Research on the Construction of Bispecific-Targeted Sustained-Release Drug-Delivery Microspheres and Their Function in Treatment of Hepatocellular Carcinoma

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ABSTRACT: Lenvatinib (LEN) is approved as one of the commonly used drugs in the treatment of hepatocellular carcinoma (HCC). It is recognized to be a novel therapeutic choice for the direct and targeted delivery of effective drugs to HCC tumor sites. The key to the proposed method lies in the requirement for efficient targeted drug delivery carriers with targeting performance to deliver effective drugs directly and safely to tumor lesions. Methods: Here, magnetic liposomes (MLs) were modified by phosphatidylinositol proteoglycan 3 (GPC3) and epithelial cell adhesion molecules (EpCAMs). Subsequently, bispecific-targeted sustained-release drug-loaded microspheres containing LEN (GPC3/EpCAM-LEN-MLs) were constructed. In addition, both cytotoxicity and magnetic resonance imaging (MRI) analyses were performed to establish a mouse model and further perform corresponding performance assessments. Results: The corresponding results showed that GPC3/EpCAM-LEN-MLs were spherical-shaped and evenly dispersed. The encapsulation and drug-loading efficiencies were 91.08% ± 1.83% and 8.22% ± 1.24%, respectively. Meanwhile, GPC3/EpCAM-LEN-MLs showed a high inhibition rate on the proliferation of HCC cells and significantly increased their apoptosis. Furthermore, MRI revealed that the system possessed the function of tracking and localizing tumor cells, and animal experiments verified that it could exert the function of disease diagnosis. Conclusions: Our experiments successfully constructed a safe and efficient bispecific-targeted sustained-release drug delivery system for HCC tumor cells. It provides a useful diagnostic and therapeutic scheme for the clinical diagnosis and targeted therapy of HCC. Moreover, it can be used as a potential tumor-specific MRI contrast agent for the localization and diagnosis of malignant tumors.

INTRODUCTION

Primary liver cancers are generally classified as hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and a mixed-tumor type of HCC–ICC. Of the three types, HCC is the most common form of primary liver cancer, accounting for 85–90%, and its 5 year survival rate is 5–30%. Liver cancers are one of the most common forms of malignant tumors. For instance, there are 830,000 deaths associated with liver cancer worldwide, and the mortality rate accounts for 8.3%, thus ranking third among all cancer-related causes of death in the world. Hence, this order seriously threatens human health and safety. Due to the occult onset of HCC, up to 80% of patients were in the advanced stage of unresectable or metastatic disease when they were first diagnosed. In general, patients with advanced HCC have rapid disease progression and poor prognosis, with a 5 year survival rate of only 12.1%, and the median survival time of advanced HCC in China is less than 1 year. There are multiple therapeutic choices for HCC, including targeted...
therapy, transhepatic arterial chemoembolization (TACE), radio-frequency ablation, tumor immunotherapy, and so forth. Great progress has been made in the diagnosis and treatment of HCC. However, due to the high metastatic and recurrence rates, there is still a poor prognosis of HCC. Even in patients with subclinical HCC (<3 cm in diameter), the 5 year recurrence rate was as high as 43.5% after hepatectomy. Lenvatinib (LEN) is an oral, multtargeted tyrosine kinase inhibitor with activity against vascular endothelial growth factor receptor 1–3, fibroblast growth factor receptor 1–4, platelet-derived growth factor receptor α, proto-oncogenes RET and KIT, and so forth. Studies in the past have documented that LEN has potent antitumor effects in various advanced solid tumors such as thyroid cancer, renal cancer, and melanoma. Meanwhile, recent research has found that LEN also exhibits good inhibitory effect on HCC, and its therapeutic effect has been proven to be not inferior to sorafenib. Moreover, it can also be used in patients with sorafenib treatment failure or intolerance. At present, LEN has become the first-line drug for the treatment of advanced HCC. According to the phase III clinical study of LEN in the treatment of HCC patients, the incidence of adverse reactions was 99% after the use of LEN or sorafenib. At present, a novel therapeutic option has been recognized to be the targeted direct delivery of effective drugs to the sites of HCC tumors. The key to the proposed method lies in the requirement for efficient targeted drug-delivery carriers with targeting performance to deliver effective drugs directly and safely to tumor lesions.

With the deepening of research, great concern has been attached to magnetic nanomaterials in view of their unique physical properties, biocompatibility, stability, and so forth. For instance, PEG-modified Fe3O4 nanoparticles can increase the water solubility and biocompatibility of drug delivery materials, so as to reduce the accompanying cytotoxicity, avoid recognition by the reticuloendothelial system, prolong the circulation time of drug-loaded microspheres in vivo, and so forth. Moreover, Fe3O4 nanoparticles can be metabolized in vivo, and the material itself can also be used to treat anemia, for example, Feraheme and other drugs, which can be used in vivo. It has become a hotspot of research to combine the advantages of two materials into composites. Specifically, magnetic nanomaterials can be used for biological imaging. Through encapsulation into nanoliposomes, the magnetic nanoparticles encapsulated in liposomes can still maintain good magnetism. Besides, liposomes can load a large number of drugs. In this regard, the nanocomposites can be applied in the field of magnetically targeted drug delivery and biological imaging, so as to exert the effect of integration of diagnosis and treatment.

**Materials and Instruments.** Human hepatoma cell lines, HUH-7 and Hep3B, and human normal cell lines, HUV-EC, BEAS-2B, and QSG-7701, were obtained from Shanghai Cell Bank of Chinese Academy of Sciences, China; RPMI-1640 culture medium, fetal bovine serum, and trypsin were obtained from Thermo Fisher Scientific (China) Co., Ltd; MLs, DAPI, CK8/18/19-FITC, CD45-PE, and magnetic separation rack were obtained from Huzhou Lieyuan Medical Laboratory Co., Ltd; anti-GPC3 and anti-EpCAM antibodies were obtained from Abcam; the Prussian Blue Iron Stain Kit (Nuclear fast red) was obtained from Beijing Solarbio Science & Technology Co., Ltd; dicytroylophosphatidylethanolamine-polyethylene glycol (DSPE-PEG), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), hexadecyl-quaternized (HQCMC), glycyl hexadecyl dimethylammonium chloride (GHDC), N-hydroxyssucinmide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCL), and agarose were obtained from JuKang (Shanghai) Biotechnology Co., Ltd; cholesterol, hydrochloric acid, and other common reagents were obtained from Sinopharm Chemical Reagent Co., Ltd; and the cell proliferation and toxicity test kit (CCK-8) was obtained from Shanghai Yisheng Biology Co., Ltd.

**Preparation of Drug-Loaded Microspheres.** Fe3O4 (1 mL, 61.06 mg/mL) solution was added into an Ep tube for 5 min magnetic separation. After sucking and discarding the solution, dichloromethane (2 mL) was added for washing, which was then transferred to a 100 mL pear-shaped flask. A mixture was prepared following the addition of LEN (1 mL, 10 mg/mL), DSPE-PEG (10 mg), cholesterol (10 mg), DOPC (250 μL, 10 mg/mL), HQCMC (100 μL, 10 mg/mL), and GHDC (10 mg). The mixture was then subjected to ultrasonic processing for 30 s using an ultrasonic cell crusher, with continuous processing for 6 min after the addition of 12 mL of water. A rotary evaporator was then used to rotate and evaporate at 0.8 MPa and room temperature (20 °C) for 30 min to remove dichloromethane. After that, the resultant solution was transferred to a 15 mL centrifugal tube, with the collection of about LEN-ML aqueous solution, and the concentration of LEN-MLs was 8.713 mg/mL. Similarly, MLs were prepared according to the above protocol, but without the addition of LEN. Subsequently, LEN-ML solution (1 mL) was taken and mixed well with NHS (0.2 mg) and EDC-HCl (0.2 mg), followed by the addition of anti-GPC3 (60 μg) and anti-EpCAM antibodies, respectively. The corresponding solutions were preserved at 4 °C with vortexing for 1 min at every 30 min. After a continued reaction for 12 h,
Characterization and Microstructure Analysis of Drug-Loaded Microspheres. After dilution in distilled water (1 mL), the diluted samples (10 μL) were subjected to the detection of the particle size and the potential of magnetic beads by using a BI-90Plus laser particle sizer/zeta potential meter. Subsequently, the sample (1 mL) was freeze-dried and prepared using the KBr pellet, and the infrared spectrum of the materials was measured by Fourier transform infrared spectroscopy. Another 10 μL of the sample was dissolved in distilled water (1 mL) for recording the UV absorption spectrum using a UV-1800 spectrophotometer. The crystallization properties of the samples were determined and analyzed by X-ray diffraction (XRD). The magnetization curve was measured using a vibrating sample magnetometer at room temperature. Furthermore, 10 μL of samples was diluted in distilled water (1 mL), 50 μL of which was coated on mica and dried naturally. The morphology of drug-loaded microspheres was then observed using an atomic force microscope. Meanwhile, GPC3/EpCAM-LEN-MLs (20 μL) bound to the fixed HUH-7 cells, which was applied on the sample, were also observed using the microscope. After drying, the sample was subjected to gold sputtering and observed under a scanning electron microscope. In addition, 50 μL of GPC3/EpCAM-LEN-MLs diluent was dropped on the copper mesh, and the morphology of drug-loaded microspheres was observed under a transmission electron microscope after drying.

Determination of the Encapsulation Efficiency, Drug-Loading Efficiency, and In Vitro Release. An UV–vis spectrophotometer was used to scan the 0.1 mg/mL LEN drug solution in the full wavelength range of 250–800 nm to determine the optimal LEN ultraviolet detection wavelength. A LEN standard solution of 0.04 mg/mL was prepared and drug-release curve. The drug-loading efficiency is calculated as follows

\[
\text{drug-loading efficiency} = \frac{\text{actual drug mass}}{\text{microsphere mass}} \times 100\%
\]

The encapsulation efficiency is calculated as follows

\[
\text{encapsulation efficiency} = \frac{\text{actual drug mass}}{\text{total drug mass}} \times 100\%
\]

The dialysis bag was kept in distilled water for over 12 h. With phosphate-buffered saline (PBS) (pH 7.4) as the release medium (600 mL), 2 mL of PBS was added to the dialysis bag, which was then placed in the PBS solution for equilibration for 12 h and stirred on a magnetic stirrer (OLAB, 85-2B) at 37 °C (50 r/min). Afterward, 500 μL of GPC3/EpCAM-LEN-MLs was supplemented into the dialysis bag. At regular intervals, the content of LEN generated at the specified time was measured using a UV-1800 UV–vis spectrophotometer, with the release medium supplemented accordingly. Each experiment was repeated three times.

Cellular Uptake Experiment. HUH-7 and QSG-7701 cells were cultured in a 5% CO2 incubator at 37 °C to the logarithmic growth stage and prepared into cell suspension (1 × 10^5 cells/mL). Then, the cells were incubated in six-well Petri dishes for 24 h, followed by the addition of 10 μL each of GPC3/EpCAM-LEN-MLs, GPC3-LEN-MLs, EpCAM-LEN-MLs, LEN-MLs, LEN, and MLs, for the continuous culture in the incubator for another 8 h. After the absorption and the culture medium was discarded, cells were washed with DPBS (pH = 7.4 and pH = 5.3) twice. Thereafter, the Prussian blue iron stain kit (nuclear fast red) was used for cell staining in each group, and subsequently the cells were digested with trypsin, placed on a glass slide, and the specific adsorption effect of the carrier on cells was observed under the microscope.

Tests for Cytotoxicity, Proliferation, and Apoptosis. One hundred microliters (100 μL) of each of HUVEC and BEAS-2B cell suspensions was inoculated in 96-well plates at a density of about 1 × 10^4 cells/well, which were cultured in an incubator containing 5% CO2 at 37 °C for 24 h. After that, 50 μL each of GPC3/EpCAM-LEN-MLs, GPC3-LEN-MLs, EpCAM-LEN-MLs, LEN-MLs, LEN, and MLs, at different concentrations, were added for 24 h of incubation. Subsequently, each well was supplemented with 15 μL CCK8 solution for continuous culture for 2 h. The optical density (OD, absorbance) was measured at a wavelength of 450 nm using a microplate reader to investigate the cytotoxicity of these materials to cells. The cell survival rate was calculated according to the following formula

\[
\text{Cell survival rate} (%) = \frac{OD(\text{untreated}) - OD(\text{blank})}{OD(\text{materials}) - OD(\text{blank})} \times 100\%
\]

Again, 100 μL each of HUH-7 and Hep3B cell suspensions were inoculated in the 96-well plate (about 1 × 10^4 cells/well). After 24 h of culture, 50 μL each of GPC3/EpCAM-LEN-MLs, GPC3-LEN-MLs, EpCAM-LEN-MLs, LEN-MLs, LEN, and MLs were added and incubated for various durations of time. After that, each well was added with 15 μL of CCK8 solution and subsequently cultured for 2 h. The OD value of each well was measured at 450 nm using a microplate reader. The cell inhibition rate was calculated according to the following formula

\[
\text{cell inhibition rate} (%) = 1 - \frac{OD(\text{materials with LEN or control}) - OD(\text{blank})}{OD(\text{untreated}) - OD(\text{blank})} \times 100\%
\]

HUH-7 and Hep3B cells in the logarithmic growth period were counted and inoculated with 1 mL culture medium containing 1 × 10^5 cells in each well of a 24-well plate. After culturing for 12 h to allow cells to adhere to the walls, 30 μL of each of GPC3/EpCAM-LEN-MLs, GPC3-LEN-MLs, EpCAM-LEN-MLs, LEN-MLs, LEN, and MLs was added with further treatment for 24 h. Thereafter, an appropriate volume of trypsin was used to digest the cells, followed by centrifugation at 3000 rpm for 5 min. The supernatant was discarded after washing with PBS for once. Then, 195 μL of Annexin V-FITC binding solution was added for the resuspension of the cells and 5 μL of propidium iodide staining solution was supplemented and mixed evenly. Further incubation was performed at room temperature in the dark for 20 min, then the mixture was placed in an ice bath in the dark by wrapping with an aluminum foil. Apoptotic cells were detected by flow cytometry.
**Material Preparation and Experimental Flow Chart**

![Flow Chart](Image)

**Figure 1.** Preparation of GPC3/EpCAM-LEN-MLs and experimental procedures.

**Experiment for the Detection of Cell Capture Efficiency.** HUH-7 cell suspension was prepared and counted, after which the cells were added into PBS and blood at a concentration of 100 cells/7.5 mL. Subsequently, cells were added with 20 μL each of GPC3/EpCAM-LEN-MLs, GPC3-LEN-MLs, EpCAM-LEN-MLs, and LEN-MLs and incubated for 20 min. Following magnetic separation on a magnetic separation rack, 20 μL of FITC-labeled anti-CK8/18/19 polyclonal antibody (anti-CK8/18/19-FITC), 20 μL of DAPI staining solution, and 20 μL of PE-labeled CD45 antibody (CD45-PE) were added and mixed evenly in the dark for 15 min for further staining. At the end of staining, using 1 mL of ddH2O the samples were washed two times. Finally, 20 μL of ddH2O was added and mixed evenly in the centrifugal tube, after which the mixed solution was applied on the poly-L-lysine-prep slides uniformly. After the droplets were dried naturally, the slides were observed and the cells were counted under a fluorescence microscope. Capture efficiency = (captured cells/added cells) × 100%.

**MRI Analysis.** HUH-7 cells (1.0 × 10⁵) were cultured in a six-well plate for 24 h. Then, 50 μL of GPC3/EpCAM-LEN-MLs and LEN-MLs at varying concentrations (0, 10, 20, 40, 60, 80, and 100 μg/mL) was added for 2 and 5 days of culture, respectively. With the culture medium sucked and discarded,
50 μL of fresh culture medium was added for cell resuspension, after which the solution was transferred into eight-strip polymerase chain reaction tubes for the MRI test. The instrument used for the test was an Intera Archiva 3.0T dual-gradient superconducting magnetic resonance scanner (Philips) using the turbo spin echo sequence of fast spin-echo T2-weighted image. The corresponding parameters included echo time (TE) = 80 ms, repetition time (TR) = 1500 ms, number of signals averaged = 8 times, thickness = 1.8 mm, a field of view of 90 × 40 mm, and an imaging matrix of 360 × 120.

**Functional and Experimental Analyses.** The HUH-7 cell suspension at a density of 5 × 10⁴ cells/μL was prepared for subsequent experiments. Twelve BALB/c nude mice aged 4–6 weeks (purchased from Shanghai Slack Laboratory Animal Co., Ltd; Institutional Animal Ethics Committee number: 2021-0832) were disinfected with the skin disinfectant Anedrian. Afterward, 100 μL of HUH-7 cell suspension was taken using 1 mL syringe and injected into the mediastinal axillary of each nude mouse. After inoculation, the weight, diet, and activity of mice were observed every 2 days. After tumor formation, two nude mice with a similar tumor size were screened every 5 days after administered with an injection of 100 μL each of LEN-MLs and GPC3/EpCAM-Len-MLs through the caudal vein. After 3 h of injection, 500 μL of blood samples was taken from each of the eyeballs of the mice. Subsequently, the mice were killed, the tumor bodies were removed and weighed, and the length (a) and width (b) of the tumors were measured using a Vernier caliper. The tumor size was calculated according to the formula of volume (V) = a × b × c/2 and meanwhile tumors were photographed. Furthermore, the collected blood samples were placed on the magnetic separation rack for 20 min of magnetic separation. After that, 20 μL of each of CK8/18/19-FITC, DAPI staining solution, and CD45-PE were added for staining and left for 15 min in the dark. At the end of staining, the samples were washed two times with 1 mL of ddH₂O and 20 μL of ddH₂O was then added to the centrifugal tube and mixed well. In the final step, the mixed solution was applied to the poly-lysine-prep slides uniformly, and the slides were observed and cells were counted under the fluorescence microscope after allowing for natural drying of the droplets.

**RESULTS**

**Material Preparation and Experimental Flow Chart.** In terms of experimental procedures, Fe₃O₄ particles and LEN were encapsulated in liposomes by DSPE-PEG and other biodegradable materials. MLs were modified by combining anti-GPC3 and anti-EpCAM to jointly construct targeted sustained-release drug-loaded bispic microspheres containing LEN. Meanwhile, the function of GPC3/EpCAM-Len-MLs was detected by cellular and animal experiments (Figure 1).

Characterization test results are displayed in Figure 2. As shown in Figure 2A, the average particle size of GPC3/ EpCAM-Len-MLs was 229.3 ± 6.2 nm (ranging from 110.1 to 412.5 nm), with a polydispersity index of 0.120, showing a small particle size. With a decrease in the particle size and an increase in the specific surface area, there will be an increase in the bioavailability of drug-loaded nanosystem. ⁳⁰ According to the zeta potential distribution results in Figure 2B, GPC3/ EpCAM-Len-MLs were positively charged with an average potential of 27.5 ± 4.3 mV. When charged in a solution, liposomes can exhibit good dispersion in the solution due to the mutual existence of electrostatic repulsion; while particles with low potential or uncharged tend to agglomerate due to the requirement for spontaneous reduction of surface energy. In view of the infrared test (Figure 2C), the infrared absorption vibrational spectrum of GPC3/EpCAM-LENO-MLs, GPC3-Len-MLs, EpCAM-Len-MLs, and MLs showed characteristic absorption peaks at 1112.7, 1652.8, 1714.1, and 2849.55 cm⁻¹, which were attributed to C=O–C in the ester bond, –CO–NH– in the amido bond, –C==O in the ester group, and stretching vibration absorption peak of –CH–, respectively. However, no absorption peak was observed for LEN. Moreover, the growth of the absorption peak of GPC3/EpCAM-Len-MLs was the most obvious, indicating the corresponding successful preparation and that GPC3/EpCAM-Len-MLs contain the above groups. Furthermore, the results of the UV test (Figure 2D) revealed that there were anti-GPC3 and anti-EpCAM absorption peaks at about 270 nm, suggesting the successful preparation of the targeted sustained-release drug-loaded bispic microspheres containing LEN. As evidenced by the test results of XRD in Figure 2E, the XRD peaks appeared at 2θ = 30.1, 35.5, 43.1, 53.7, 57.2°, corresponding to the crystal planes (220, 311, 400, 422, 511, and 440) of cubic-phase Fe₃O₄. In addition, it indicates that the drug-loaded microspheres have a Fe₃O₄ crystal structure, suggesting that the prepared samples have the characteristics of magnetic particles and the potential function of the MRI contrast agent. The VSM test results (Figure 2F) showed that the saturation magnetization of MLs was 50.1 Am²/Kg, and that of LEN-MLs after drug loading decreased to 48.2 Am²/Kg. There was an evident decrease in the saturation magnetization after antibodies were coupled to the surface of the microspheres. It was observed that the saturation magnetization of GPC3/EpCAM-Len-MLs, GPC3-Len-MLs, and EpCAM-Len-MLs was around 30 Am²/Kg, indicating a strong saturation magnetization and no hysteresis, however. It confirms that the prepared drug-loaded microspheres have good superparamagnetism.

Observation under AFM (Figure 3A) showed that EpCAM-MLs, GPC3-Len-MLs, and GPC3/EpCAM-Len-MLs were all irregularly spherical-shaped, without agglomeration, and had the characteristics of liposome vesicles. Further observation under SEM (Figure 3B) revealed that a large number of drug-loaded microspheres were adsorbed and aggregated on the cell surface, with the corresponding particle size ranging between 100 and 400 nm. It was consistent with the particle size test results of drug-loaded microspheres. The imaging results obtained using the transmission electron microscope (Figure 3C) indicated that the drug-loaded microspheres were spherical with a diameter of about 200 nm.

**Determination of the Encapsulation Efficiency, Drug-Loading Efficiency, and In Vitro Release.** The UV scanning curve of LEN shows that there are absorption peaks at 276, 481, and 554 nm, but there may be interference from drug excipients at 250 nm. Therefore, the optimum UV detection wavelength was selected to be 481 nm with a higher peak (Figure 4A). The prepared concentrations of LEN-MLs, GPC3-Len-MLs, EpCAM-Len-MLs, and GPC3/ EpCAM-Len-MLs were 8.713, 9.233, 9.233, and 9.233 mg/mL, respectively. Taking the drug concentration as the abscissa (unit: μg/mL) and the OD value as the ordinate, the standard curve equation is as follows: y = 0.1163 × −0.1099, with a good linear relationship (R² = 0.9992) (Figure 4B). The free
drug concentration of LEN was 74.36 μg/mL in GPC3/EpCAM-LEN-ML solution. In other words, the drug concentration of LEN encapsulated by GPC3/EpCAM-LEN-MLs was 758.94 μg/mL. According to the formula, the encapsulation efficiency was 91.08% ± 1.83%, and the drug-loading efficiency was 8.22% ± 1.24% (Figure 4C,D). Furthermore, according to the in vitro release results in Figure 4E,F, when pH = 7.4, the drug-release rate of LEN reached 85% within 24 h and gradually stabilized after 24 h, indicating a fast release rate of naked drugs. Meanwhile, the release rate of GPC3/EpCAM-LEN-MLs, GPC3-LEN-MLs, EpCAM-LEN-MLs, and LEN-MLs was about 30% within 24 h; besides, with the extension of time, there was a slow increase in the release rate gradually, and finally about 70% of the drugs were released. When pH = 5.3, the drug-release rate was accelerated.

In addition, there was a significant prolongation in the drug-release time of LEN encapsulated by microspheres, indicating that the prepared drug-loaded microspheres have a sustained-release effect.

### Cellular Uptake

According to the results of the Prussian blue staining (Figure 4G), there were free microspheres labeled with blue around the HUH-7 and QSG-7701 cells in Cell + MLs and Cell + LEN-MLs groups; while a large number of blue-labeled microspheres were observed on the HUH-7 cell surface in Cell + GPC3-LEN-MLs, Cell + EpCAM-LEN-MLs, and Cell + GPC3/EpCAM-LEN-MLs groups, with only a small amount of free microspheres; while blue-labeled microspheres were not observed on the QSG-7701 cell surface in Cell + GPC3-LEN-MLs, Cell + EpCAM-LEN-MLs, and Cell + GPC3/EpCAM-LEN-MLs groups, there are a large number of free microspheres. It suggests that most of the drug-loaded microspheres in the targeted drug-loaded microspheres groups can specifically target and recognize HUH-7 cells, resulting in the enrichments on the cell surfaces. These results support the fact that the antibodies in the targeted drug-loaded microspheres can specifically bind to the receptors on the surfaces of HUH-7 cells, with the implications that they can specifically target and recognize HUH-7 cells.

### Results of Cytotoxicity, Proliferation, and Apoptosis Tests

The biosafety of carrier materials is of great significance in a drug delivery system. Figure 5A,B shows the results of the toxicity tests of drug-loaded microspheres on HUVEC and BEAS-2B cells. It was found that the cell survival rates decreased with an increase in the concentrations of drug-loaded microspheres. The safe concentration of the drug-loaded microspheres was 100 μg/mL because the cell survival rate at this concentration was over 85%; in fact, the cell viability in the ML group was over 95% with low cytotoxicity. Figure 5C shows the proliferation inhibition results of drug-loaded microspheres on HUH-7 and Hep3B cells. GPC3/EpCAM-LEN-MLs had a high inhibition rate of proliferation on HUH-7 and Hep3B cells, while LEN-MLs showed the lowest inhibition rate on proliferation. As for its potential reason, GPC3/EpCAM-LEN-MLs have a better targeting effect and can effectively identify HUH-7 and Hep3B cells and enrich on the cell surface; whereas when LEN is encapsulated by the carrier, it has no specific targeting or recognition function, and the drug will be released slowly, resulting in the minimum inhibition rate of LEN-MLs on the proliferation of HUH-7 and Hep3B cells. MLs have no effect on the proliferation of cells because they are not loaded with drugs. The effects of drug-loaded microspheres on the apoptosis of HUH-7 and Hep3B cells can be observed in Figure 5E,F. The results revealed that GPC3/EpCAM-LEN-MLs could significantly increase the apoptosis of HUH-7 and Hep3B cells. According to the aforementioned results, GPC3/EpCAM-LEN-MLs can specifically target, recognize, and inhibit HCC cells.

### Cell Capture Efficiency and MRI Analysis

In the PBS system (Figure 6A), the average capture efficiency of GPC3/EpCAM-LEN-MLs was 93.02% ± 2.08% for HUH-7 cells, which was significantly higher than that of GPC3-LEN-MLs, EpCAM-LEN-MLs, and LEN-MLs. It suggested that GPC3/EpCAM-LEN-MLs had a good targeting and recognition functions on tumor cells. Furthermore, in the blood simulation system (Figure 6B), the average capture efficiency of GPC3/EpCAM-LEN-MLs reached 90.20% ± 1.51% for HUH-7 cells, also much higher than that of GPC3-LEN-MLs, EpCAM-LEN-MLs, and LEN-MLs, exhibiting the relatively good specificity of GPC3/EpCAM-LEN-MLs. According to the results of the MRI analysis in Figure 6C, obvious MRI signals were observed following coculture of GPC3/EpCAM-LEN-MLs, LEN-MLs, and HUH-7 cells for 2 and 5 days, respectively. The targeted binding of the GPC3/EpCAM-LEN-ML group to tumor cells resulted in a weakened MRI signal. Therefore, the MRI signal of the GPC3/EpCAM-LEN-ML group was lower than that of the LEN-ML group; and these signals enhanced gradually with the increase in the concentrations of GPC3/EpCAM-LEN-MLs, suggesting a protective effect of the microspheres on magnetic particles. The established system could still be used as the MRI contrast agent after coculturing for 5 days.
was no obvious change in the magnetic response function and characteristics of the drug-loaded microspheres. Simultaneously, it could delay the absorption of drug-loaded microspheres by cells.

**Functional and Experimental Analyses.** The change in the tumor volume in nude mice is shown in Figure 6D. The tumor volumes increased in a time-dependent manner. Figure 6E shows the immunofluorescence staining of tumor cells isolated from the blood of nude mice. As shown in the figure, anti-CK8/18/19-FITC bispecific antibodies recognize tumor cell surface antigens and the labeled cell membrane (green), DAPI label the nucleus (blue), and CD45-PE recognize leukocyte surface antigens. Therefore, it can identify tumor cells and tumor cell counting. The relationship between the cell count and the tumor volume is shown in Figure 6F. The relationship between the cell count and the tumor weight is shown in Figure 6G. It was found that GPC3/EpCAM-LEN-ML group had an increased tumor cell count in the blood along with the enlargement of the tumor volume and weight in nude mice, and only a few nonspecifically adsorbed tumor cells were detected in the LEN-ML group. It shows that GPC3/EpCAM-LEN-MLs have specific target recognition function.
and can specifically recognize and capture tumor cells in the blood. These results support the fact that there can be a gradual increase in the tumor cell count from the blood of nude mice with the increase in the tumor volume and weight, and GPC3/EpCAM-LEN-MLs can exert the function of effectively recognizing tumor cells in vivo. Therefore, GPC3/EpCAM-LEN-MLs may have potential clinical applications/values in the field of HCC diagnosis and treatment.

**DISCUSSION**

HCC is the major type of primary malignant tumor of the liver, showing preponderantly high incidence and mortality rates. At present, chemotherapy is one of the most effective therapeutic options for the treatment of HCC. However, the majority of chemotherapeutic agents used for liver cancer have the shortcomings of the lack of targets, multidrug resistance, and low bioavailability, which therefore restrict their clinical applications to a considerable extent. In this regard, it has been a hotspot of research in the medical field concerning the development of a novel drug delivery system that targetedly delivers effective drugs directly to tumor sites.17–21 Studies in the past have documented that drug-loaded microspheres are an ideal drug carrier, with the advantages of higher drug loading, slow, but continuous drug release, better therapeutic effects with lower toxicity and side effects, and so forth.21–23 For instance, Ma et al.24 prepared norcantharidin-loaded lipid microspheres with a measured drug encapsulation efficiency of >80%. Sharma et al.25 loaded polyvinyl alcohol-hydrogel microspheres with Lipiodol, prepared radiopaque particles, and found that the prepared particles were able to be imaged with conventional intraprocedural fluoroscopy and computed tomography during TACE. Meanwhile, as proposed by Hagan et al.,26 Vandetanib had suitable characteristics for intra-arterial delivery and site-specific sustained drug release into liver tumors. Liu et al.27 prepared a drug-loaded contrast agent liposome with dual controlled release ability, which is highly cytotoxic at high concentrations, and the safe use concentration is 1.25 mM, which has obvious inhibitory effect on tumor cells. Furthermore, Song et al.28 constructed an EpCAM-targeted long-circulating drug delivery system with an average capture efficiency of 84.2% for SK-BR-3 cells. In our study, it was found that GPC3/EpCAM-LEN-MLs had high drug-loading and low side effects, with the safe use concentration being 100 μg/mL. In addition, GPC3/EpCAM-LEN-MLs had a high inhibition rate of the proliferation of HCC cells and could significantly increase the apoptosis of the cells. GPC3/EpCAM-LEN-MLs possess the function of slow drug release. Through encapsulation of drugs in microspheres, the drug-release time was significantly prolonged, the release rate was about 30% within 24 h, and the drug release lasted for more than 5 days, with the average capture efficiency of HUH-7 being 90.20%. Simultaneously, experimental in vivo and in vitro data indicated that GPC3/EpCAM-LEN-MLs could specifically target and recognize HCC cells, effectively aggregate drugs on the surface of tumor...
cells, and ensure a slow release. Eventually, it can achieve a better targeted therapeutic effect.

Surgical resection is one of the most effective therapeutic approaches for liver cancers. However, it is practicable in only 10−15% of patients when diagnosed because most patients are progressed into locally advanced stage or have distant metastasis. Encouragingly, the 5 year survival rate after radical surgery can reach >70% in liver cancer patients with tumors of diameter <1 cm and no lymph node metastasis or local infiltrations. Therefore, early and timely diagnosis of liver cancer is of great significance for improving the success rate of operations and significantly increasing the survival rates of patients. Furthermore, for patients with obvious clinical symptoms, noninvasive imaging is the most convenient and effective choice for diagnosing liver cancer. Among various available clinical imaging methods, MRI is becoming one of the most important imaging techniques for the screening, diagnosis, and treatment evaluation of liver cancer in the clinical setting. MRI is a comprehensive imaging technique that can be used without ionizing radiation. It can be used for quantitative analysis of morphological and functional imaging based on its advantages of high resolution for soft tissues, multiparameters, and multisequence imaging. MRI is sensitive and accurate in the diagnosis of typical HCC with a tumor diameter >1 cm. Nevertheless, it remains a challenge for MRI to identify benign and malignant liver nodules less than 1 cm in diameter, which is mainly attributed to low tumor contrast or lack of specificity of MRI contrast agents. In a prior
research conducted by Lee et al., iron oxide-containing embospheres were prepared, which could be detected by a dedicated MRI when injected intra-arterially in an animal model of liver cancer. Ma et al. prepared a bispecific MRI molecular probe for liver cancer and found in vitro that the bispecific probe had higher targeting efficiency and sensitivity of MRI to HCC cells than single-targeted or nontargeted molecular probe. Sun et al. prepared a tumor-targeted MRI and drug-loaded nanoparticles, with different concentrations studied using MR tubes, providing intuitional views by distinguishing the brightness (T1) and darkness (T2) of the images. With an increasing concentrations of PYFGN, T1- and T2-weighted MR images gradually brightened and darkened, respectively.

Our study prepared and utilized the dual-antigen-specific targeted drug-loaded microspheres with higher complexity, which could accurately identify the molecular characteristics of HCC cells and further improve the accuracy of diagnosis and imaging quality of HCC lesions. Our experiment coupled anti-GPC3 and anti-EpCAM antibodies to drug-loaded microspheres containing drugs and superparamagnetic iron oxide nanoparticles. Further in vivo and in vitro experiments were carried out to explore the binding properties of dual-antibody-coupled drug-loaded microspheres to cells, so as to examine the antigen-targeting capability and the potential as the MRI contrast agent for HCC. The corresponding results revealed that GPC3/EpCAM-LEN-MLs could efficiently recognize tumor cells in vivo and had a cell capture efficiency of 90.20% in the blood simulation systems, indicating high specificity. Meanwhile, obvious MRI signals were observed following the coculturing of GPC3/EpCAM-LEN-MLs and HCC cells for 2 and 5 days, respectively; suggesting a protective effect of the microspheres on magnetic particles. The established system could still be used as the MRI contrast agent even after coculturing for 5 days. No obvious change was noticed in the magnetic response function and characteristics of drug-loaded microspheres, accompanied by a delayed absorption of drug-loaded microspheres by cells. Collectively, GPC3/EpCAM-LEN-MLs can be used as a potential tumor-specific MRI contrast agent for the localization and diagnosis of malignant tumors. On this basis, the established delivery system can overcome the problem of tumor heterogeneity and improve the sensitivity for the early clinical diagnosis of HCC. Significantly, the present study provides preliminary research evidence for promoting in vivo or clinical studies in the future. Future experimental studies are expected to be performed to assist further validation in multiple cell lines, including an improvement in drug-loading efficiency and bioavailability.

CONCLUSIONS

In our study, a long-circulating drug delivery system containing LEN for HCC cells is constructed jointly based on the modification of MLs by combining anti-GPC3 and anti-EpCAM. GPC3/EpCAM-LEN-MLs possess the advantages of long circulation, targeting, biocompatibility, and strong inhibitory effects on the HCC cells, which can also track tumor cells within 5 days as indicated by MRI. In view of these findings in our study, the established system may provide a useful diagnosis and treatment scheme for the clinical diagnosis and targeted therapy of HCC. GPC3/EpCAM-LEN-MLs may also have a potential role as a tumor-specific MRI contrast agent in the localization and diagnosis of malignant tumors. Thus, we believe this multifunctional nanoplatform could be a potential nanotheranostic in the future for accurate diagnosis and targeted therapy for HCC.

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H.Z.L., X.Y.H., and H.X.Y. designed and coordinated the study; H.Z.L., L.F., Z.J.T. and S.X.J. performed the experiments, acquired and analyzed data, and contributed to the revised manuscript; H.Z.L., H.X.Y., and X.Y.H. interpreted the data; H.Z.L., H.X.Y., and X.Y.H. wrote the manuscript; all authors approved the final version of the article.

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Notes

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REFERENCES

(1) Miranda-Filho, A.; Bray, F.; Charvat, H.; Rajaraman, S.; Soerjomataram, I. The world cancer patient population (WCPP): an updated standard for international comparisons of population-based survival. Cancer Epidemiol. 2020, 69, 101802–101805.

(2) Allemani, C.; Matsuda, T.; Di Carlo, V.; Harewood, R.; Matz, M.; Nikšić, M.; Bonaventure, A.; Valkov, M.; Johnson, C.J.; Esteve, J.; et al. Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet 2018, 391, 1023–1075.
carcinoma, endometrial cancer, and other selected advanced solid cancers in 185 countries.

Lancet 2015, 385, 977–1010.

Global surveillance of cancer survival 1995–2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2).

Wang, X.-S.; Bannon, F.; Ahn, J. V.; Johnson, C. J.; Bonaventure, A.; et al. Global surveillance of cancer survival 1995–2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2).

Lancet 2015, 385, 977–1010.

Taylor, M. H.; Lee, C.-H.; Makker, V.; Rascio, D.; Dutcus, C. E.; Wu, J.; Stepian, D. E.; Shumaker, R. C.; Motzer, R. J. Phase IB/II trial of lenvatinib plus pembrolizumab in patients with advanced renal cell carcinoma, endometrial cancer, and other selected advanced solid tumors.

J. Clin. Oncol. 2020, 38, 1154–1163.

Kudo, M.; Finn, R. S.; Qin, S.; Han, K.-H.; Ikeda, K.; Piscaglia, F.; Baron, A.; Park, J.-W.; Han, G.; Jassem, J.; et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial.

Lancet 2018, 391, 1163–1173.

Hiraoka, A.; Kumada, T.; Kariyama, K.; Takaguchi, K.; Atsukawa, M.; Itobayashi, E.; Tsuji, K.; Tajiri, K.; Hirooka, M.; Shimada, N.; et al. Clinical features of lenvatinib for unresectable hepatocellular carcinoma in real-world conditions: multicenter analysis.

Cancer Med. 2018, 8, 137–146.

Zhong, T.; Lip, H.; He, C.; Cai, P.; Wang, Z.; Henderson, J. T.; Rauth, A. M.; Wu, X. Y. Multitargeted Nanoparticles Deliver Synergistic Drugs across the Blood-Brain Barrier to Brain Metastases of Triple Negative Breast Cancer Cells and Tumor-Associated Macrophages.

Adv. Healthcare Mater. 2019, 8, 1900543.

Chen, Y.; Li, X.; Xiao, H.; Xiao, J.; Li, B.; Chen, X.; Wang, Y.; Cheng, D.; Shuai, X. Reduction and pH dual-sensitive nanovesicles co-delivering doxorubicin and gefitinib for effective tumor therapy.

RSC Adv. 2018, 8, 2082–2091.

Yao, Y.; Saw, P. E.; Nie, Y.; Wong, P.-F.; Jiang, L.; Ye, X.; Chen, J.; Ding, T.; Xu, L.; Yao, H.; et al. Multifunctional supramolecular micelles for targeted drug delivery and effective breast cancer therapy.

J. Mater. Chem. B 2019, 7, 576–585.

Yu, L.; Wang, Z.; Mo, Z.; Zou, B.; Yang, Y.; Sun, R.; Ma, W.; Yu, M.; Zhang, S.; Yu, Z. Synergetic delivery of triptolide and Cel6 with light-activatable liposomes for efficient hepatocellular carcinoma therapy.

Acta Pharm. Sin. B 2021, 11, 2004–2015.

He, M.; Yu, L.; Yang, Y.; Zou, B.; Ma, W.; Yu, M.; Lu, J.; Xiong, G.; Yu, Z.; Li, A. Delivery of triptolide with reduction-sensitive polymer nanoparticles for liver cancer therapy on patient-derived xenografts models.

Chin. Chem. Lett. 2020, 31, 3178–3182.

Yang, Y.; Yu, Y.; Chen, H.; Meng, X.; Ma, W.; Yu, M.; Li, Z.; Li, C.; Liu, H.; Zhang, X.; et al. Illuminating Platinum Transportation while Maximizing Therapeutic Efficacy by Gold Nanoclusters via Simultaneous Near-Infrared-II Imaging and Glutathione Scavenging.

ACS Nano 2020, 14, 13536–13547.

Mahmoud, N. N.; Sabbah, D. A.; Abu-Dahab, R.; Abuarqoub, D.; Abdallah, M.; Khalil, A.; Khalil, E. A. Cholesterol-coated gold nanorods as an efficient nano-carrier for chemotherapeutic delivery and potential treatment of breast cancer: in vitro studies using the MCF-7 cell line.

RSC Adv. 2019, 9, 12718–12731.

Wang, X.; Deng, A.; Cao, W.; Li, Q.; Wang, L.; Zhou, J.; Hu, B.; Xing, X. Synthesis of chitosan/poly (ethylene glycol)-modified magnetic nanoparticles for antibiotic delivery and their enhanced anti-biofilm activity in the presence of magnetic field.

J. Mater. Sci. 2018, 53, 6433–6449.

Sharma, A.; Cornejo, C.; Mihalic, J.; Geyh, A.; Bordelon, D. E.; Korangath, P.; Westphal, F.; Gruettner, C.; Ivkov, R. Physical characterization and in vivo organ distribution of coated iron oxide nanoparticles.

Sci. Rep. 2018, 8, 4916.
(34) Dozie-Nwachukwu, S. O.; Danyuo, Y.; Obayemi, J. D.; Odusanya, O. S.; Malatesta, K.; Soboyejo, W. O. Extraction and encapsulation of prodigiosin in chitosan microspheres for targeted drug delivery. *Mater. Sci. Eng., C* 2017, 71, 268–278.

(35) Ma, J.; Teng, H.; Wang, J.; Zhang, Y.; Ren, T.; Tang, X.; Cai, C. A highly stable norcantharidin loaded lipid microspheres: Preparation, biodistribution and targeting evaluation. *Int. J. Pharm.* 2014, 473, 475–484.

(36) Sharma, K. V.; Dreher, M. R.; Tang, Y.; Pritchard, W.; Chiesa, O. A.; Karanian, J.; Pereygo, J.; Orandi, B.; Woods, D.; Donahue, D.; et al. Development of "imageable" beads for transcatheter embolotherapy. *J. Vasc. Interv. Radiol.* 2010, 21, 865–876.

(37) Hagan, A.; Phillips, G. J.; Macfarlane, W. M.; Lloyd, A. W.; Crzuczman, P.; Lewis, A. L. Preparation and characterisation of vandetanib-eluting radiopaque beads for locoregional treatment of hepatic malignacies. *Eur. J. Pharm. Sci.* 2017, 101, 22–30.

(38) Liu, C.; Ewert, K. K.; Wang, N.; Li, Y.; Safinya, C. R.; Qiao, W. A multifunctional lipid that forms contrast-agent liposomes with dual-control release capabilities for precise MRI-guided drug delivery. *Biomaterials* 2019, 221, 119412.

(39) Song, C.; Gao, C.; Zhao, J.; Wang, Z. Construction of long-circulation EpCAM targeted drug delivery system and its application in the diagnosis and treatment of breast cancer. *J. Biomater. Appl.* 2021, 35, 947.

(40) Wang, M.; Wei, C.; Shi, Z.; Zhu, J. Study on the diagnosis of small hepatocellular carcinoma caused by hepatitis B cirrhosis via multislice spiral CT and MRI. *Oncol. Lett.* 2018, 15, 503–508.

(41) Aziz-Tiwari, H.; Kalb, B.; Chundru, S.; Sharma, P.; Costello, J.; Gussner, R. W.; Martin, D. R. MRI of hepatocellular carcinoma: an update of current practices. *Diagn. Interv. Radiol.* 2014, 20, 209–221.

(42) Elsayes, K. M.; Hooker, J. C.; Agrons, M. M.; Kielar, A. Z.; Tang, A.; Fowler, K. J.; Chernyak, V.; Bashir, M. R.; Kono, Y.; Do, R. K.; Mitchell, D. G.; Kamaya, A.; Hecht, E. M.; Sirlin, C. B. 2017 version of LI-RADS for CT and MR imaging: an update. *Radiographics* 2017, 37, 1994–2017.

(43) Lee, K.-H.; Liapi, E.; Vossen, J. A.; Buijs, M.; Ventura, V. P.; Georgiadis, C.; Hong, K.; Kamel, I.; Torbenson, M. S.; Geschwind, J.-F. H. Distribution of Iron Oxide-containing Embosphere Particles after Transcatheter Arterial Embolization in an Animal Model of Liver Cancer: Evaluation with MR Imaging and Implication for Therapy. *J. Vasc. Interv. Radiol.* 2008, 19, 1490–1496.

(44) Ma, X.-H.; Wang, S.; Liu, S.-Y.; Chen, K.; Wu, Z.-Y.; Li, D.-F.; Mi, Y.-T.; Hu, L.-B.; Chen, Z.-W.; Zhao, X.-M. Development and in vitro study of a bi-specific magnetic resonance imaging molecular probe for hepatocellular carcinoma. *World J. Gastroenterol.* 2019, 25, 3030–3043.

(45) Sun, X.; Du, R.; Zhang, L.; Zhang, G.; Zheng, X.; Qian, J.; Tian, X.; Zhou, J.; He, J.; Wang, Y.; Wu, Y.; Zhong, K.; Cai, D.; Zou, D.; Wu, Z. A pH-responsive yolk-like nanoplatform for tumor targeted dual-mode magnetic resonance imaging and chemotherapy. *ACS Nano* 2017, 11, 7049–7059.