pegylated interferon and ribavirin (antiviral ribonucleoside analog) is available for treating chronic hepatitis C.[1,2] However, the treatment is effective in only 50% of the patients; the treatment is costly and takes a long time.[3] Moreover, clinical application of ribavirin is restricted because of ribavirin-induced hemolytic anemia.[4] In 2011, triple therapy with pegylated interferon, ribavirin, and telaprevir (HCV protease inhibitor) was approved for human use.[5] However, ribavirin is certainly essential to exhibit the beneficial effects of these treatments. Thus, this research focused on the development of a carrier for ribavirin to improve its therapeutic effect and safety.

Many conjugates between antiviral drugs and carrier molecules, such as asialofetuin,[6] albumin modified with lactose,[7] polylysine modified with lactose,[8] high-density lipoprotein modified with lactose,[9] hemoglobin,[10] and dextran,[11] have been developed for liver-specific delivery and for the treatment of various hepatic diseases caused by viral infection.[3,12] In the conjugates-based delivery systems, however, the drugs must be chemically cleaved from the carrier molecules after accumulation in the liver, because they were covalently attached to the carriers. On the contrary, colloidal carriers (e.g., liposomes, nanoparticles, and polymeric micelles) can generally entrap drugs without chemical coupling.[3]

In the current combination therapy, ribavirin is orally administered daily, whereas carriers for targeting to the liver have been generally developed as injections. Thus, to prevent the reduction in quality of life, it may be necessary to develop a pharmaceutical drug with long-term therapeutic effect as well as specific targeting properties.

In our previous reports, we described the preparation of polymeric nanoparticles from biodegradable polymers such as poly(lactic acid) and poly(lactic acid-co-glycolic acid).[13,14] The water-soluble drugs could be efficiently encapsulated in the nanoparticles by a unique technique involving the use of metal ions.[13–15] The nanoparticles were prepared from poly(D,L-lactic acid) (PLA), poly(ethylene glycol)-*block*-PLA copolymer, and betamethasone phosphate (BP) in the presence of zinc using the oil-in-water solvent diffusion method.[14] BP was more efficiently encapsulated in the nanoparticles than hydrophobic betamethasone derivatives despite its hydrophilic properties, because zinc was used as an ionic bridge between a terminal carboxyl group of the PLA chain and a phosphate group of BP.[13,14] Similarly, ionizable prostaglandin E1 could also be encapsulated in solid nanoparticles by formation of an ionic bridge between the carboxyl groups of PLA and prostaglandin E1 with iron (III) ion.[15] Furthermore, the nanoparticles encapsulating BP were accumulated in the inflamed lesion *in vivo* and BP was gradually released at the site.[16,17] High anti-inflammatory activity of nanoparticles has also been shown using experimental animal models of inflammation.[17–19]

Here, we designed biodegradable solid nanoparticles encapsulating ribavirin with three characteristics in terms of pharmacokinetics. First, stable retention of ribavirin by the nanoparticles in systemic circulation can prevent

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accumulation of ribavirin in erythrocytes and excretion from the kidneys. Because hemolytic anemia is related to extensive ribavirin accumulation in erythrocytes,[20] ribavirin must be physically and stably encapsulated in the nanoparticles in blood circulation to reduce the side effects. Second, nanoparticles formed from biodegradable polymers can be used to achieve a long-term therapeutic effect by slow release of ribavirin along with degradation of the polymers. Third, liver-specific delivery could enhance the therapeutic efficacy. Asialoglycoproteins with clustered galactose moieties selectively bind to the asialoglycoprotein receptor on the surface of hepatocytes and are internalized via receptor-mediated endocytosis.[21] Thus, many carriers with ligand molecules consisting of galactose have been widely exploited for liver-specific drug delivery.[22–24] Arabinogalactan (AG) is a polysaccharide with a galactan core and side chains of galactose and arabinose, and is used as a ligand molecule for liver targeting.[25–28] Thus, nanoparticles modified with AG on their surfaces could be internalized by receptor-mediated endocytosis in hepatocytes. Here, we prepared ribavirin-loaded biodegradable nanoparticles coated with AG, and evaluated their biodistribution *in vivo*.

MATERIALS AND METHODS

Materials and Animals

Poly(D,L-lactic acid) (Mw = 7100 Da, Mn = 5500 Da) was supplied from Taki Chemical Company, Ltd. (Kakogawa, Japan). Iron (III) chloride anhydrous was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). AG from larch wood was purchased from Tokyo Chemical Industry Company, Ltd. (Tokyo, Japan). Poly(L-lysine) (PLL) hydrobromide was purchased from Sigma–Aldrich (St. Louis, Missouri). 1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (ribavirin) was purchased from Funakoshi Company, Ltd. (Tokyo, Japan), and ribavirin 5′-monophosphate (RMP; Supplemental Fig. S1) was synthesized from ribavirin according to a reported method.[29]

Female C57BL/6N mice (6-week old) were obtained from Charles River Laboratories, Inc. (Yokohama, Japan) and CLEA Japan, Inc. (Tokyo, Japan). Mice were allowed free access to water and rat chow, and were housed under controlled environmental conditions (constant temperature, humidity, and a 12-h dark-light cycle). Experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Keio University and Nihon University.

Synthesis of Arabinogalactan–Poly(L-Lysine) Conjugate

Arabinogalactan was purified by dialysis using Spectra/Por7 dialysis membrane (MWCO: 1000 Da; Spectrum Lab., Inc., Rancho Dominguez, California) for 3 days, and recovered by lyophilization. A conjugate of AG and PLL (AG–PLL) was synthesized by reductive amination in the presence of sodium cyanoborohydride, according to a reported method with minor revision.[28] PLL hydrobromide (100 mg), AG (600 mg), and sodium cyanoborohydride (300 mg) were dissolved in 8 mL of water, and the pH of the solution was adjusted to 8.5 by adding 4 M sodium hydroxide. After incubation for 18 h at 60°C, the resulting conjugates were precipitated three times in excess volume of ethanol by centrifugation at 20,000*g* for 10 min. Finally, AG–PLL was obtained by lyophilization. The molecular weight of AG–PLL was determined by size-exclusion chromatography (SEC) on TSKgel α-4000 and TSKgel G3000PW$_{XL}$ columns (Tosoh Company, Tokyo, Japan) coupled with a refractive index detector using aqueous solution of 0.5 M acetic acid and 0.2 M sodium sulfate as the mobile phase. The instrument was calibrated with monodispersed PEG standards (Tosoh Company). The composition of AG–PLL was also evaluated by ^{1}H-NMR in D_2O.

Preparation of Nanoparticles

Nanoparticles were prepared using the oil-in-water solvent diffusion method in the presence of iron (III) as previously reported with minor revision.[15] PLA (25 mg) was dissolved in 600 μL of dimethyl sulfoxide (DMSO). Then, 45 μL of 0.5 M iron (III) chloride anhydrous DMSO solution, 50 μL of 200 mg/mL AG–PLL aqueous solution, 25 μL of 200 mg/mL RMP aqueous solution, 100 μL of 50 mg/mL diethanolamine (DEA) DMSO solution, and 200 μL of DMSO were added in this order. The resulting mixture was allowed to stand for 10 min at room temperature. The mixture was added drop by drop to 25 mL of 0.2 mg/mL polysorbate 80 aqueous solution with continuous stirring at 700 rpm. Subsequently, 300 μL of sodium citrate aqueous solution (0.5 M, pH 7.2) was immediately added. The resulting nanoparticles were purified using a Minimate™ TFF capsule with a 300-kDa Omega membrane (Pall Company, Port Washington, New York) and condensed using a Centriprep Centrifugal Filter Unit (YM-50, MWCO:50000 Da; EMD Millipore Corporation, Billerica, Massachusetts). Nanoparticles formed from PLA homopolymer alone without AG--PLL were prepared similarly. Forty-five microliters of 0.5 M iron (III) chloride anhydrous DMSO solution, 25 μL of 200 mg/mL RMP aqueous solution, and 100 μL of 50 mg/mL DEA DMSO solution were added in this order to 2400 μL of DMSO solution in which 25 mg of PLA was dissolved. After standing for 10 min at room temperature, the nanoparticles were prepared as mentioned above.

Furthermore, nanoparticles with fluorescent dye (rhodamine B) were prepared according to a method previously reported.[15] The mixture of 25 mg of PLA and 1 mg of a conjugate of PLA and rhodamine B isothiocyanate was dissolved in 600 μL of DMSO. After mixing with 50 μL of 200 mg/mL AG–PLL aqueous solution, the mixture was added drop by drop to 25 mL of 0.2 mg/mL polysorbate 80 aqueous solution with continuous stirring at 700 rpm. In addition, the rhodamine-loaded nanoparticles formed from PLA homopolymer alone were prepared. The mixture of 25 mg of PLA and 1 mg of a conjugate of PLA and rhodamine B isothiocyanate was dissolved in 2800 μL of acetone, after which the solution was added to 25 mL of 0.2 mg/mL polysorbate 80 aqueous solution. The obtained nanoparticles containing the dye were purified as mentioned above.

Evaluation of Nanoparticle Characteristics

The particle size was determined by dynamic light scattering (nano Partica SZ-100; HORIBA, Ltd., Kyoto, Japan). The zeta potential of the particles was also determined in 10 mM sodium phosphate buffer solution (pH 7.0) under a constant applied voltage (10 V).

The RMP content in the nanoparticles was determined using HPLC. To an aliquot (100 μL) of the purified nanoparticles suspension, 300 μL of tetrahydrofuran and 600 μL of sodium