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Preparation, characterization and controlled release of liver-targeting nanoparticles from the amphiphilic random copolymer

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ABSTRACT

Liver-targeting ribavirin-conjugating nanoparticles were successfully constructed via self-assembly of the lactose-functionalized amphiphilic random copolymer, which was facilely prepared by a two-step chemoenzymatic synthetic route. Aggregation morphology of the resulting self-assemblies observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) was regularly spherical shape, and hydrodynamic diameter determined by dynamic light scattering (DLS) was 174 ± 27 nm. Critical aggregation concentration (CAC) was measured by fluorescence probe technology using pyrene as the hydrophobic molecule, and the CAC value was about 0.1 mg/L. Cell cytotoxicity tests performed by MTT assay showed that the nanoparticles had effective growth-inhibitory activity in hepG2 human hepatoma cells. Moreover, ribavirin could be slowly released from the copolymer with pseudo zero-order kinetics in different incubation media. The targeting nanoparticles self-assembled from amphiphilic random copolymers could be used as novel potential drug delivery vehicles.

1. Introduction

In recent years, design and synthesis of polymeric micelles or nanoparticles suitable for biomedical applications have been an intense field of research, among which, the most challenging issues are how to prolong the circulation of the micelles or nanoparticles in blood and enhance their targeting functionalities to the specific site of interest [1,2]. A reliable strategy for achieving the long blood circulation is to design polymeric micelles or nanoparticles containing covalently bound drug. Therefore, polymeric micelles or nanoparticles generated through the directed-assembly of drug-containing amphiphilic polymers have emerged as a promising new class of polymer therapeutics [3–5]. The introduction of targeting ligands such as antibodies, sugars, folic acid, or RGD on the surface of the micelles or nanoparticles can effectively realize the specific recognition of these micelles or nanoparticles to certain site of interest, and thus enhances their targeting ability [6–10]. Bertin et al. prepared core–shell polymeric nanoparticles, which allowed for the surface conjugation of DNA and tumor-targeting antibodies, by self-assembly of amphiphilic block copolymers containing small-molecule drug segments and tosylated hexaethylene glycol segments [11]. Bae et al. designed folate-functionalized adriamycin-containing polymeric micelles self-assembled from a amphiphilic block copolymer, folate–poly(ethylene glycol)–poly(aspartate hydrazone adriamycin) [12].

Hepatitis C virus (HCV) infection is the leading cause of sporadic, post-transfusion, non-A, non-B hepatitis [13]. An estimated 170 million people worldwide are thought to be infected with hepatitis C virus and up to 80% of infected individuals turn chronic infection [14–16]. Chronic HCV is the single most common indication for orthotopic liver transplantation worldwide, and long term chronic HCV infection can lead to liver cirrhosis and to hepatocellular carcinoma [17–19]. However, treatment of HCV infection remains problematic. Currently, the recommended therapy is a combination of interferon alpha (IFN-α) or pegylated IFN-α and nucleotide analogue ribavirin, which has only a success rate of $<60\%$ [20–22]. Furthermore, this combination treatment is limited by severe side effects. The major toxicity is a dose-dependent hemolytic anemia that is caused by high ribavirin accumulation in red blood cells, which results in a ribavirin dose reduction or premature cessation of therapy [23]. Constructing liver-targeting ribavirin-conjugating polymeric micelles or nanoparticles is a strategy for improving drug efficacy and reducing systemic side effects.

However, most of the previous studies about polymeric micelles or nanoparticles have been focused on amphiphilic block copolymers, whose synthesis often requires nontrivial conditions [24]. Moreover, the preparation of these block copolymers linking drug and targeting ligands always requires a somewhat complex

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synthetic route [12]. Therefore, it is significant to seek more facile protocol for the formation of targeting drug-conjugating polymeric micelles or nanoparticles.

Herein, we wish to achieve the formation of liver-targeting ribavirin-linking nanoparticles by self-assembly of the amphiphilic random copolymer, which could be facilely prepared by a two-step chemoenzymatic synthetic route. The aggregation morphologies, size distribution, and critical aggregation concentration of the resulting nanoparticles were, respectively, characterized by transmission electron microscopy, scanning electron microscopy, dynamic light scattering, and fluorescence probe technology. We further evaluated liver-targeting function of the nanoparticles by cell cytotoxicity tests, and investigated in vitro release behaviors of ribavirin in different incubation media by UV–vis spectrophotometer.

2. Experimental

2.1. Materials

Lipase acrylic resin from *Candida antarctica* (E.C. 3.1.1.3, 10,000 U/g, CAL-B) was purchased from Sigma. Alkaline protease from *Bacillus subtilis* (E.C. 3.4.21.14, a crude preparation of the alkaline serine protease, 100 U/mg, Subtilisin) was purchased from Wuxi Enzyme Co. Ltd. (Wuxi, PR China). α,α′-Azobis-(isobutyronitrile) (AIBN) was purchased from Fluka and purified by re-crystallization in ethanol and dried at room temperature under vacuum. Ribavirin (raw drug) was supplied by Xinxiang Tuoxin Biochemical Science & Technology Co. Ltd. (Xinxiang, PR China). 5′-*O*-Vinyladipyl-ribavirin (VAR) was synthesized and purified as described in the paper [25]. 6-*O*-Vinylsebacyl-lactose (VSL) was prepared and purified according to the literature [26]. *N*,*N*-Dimethylformamide (DMF) was HPLC grade. Dimethyl sulfoxide (DMSO), methanol, and all other chemicals were analytical grade.

2.2. Characterization methods

FTIR spectra were recorded on a Nicolet Nexus 670 FTIR spectrophotometer at room temperature in the range of 4000–400 cm^{-1}. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra measurements were run on a Bruker AMX-500 MHz FT-NMR spectrometer using DMSO-d_6 as the solvent and tetramethylsilane (TMS) as an internal standard. Polymer molecular weights were measured by gel permeation chromatography (GPC). The GPC columns were standardized with near-monodisperse polystyrene from Aldrich in molecular weights ranging from 7.0×10^5 to 1920 Da. DMF was used as the mobile phase at a flow rate of 1.0 mL/min.

2.3. Enzymatic synthesis of vinyl ribavirin derivative and lactose derivative

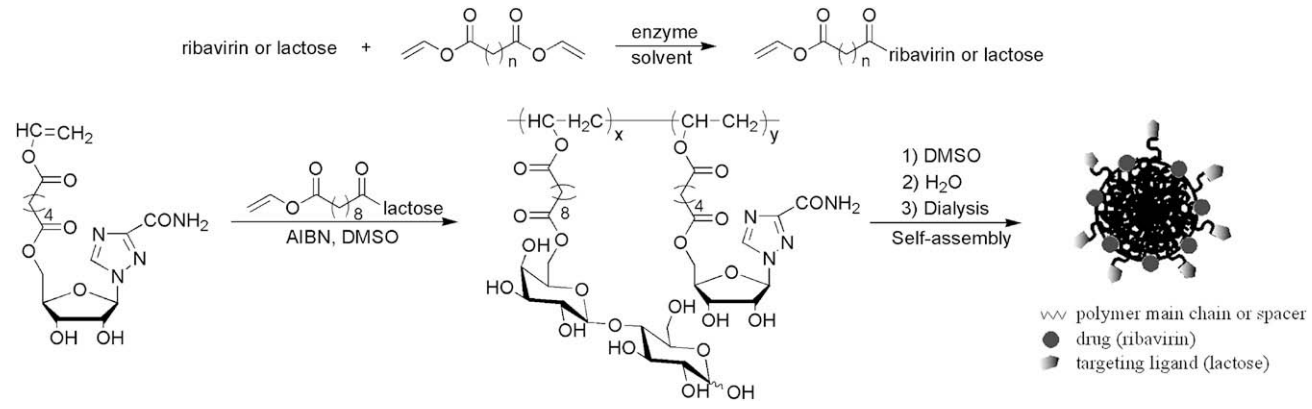
A general procedure: the reaction was initiated by adding CAL-B to anhydrous acetone containing ribavirin and divinyl adipate or adding Subtilisin to pyridine containing lactose and divinyl sebacate [25, 26]. The suspension was then kept at 50 °C and stirred at 250 rpm. The reaction process was monitored using TLC, and reaction was terminated by filtering the enzyme. The product was separated by silica gel chromatography.

2.4. Synthesis of poly(5′-O-vinyladipyl-ribavirin-co-6-O-vinylsebacyl-lactose)

Poly(5′-*O*-vinyladipyl-ribavirin-*co*-6-*O*-vinylsebacyl-lactose) [poly(VAR-*co*-VSL)] was prepared by adding VAR (398 mg, 1.0 mmol) and VSL (570 mg, 1.0 mmol) into a 10-mL polymerization tube containing DMSO (1.00 mL) and AIBN (20.0 mg). The mixture was degassed by three freeze–thaw cycles, and then stirred under nitrogen at 70 °C for 24 h. The resulting product was repeatedly precipitated in methanol and dried under vacuum to afford a light yellow solid poly(VAR-*co*-VSL) (328 mg, 34%). $M_n = 17{,}000$, $M_w/M_n = 2.65$. IR (KBr): ν (cm^{-1}) 3432, 2932, 2860, 1736, 1686, 1466, 1288, 1176, 1138, 1076. ^1H NMR (500 MHz; DMSO-d_6; Me_4Si) δ 8.83 (5-H of ribavirin), 7.84 (NH_2 of ribavirin), 7.64 (NH_2 of ribavirin), 6.68, 6.38 (1-OH of lactose), 5.94 (1′-H of ribavirin), 5.67 (2′-OH of ribavirin), 5.38 (3′-OH of ribavirin), 5.29–2.75 (CHO; 1-H, 2-H, 3-H, 4-H, 5-H, 6-H, 2-OH, 3-OH, 6-OH, 2′-OH, 3′-OH, 4′-OH, 1′-H, 2′-H, 3′-H, 4′-H, 5′-H and 6′-H of lactose; 2′-H, 3′-H, 4′-H and 5′-H of ribavirin), 2.38–1.23 (CH_2). ^{13}C NMR (125 MHz; DMSO-d_6; Me_4Si) δ 173.4, 173.0, 172.5 (C═O), 160.8 (C-3 of ribavirin), 158.2 (C-6 of ribavirin), 146.2 (C-5 of ribavirin), 104.0 (C-1′ of lactose), 97.2, 92.5 (C-1 of lactose), 91.8 (C-1′ of ribavirin), 82.2 (C-4′ of ribavirin), 81.3, 80.4 (C-4 of lactose), 74.6 (C-2′ of ribavirin), 75.3, 75.2, 75.1, 73.3, 72.8, 71.8, 70.8, 70.2, 68.7, 64.2, 61.0 (C-6, C-5, C-3, C-2, C-6′, C-5′, C-4′, C-3′, C-2′ of lactose), 70.9 (C-3′ of ribavirin), 63.7 (C-5′ of ribavirin), 33.8, 33.4, 29.1, 24.8, 24.2 (CH_2).

2.5. UV measurement of pyrene/poly(VAR-co-VSL)

Self-assembly of the resulting amphiphilic random copolymer poly(VAR-*co*-VSL) was preliminarily proved by UV–vis absorption spectra, which were recorded on an Analytikjena SPECORD 200



Scheme 1. Chemoenzymatic synthesis of lactose-functionalized ribavirin-conjugating amphiphilic random copolymer and preparation of nanoparticles by water addition to copolymer solution in DMSO followed by dialysis against water.

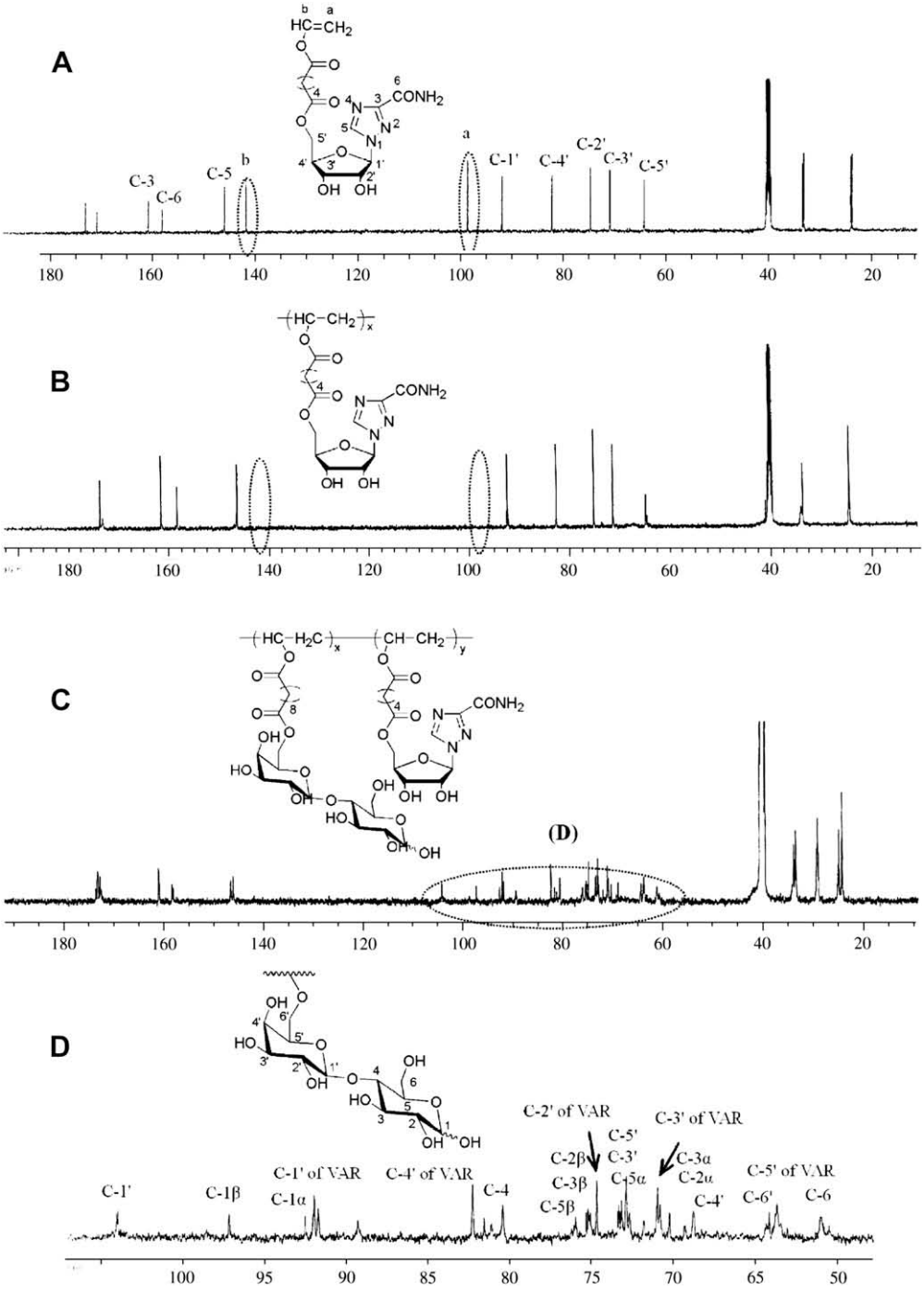


Fig. 1. ^{13}C NMR spectra of (A) VAR, (B) PVAR, and (C) poly(VAR-*co*-VSL).

UV–vis spectrophotometer. The sample was prepared as follows: poly(VAR-*co*-VSL) aqueous solution (0.50 mg/mL, 5.00 mL) was firstly added to the vial containing a known amount of pyrene, and the final concentration of pyrene was 10^{-4} M. The resulting mixture was then heated at 50 °C for 5 h to reach the equilibrium of pyrene in the copolymer aqueous solution. Finally, the mixture was cooled overnight at room temperature. The absorption spectra were recorded ranging from 245 to 400 nm.

2.6. CAC measurement

Nine samples of the copolymer aqueous solution with concentrations ranging from 1×10^{-5} to 0.5 mg/mL were prepared and then left to equilibrate with a constant pyrene concentration of 6×10^{-7} M for 12 h at room temperature. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. The excitation wavelength was 339 nm and the emission spectra were recorded from 360 to 460 nm. Both excitation and emission bandwidths were 3 nm. In the emission spectra of pyrene/copolymer, the intensity ratio of the first band (I_{373}) to the third band (I_{384}) was analyzed as a function of copolymer concentration. A CAC (critical aggregation concentration) value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration [27].

2.7. Nanoparticle preparation

The ribavirin-containing copolymer was dissolved in DMSO at an initial concentration of 0.01%. Then, a given volume of ultrapure water (15% of the solution) was added into the copolymer/DMSO solutions with stirring. The resulting solution was dialyzed using dialysis bag (MWCO = 3500) against ultrapure water for 2 days to remove DMSO from the solution.

2.8. Transmission electron microscopy measurement

Transmission electron microscopy (TEM) measurement was performed with a JEM 200CX instrument operated at 100 kV. The nanoparticle solution was dropped onto carbon-coated copper grids and dried at room temperature before measurement.

2.9. Scanning electron microscopy measurement

Scanning electron microscopy (SEM) measurement was carried out with a Sirion-100 instrument (FEI, USA) at the acceleration voltage of 25.0 kV. Sample was prepared by loading 80 μL of the aggregate solution onto a glass slide. The glass slide was then dried and a thin layer of Au was coated on the sample surface before measurement.

2.10. Dynamic light scattering measurement

Dynamic light scattering (DLS) measurement was carried out in the nanoparticle aqueous solution using a Nanoseries (Malvern, UK) zetasizer at a scattering angle of 90° under 25 °C. All samples were passed through a 0.45 μm pore size filter before measurement.

2.11. Cell cytotoxicity assay

HepG2 liver carcinoma cell line was provided by Biochemistry Institute of Zhejiang University and cultured in RPMI 1640 medium containing 10% fetal calf serum in a cell culture incubator at 37 °C under 5% CO_2 [28]. Cells were firstly seeded in serum-containing RPMI 1640 medium to 96-well plates until 70–80% confluent. Then, the cells were exposed to serum-free RPMI 1640 medium containing various concentrations (1.5, 3.0, 7.5, 15 mg/L ribavirin equivalent concentration, respectively) of the polymers for 72 h at 37 °C. PBS (phosphate buffer solution) was chosen as the control. The cytotoxic effects of the ribavirin-containing polymers were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide) dye reduction assay.

2.12. In vitro release experiment

In vitro release behavior of ribavirin from the copolymer was studied by dialysis experiment. Firstly, the resulting lactose-functionalized ribavirin-containing copolymer poly(VAR-*co*-SLA) (10.0 mg) was added to 1.00 mL incubation medium (pH = 1.2, 0.2 M HCl/NaCl/glycine solution, or pH = 7.4, 0.1 M phosphate buffer solution) and subsequently placed into a dialysis bag (MWCO = 3500). The dialysis bag was then placed into a 10-mL bottle with 5.00 mL corresponding medium and stirred at 37 °C under 100 rpm. At set time intervals, the whole medium (5.00 mL) was taken and replaced with the same volume of fresh solution. Lastly, the concentration of ribavirin released from the copolymer in different media was determined by an Analytikjena SPECORD 200 UV–vis spectrophotometer at 207 nm.

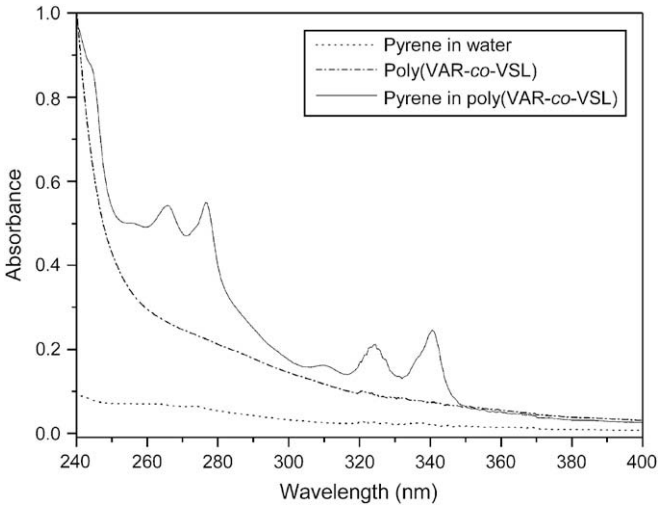


Fig. 2. UV absorption spectra of pyrene in aqueous solution of poly(VAR-*co*-VSL), pyrene in water, and aqueous solution of poly(VAR-*co*-VSL).

3. Results and discussion

3.1. Synthesis and characterization of the amphiphilic random copolymer

In this study, we developed a facile and effective method for the construction of liver-targeting drug-conjugating nanoparticles via self-assembly of the lactose-functionalized amphiphilic random copolymer. The functional drug-containing amphiphilic random copolymer could be easily prepared by a two-step chemoenzymatic synthetic route in ordinary conditions. Because of high selectivity and mild reaction conditions, it is convenient to achieve the selective synthesis of polymerizable multifunctional substrate derivatives such as drug and sugar derivatives by enzymatic approaches. The radical copolymerization of the resulting polymerizable drug derivatives with properly selected comonomers could provide an enormous variability in composition and properties of the resulting drug-containing polymers.

Herein, a lactose-functionalized ribavirin-containing amphiphilic random copolymer poly(VAR-*co*-VSL), in which ribavirin was

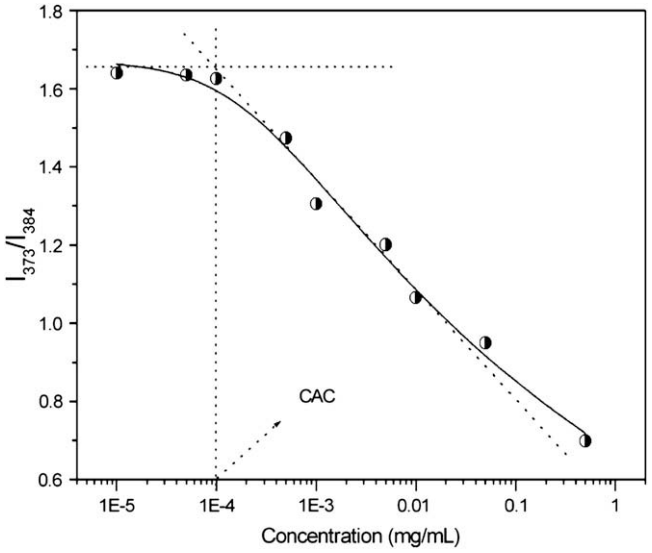


Fig. 3. The value of I_{373}/I_{384} in the emission spectra of pyrene/poly(VAR-*co*-VSL) as a function of poly(VAR-*co*-VSL) concentration.

chosen as a model drug and lactose was employed as the targeting ligand, was facilely prepared by combining enzymatic synthesis with radical polymerization (Scheme 1). By enzymatic transesterification of ribavirin or lactose with divinyl dicarboxylates, controllable selective acylation at the primary hydroxyl of ribavirin or lactose could be easily achieved without protection/deprotection steps to afford polymerizable vinyl ribavirin derivatives or vinyl lactose derivatives. Based on our previous report about the effect of the linker structure on release rate and targeting ability of drug–polymer conjugates [29], we chose 5′-*O*-vinyladipyl-ribavirin (VAR) and 6-*O*-vinylsebacyl-lactose (VSL) as comonomers for further synthesis of the copolymer.

The lactose-functionalized ribavirin-containing amphiphilic random copolymer was then prepared by AIBN-initiated radical copolymerization of VAR with VSL and fully characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and GPC. From FTIR spectrum data of the copolymer, it could be found that vinyl group absorption present in comonomers was absent in the corresponding copolymer (see Supplementary data). ^{13}C NMR (Fig. 1) and ^{1}H NMR data of the copolymer revealed the disappearance of vinyl groups and the existence of ribavirin and lactose groups. From the ^{1}H NMR spectrum, the molar ratio of VAR to VSL comonomers in the copolymer could be approximately calculated. Thus, the loading capacity of ribavirin in the copolymer could be estimated. According to this method, the content of ribavirin in the copolymer was calculated as 30.7 wt%. Furthermore, the copolymer containing galactose and ribavirin [poly(VAR-*co*-VGA)], the copolymer containing glucose and ribavirin [poly(VAR-*co*-VGL)], and the sugar-free polymer (PVAR) were, respectively, prepared and characterized by the same methods (see Supplementary data).

3.2. Formation and characterization of the nanoparticles

The resulting lactose-functionalized ribavirin-linking random copolymer was an amphiphilic polymer, which contained hydrophobic main chains and hydrophilic pendants. Therefore, certain selective polar solvents may trigger self-assembly of the copolymer. The hydrophobic components were preferred to be tucked in the interior of an assembly, and the hydrophilic parts were exposed to the bulk solvent (Scheme 1). Self-assembly of the copolymer was preliminarily proved by UV–vis absorption spectra using pyrene as the guest molecule. From the UV absorption spectrum of pyrene in water, it could be found that pyrene was a strong hydrophobic compound (Fig. 2). If the amphiphilic random copolymer does indeed form the core–shell aggregates in water, the copolymer should be able to act as nanocontainers for pyrene molecules in water. As expected, an UV absorbance of 0.24 was investigated at 341 nm when 10^{-4} M pyrene was dispersed in the aqueous solution of the copolymer poly(VAR-*co*-VSL).

The self-assembling ability of the amphiphilic random copolymer poly(VAR-*co*-VSL) in water was also confirmed by the critical aggregation concentration (CAC) measurement using pyrene as the hydrophobic fluorescent probe. In emission spectra of pyrene, the value of I_{373}/I_{384} emission intensity ratio was very sensitive to the polarity of the medium surrounding pyrene molecules [30]. The larger the value was, the bigger the polarity of the medium was. From the plot of fluorescence intensity ratio (I_{373}/I_{384}) versus poly (VAR-*co*-VSL) concentration (Fig. 3), an abrupt decrease of I_{373}/I_{384} value could be observed at a critical concentration, indicating the formation of aggregates and the transfer of pyrene into the hydrophobic interior of aggregates [31]. The critical concentration was defined as CAC. From this figure, we could find that the CAC value of poly(VAR-*co*-VSL) in water was about 0.1 mg/L, which allowed their use in very dilute aqueous media such as body fluids.

The CAC measurement of poly(VAR-*co*-VSL) further proved the formation of self-assemblies from the amphiphilic random copolymer. Aggregation morphology of the self-assemblies was investigated by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Fig. 4B and C). Apparently, the self-assemblies were well dispersed as individual nanoparticles with regularly spherical shape in the aqueous phase. From the TEM image, the average diameter of the nanoparticles from poly(VAR-*co*-VSL) was about 135 nm in the dry state.

The size of the nanoparticles was also measured by dynamic light scattering (DLS). The hydrodynamic diameter of the nanoparticles was 174 ± 27 nm in the aqueous solution of poly(VAR-*co*-VSL) (Fig. 4A), which was in agreement with the TEM result.

3.3. Cell cytotoxicity of the nanoparticles

It is well known that terminal galactose and *N*-acetylgalactosamine can mediate efficient liver targeting via the asialoglycoprotein receptor (ASGPR) of hepatocytes [32–35]. In order to verify whether the resulting lactose-functionalized nanoparticles had targeting function against living cells, cell cytotoxicity of the nanoparticles from poly(VAR-*co*-VSL) was investigated in hepG2 human hepatoma cells, which overexpressed galactose-binding asialoglycoprotein receptor. Phosphate buffer solution (PBS) was chosen as the control. Evidently, the lactose-functionalized nanoparticles from poly(VAR-*co*-VSL) showed effective growth-inhibitory activity in hepG2 cells at the ribavirin equivalent concentration of 6 μg/mL (Fig. 5). Similarly, cell cytotoxicity effects of the nanoparticles from sugar-free polymer PVAR, glucose-containing copolymer poly(VAR-*co*-VGL), and galactose-containing copolymer poly(VAR-*co*-VGA) on hepG2 cells were, respectively, determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye reduction assays. The nanoparticles from PVAR or poly(VAR-*co*-VGL) had no evident effect on the hepG2 cell viability.

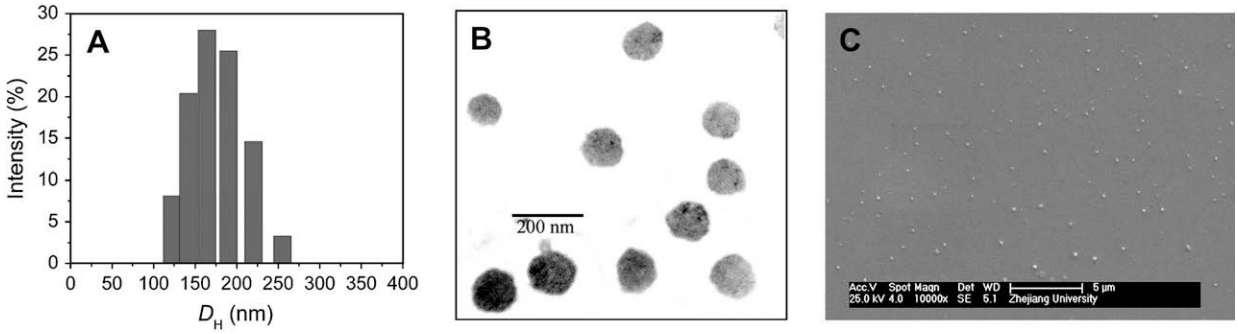


Fig. 4. (A) Size characterization of the nanoparticles from poly(VAR-*co*-VSL) by DLS (D_H: hydrodynamic diameter). (B) TEM image of the nanoparticles from poly(VAR-*co*-VSL). (C) SEM image of the nanoparticles from poly(VAR-*co*-VSL).

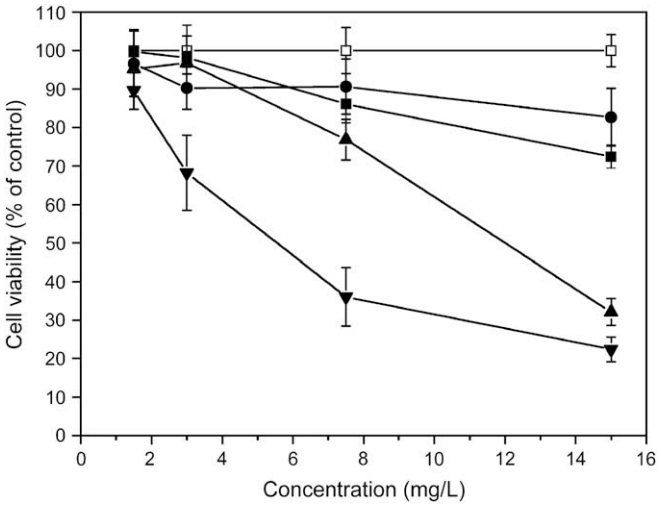


Fig. 5. Effects of □ PBS, ● poly(VAR-*co*-VGL), ■ PVAR, ▲ poly(VAR-*co*-VGA), and ▼ poly(VAR-*co*-VSL) on HepG2 cell viability. Cells were, respectively, incubated with the polymers (1.5, 3.0, 7.5, 15 mg/L ribavirin equivalent concentration, respectively) for 72 h. PBS was chosen as the control. Data are shown as mean ± SD ($n = 4$).

The growth of hepG2 cells was inhibited by the nanoparticles from poly(VAR-*co*-VGA) at the ribavirin equivalent concentration of 12 μg/mL. It was obvious that the lactose-functionalized nanoparticles self-assembled from the amphiphilic random copolymer poly(VAR-*co*-VSL) showed an enhanced liver-targeting function than the galactose-installing nanoparticles from poly(VAR-*co*-VGA).

3.4. In vitro release of ribavirin

In order to investigate whether ribavirin could be slowly released from the system, in vitro release behaviors of ribavirin from poly(VAR-*co*-VSL) in different media were studied. Small-molecule ribavirin (raw drug) was chosen as the control. Two solutions, pH = 7.4 phosphate buffer solution (simulated extracellular fluids) and pH = 1.2 hydrochloric acid solution (simulated gastric juice), were chosen as incubation media. The release process of ribavirin in different incubation media was monitored by UV, and the curves are shown in Fig. 6. In pH = 7.4 phosphate buffer solution, ribavirin (raw drug) was quickly released and the cumulative released amount was up to 100% after 2 h. Comparatively, ribavirin was slowly released from poly(VAR-*co*-VSL) with pseudo zero-order kinetics in the two incubation media. The cumulative released ribavirin was 63 and 38% after 7 days in pH = 1.2 hydrochloric acid solution and pH = 7.4 phosphate buffer solution, respectively. Moreover, the liberation rate of ribavirin was relative with pH value of incubation medium. In pH = 1.2 hydrochloric acid solution, the liberation rate of ribavirin was 9.1%/day, which is faster than in pH = 7.4 phosphate buffer solution (5.4%/day) because the ester bonds binding ribavirin to macromolecular carrier could more easily undergo hydrolysis in acidic medium.

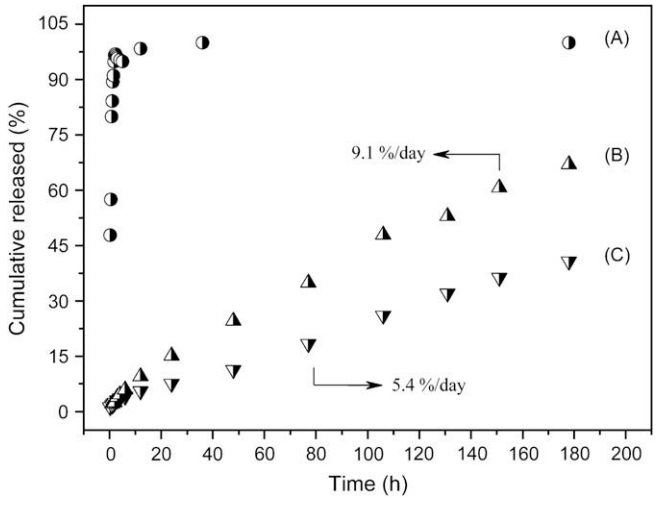


Fig. 6. In vitro release of (A) ribavirin (raw drug) in pH = 7.4 phosphate buffer solution, (B) poly(VAR-*co*-VSL) in pH = 1.2 hydrochloric acid solution, and (C) poly(VAR-*co*-VSL) in pH = 7.4 phosphate buffer solution.

4. Conclusions

In this study, a convenient and effective protocol for constructing liver-targeting drug-conjugating nanoparticles was developed by the self-assembly of facilely synthesized lactose-functionalized ribavirin-containing amphiphilic random copolymer. The resulting self-assemblies could be well dispersed as spherical nanoparticles in water, whose hydrodynamic diameter was 174 ± 27 nm. The lower CAC value (0.1 mg/L) of the nanoparticles allowed their use in very dilute aqueous media such as body fluids. Moreover, the nanoparticles had effective growth-inhibitory activity in hepG2 human hepatoma cells. Ribavirin could be slowly released from the system with pseudo zero-order kinetics in different incubation media. Further studies about in vivo disposition of the lactose-functionalized nanoparticles are being conducted in our laboratory.

Acknowledgements

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.polymer.2008.09.006.

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