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LyP-1 Ultrasonic Microbubbles Targeting to Cancer Cell as Tumor Bio-acoustics Markers or Drug Carriers: Targeting Efficiency Evaluation in Microfluidic Channels

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Abstract: Using ultrasonic contrast microbubbles as acoustic biomarkers and drug carrier vehicles by conjugating tumor specific antibody to microbubbles has shown great potential in ultrasonic tumor molecular imaging or drug-delivery and therapy. Microbubble probe targeting efficiency is one of the major challenges. In this study, we developed a novel method to evaluate the targeting capability and efficiency of microbubbles to cells, and more specifically, microbubbles binding LyP-1 (a cyclic nonapeptide acid peptide) target to cancer cell within a microfluidic system. The micro cell sieves within the microfludic channels could trap the tumor cells and enhance the microbubble's interaction with the cell. Assisted with the controllable fluid shear stress, the microbubble's targeting to the cell and the corresponding affinity efficiency could be quantitatively evaluated under a florescent microscope. The system provides a useful low-cost high efficient *in vitro* platform for studying microbubble-cell interaction for ultrasonic tumor molecular imaging or drug-delivery and therapy.

Keywords---Targeted Microbubbles, Ultrasound Molecular Image, Drug delivery

I. INTRODUCTION

A. *Ulrasound molecular imaging*

Molecular biology is a subject to study the relationship and reciprocity between biological macromolecules. And the molecular imaging is a significant technology to study the molecular biology. Ultrasound molecular imaging (UMI) is a kind of imaging method that involves the noninvasive detection and therapy of molecular mediators of disease using contrast-enhanced ultrasound depend on the detection of specific site-target microbubble. In other words, UMI mainly observe the image of targeted zone on tissue, cellular and sub-cellular level aim to reflect the pathophysiological change of disease zone. So that the most important role for ultrasound molecular imaging in the research is describing of disease pathophysiology *in vivo* and provides a way for a continued investigation of pathologic and processes in disease research which is benefit for early diagnoses and therapy[1].

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Compare with classical imaging technologies, the ultrasound molecular imaging has a potential application on the clinical therapy especially in thrombus, inflammation, tumors, and diseases in the reticuloendothelial and lymphatic system. The microbubbles packing drug or gene can compress and expand under the ultrasound pulse. When the pulse energy is high, the strong microbubble oscillation and crash can destroy the peripheral micro vessels which may benefit drug or gene's release into capillary vessels and improve tumor therapy. Meanwhile, ultrasound parameters used for exciting the microbubble such as pulse time, intensity, and the concentration of the contrast agent should be optimized to avoid the tissue damage [2].

B. *Microbubble Targeting*

The ultrasound contrast agent involves a wide variety of designs, from liposomes to liquid nanoemulsions, microemulsions and human albumin, to the dispersions of gas-filled microbubbles. The gas filled microbubbles with specific probe provide a sensitive and high-resolution ultrasound agent that can be monitored by the ultrasound imaging. And those nonmicrobubble agents have also been developed for specific targeting purposes [1]. The most common targeting methods always utilize some site-specific adhesion molecules such as the monoclonal antibodies, peptides, or polysaccharides to stick onto the shell of the microbubble or liposome [3].Thus, the microbubbles with specific molecular probe have possessed the specific disease target capability (Figure 1). Although this property indicates a potential application on the non-invasive disease treatment, there are also some disadvantages including some operator dependence in image acquisition and interpretation, and the biggest challenge is the developing molecularly targeted microbubble contrast agents.

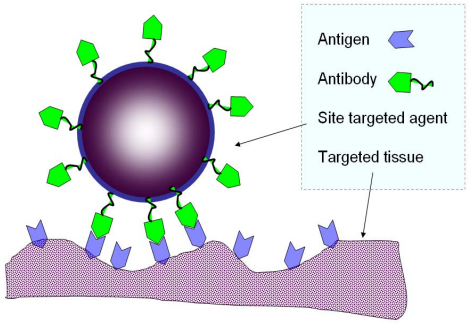

Figure1 Illustration of Targeted microbubble model for ultrasound molecular imaging

C. *LyP-1*

Compare to other molecular imaging modalities, ultrasound molecular imaging has several superiorities such as low poisonous, enhancing drug delivery and cost effective. However, the only disadvantage is lack of molecular probes [4]. The Ultrasound specific cancer molecular markers can be used to distinguish the tumor blood vessels from normal blood vessels. Many of these tumor vessel markers are related to angiogenesis, but some are only selective for certain tumors [5]. Several molecular/protein sequence have been selected as the probe in the previous studies of targeted contrast agents. In this study, we select LyP-1 as the tumor probe which has not been used as UCA target probe before. LyP-1 (CGNKRTRGC) is a cyclic nonapeptide acid peptide selected from a phage-displayed peptide library [6]. Previous research has demonstrated that the LyP-1 peptide is good cancer marker. It not only is a marker for the lymphatic vessels but also binds to tumor cells, so it offers a potential to selectively target both tumor lymphatics and tumor cells [7].

II. EXPERIMENTAL METHOD AND RESULTS

A. *Cell Culture*

MDA-231 human breast carcinoma cells are seeded in the cell laboratory, and cell culture reagents were purchased from Hyclone Corp. The cell line were manipulated under sterile tissue culture hoods and maintained in a 5% CO2 humidified incubator at 37 °C. The MDA-231line was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS).once the cells were confluent, they were trypsinized (0.25% in EDTA, Sigma) and passaged at a 1: 3 sub-culture ratio.

B. *Microfluidic Array Fabrication*

Microfabrication and microfluidic technologies have been recognized as potential platforms for cell-based biosensing and drug screening [8]. Microfluidic networks consist micromachined or molded channels with micron dimensions, have the capability to perform experiments in a high-throughput manner in nanolitre or picolitre volumes using integrated components such as valves, pumps and gradient generators [9]. In recent years, microfluidics has been applied to several cell-based biological applications including mammalian cell patterning[10], monitoring cellular responses to chemical gradients[11],cellular differentiation[12], and dynamic gene expression[13].

In our experiment, a fluid chamber is used to study the microbubble-LyP-1's ability to target the cancer cell. The flow channels in this device are 20μm (h) × 200μm (w), and the diameter of the chamber with eight micro sieves is 800μm.Figure 2 shows the schematic of microfluidic array.

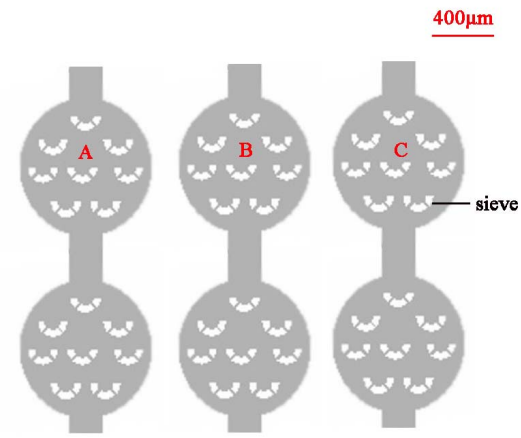

Figure2 Image of three microfluidic arrays with single chamber containing eight micro cell sieves for cell trapping.

C. *Microfluidic Device Cell Seeding*

Firstly, the microfluidic device should be autoclaved, rinsed with PBS. The channels downside glass surface was pre-coated with 20 mg/ml gelatin for 1 hour to enhance cells attachment. Gelatin was removed by rinsing with PBS. Then all cell types were spun down in a centrifuge (1000 rpm, 5 minutes). A vertical array valve was actuated to load the cells into the circular cell culture chambers; each cell line was injected into the device through the inlets using a channel roller pump. A tip was used to change out the media, replenishing critical metabolites and removing potentially toxic waste products [14].Figure 3 shows the experimental cell capture results using cell using MDA-231 human breast cancer cell.

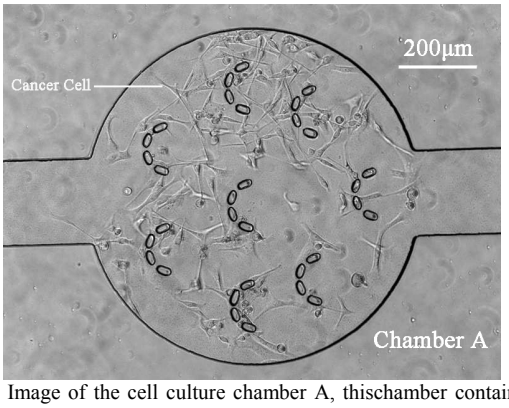

Figure3 Image of the cell culture chamber A, thischamber contains eight micro cell sieves that have trapped the tumor cell. And the cell have attached onto the glass surface. (100x)

D. *UCA for Targeting*

In our experiment, a strong binding between microbubbles and LyP-1 using the high affinity interaction between avidin and biotin is obtained for it is the very strong non-covalent interaction between protein and ligand. The experimental procedures are shown as below:

1. The microbubbles are made of chloroform solution of lipid formulation comprising, DSPC (Avanti), DSPE-PEG2000-Biotin (Avanti) and DSPE-PEK-2000(Avanti)

(90:5:5) [15], and evaporate the chloroform by flowing dry N2 gas. For wiping off the chloroform completely, we put the tube covering by a double layer of apertured Parafilm into a vacuum oven for two hours.

2. Add Tris diluents (80% 0.1M TRIS, 10% Glycerol, 10% 1.2 Propanediol, PH=7.4).Pre-warming the buffer and insert the tube into an ultrasound water tank by 20 kHz sonication about 5-15 min until all phosphatide dissolved in the solution. Move the lucent phosphatide solution to a sealed vial (2ml) and Force 10 mL SF6 gas into the liposome solution by a needle.
3. To make microbubbles, we need a vibrator to fabricate microbubbles with 45 seconds sudden vibration. After10 mins placement, remove the unformed phosphatide by 500r 3min centrifugalization.
4. Add FITC-Avidin PBS solution into the microbubbles solution and incubate 15mins. Remove the superfluous FITC-Avidin by 500r 3min centrifugalization 3 times.
5. Finally, mix 100μl Biotin-LyP-1 solution (1mg/ml) with collected microbubble incubating for 10mins. Acquire the final targeting microbubble by wash 3-4 times in floating method aim to wash out the non-binding LyP-1.
6. Microbubbles' sizes were visually confirmed using a size-calibrated Olympus IX-71 inverted microscope under bright-field illumination.
7. Inject microbubbles into the flow channel, and then they will flow through the chambers in which the breast cancer cells MDA-231 grow.

E. Results

Targeting microbubbles be manufactured by affinity between antibody and antigens. After 45 seconds mechanical vibration, the volume of microbubbles solution has increased about 20% comparing to previous liposome solution. Microbubbles evaluated under microscope. Figure4 shows the fluorescent image of microbubbles-LyP-1 with average diameter of 3μm.

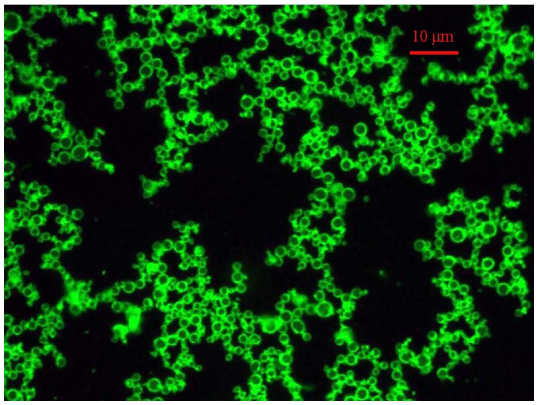

Figure4 Fluorescent image of Microbubbles-LyP-1 (200x)

In contrast to targeting microbubbles, we inject some nontarget microbubbles through the cell culture chamber. (Figure 5)

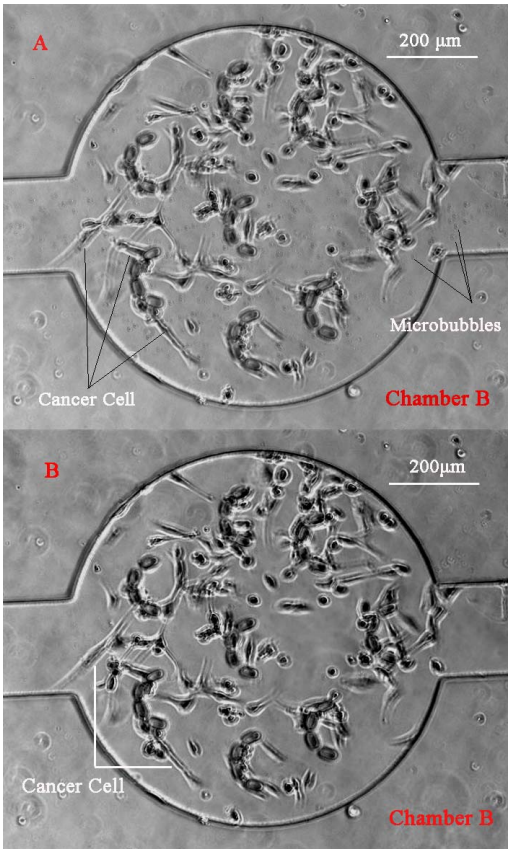

Figure5 A Image of cell culture chamber with non-target hydro-microbubbles flow though. (100x)
B Inject PBS solution instead of microbubbles, and there are no microbubbles left. (100x)

After 5mins washing by non-targeting microbubbles flow, (Figure 5 A) some cancer cells die. A part of dead cells and non-targeting microbubbles can be washed our by the flow, and the rest cells stay in the chamber. Figure 5 B shows there are no microbubbles targeted on the cancer cells after wash. It demonstrates that the nontarget microbubbles can not be trapped by the sieves.

The targeting ability of microbubble-(LyP-1) is evaluated by adding target microbubbles solution into another microfluidic channel. And observe cell culture chamber C at same time. Figure 6 A shows the bright image of chamber C before injecting the target microbubbles. And the fluorescent image (Figure 6 B) shows that microbubbles-(LyP-1) adherent to the cancer cell. It's clear that many targeted bubble's sticking on the cell and some moving bubbles are flowing through the chamber with a long fluorescent trace shown in the image. we has modulated speed and concentration of microbubbles solution aim to observe the targeting of microbubbles-(LyP-1) in different conditions. And the result shows a great targeting ability of microbubbles.

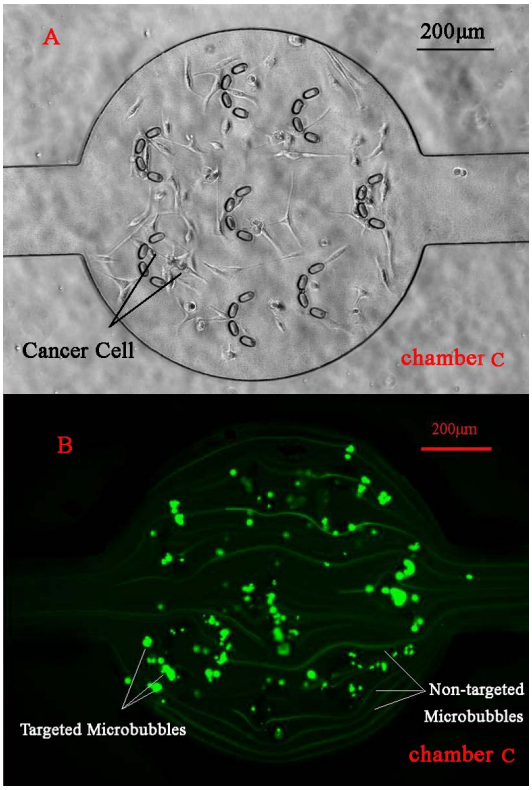

Figure6 A Image of an cell culture chamber in bright scope ;(100x)
B Fluorescent image of the fluid chamber in which the cancer cells grow injecting the targeting mircobubbles into the chamber. (100x)

III. CONCLUSION

We have developed a novel microbubble targeting study platform within a microfludic system. Assisted with the controllable fluid shear stress, the microbubble's targeting to the cell and the corresponding affinity efficiency could be quantitatively evaluated under a florescent microscope. The microfluidic chip has eight fluid chambers and eight fluid channels with eight sieves within a chamber for holding the flowing cell. Within the system, many parameters could be adjust flexibly such as the the concentration of microbubbles, the speed of microbubbles flow which are essential for the targeting efficiency study. The system provides a useful low-cost and high efficient platform for studying microbubble-cell interaction for ultrasonic tumor molecular imaging or drug-delivery and therapy. Compare to the flow cytometry which always been used to evaluate the target ability of microbubbles, Using microfluidic system is more flexibly so as to simulate the real situation in human blood circulation by modulating the parameters. In addition, the microfluidic system is less expensive and simpler than other methods. We also have demonstrated that LyP-1 is a sensitive targeted probe ultizing this platform. In our research, LyP-1 has shown a great targeting ablity, and it can be utiltized as a useful probe to target the cancer cell in ultrasound molecular imaging. In the comparision of other UCA probes, LyP-1 is more sensitive and specific to kinds of cancer cells which indicate LyP-1 has a protential application in the clinical threapy of cancer.

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