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論文題目	Development of novel phospholipids-based ultrasound contrast agents intended for drug delivery and cancer theranostics （ドラッグデリバリーとがん･セラノスティクスを志向した新規リン脂質基盤型超音波造影剤の開発）		

（論文内容の要旨）

Theranostics is a term that refers to the combination of therapy and diagnostics so that for example the same particle can both be used for finding a tumour and deliver drugs to treat it. Ultrasound (US) imaging is well-known and safe diagnostic tool that is widely used in many different applications. A limitation of US imaging is the difficulty in differentiating the blood vasculature from the surrounded tissues and therefore US contrast agents (UCAs) are often used for enhancing the contrast signal in the blood vasculature. UCAs are usually made of hydrophobic gases such as perfluorocarbons (PFCs) stabilized in bubble form by biocompatible shells. Recently, not only gas cored carriers (such as micro- and nano-sized bubbles) but also liquid nanodroplets that can form gas bubbles *in vivo* (also called phase shift acoustic nanodroplets (PSANDs)) have been proposed. Gas form UCAs usually have poor *in vivo* stability that can be improved by using liquid droplets instead. After being used for diagnosis for many years, UCAs have recently also been used for enhancing the delivery of drugs and genes through the cavitation effects when combined with therapeutic US (TUS). However, most of the reported UCAs were investigated for imaging or for therapy separately. For theranostic applications, the balance between both therapeutic and diagnostic characteristics will be critical. For instance, theranostic UCAs should give image enhancement and then often activated by TUS at the target site. This means they need high contrast signal and sufficient drug payload in one carrier and the stability has to be good enough for reaching the target but not too good for the TUS activation. To solve this dilemma I focused on two main aspects of perfluorocarbon carrier systems, first, the employment of phospholipids for stabilizing the UCAs and second the utilization of different hydrophobic PFCs.

Phospholipids are biocompatible amphiphilic molecules that are the main component in cell membranes and have been frequently used in the biomedical research. Compared to other shell materials (i.e. polymers that form rigid shells), phospholipids can maintain better stability and resonant properties in UCAs due to their high flexibility and ability to adapt when an US wave leads to bubble oscillation. Moreover, drug and nucleic acids can be loaded into phospholipids shells easily by using the charge properties that leads to complex formation between drug molecules and phospholipids' heads. The other component, the PFCs are essential for US contrast signal enhancement. They have different physicochemical properties depending on structure and molecular weight that consequently affect UCAs size distribution, stability, and echogenicity.

I have developed several types of novel phospholipid-based UCAs. I aimed to show the merits and limits of each formulation and how we can improve these limits for better theranostic use. This included *in vitro* and *in vivo* evaluation of theranostic characteristics of these UCAs and based on that, the potential use of these carriers was then investigated for the purpose of gene delivery and cancer theranostics.

Chapter 1. Formulation and evaluation of nano- and micro-sized phospholipids-based theranostic ultrasound contrast agents

A type of UCAs was developed by using mechanical agitation of lipids dispersion in the presence of perfluoropropane gas (PFP). The focus was on improving the size distribution and

stability. Mechanically formed bubbles (MFBs) were composed of the zwitterionic phospholipid distearoylphosphatidyl choline (DSPC) with a portion of polyethylene glycol (PEG) engraftments. MFBs with PFP had a smaller size (~400 nm) compared to those made with perfluorobutane or nitrogen gases. Also, MFBs with PFP were found to be stable with uniform size for 24 h at room temperature (RT) and at 4 °C bubbles could be preserved more than 50 h. By using a similar method, doxorubicin loaded bubbles (DLBs) were also prepared. The DLBs were prepared by mechanical agitation of phospholipid dispersion in the presence of PFP gas. The anionic phospholipid distearoylphosphatidyl glycerol (DSPG) was selected to bind doxorubicin to the bubbles by electrostatic interaction. Drug loading was ≥92% and bubbles had an average size of about 1 µm. The PFP was retained in the bubbles at least 30 min at RT. *In vitro* ultrasonography also showed that DLBs have high signal even after 10 min and when TUS irradiation was applied most of the bubbles were destroyed. This indicated that DLBs were presumably destroyed due to cavitations effects. PSANDs were also prepared by using a similar phospholipid composition as in the MFBs. The PSANDs were prepared in a two step process that consisted of mixing liposomes with liquid perfluoropentane (PFPn) or perfluorohexane (PFH) followed by bath sonication. PSANDs had average size of around 200 nm. PFH/ PFPn leakage was then tested *in vitro* at 37 °C. PFH/ PFPn were retained in PSANDs at least for 1 h. The PSANDs could be turned from liquid to gas by TUS irradiation. The extent of the phase shift depended more on the US frequency than the intensity. Maximum contrast enhancement was achieved with US intensity about 2 W/cm^2 for PFPn, and 5 W/cm^2 for PFH PSANDs.

These results suggest that UCAs with proper size and high drug loading can be prepared with fairly simple means. Therefore, these UCAs can be easily and effectively be used in the field of drug delivery and cancer theranostic.

Chapter 2. Application of phospholipid-based ultrasound contrast agents for gene delivery and cancer theranostics

Based on the findings presented in Chapter 1, two main applications were decided. The first application was employment of MFBs for gene transfection. MFBs were mixed with plasmid DNA and intravenously injected into mice followed by TUS irradiation on the left limb muscles. The gene expression was significantly higher than in the mice treated with plasmid DNA and TUS only. Moreover, aged MFBs that left for 24 h at RT or at 4 °C for several days were found to still be functional in enhancing the gene expression in mice limb muscles. The second application was the utilization of the DLBs as a theranostic agent in tumor bearing mice. The inhibitory effect on the proliferation of murine B16BL6 melanoma cells *in vitro* was enhanced using a combination of TUS irradiation and DLBs compared to that with only DLBs treatment. Moreover, *in vivo*, DLBs in combination with TUS significantly inhibited the growth of B16BL6 melanoma tumor in mice. Additionally, ultrasonography showed high contrast enhancement of the DLBs in the tumor vasculature. Finally, I evaluated the stability of (PFH) PSANDs in mice. Gas chromatography analysis showed that PFH can be sustained in circulation as well as in tumour for 15 min after intravenous injection. Moreover, the ultrasonography imaging in mouse carotid artery indicated that droplets could shift to bubbles after TUS irradiation leading to a contrast signal enhancement at the imaging site.

In conclusion, this research is one of the first attempts in highlighting the theranostic potential of UCAs. Accordingly, I have developed several methods for producing different theranostic UCAs starting with phospholipids and (gas/liquid) perfluorocarbons. These UCAs have been tested *in vitro* and *in vivo* and have been shown to be effective as therapeutic and diagnostic agents in applications like gene delivery and cancer treatment.

（論文審査の結果の要旨）

近年、医療機器と薬物あるいはドラッグデリバリーシステム（ＤＤＳ）技術を組み合わせ、疾患の診断と治療を同時に行うことを目指すセラノスティックス（theranostics）が、医療分野で大きな注目を集めている。多くの医療技術の中でも超音波診断は安全性の高い技術として多用されているが、血管腔と周囲組織との区別がつきにくいことからシグナル増強を目的として超音波造影剤が併用される。造影剤としては、生体適合性のある殻で安定化した perfluorocarbon（PFCs）などの疎水性ガスの気泡が用いられてきたが、最近では超音波音響エネルギーで気液転移するナノ液滴（phase shift acoustic nano-droplets: PSANDs）が開発され、気泡が有する体内での不安定性の問題を解決する方法として注目を集めている。また、超音波の照射を薬物や遺伝子の細胞導入に利用する試みも進んでいる。しかしながら、これらの試みは診断あるいは治療のいずれかに着目したものが殆どで、両者の並立を目指した場合、例えば液滴などをキャリアとして用いると、標的部位への送達と治療的超音波照射への応答という生体内安定性に関して相反する性質が要求され、かつ薬物を十分量担持しつつ高いコントラストシグナルを与えないといけないという、非常に実現が難しい機能の設計が求められるため研究は進んでいない。申請者はこうした問題の解決を目指し、リン脂質を安定化に利用した PFCs 製剤を開発し、その製剤特性を評価するとともに、診断と治療への応用の可能性を評価した。

開発したキャリア製剤は、機械的攪拌で調製が可能な perfluoropropane（PFP）の気泡（MFBs）とこれに抗がん剤 doxorubicin を包含させた製剤、リン脂質からなるリポソームに perfluoropentane（PFPn）あるいは perfluorohexane（PFH）の液滴を内封させた PSANDs で、それぞれの粒子径、製剤としての安定性及び薬物保持率等を評価した。平均分子量 2000 の polyethylene glycol（PEG）鎖導入 distearoylphosphatidylethanolamine（PEG-2000-DSPE）と distearoylphosphatidylcholine（DSPC）を用いて調製した MFBs は、平均分子径が 400nm 以下で室温でも 24 時間後まで、4℃では 50 時間以上安定にその粒子径を保った。さらに、これに doxorubicin をリモートローディング法を用いて保持させると、得られた MFBs は平均直径 1μm、薬物含有率 92%以上で、30 分以上 PFP ガスを保持する一方、in vitro においては 10 分後も高いコントラストシグナルを示し、また治療的超音波の照射により速やかに崩壊した。一方、PSANDs は平均粒子径が 200 nm で、37℃において 1 時間以上ガスの放出は認められなかった。これらの結果は、両製剤においてセラノスティックスとして期待した特性が得られていることを示すものである。

これらの知見を踏まえて、次に各製剤の診断及び治療への応用の可能性を検討した。最初に、遺伝子導入に対する MFBs と治療的超音波照射併用の効果を評価するため、MFBs と plasmid DNA の混合物をマウスに静脈注射後治療的超音波照射を行った

結果、超音波照射部位に選択的に遺伝子発現がみられることが確かめられた。次にdoxorubicinを保持したMFBsの評価を*in vitro*及び*in vivo*で行い、共に治療的超音波照射との併用によって、B16BL6メラノーマに対して高い抗腫瘍効果が得られることを確認した 。さらに、*in vivo*実験において高コントラストの腫瘍血管像が得られた。一方、PSANDsについては、PFH含有PSANDsを静脈注射後15分まで、PSANDsが血管内腔及び腫瘍に存在することが示唆され、かつ治療的超音波照射によって液滴が気泡化しコントラストシグナルを増強して鮮明な超音波造影像を与えることが確かめられた。

以上、申請者は、超音波照射と組み合わせることにより診断と治療を同時に行うことができる製剤の開発に取り組み、リン脂質を安定化剤としてPFCsを気泡あるいは微小液滴化することによって、診断あるいは抗がん剤や遺伝子医薬品と組合した治療が可能となることを示した。以上の知見は、今後セラノスティックス概念に基づく治療技術を構築する上で有益な基礎的情報を提供するものと考える。

よって、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、平成２８年８月２５日、論文内容とそれに関連した事項について諮問を行った結果、合格と認めた。

なお、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、（当分の間）当該論文の全文に代えてその内容を要約したものとすることを認める。

要旨公表可能日：　　年　　月　　日以降