

# Preparation of human hepatocellular carcinoma-targeted liposome microbubbles and their immunological properties

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## Abstract

**AIM:** To prepare the human hepatocellular carcinoma (HCC)-targeted liposome microbubbles and to investigate their immunological properties.

**METHODS:** Human hepatocarcinoma specific monoclonal antibody HAb18 was attached to the surface of home-made liposome microbubbles by static attraction to prepare the targeted liposome microbubbles. The combination of HAb18 with liposome microbubbles was confirmed by the slide agglutination test and immunofluorescent assay. Their immunological activity was measured by ELISA. Rosette formation test, rosette formation blocking test and immunofluorescent assay were used to identify the specific binding of targeted liposome microbubbles to SMMC-7721 hepatoma cells, and cytotoxicity assay was used to detect their effect on human hepatocytes.

**RESULTS:** The targeted liposome microbubbles were positive in the slide agglutination test and immunofluorescent assay. ELISA indicated that the immunological activity of HAb18 on the liposome microbubbles was similar to that of free HAb18. SMMC-7721 cells were surrounded by the targeting liposome microbubbles to form rosettes, while the control SGC-7901 gastric cancer cells were not. Proliferation of SMMC-7721 cells and normal human hepatocytes was not influenced by the targeted liposome microbubbles.

**CONCLUSION:** The targeted liposome microbubbles with a high specific biological activity have been successfully prepared, which specifically bind to human hepatocarcinoma cells, and are non-cytotoxic to hepatocytes. These results indicate that the liposome microbubbles can be used as a HCC-targeted ultrasound contrast agent that may enhance ultrasound images and thus improve the diagnosis of HCC, especially at the early stage.

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## INTRODUCTION

Targeted ultrasound imaging is a promising method of imaging diagnosis<sup>[1]</sup>. This technique can significantly improve the

sensitivity and specificity of ultrasonic diagnosis. At present, several targeted ultrasound contrast agents that can enhance imaging of specific tissues, such as thrombus-specific and inflammatory tissue-specific targeted ultrasound contrast agent, have successfully been developed abroad<sup>[2-15]</sup>, while study on hepatocellular carcinoma-targeted ultrasound contrast agent has not been reported.

In this study, liposome microbubbles containing fluorocarbon gases were prepared in our laboratory. Then human hepatocarcinoma specific monoclonal antibody HAb18 was attached to the surface of home-made liposome microbubbles to prepare targeted liposome microbubbles. Finally the immunological properties of targeted liposome microbubbles were investigated.

## MATERIALS AND METHODS

### Materials

Human hepatocarcinoma specific monoclonal antibody HAb18 was kindly provided by Research Centre of Cell Engineering, the Fourth Military Medical University. Rabbit anti-mouse serum was purchased from Beijing Zhongshan Biotechnology Co., Ltd, China. FITC conjugated sheep anti-mouse IgG and HRP conjugated sheep anti-mouse IgG were purchased from Huamei Bioengineering Company, China. MTT was purchased from Sigma. Human hepatoma cell line SMMC-7721 and human gastric carcinoma cell line SGC-7901 were maintained in our laboratory. Primary cultures of normal human hepatocytes were established from human liver tissue collected from person who died in accident.

### Preparation of liposome microbubbles

The lipid film prepared previously in our laboratory and some mediators were mixed in proportion<sup>[16]</sup>. Then the resulting mixture was sonicated, into which perfluoropropane gas was injected. The concentration and size of the microbubbles were measured under microscope. Zeta potential of the microbubbles was determined by the Chongqing Comed Nanopharma Co., Ltd, China.

### Preparation of targeted liposome microbubbles

Human hepatocarcinoma specific monoclonal antibody HAb18 was added to the liposome microbubble suspension in proportion and mixed for 2 h at pH4.0, 4 °C<sup>[17]</sup>. After the mixture was separated into 2 distinct layers, the lower layer was discarded and the upper layer was washed three times with phosphate-buffered saline (PBS) to elute the free HAb18.

### Identification of targeted liposome microbubbles

**Slide agglutination test** A drop of rabbit anti-mouse serum or normal saline was mixed with a drop of targeted liposome microbubbles or liposome microbubbles respectively for about 5 min. The results were observed under a ×100 field of microscope.

**Immunofluorescent assay** A 100 μL of FITC conjugated sheep anti-mouse IgG was put into 200 μL of targeted liposome microbubbles and mixed for 30 min at 4 °C. After the mixture was separated into 2 distinct layers, the lower layer was discarded and the upper layer was washed three times with PBS to elute the free FITC conjugated sheep anti-mouse IgG. Then the microbubbles were observed under fluorescence microscope.

### Assessment of immunological activity of targeted liposome microbubbles

SMMC-7721 cells ( $1 \times 10^5$ ) were placed in each well of a 96-well ELISA plate and cultured overnight. The cells were fixed in 0.25 g/L glutaraldehyde for 20 min. A 30 g/L of defatted milk powder was added and incubated for 1 h at 37 °C. Different concentrations of targeted liposome microbubbles or free HAb18 were added and incubated for 1.5 h at 37 °C. HRP conjugated sheep anti-mouse IgG was added and incubated for 1.5 h at 37 °C. O-phenylenediamine (OPD) and  $H_2O_2$  were added and incubated for 10 min at 25 °C. A 2 mmol/L of  $H_2SO_4$  was added to terminate the reaction. The optical absorbance (A) value at 490 nm was detected with ELISA.

### Specific binding of targeted liposome microbubbles to SMMC-7721 cells

Rosette formation test<sup>[18]</sup> Log phase SMMC-7721 cells and SGC-7901 cells were digested with 2.5 g/L trypsin. The isolated cells were adjusted to a concentration of  $2 \times 10^5$ /mL in PBS respectively. One hundred  $\mu$ L of targeted liposome microbubbles or liposome microbubbles was added to 200  $\mu$ L of SMMC-7721 or SGC-7901 cell suspension and mixed for 30 min at 25 °C. The resulting mixture was observed under microscope and the cells that formed rosette were counted.

Rosette formation blocking test SMMC-7721 cells were mixed with different concentrations of HAb18 for 30 min at 25 °C. The mixture was centrifuged and washed with PBS to elute free HAb18. Then targeted liposome microbubbles were added to cell suspension and mixed for 30 min at 25 °C. Cells forming rosette were counted under microscope.

Immunofluorescent assay one hundred  $\mu$ L of targeted liposome microbubbles combined with FITC conjugated sheep anti-mouse IgG and 200  $\mu$ L of SMMC-7721 cells or SGC-7901 cells were mixed for 30 min at 25 °C. The suspension was observed under fluorescence microscope.

### Cytotoxicity assay of targeted liposome microbubbles

SMMC-7721 cells or human hepatocytes ( $1 \times 10^5$  per well) were added into a 96-well culture plate. About three hours later different concentrations of targeted liposome microbubbles were added, while PBS was used as control. Three wells were used for each concentration. They were cultured in a  $CO_2$  incubator at 37 °C for 48 h, then MTT (2 g/L, 50  $\mu$ L/well) was added and incubated at 37 °C for 6 h. The medium was discarded, and 100  $\mu$ L/well DMSO was added. The culture plate was shaken for 10 min and the optical absorbance (A) value at 570 nm was detected with ELISA.

## RESULTS

### Preparation of liposome microbubbles

The average diameter of liposome microbubbles ranged from 2 to 5  $\mu$ m, the concentration was  $7 \times 10^9$  bubbles/mL (Figure 1). The mean zeta potential was -71.2 mV. Animal experiment demonstrated that the microbubbles could significantly enhance the ultrasound images of several tissues.



**Figure 1** Light micrograph of liposome microbubbles under a  $\times 200$  field of microscope.

### Preparation and identification of targeted liposome microbubbles

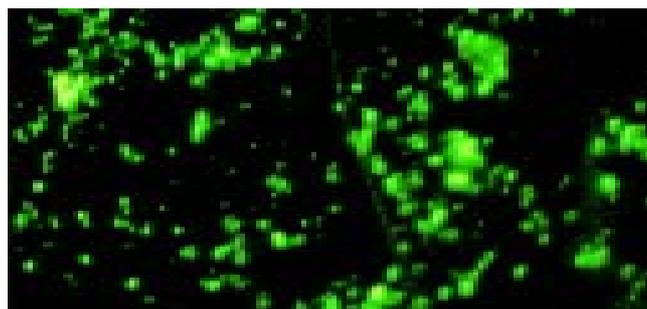
The average diameter of targeted liposome microbubbles was similar to that of liposome microbubbles. HAb18 did not affect the size of microbubbles. The concentration was  $5 \times 10^8$  bubbles/mL.

After rabbit anti-mouse serum was added, targeted liposome microbubbles agglutinated (Figure 2), but liposome microbubbles did not. While normal saline (NS) did not lead to agglutination of two kinds of microbubbles.

The surfaces of targeted liposome microbubbles gave out bright yellow-green fluorescence after they were stained with fluorescent agents (Figure 3), whereas liposome microbubbles did not give out any fluorescence.



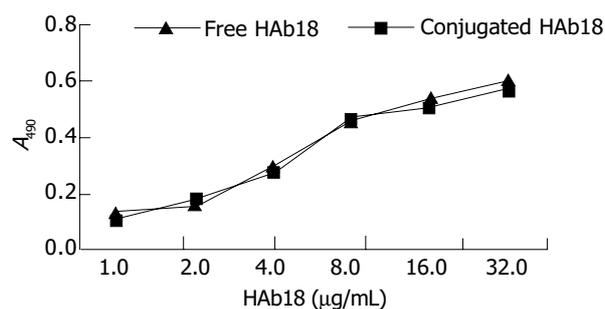
**Figure 2** Direct agglutination of targeted liposome microbubbles in the presence of rabbit anti-mouse serum.



**Figure 3** Immunofluorescence staining of targeted liposome microbubbles with FITC conjugated sheep anti-mouse IgG. Targeted liposome microbubbles were stained in yellow-green under a  $\times 400$  field of fluorescence microscope.

### Assessment of immunological activity of targeted liposome microbubbles

ELISA indicated that the immunological activity of HAb18 on liposome microbubbles was similar to that of free HAb18. There were no significant differences in optical absorbance (A) value between free HAb18 ground and conjugated HAb18 ground (Figure 4).



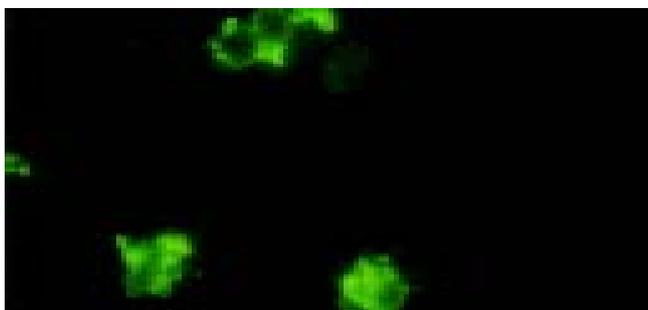
**Figure 4** Activity assay of free HAb18 and conjugated HAb18 on microbubbles by ELISA.

### Specific binding of targeted liposome microbubbles to SMMC-7721 cells

After targeted liposome microbubbles and SMMC-7721 cells or SGC-7901 cells were mixed for 30 min, SMMC-7721 cells were surrounded by targeted liposome microbubbles to form rosettes and the rate of rosettes reached 90% (Figure 5), while SGC-7901 cells were negative. Rosettes were not observed in the mixture of liposome microbubbles and SMMC-7721 cells under microscope. After SMMC-7721 cells were pretreated with different concentrations of HAb18, the rate of rosettes declined significantly, even dropped to 0. Immunofluorescent assay indicated that SMMC-7721 cells were surrounded by the microbubbles and gave out bright yellow-green fluorescence to form rosettes (Figure 6), whereas control cells were negative.



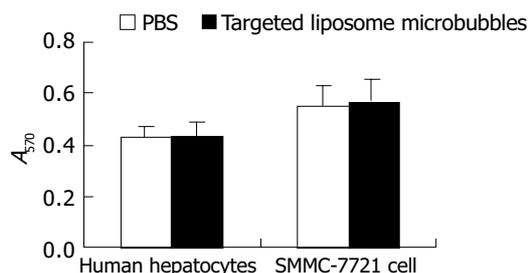
**Figure 5** Light micrograph of the binding of targeted liposome microbubbles to SMMC-7721 cells under a  $\times 400$  field of microscope. SMMC-7721 cells were surrounded by targeted liposome microbubbles to form rosettes.



**Figure 6** Immunofluorescent assay for identification of the binding of targeted liposome microbubbles to SMMC-7721 cells. SMMC-7721 cells were surrounded by the microbubbles and gave out bright yellow-green fluorescence.

### Cytotoxicity assay of targeted liposome microbubbles

The result of MTT assay showed that the proliferation of human hepatocytes and SMMC-7721 cells were not influenced by targeted liposome microbubbles. There were no significant differences in average optical absorbance ( $A_{570}$ ) value between experiment group and control group (Figure 7).



**Figure 7** Effect of targeted liposome microbubbles on the proliferation of human hepatocytes and SMMC-7721 cells.

### DISCUSSION

It has been nearly thirty years since the conception of ultrasound contrast imaging was suggested. Especially in recent years it has been developed rapidly and a number of ultrasound contrast agents have been described, but most of these are nontargeted. Now several targeted contrast agents that can bind to a specific acceptor have been developed<sup>[2-15,19]</sup>, and it has been demonstrated that such agents can enhance ultrasound images of chosen target tissues. Skyba *et al.*<sup>[17,20-25]</sup> observed that ultrasound could induce intravascular microbubble destruction and bioeffects, and found that the application of ultrasound to thin-shelled microbubbles flowing through small microvessels ( $<7 \mu\text{m}$  in diameter) could produce vessel wall ruptures and the microspheres in vessel could go out through the ruptured walls. Thus antibody-loaded ultrasound contrast microbubbles could also cross the ruptured microvessels and bind specifically to the target tissues.

At present, hepatocellular carcinoma (HCC) is one of the most common malignant tumors with a high incidence and mortality in the world<sup>[26]</sup>. This cancer severely threatens our health and needs to be diagnosed as early as possible, but differential diagnosis of HCC at its early stage is still difficult. So it is important to find a specific and sensitive diadynamic method for it. Studies<sup>[27]</sup> have demonstrated that human hepatocarcinoma specific monoclonal antibody HAb18 could specifically bind to hepatocellular carcinoma cells and can be used for site targeting. If the antibody was used as a target carrier to prepare targeted ultrasound contrast agent, the contrast agent could assemble in a higher concentration of hepatoma tissues and the aim of target imaging could be achieved.

In this study, we prepared and identified targeted liposome microbubbles and investigated their immunological properties. The results indicated that human hepatocarcinoma specific monoclonal antibody HAb18 was firmly attached to the surfaces of liposome microbubbles by static attraction and the immunological activity of HAb18 on liposome microbubbles was similar to that of free HAb18. These demonstrate that it is feasible to prepare targeted liposome microbubbles by static attraction. This method is simple and inexpensive. Moreover, the activity of the antibody can be retained completely. Besides, in order to investigate the properties of targeted liposome microbubbles bound specifically to target cells, rosette formation test and rosette formation blocking test as well as immunofluorescent assay were performed. These tests indicated that targeted liposome microbubbles could bind to human hepatocarcinoma cells specifically and effectively, and the binding was mediated by HAb18.

In order to evaluate the safety of targeted liposome microbubbles, we performed cytotoxicity assay. The result indicated that the microbubbles did not influence the proliferation of human hepatocytes and SMMC-7721 cells. These results lay a good foundation for further study.

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