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Galactosylated Chitosan (GC)-*graft*-Poly(vinyl pyrrolidone) (PVP) as Hepatocyte-Targeting DNA Carrier: *In Vitro* Transfection

In-Kyu Park[1], Hu-Lin Jiang[1], Seung-Eun Cook[1], Myung-Haing Cho[1,2], Su-Il Kim[1], Hwan-Jeong Jeong[3], Toshihiro Akaike[4], and Chong-Su Cho[1]

[1]School of Agricultural Biotechnology, [2]College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea, [3]Department of Nuclear Medicine, Wonkwang University School of Medicine, Iksan 570-749, Korea, and [4]Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama 226-8501, Japan

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Galactosylated chitosan-*graft*-poly(vinyl pyrrolidone) (GCPVP) was synthesized and characterized for hepatocyte-targeting gene carrier. GCPVP itself as well as GCPVP/DNA complex had negligible cytotoxicity regardless of the concentration of GCPVP and the charge ratio, but GCPVP/DNA complex had slightly cytotoxic effect on HepG2 cells only in the case of the higher charge ratio and 20 mM of Ca^{2+} concentration used. Through the confocal laser scanning microscopy, it is shown that the endocytosis by interaction between galactose ligands of GCPVP and ASGPR of the hepatocytes was the major route of transfection of GCPVP/F-plasmid complexes.

Key words: Galactosylated chitosan, Poly(vinyl pyrrolidone), Hepatocyte, Gene delivery

INTRODUCTION

Gene therapy has the potential to treat devastating inherited diseases for which there is little hope of finding a conventional cure. While recombinant viruses are the most efficient gene delivery vectors currently available, polymeric vectors have several advantages that make them a promising alternative (Crystal, 1995; Tripathy *et al.*, 1996). Until now, non-viral gene carriers, such as polyethylenimine, poly(L-lysine), chitosan, etc., have been studied for the successful gene therapy (Niidome *et al.*, 2002).

Due to its non-immunogenecity, good biocompatibility and non-toxicity, chitosan has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical materials for artificial skin and wound healing bandage applications (Dodane *et al.*, 1998; Jiang *et al.*, 2004). Also, chitosan and its derivatives have been used actively as gene carriers (Erbacher *et al.*, 1998; Lee *et al.*, 1998).

In our previous studies (Park *et al.*, 2000; Park *et al.*, 2001), galactosylated chitosan (GC)-*graft*-dextran or poly (ethylene glycol) (PEG) were synthesized and characterized as hepatocyte-targeting gene carriers. The transfection of the complexes only occurred in the cells with asialoglycoprotein receptors (ASGPR), indication of receptor-mediated delivery of the DNA into the hepatocyte. But transfection efficiency of the carriers is still low compared with that of lipofection. Recently, we synthesized GC conjugated with poly(vinyl pyrrolidone) (PVP) as a hydrophilic group (Park *et al.*, 2003a). PVP is often used as a medicinal additive or polymeric modifier of bioactive proteins (Kamada *et al.*, 1999; Mu *et al.*, 1999). The PVP was found to be retained in blood better than PEG. PVP-conjugated TNF had more than 200- and 5-fold higher antitumor efficacy than native TNF and PEG-conjugated TNF (Mu et al., 1999). The synthesized GC-*graft*-PVP (GCPVP)/DNA complexes had the smallest particle size distribution, due to the additional secondary interaction between PVP and DNA as well as ionic interaction between chitosan and DNA, among the hydrophilic GC derivatives such as GC-*graft*-dextran, GC-*graft*-PEG or GC-*graft*-PVP.

In this study, *in vitro* transfection and hepatocyte-specific delivery of GCPVP was evaluated for gene carrier. Effect of calcium ion on the transfection of the complexes was also investigated, because the calcium ion is required for

Correspondence to: Chong-Su Cho, School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea
Tel: 82-2-880-4636, Fax: 82-2-875-2494
E-mail: chocs@plaza.snu.ac.kr

binding between ASGPR and galactose of asialoglycoprotein (Park *et al.*, 2003b; Park *et al.*, 2003c)

Materials and methods

Materials

Chitosan 10K (degree of deacetylation : 90.8%) was kindly donated by JAKWANG Co., Ltd. (Ansung, Korea). Fluorescein isothiocyanate was purchased from Dojindo Laboratory (Tokyo, Japan). DNA (sodium salt, from salmon testes), *N*-vinyl pyrrolidone, 2,2'-azobisisobutyronitrile (AIBN), 3-mercaptopropionic acid, and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade and used without further purification. pEGFP-N2 (4.7 kb) encoding green fluorescent protein driven by immediate early promoter of CMV was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The plasmids were propagated in *Escherichia coli* and purified by chromatography (Megaprep Kits, Qiagen, Chatsworth, CA).

Synthesis of monocarboxylic terminated-poly(vinyl pyrrolidone) (PVP)

PVP with single terminus carboxylic group was radically polymerized with modification as previously reported (Kamada *et al.*, 1999; Mu *et al.*, 1999). Briefly, *N*-vinyl pyrrolidone (27 mmol) dissolved in dimethylformamide (7 mL) using AIBN (1.2 mmol) as an initiator and 3-mercaptopropionic acid (2.7 mmol) as a chain transfer agent, was reacted at 60°C for 24 h. The synthesized PVP was dialyzed against distilled water for 4 days using Spectra/Por7 membrane (MWCO = 3,500). The number-average molecular weight of the resulting PVP determined by MALDI-TOF mass spectroscopy (Voyager™ RP, Perseptive Biosystems, USA) was 5,500.

Preparation of GC and GCPVP

The GC were synthesized as the similar method previously reported (Park *et al.*, 2000; Park *et al.*, 2001). Briefly, chitosan was coupled with lactobionic acid (TCI, Japan) *via* an active ester intermediate using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Dojindo, Japan). Chitosan and equivalent mole of LA were dissolved in 10 mM *N,N,N',N'*-tetramethylethylenediamine (TEMED)/HCl buffer solution (pH 4.7). EDC was added to this solution and stirred for 72 h at room temperature. The resulting GC was dialyzed for 4 days using Spectra/Por7 membrane (MWCO = 3,500) against distilled water. The composition of galactose group in GC was determined by NMR spectroscopy (Bruker, 600 MHz, Germany).

The GC was chemically conjugated with PVP *via* the formation of an amide bond between the amino group of GC and the terminal carboxyl group of the PVP. The terminal carboxyl group of PVP was activated by the *N*-hydroxysuccinimide (NHS)/EDC dissolved in buffer solution (0.1 M 2-morpholinoethanesulfonic acid, 0.5 M NaCl, and pH 6.0). The GC was added to the above reaction mixture and allowed to react overnight at room temperature. The reaction was quenched by adding hydroxylamine (Fluka, Switzerland) to a final concentration of 10 mM.

The resulting GCPVP was purified by ultrafiltration in a stirred Advantec ultrafiltration cell (UHP-76K, Toyo Roshi Kaisha Ltd., Japan) fitted with ultrafiltration disk membrane (NMWL:10,000, diameter: 76 mm, Sigma). Advantec membrane (76 mm, MWCO: 20,000) was used in case of chitosan 50K. The chemical composition of PVP in GCPVP could not be measured by NMR spectroscopy because the chemical shift of acetamide group of chitosan was overlaped with those of methylene groups of pyrrolidone side chain of PVP. Free amino groups of GCPVP in solution was determined by ninhydrin assay using glucosamine standard (Crrotto *et al.*, 1993). The chemical structure of synthesized GCPVP was shown in Fig. 1.

Preparation of GCPVP/DNA complex

The GCPVP/DNA (+/-) charge ratio was expressed as

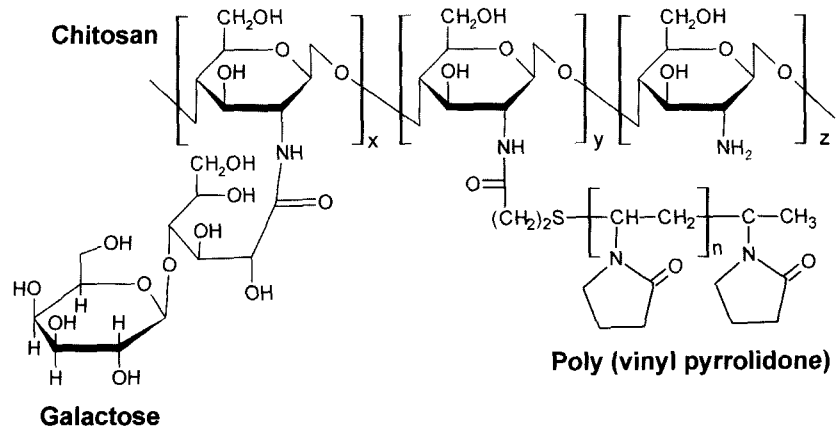

Fig. 1. The chemical structure of galactosylated chitosan-*graft*-poly(vinyl pyrrolidone) (GCPVP)

the ratio of mole of the amino group of chitosan to mole of the phosphate group of DNA. Complexes were induced to self-assemble in 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 150 mM NaCl by mixing DNA (30 μg/mL) with appropriate polymer solution at desired charge ratio, and DNA concentration was adjusted to 10 μg/mL with PBS and left to stand for 30 min at room temperature before use.

Evaluation of cytotoxicity

Hep G2 cells were seeded in triplicate at a density of 5×10^4 cells/well into 96-well microtitre plates. Cells were incubated for 24 h prior to the addition of GCPVP. GCPVP-untreated cell in media was used as a positive reference. The cell viability was measured by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA). The absorbance was recorded at 540 using a Titertek plate-reader. A reference wavelength (620 nm) was used to reduce background contributed by excess cell debris and other nonspecific absorbance. The cytotoxicity of GCPVP containing Ca^{2+} was also measured.

Transfection protocol

HeLa and Hep G2 were grown in media containing 10% fetal bovine serum at 37°C under 5% CO_2 atmosphere. HeLa and Hep G2 cells were seeded at a density of 5×10^4/mL in 22 mm culture plate on the day before transfection and grown to 60~70% confluency. GCPVP/pEGFP-N2 complexes diluted with serum-free media were added to culture plate and incubated for 6 h at 37°C under a 5% CO_2 atmosphere. The serum-free media were then replaced with fresh media containing serum and incubated for additional 24 h at 37°C under a 5% CO_2 atmosphere. After incubation, the cells transfected with pEGFP were observed under inverted fluorescent microscope.

FITC labeling of plasmid

FITC-labeled plasmid was prepared by Ishii's method (Ishii *et al.*, 2000). FITC (0.257 mmol) was reacted with 2-(4-aminophenyl)-ethylamine (0.257 mmol) in 1.5 mL of DMF overnight at 25°C with stirring, resulting in the formation of FITC-aniline. FITC-diazonium salt was then prepared by reacting FITC-aniline (25.7 μmol in 150 μL of DMF) with sodium nitrite (110 μmol) in 2 mL of 0.5 M HCl for 5 min at 0°C with constant stirring. The reaction was quenched by adding 1 mL of 1 M NaOH. Then, the solution of FITC-diazonium salt (25 mol) was mixed with the solution of plasmid (2 mg) in 15 mL of 0.1 M borate buffer (pH 9.0). The reaction was carried out for 15 min at 25°C with stirring. The FITC-labeled plasmid (F-plasmid) was isolated by ethanol precipitation and gel-exclusion chromatography (Sephacryl S-200, Amersham Pharmacia Biotech). The average number of FITC linked to one plasmid determined by absorbance at 260 nm and fluorescence intensity (Ex=495 nm and Em=520 nm) was 6.9. The ordered structure of the F-plasmid confirmed by 1% agarose gel electrophoresis was as same as non-labeled plasmid.

Primary hepatocyte isolation and culture

The female ICR mice employed in this study were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The liver was perfused with 1.25×10^{-2} wt% collagenase solution. After the liver had been excised, parenchymal hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50 g for 90 s. The dead parenchymal hepatocytes were removed by density gradient centrifugation on Percoll (Pharmacia, Piscataway, NJ). The viable parenchymal hepatocytes were suspended in Williams' E (WE) medium containing antibiotics and plated on collagen-coated glass coverslips in 6-well plates (Iwaki Glass Co., Tokyo, Japan) at 2×10^5 cells per well. The hepatocytes were incubated at 37°C for 3 h. Then, the old medium was removed and serum-free WE medium containing R-GPC/F-plasmid complexes was added to cells. Cells were rinsed and treated with 1 mL of ethanol for 30 min at 20°C. After being rinsed twice with 0.1 M PBS, the coverslips were enclosed in 1 mL of glycerol and visualized by confocal laser sccaning microscope (Micro Systems LSM 410, Carl Zeiss, Germany). Propidium iodide (PI) (1 μg/mL) was treated as the fluorescent stain for chromosome.

RESULTS AND DISCUSSION

The basic concept underlying gene therapy is that human disease can be treated by the transfer of the therapeutic genes into specific cells of a patient in order to correct or supplement defective genes responsible for disease development. Gene delivery to liver parenchymal cells, hepatocytes is an attractive proposition for the treatment of a variety of diseases. The fact that the liver parenchymal cells have the abundant ASGPR which is specific for hepatocytes makes hepatocyte-targeted gene delivery plausible (Stockert, 1995).

GCPVP had been synthesized and its physicochemical properties had been examined previously, which revealed that GCPVP could have the potential for hepatocyte-targeting gene carrier. Therefore, *in vitro* transfection of GCPVP was characterized for hepatocyte-specific gene carrier in this study.

Fig. 2 shows the cytotoxicity of GCPVP itself and GCPVP/DNA complex containing Ca^{2+} at the various charge ratios against HepG2 cells. GCPVP itself and GCPVP/DNA complex had negligible cytotoxic effects regardless of the concentration of GCPVP and the charge

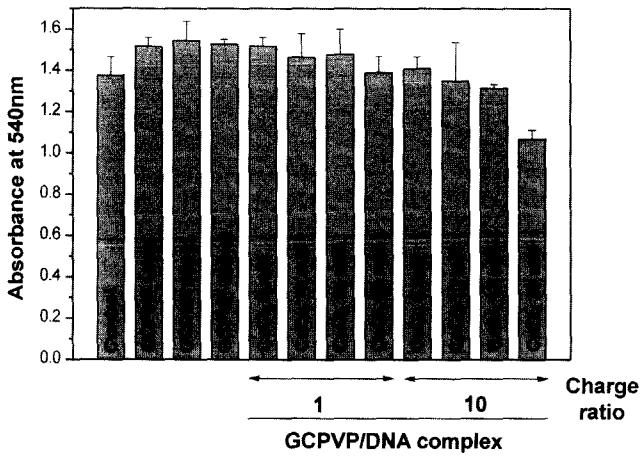

Fig. 2. Cytotoxicity of GCPVP itself and GCPVP/DNA complex containing Ca^{2+} + at the various charge ratios against HepG2 cells.

ratio, but GCPVP/DNA complex had slightly cytotoxic effect on HepG2 cells at higher charge ratio and Ca^{2+} concentration of 20 mM used.

The efficacies of GCPVP/DNA complex in gene transformation of Hep G2 cells bearing ASGPR and HeLa ones without ASGPR are shown in Fig. 3. The plasmid DNA used in this experiment is pEGFP which produces the green fluorescence proteins in cytoplasm. Hep G2 cells, which were transfected with GCPVP/DNA complex and Lipofectamine Plus/DNA complex as a control, efficiently produced green fluorescence under UV irradiation. On the other hand, HeLa cells were transfected with Lipofectamine Plus/DNA but not with GCPVP/DNA complex, suggesting that GCPVP/DNA complex transfected the ASGPR-bearing cells selectively through the specific interaction between ASGPR and galactose ligand.

It is well known that the galactose moiety binds to the hepatocyte cell surface mediated by ASGPR in a Ca^{2+}-dependent manner (Kim *et al.*, 2000, 2001). Calcium effect on transfection efficiency of GCPVP was investigated to clarify whether the transfection of GCPVP was dependent upon the calcium concentration (Fig. 4). The transfection efficiency of GCPVP was decreased with an increase of calcium concentration and/or the charge ratio of GCPVP/DNA complex. Also, the calcium ion was precipitated onto the cells especially at the higher concentration, which might have the negative effect on the cytotoxicity and transfection. However, the transfection of GCPVP/DNA without calcium ion was drastically decreased compared to that with calcium ion. It is thought that the optimal condition of GCPVP transfection was at 2 mM of Ca^{2+} concentration.

The hepatpcyte-specific delivery of GCPVP/fluorescein-labeled plasmid (F-plasmid) complex was examined by confocal laser scanning microscopy (Fig. 5). PI was used for nucleic acid staining, because PI binds to DNA by intercalating between the bases with little or no sequence preference and enhance the fluorescence 20- to 30-fold once the dye is bound to nucleic acids. Binding of the GCPVP/F-plasmid complex at the plasma membrane of hepatocytes occurred even at 30 min after transfection and GCPVP/F-plasmid complexes was internalized into the interior of the cell through endosome leading to be distributed in the cytoplasm after 1 h and continued to accumulate in the cytoplasm by 3 h-post transfection. However, there is no indication of the presence of F-plasmid or GCPVP/F-plasmid complex in the nucleus. This indicates that the slow nuclear trafficking of plasmid followed by rapid escape from endosome is thought to be

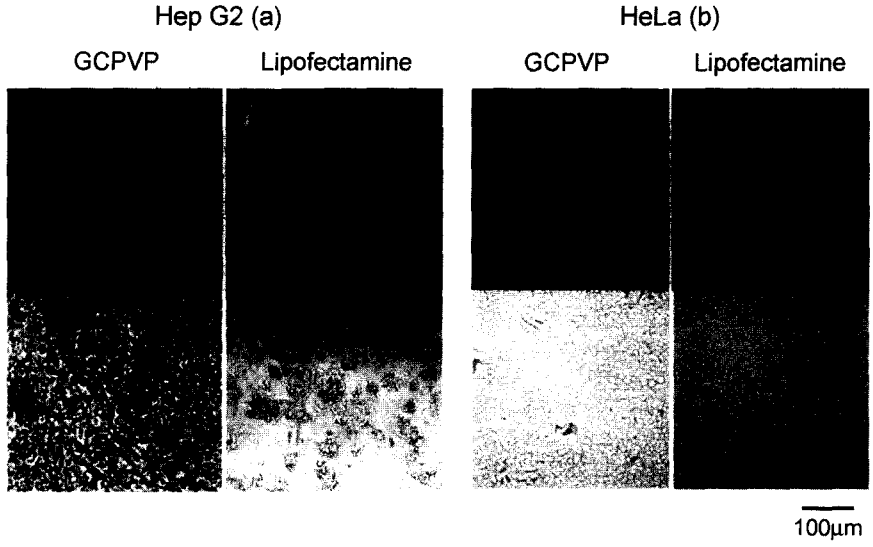

Fig. 3. Fluorescence and phase-contrast micrographs of HepG2 cells (a) and HeLa cells (b) transfected with GCPVP/DNA(pEGFP N2) complex (charge ratio 5) with 2mM Ca^{2+} or lipofectamine Plus/DNA complex.

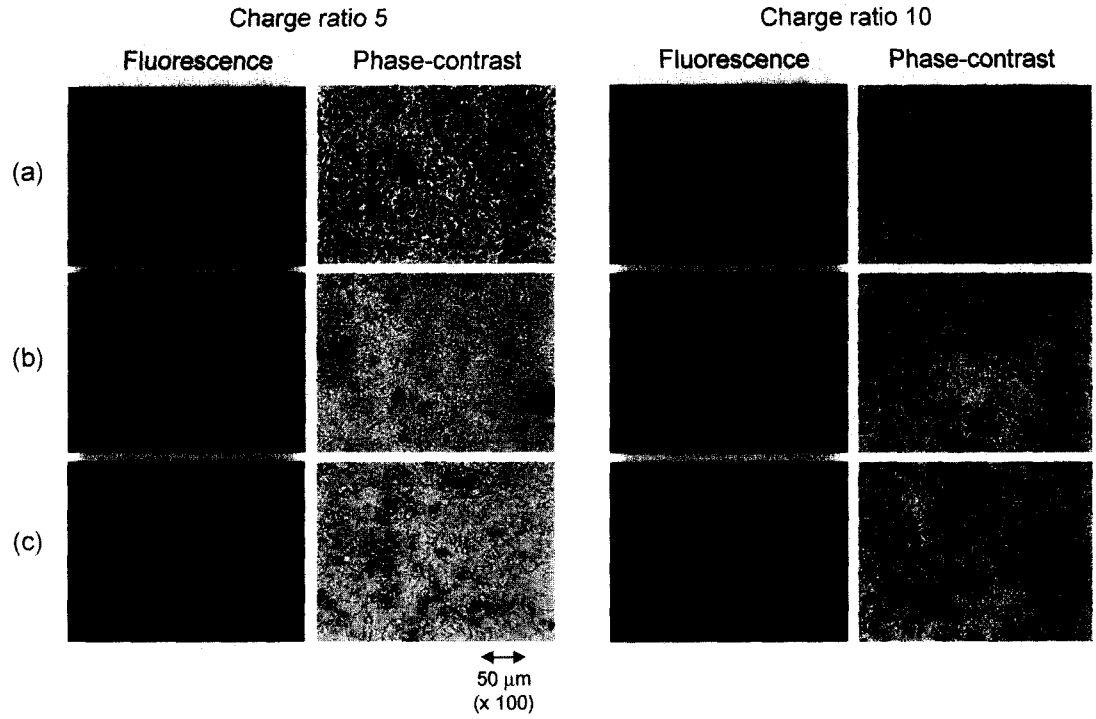

Fig. 4. Effect of Ca^{2+} concentration 2 μmol (a), 4 μmol (b), and 6 mol (c) on the transfection of GCPVP/DNA complex according to the charge ratio.

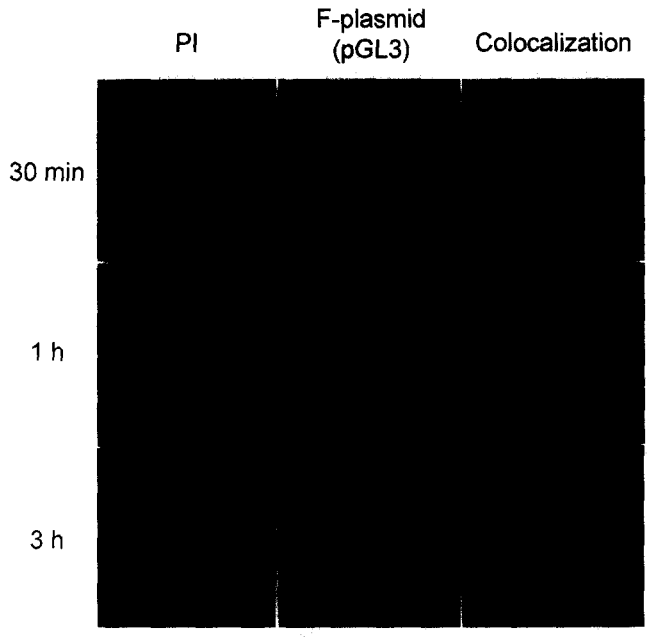

Fig. 5. Confocal laser scanning microscopy of GCPVP/F-plasmide complex transfected into primary hepatocytes according to the time schedule (charge ratio (+/-) = 5 and concentration of plasmid 10 μg/mL).

a rate-limiting step and cause a low transgene expression in GCPVP-mediated transfection. On the other hand, F-plasmid itself and CPVP/F-plasmid complex without galactose ligand were almost never internalized into the hepatocytes (data not shown), indicating that the endocytosis by interaction between galactose ligands of GCPVP and ASGPR of the hepatocytes was the major route of transfection of GCPVP/F-plasmid complexes. This tendency was the same as the data of GCP/F-plasmid complex (Park *et al.*, 2003d).

CONCLUSIONS

Galactosylated chitosan-*graft*-poly(vinyl pyrrolidone) (GCPVP) was synthesized and investigated for hepatocyte-targeting gene carrier. GCPVP was shown to be the promising non-toxic hepatocyte-targeting gene carrier. However, lower efficiency of GCPVP should be overcome through both the precise design of the carrier and the effective control of intracellular trafficking following hepatocyte-specific delivery

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