# ภาคผนวก

iGRC2013
Chiang Mai University, THAILAND

PROCEEDINGS: International Graduate Research Conference 2013
20 December 2013

## Improving Microbubble Contrast Agent Stabilizing for Ultrasound Persistency by Addition of Ferric Chloride in The Lipid Layer

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**Abstract**

Microbubble contrast agents are used to improve the quality of ultrasound images. The present report addresses the need to improve stability of microbubbles. Microbubble types PC:PG(1:1), (5:1), (10:1) and PC:PG:FeCl(1:1:1) were constructed and characterized. The diameters of all microbubble types were between 200-250 nanometers. Among the three types of PC:PG microbubbles, PC:PG(1:1) was most stable at 37°C for up to 21 days. Therefore, PC:PG(1:1) was selected for modification by addition of ferric chloride into the lipid layer of the microbubble to obtain microbubble type PC:PG:FeCl(1:1:1). The presence of ferric chloride in the lipid layer can improve ultrasound (8 MHz) persistency. Cell culture studies demonstrated that these microbubbles can be taken up by small-cell lung cancer cells without apparent toxicity. The present study demonstrates that microbubbles PC:PG(1:1) and PC:PG:FeCl(1:1:1) are potential agents that can be used for improved ultrasound imaging and for drug delivery.

**Keywords**

Microbubble, Ferric Chloride, Lipid Layer, Ultrasound

**Introduction**

Ultrasound imaging is a powerful, noninvasive diagnostic modality [1-3] Ultrasound contrast agents (USCA) are used to improve image quality. Microbubbles have been useful to improve ultrasound image quality, but stability issues remain [4-5]. In the present study, the biomembrane system USCA microbubbles were altered by addition of ferric chloride and the modified microbubbles were characterized by their physical properties, stability, and image improvement.

**Methodology/Experimental design**

1. Microbubble preparation

The phospholipids (L-α-phosphatidyl choline (PC) and 1.2-dipalmitoyl-sn-glyero-3-phospho-rac-(2-glycerol) sodium salt (PG)) were used for microbubble formation at a ratio of PC:PG 1:1, 5:1, and 10:1 (0.5 mM phospholipid final concentration). In the first step: both lipids were solubilized in trichloromethane. When a clear lipid solution was obtained, the solvent was evaporated under pressure of 296 mbar at 25°C and 84 rpm to form a thin lipid film. Next, microbubbles were formed by hydration with 0.01mM Tris-HCl pH 7.4 using a freeze thaw technique [6]. During freeze thaw, the microbubbles were filled by nitrogen gas. Modified microbubbles were generated by mixing the lipid mixture with a 0.25 mM final concentration of ferric chloride ($FeCl_3$) prior to thin lipid film formation at a ratio of PC:PG:FeCl as 1:1:1. After preparation, microbubbles were stored in the dark at 37°C.

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2. Physical characterization

The physical properties of the synthesized microbubbles were characterized by measurement of their diameter and zeta-potential using dynamic light scattering (DLS) and flowcytometry. One hundred µL of each sample was suspended in 1 mL of Tris-HCI pH 7.4 weremeasured at 25°C. DLS data were analyzed using Malvern software. Flowcytometric data were analyzed using Flowing software 2.5.0.

3. Stability of vesicular formation

Microbubble vesicular formation was analyzed with the fluorescence probe 1,6-diphenyl-1-3,5-hexatriene (DPH) [7-9]. Ten µL of each microbubble were suspended in 2 mL of buffer (Tris-HCI pH-7.4, 37°C), then 0.1 µM of DPH was added to the microbubble suspension. The fluorescence intensity of DPH at 427 nm (excitation at 370 nm) was immediately recorded by a spectrofluorometer (Perkin Elmer LS55). All samples were characterized for up to 21 days.

To explore the effects of ultrasound on vesicular formation by PC:PG(1:1) and PC:PG:FeCl(1:1:1), both microbubble types were exposed to 8.0 MHz ultrasound for 10, 30, and 60 min, and the vesicular formation was then determined.

4.Cytotoxicity assay

Two human small cell lung cancer cell lines were used: a doxorubicin-sensitive cell line (GLC4) and a doxorubicin-resistant cell line (GLC4/Adr). Both cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, 5% penicillin-streptomycin in a humidified atmosphere of 95%, 5% $CO_2$ at 37°C for 3 days before the experiments were carried out. Cells ($5\times10^5$) were incubated in the absence and presence of various concentrations of microbubbles up to 10 µM for 72 hours. At 72 hours, the amount of untreated and microbubble-treated cells were counted with a flowcytometer (Beckman-Coulter, Miami, FL, USA). Cytotoxicity was determined by inhibition of proliferation of the microbubble-treated cells compared to that of the untreated control cells.

5.Uptake of microbubble vesiclesby lung cancer cells

The GLC4 cell line was used to measure vesicular uptake. Cells ($5\times10^5$ in 2 mL complete medium) were seeded in a 3.5 cm diameter petri-dish for 24 hours in order to become adherent. The culture medium was then discarded and the cells were washed twice and maintained in 1 mL phosphate buffer saline solution pH 7.4. DPH-microbubbles (DPHM) were prepared by loading the microbubbles with 1 µM DPH for 1h. The fluorescence intensity of DPH decreased due to energy transfer from DPH to the lipid layer in the microbubble. DPHM solution (50 µL) was gently added to the petri-dish. Cells, pre- and post-DPHM addition, were observed using a fluorescence microscope (Floid™ Cell Imaging Station, Life Technologies, USA). The blue channel, representing the fluorescence of DPH, was analyzed with ImageJ software.

6.*In vitro*ultrasound image

Ultrasound images were obtained with an agarose gel (10 mg/mL) phantom system using the B-mode of a Toshiba Famio8, with a transducer PLQ805A linear array frequency range of 6.0-12.0 MHz. The mean gray value (MGv) (range 0-255) of the region of interest (ROI) of the images (8-bit) was analyzed with ImageJ software.

**Results and Discussion**

1. Microbubble size and Zeta potential

Using flowcytometry, the microbubble diameters of PC:PG(1:1), (5:1), (10:1) and PC:PG:FeCl(1:1:1) were determined to be 203.0 ± 134.8, 232.0 ± 145.6, 247.7 ± 147.2 and 204.4 ± 141.6 nm, respectively. The zeta-potential represents the electrostatic or charge repulsion or attraction between microbubbles, and was measured by DLS. The zeta potential of

PC:PG(1:1) and PC:PG:FeCl(1:1:1) were determined to be -30.0 ± 3.3 and -28 ± 0.6 mV, respectively.

2. Microbubble stability

The DPH fluorescent probe was used to investigate membrane dynamics. DPH is negligibly fluorescent in aqueous phase but is highly fluorescent in organic solvent or in a membrane core. DPH interacts with membrane lipids in the hydrophobic region either in perpendicular or parallel orientation to the membrane plane [7]. The fluorescence intensity of DPH is due not only to DPH-lipid interaction but also to the energy transfer rate of DPH to an environment that can cause diminution of fluorescence intensity [8-9]. With these properties, the fluorescence intensity of DPH represents the lipid-lipid interaction and arrangement during vesicular membrane formation of microbubbles. The day after vesicular formation, the fluorescence intensity of PC:PG(1:1), (5:1), and (10:1) were similar. After storage at 37°C, the fluorescence intensity of microbubble types (5:1) and (10:1) decreased(Fig. 1).

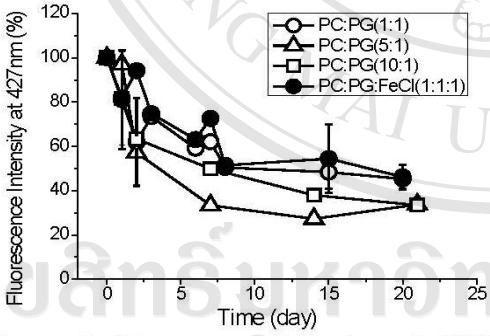


Figure 1 Fluorescence intensity of DPH incorporated in the membranesof microbubblesPC:PG(1:1) (○),(5:1) (△),(10:1)(□) and PC:PG:FeCl (●).

After vesicular formation, all microbubble types had similar lipid arrangement, but the microbubble type (5:1) and (10:1) were less stable at 37°C due to unsuitable stoichiometry.

To improve microbubble stability and specificity, PC:PG(1:1) was selected for modification with ferric chloride (PC:PG:FeCl) at a ratio of 1:1:1. After formation, PC:PG:FeCl was tested for vesicular formation. PC:PG:FeCl had lower fluorescence intensity than PC:PG(1:1). This observation can be explained by the lipid-lipid layer becoming more tightly bound by either the ferric or chloride ion, which caused energy transfer from DPH to the hydrophobic region of the lipid. However, the degeneration rate of PC:PG:FeCl was the same as PC:PG(1:1) (Fig. 1).

Ultrasound (8 MHz) treatment of microbubble types PC:PG(1:1) and (PC:PG:FeCl) demonstrated that PC:PG(1:1) was more degenerated than the modified microbubble (PC:PG:FeCl) (p-value<0.01) (Fig.2). Thus, ferric chloride results in increasing the stability of the microbubble to ultrasound.

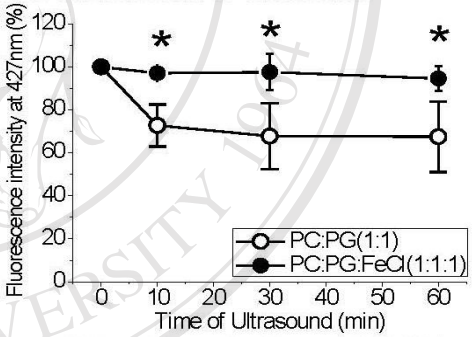


Figure 2The effect of ultrasound(8MHz) on the alteration of fluorescence intensity due to DPH incorporation in membranes of microbubbles PC:PG(1:1)(○)and PC:PG:FeCl (●)(*p-value<0.01)

3. Cytotoxicity assay

The influence of microbubbles on cancer-cell proliferation was investigated. After cells were incubated with microbubbles for 72 h, there was no significant cellular toxicity of any microbubble type on either cell line up to a concentration of 10 μM.

4. The incorporation of DPH-labeled microbubbles by small-cell lung cancer cells

After adding additional DPH-labeled microbubbles (DPHM) to the cell culture, the bright-light and fluorescence microscopy using the blue channel was performed at 5, 10, and 20 min (Fig. 3A,B,C). The images were analyzed for area by integration of the blue intensity profile from the ROI (Fig.3D).The maximum increase in area determined by integration was at 10 min. These data suggest that the DPHMs attached and integrated in the cell membrane whereby the DPH molecules were detached from the DPHMs resulting in re-fluorescence of DPH by 5 min (Fig.3E).

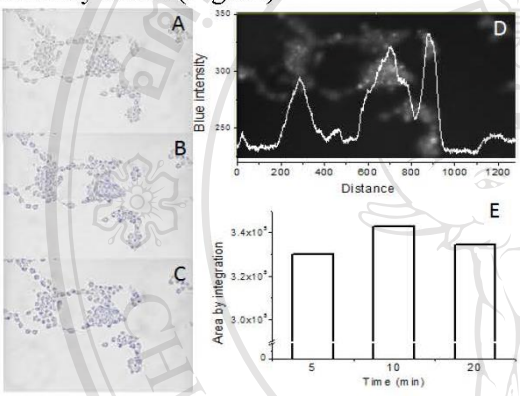


Figure 3 Fluorescence micrograph (20x) of GLC4 cellspost-DPHM addition: Mix channel(A) 5, (B) 10, (C) 20 min, and blue-channel image overlay byintensity profile (D) andarea by integration of blue-channel intensity profile (E)

5.*In vitro* ultrasound imaging

The ultrasound images of the agarose gel system loaded with air (lane 1), microbubbles PC:PG(1:1) (lane 2) and water (lane 3) were recorded as shown in Fig.4. The MGv was determined indicating that the microbubbles slightly enhanced the ultrasound image signal compared with water and agarose gel (Fig.5).

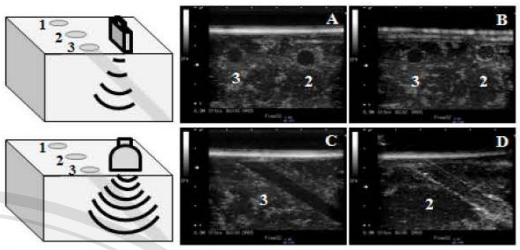


Figure 4 Ultrasound images of the agarose gel system: Transverse view load with (A) water (lane 2, 3), (B) microbubbles (lane 2) and water (lane 3) and axial view load with (C) water (lane 3) and (D) microbubbles (lane 2)

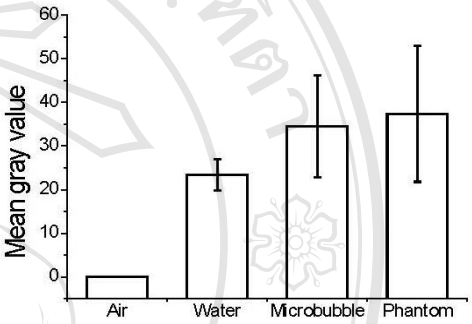


Figure 5The mean gray value of ROI of air, water, microbubbles and agarose gel phatom in ultrasound images

## Conclusion

Microbubbles PC:PG(1:1) and PC:PG:FeCl(1:1:1) were constructed. The combination of ferric chloride into the lipid layer of microbubbles improves stability and ultrasound persistence.

## Acknowledgement

The authors thank the National Research University Project under Thailand's Office of the Commission on Higher Education (CHE) for financial support.

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