Porphyrin Microparticles for Biological and Biomedical Applications

by

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Lipids are one of the critical building blocks of life, forming the plasma membrane of cells. In addition, porphyrins also play an equally important role in life, for example, through carrying oxygen in blood. The importance of both these components is evident through the biological and biomedical applications of supramolecular structures generated from lipids and porphyrins. This thesis investigates new porphyrin microparticles based on porphyrin-lipid architecture and their potential applications in biology and medicine. In Chapter 1, a background on lipid and porphyrin-based supramolecular structures is presented and design considerations for generating multifunctional agents. Chapter 2 describes the generation of a monolayer porphyrin microparticle as a dual-modal ultrasound and photoacoustic contrast agent and subsequently, a trimodal ultrasound, photoacoustic and fluorescence contrast agent. Chapter 3 examines the optical and morphological response of these multimodality ultrasound-based contrast agents to low frequency, high duty cycle ultrasound that causes the porphyrin microparticles to convert into nanoparticles. Chapter 4 examines the generation of bilayer micrometer-sized porphyrin
vesicles and their properties. Chapter 5 presents a brief summary and potential future directions. Although these microscale structures are similar in structure, the applications of these structures greatly differ with potential applications in biology and also imaging and therapy of disease. This thesis aims to explore and demonstrate the potential of new simplified, supramolecular structures based on one main building block, porphyrin-lipid.
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Abbreviations

RNA - ribonucleic acid

US – ultrasound

MRI – magnetic resonance imaging

PDT – photodynamic therapy

BPD-MA - benzoporphyrin derivative monoacid

IONP – iron oxide nanoparticles

PET – positron emission tomography

PA – photoacoustic

CT – computed tomography

NIRF – near infrared fluorescence

$^{64}$Cu – copper-64

HSA – human serum albumin

DOTA - 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

NHS – N-Hydroxysuccinimide

siRNA - small interfering RNA

PEG – polyethylene glycol

PAV - prednisolone acetate valerate
RGD - Arg-Gly-Asp

SPECT - single-photon emission computed tomography

PTT – photothermal therapy

FDG – fluorodeoxyglucose

GPV – giant porphyrin vesicle

DNA - deoxyribonucleic acid

pMB – porphyrin shell microbubble

SD – standard deviation

egg PC - egg phosphatidylcholine

chol – cholesterol

pyro - pyropheophorbide

TRITC – tetramethylrhodamine

NaCl – sodium chloride

FRAP – fluorescence recovery after photobleaching

DPPC - 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

POPC - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

DMPC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine

DSPC - 1,2-distearoyl-sn-glycero-3-phosphocholine

PBS - phosphate buffered saline
PEG40S - polyoxyethylene-40 stearate

SF6 - sulfur hexafluoride

PFB – perfluorobutane

PFP – perfluoropropane

FBS – fetal bovine serum

DSPE-mPEG2000 - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]

pNP – porphyrin shell nanoparticles

BChl - bacteriochlorophyll

DPPA - 1,2-dipalmitoyl-sn-glycero-3-phosphate

DPPE-mPEG5000 - 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000]

TEM – transmission electron microscopy

APS - Ammonium persulfate

TEMED - N,N,N',N'-tetramethylethylenediamine

EPR - enhanced permeability and retention

BBB – blood brain barrier
Chapter 1
Porphyrin Lipid Supramolecular Structures and Designing Multifunctional Biomedical Agents

1.1 Acknowledgements

Sections of this chapter are from review articles entitled “Porphysome nanotechnology: a paradigm shift in lipid-based supramolecular structures,” published in Nano Today\textsuperscript{1}, “Engineering multifunctional nanoparticles: all-in-one versus one-for-all”, published in WIREs Nanomedicine and Nanobiotechnology\textsuperscript{2} and “Organic biophotonic nanoparticles: porphysomes and beyond,” published in IEEE Journal of Selected Topics in Quantum Electronics\textsuperscript{3}. Under the supervision of Dr. Gang Zheng, my contribution to these review papers included planning and writing of the manuscript text and figures.

1.2 Lipid and porphyrin supramolecular structures

1.2.1 Lipid supramolecular structures

1.2.1.1 Lipid bilayer structures

The amphipathic nature of lipids enables the self-assembly of various supramolecular structures via the presence of a hydrophilic headgroup and hydrophobic acyl chains. These structures can be categorized according to the presence of a lipid bilayer or monolayer (Figure 1.1). Lipid bilayers are approximately 4-5 nm in thickness\textsuperscript{4} (Figure 1.2a) and the aqueous core of the lipid bilayer can be filled with hydrophilic cargo. Nanometer sized lipid bilayer particles are known as liposomes (Figure 1.2b). Liposomes have been utilized as carriers for hydrophilic drugs and imaging agents due to the high loading capacity of their core. Furthermore, since the bilayer is also hydrophobic, liposomes can also carry hydrophobic drugs. Liposomes have been developed for a number of biomedical applications in therapy and imaging of disease and have received clinical success. Liposomes have been clinically approved to deliver chemotherapy drugs, such as doxorubicin (Doxil\textsuperscript{®}, Janssen Biotech) used to treat ovarian cancer, breast cancer and Kaposi’s sarcoma\textsuperscript{5}. Several other drug loaded liposomes have also been approved or are in clinical trial for various cancers, details of which can be found in the following reference\textsuperscript{6}. The
clinical success of these liposomes has been based on their high biocompatibility, biodegradation and clearance properties, in addition to their high therapeutic efficacy or contrast enhancement capabilities. The organic nature of lipids has the ability to avoid eliciting an undesired immune response\(^7\) and ensure safe and timely clearance from the body\(^8,9\). However, the clinical role of the lipids themselves has been restricted as simple carriers used to increase the circulation time of the agent and prevent interaction of the cargo with blood components. The lipids serve as building blocks to form a vehicle to deliver the therapy or imaging agents, but they themselves are not intrinsically therapeutic or imaging agents.

Lipid bilayers also mimic the plasma cell membrane. When reconstituted as artificial cell membranes, they are termed giant vesicles. Giant vesicles range in size from 10 to 200 µm, composed of a lipid bilayer (Figure 1.2c). Due to their large size, they are visible under a microscope, allowing for real-time monitoring of changes in bilayer behaviour, and possess a membrane curvature similar to cells. Giant vesicles have been utilized in biomimetic chemistry, biomembrane physics and the development of an artificial cell\(^10\).

While giant vesicles have not been applied for biomedical applications, they have been utilized for a number of biology, physics and chemistry-based investigations. Phospholipid-enclosed compartments also play a central role in cellular and sub-cellular homeostasis, with the bilayer serving as the general barrier between external and internal biomolecules and chemicals. Putative prebiotic bilayers, in the form of giant vesicles, have been recreated in the context of understanding how cells came to control the passage and production of biomolecules by mimicking the self-enclosed lipid matrix of the plasma membrane\(^11,12\). Giant vesicles have been filled with various chemical compounds and allowed to react inside the compartment to control the maintenance of the vesicle structure, and the growth and reproduction of the vesicle. For example, giant vesicles have been loaded with enzymes, genes, ribosomes, transfer ribonucleic acid (RNA) and small molecules to generate a “semisynthetic minimal cell”\(^13\). Furthermore, the expression of proteins within giant vesicles has been of growing interest. The activity of a synthesized protein inside such a compartment may alter the dynamics of the system, such as the controlled exchange of solutes between the interior of the vesicle and the surrounding environment, or transform them into cell-like structures, such as the reconstitution of the cytoskeleton\(^14,15\). All of these studies aim to generate a functional cell from its molecular
components and understand the associated kinetics, dynamics and processes involved in the origins, maintenance and functionality of cells.

Giant vesicles have an internal volume on the order of picolitres\(^{12}\) which has led to the use of them as microreactors to perform simplified reactions within a confined space\(^{16-19}\). For example, Michel et al.\(^{20}\) utilized giant vesicles to control the mineralization process of calcium phosphate, a biomineral that has a role in mechanical and calcium storage in living organisms. Giant vesicles have also been used for the formation of size-controlled hydrogels\(^{21}\). These are just a couple examples of the broad range of research being conducted using giant vesicles in physics, biology and chemistry.
Figure 1.1. Schematics of a lipid bilayer (a) and lipid monolayer (b).

Figure 1.2. Schematic representations of a) a lipid bilayer, b) a liposome and c) a giant vesicle.
1.2.1.2 Lipid monolayer structures

Phospholipids may also form lipid monolayer nano- or microstructures, encapsulating a hydrophobic core (Figure 1.3). This core may be a variety of materials such as a gas or oil. When the core is oil, these structures are identified as emulsions such as nanoemulsions or microemulsions and when encapsulating a gas, they are nanobubbles or microbubbles. These lipid-based emulsions incorporated different hydrophobic drugs or imaging agents into the oil core, which increases the loading capacity of the particle compared to a particle possessing a hydrophilic core\(^{22}\).

By changing the core of lipid monolayer structures to a gas, nanobubbles (typically submicrometer in size) and microbubbles (typically 1-10 µm in size) could be generated and have been developed as ultrasound (US) active agents. Nanobubbles in particular have been utilized for drug and gene therapy, in which the delivery occurs using an external US trigger\(^{23}-^{26}\). Microbubbles have also been developed for this purpose\(^{27,28}\), however, microbubbles are well recognized for their development as US contrast agents\(^{29}\). Microbubbles may be formed from a lipid shell encapsulating a gas. This lipid shell decreases the surface tension, which is responsible for driving the gas into the surrounding medium, and it also acts a barrier for the gas as it attempts to leave the core\(^{30}\). Microbubbles typically contain insoluble gases such as perfluorocarbon gas, which further enhance the lifetime of the microbubble\(^{31,32}\). Due to the presence of gas within the core of microbubbles, microbubbles exhibit an acoustic impedance mismatch with surrounding fluid and tissue. This results in a significant amount of US scattering in response to an incoming acoustic wave, in which microbubbles then appear bright on an ultrasound image\(^{33}\). Furthermore, microbubbles have the ability to expand and contract when insonified by an acoustic wave, thereby generating microbubble-specific nonlinear signals that can be detected using specific US imaging contrast algorithms\(^{34}\). They have been implemented clinically as US contrast agents to investigate blood flow in the heart, liver and kidneys for diagnostic purposes\(^{35}\).
Figure 1.3. Schematic representation of a) a monolayer of lipids, and nano- and micro-structures that may be formed (b, c).
1.2.2 Porphyrin supramolecular structures

1.2.2.1 Porphyrins

Porphyrins are also another group of organic compounds that have received clinical success. Porphyrins have been one of the most extensively investigated molecules for biomedical applications including fluorescence imaging, magnetic resonance imaging (MRI) and nuclear imaging. Porphyrins are composed of four pyrrole subunits forming a 22π electron system, which is the root of their photophysical properties. When excited via the absorption of light, the porphyrin may return to its ground state through several pathways, such as radiative decay (e.g. fluorescence emission), non-radiative decay (e.g. heat) or through intersystem crossing to an excited triplet state in which energy transfer occurs to an acceptor molecule, generating radical species or singlet oxygen. Porphyrins also behave as an intrinsic metal chelator in which they may possess a central metal atom, enabling their applications in MRI, nuclear medicine and radiotherapy.

Porphyrins have achieved clinical success as photosensitizers in photodynamic therapy (PDT) for cancer and ophthalmic diseases. PDT consists of the administration of light and a photosensitizing agent in the presence of oxygen to generate cytotoxic singlet oxygen. Porphyrins’ favourable photophysical properties, including long wavelength absorption and high singlet oxygen quantum yield, and low in vivo toxicity, have all contributed to their clinical success. For example, benzoporphyrin derivative monoacid (BPD-MA) derived from protoporphyrin IX dimethyl ester was demonstrated to be a potent photosensitizer in vitro, preclinically in vivo, and in clinical trials. However, as many porphyrins exhibit a high level of lipophilicity, including BPD-MA, they have a tendency to aggregate in aqueous solution, therefore, limiting its bioavailability. To circumvent this problem, porphyrins have been incorporated into liposomes for improved delivery, solubility and efficacy.

1.2.2.2 Porphyrin liposomes

To improve the bioavailability of BPD-MA, and ensure that it remains in its monomeric form upon intravenous administration, liposomes were used as a delivery vehicle carrying BPD-MA within the lipid bilayer membrane. Liposomal BPD-MA, marketed as Visudyne® (Valeant Pharmaceuticals International, Inc.), had enhanced tumor accumulation and greater in vivo photosensitizer potency over its non-encapsulated form, which has contributed to its clinical
success and implementation for age-related macular degeneration\textsuperscript{56,59-61}. Visudyne is one example of many porphyrins that have been incorporated into liposomal nanostructures in order to improve \textit{in vivo} performance. A significant amount of work is currently being dedicated to formulation optimization and targeting of liposome-based photosensitizer nanostructures to further improve their PDT efficacy, expanding their applications to other indications including various cancers\textsuperscript{62,63,64,65}, dermatologic diseases\textsuperscript{66-68} and microbial infections\textsuperscript{69,70}.

However, the incorporation of porphyrins within a liposomal membrane is limited to only a small molar fraction of the total lipid amount\textsuperscript{71}. In specific biomedical applications which do not require high porphyrin loading for a significant therapeutic efficacy or imaging contrast, such as PDT and fluorescence imaging, this low loading capacity of the membrane is sufficient to produce the desired effects. However, this limits the broad applications of these organic nanoparticles requiring greater porphyrin content.

1.2.2.3 Porphyrin-lipid supramolecular structures

Alternative methods have been investigated to incorporate porphyrins into liposomes by direct conjugation of the porphyrin to phospholipids. Komatsu et al. conjugated a porphyrin to the acyl chains of phospholipids, resulting in a 4:1 phospholipid:porphyrin molecule ratio (Figure 1.4a), and demonstrated that this construct could form liposome-like structures with distinct photophysical properties such as a red-shifted Soret band, strong fluorescence and short triplet state lifetime\textsuperscript{72,73}. Riske et al. also synthesized a porphyrin-lipid conjugate by attaching a porphyrin to the head groups of two phospholipids, resulting in a 2:1 phospholipid: porphyrin molecule ratio (Figure 1.4b)\textsuperscript{74}. They used this porphyrin-lipid to form giant vesicles and investigated the effect of light irradiation on the shape of the vesicle induced by singlet oxygen generation. Although a novel approach for greater porphyrin incorporation into lipid based supramolecular structures, the generated structures still only incorporated 0.5-10 mol\% porphyrin-lipid.

Recently, Lovell et al. synthesized a new type of porphyrin-lipid, which consisted of the conjugation of a porphyrin to the glycerol backbone of the phospholipid, resulting in a 1:1 phospholipid: porphyrin molecule ratio (Figure 1.4c)\textsuperscript{71}. This porphyrin-lipid has the ability to form similar supramolecular structures as regular phospholipids due to their amphipathic property resulting from the hydrophobicity of the acyl chain and porphyrin, and the hydrophilic
head group. These liposome-like structures formed from porphyrin-lipid were termed ‘porphysomes’, generate from 95 mol% porphyrin-lipid. Lovell et al. further discovered that the dense concentration of porphyrins in porphysomes provided unprecedented photonic properties, unlocking a new platform of organic lipid-based structures for thermal-based therapy, imaging and biomedical applications.
**Figure 1.4.** Schematic of different porphyrin-lipids. a) 4:1 phospholipid: porphyrin, with the porphyrin conjugated to the acyl chain of four phospholipids. Reprinted with permission from\textsuperscript{72}. Copyright 2002 John Wiley and Sons. b) 2:1 phospholipid: porphyrin, with the porphyrin conjugated to the head group of two phospholipids. Reprinted with permission from\textsuperscript{74}. Copyright 2009 Elsevier. c) 1:1 phospholipid: porphyrin, with the porphyrin conjugated to the glycerol backbone of the phospholipid. Reprinted with permission from\textsuperscript{71}. Copyright 2011 Nature Publishing Group.
1.2.2.4 Porphysomes

The high packing density of porphyrins achieved in each porphysome (~80 000 porphyrins per 100 nm vesicle) introduced biophotonic properties to organic nanoparticles, not previously possible. In their monomeric form, porphyrins behave as fluorophores and photosensitzers. For example, pyropheophorbide, the porphyrin used in porphysomes, has a combined fluorescence and singlet oxygen quantum yield close to one\textsuperscript{75}. However, when porphyrin-lipid is assembled into a nanovesicle structure (Figure 1.5a, b), the high and close packing density of the porphyrins cause the absorbed light to be predominantly released as heat instead of fluorescence and singlet oxygen generation. As a result, porphysomes are the first organic nanoparticle to have an extinction coefficient as high as inorganic nanoparticles (2.9x10\textsuperscript{9} M\textsuperscript{-1}cm\textsuperscript{-1}), and generate temperatures rivalling inorganic gold nanoparticles (Figure 1.5c).

In addition to porphysome nanovesicles, intrinsically photonic porphyrin-lipid has also been used in place of a regular phospholipid in other nanoparticles. As a result, the porphyrin-lipid imparts inherent photonic properties to these supramolecular structures. Porphyrin nanodiscs (10-30 nm in size) were developed with apolipoprotein A1 and porphyrin-lipid with structurally dependent fluorescence and singlet oxygen generation properties\textsuperscript{76}. While intact, the fluorescence is quenched and singlet oxygen generation is minimal, however, upon disruption of the porphyrin nanodiscs, the fluorescence is restored and singlet oxygen production is significantly increased, providing an activatable nanodisc for fluorescence and photodynamic therapy. The small size of the porphyrin nanodiscs allows for deeper tumor penetration and a diffusion coefficient 5-fold higher than a protein-free control which is promising for less permeable tumors\textsuperscript{76}. 
Figure 1.5. Porphysome nanovesicles. a) Schematic of porphyrin-lipid self-assembly into porphysome nanovesicles. b) Transmission electron microscopy image of porphysomes. c) Thermal images of solutions containing liposomes, gold nanorods or porphysomes and irradiated with a 673 nm laser. Reprinted with permission from\textsuperscript{71}. Copyright 2011 Nature Publishing Group.
1.3 Engineering multifunctional biomedical agents

1.3.1 Design concepts

Multifunctional agents, both in the form of nanoparticles and microparticles, require imparting targeting, imaging and therapeutic properties to a single agent. To accomplish this, one of two approaches can be used: 1) all-in-one and 2) one-for-all. The majority of reported multifunctional agents follow an all-in-one approach, where components, such as small molecules or nanoparticles, that possess a specific singular function such as a drug or imaging contrast agent are combined, resulting in multiple single components packaged in a single agent (Figure 1.6). This is often accomplished by either encapsulating agents within the core, conjugating or adsorbing agents to the surface of the agent, or a combination of these methods. As different imaging modalities often provide complementary information pertaining to the advantages and disadvantages of the partnering modalities, it is often desired to have a multifunctional agent with multimodal imaging capabilities. Furthermore, there may be a synergistic effect exhibited by the combinational administration of specific drugs, and therefore it may be desired to deliver multiple drugs simultaneously. As a result, the schematic shown in Figure 1.6 of an all-in-one approach can become increasingly complex with the addition of multiple imaging agents and drugs.
Figure 1.6. Schematic representation of “all-in-one” and “one-for-all” design approaches for multifunctional agents. All-in-one (left) involves combining different agents (drug loading, imaging, etc.) into a single compartment comprising of many different parts. A one-for-all approach (right) is based on a single building block that possesses intrinsic multifunctional properties.
1.3.2 All-in-one multifunctional agents

Of the growing number of multifunctional nanoparticles currently being reported, specific examples were selected to illustrate the concept of designing all-in-one multifunctional nanoparticles using inorganic and/or organic based nanoparticles demonstrated with iron oxide nanoparticles (IONP) and liposomes.

IONPs are attractive materials to design multifunctional nanoparticles because of their biocompatibility, facile surface modification and functionality, and intrinsic MRI and thermal enhancement capabilities. They are conventionally used as MRI contrast agents as they have been shown to decrease the T2 relaxation time of water. The surface of IONPs is often coated with polymers, such as dextrans, polyethylene glycol or albumin, to increase stability and biocompatibility in vivo and stabilize the colloid solution. These coatings can also be functionalized to bind different materials such as targeting moieties, imaging agents or drugs for additional functionality. IONPs have been functionalized with peptides, antibodies and small molecules for targeted delivery to the desired site.

Multimodal MRI contrast agents have been developed to obtain complementary information from other imaging modalities with MRI. These multimodal imaging probes are most often designed by coating the IONPs with a material, such as a functionalized polymer, that will enable surface conjugation of an additional imaging agent (for example, dyes or quantum dots for optical imaging), targeting moiety or metal chelator for multimodal imaging such as MRI/positron emission tomography (PET), MRI/photoacoustic (PA), MRI/optical imaging. The aim of these multimodal imaging probes are to provide complementary information obtained from multiple imaging modalities. MRI/optical imaging probes were developed with the potential to correlate preoperative diagnostic images with pathological feedback from intraoperative optical devices. MRI/PET probes have also been developed to co-register functional and molecular information from PET with anatomical and functional MR images, providing similar information to PET/ computed tomography (CT) but with the added benefit of significantly reducing radiation exposure.

The design of many of these multimodal imaging probes involves a sequential cumulative synthesis in which one property is added to the core nanoparticle in each step. Using a triple modality imaging probe for MRI/PET/ near infrared fluorescence (NIRF) imaging as an
example, IONPs labeled with copper-64 ($^{64}$Cu)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) for PET and Cy5.5 dye for fluorescence were imaged in a U87MG xenograft bearing mouse with the three imaging modalities (Figure 1.7). IONPs were first prepared by coating the nanoparticles with dopamine, which allowed insertion into human serum albumin (HSA) to form stable nanoparticles under physiological conditions and enabled conjugation of imaging agents. Next, a fluorescent dye, Cy5.5-N-Hydroxysuccinimide (NHS), was then added to the HSA-IONPs to add the optical property to the multimodal imaging probe. Subsequently, DOTA-NHS was added for chelation of $^{64}$Cu for PET imaging. This function-by-function procedure requires multiple purification and buffer-exchange processes to form these multimodal imaging probes, methodologies that are common to an all-in-one design approach for multifunctional nanoparticles.

In a similar manner, IO core multimodal imaging contrast agents have also been expanded to deliver therapeutics by encapsulating within or functionalizing drugs or small interfering RNA (siRNA) to the surface coat for simultaneous therapy and imaging of drug delivery and evaluation of treatment. Yu et al. reported thermally cross-linked superparamagnetic IONPs conjugated with Cy5.5 dye as a dual modality MR/optical probe and also further developed this probe to incorporate anti-cancer drug, doxorubicin, into the polymer shell, bound by the electrostatic interactions between positively charged doxorubicin and the negatively charged shell.

Other approaches to all-in-one multifunctional nanoparticles use several components to encapsulate nanoparticles within other materials. Gianella et al. developed multifunctional nanoemulsions for multimodal imaging and delivery of water insoluble therapeutic drugs. They used a water-in-oil emulsion method to generate nanoparticles formed from a layer of polyethylene glycol (PEG) conjugated lipids encapsulating soybean oil and oleic acid coated IO nanocrystals for MRI. These nanoemulsions also incorporated Cy7 dye for fluorescence imaging, glucocorticoid prednisolone acetate valerate (PAV) for therapy and/or targeted tumor angiogenesis using an Arg-Gly-Asp (RGD) peptide, specific for $\alpha_v\beta_3$ integrin (Figure 1.8). In vivo MRI and fluorescence images confirmed good accumulation in subcutaneous tumors of mice and a decrease in tumor volume using PAV carrying nanoemulsions.
Figure 1.7. Trimodal iron oxide nanoparticles (IONPs) for optical, PET and MR imaging. a) Schematic representation of trimodal imaging probe. b) Transmission electron microscopy image of IONPs in water. In vivo optical (c), PET (d) and MR (e) images of a mouse injected with trimodal IONPs. White arrows indicate tumor region. Reprinted from Xie et al. Biomaterials 2010, 31:3016-3022. Copyright 2010, with permission from Elsevier.
Figure 1.8. Iron oxide (IO) nanoemulsions for optical, MR imaging and therapy. a) Schematic of nanoemulsion. b) Transmission electron microscopy image of IO nanoemulsions. c) In vivo T2-weighted MR images of nanoemulsions encapsulating PAV +/- IO. d) In vivo NIRF images of nanoemulsions labeled with Cy-7. e) Photographs of mice injected with nanoemulsions +/- PAV. Red circles indicate tumor region. Reprinted (adapted) with permission from reference\textsuperscript{94}. Copyright 2011 American Chemical Society.
Liposomes have typically been utilized as delivery vehicles for therapeutic and imaging agents as they can carry both hydrophobic and hydrophilic agents. Zheng et al. developed multimodal liposomes for CT/MRI with the potential goal of enhancing target delineation and guiding radiation therapy\textsuperscript{95}. CT agent, iohexol, and MRI agent, gadoteriol, were co-encapsulated within liposomes and demonstrated the ability to simultaneously provide CT and MR contrast enhancement in rabbits with over a 200% MR signal enhancement and 60% CT signal enhancement maintained over at least 3 hr\textsuperscript{95} and provide prolonged circulation time of iohexol and gadoteriol over the free agents\textsuperscript{96} (Figure 1.9). Alternative approaches to using liposomes as imaging and therapeutic agents aim to reserve the large aqueous core for maximal drug loading. For such an approach, the imaging agents are largely confined to the lipid bilayer. Li et al. developed multifunctional liposomes for MR, NIRF, single-photon emission computed tomography (SPECT) and PET imaging by conjugating the imaging agents to the lipid bilayer and also demonstrated that these liposomes were able to load doxorubicin into the large aqueous core\textsuperscript{97}.

An all-in-one approach enables the generation of multifunctional nanoparticles that may combine the advantages found with specific nanomaterials and/or also produce a synergistic effect. For example, a study by Ashley et al. developed nanoporous particle-supported lipid bilayers to combine the properties of liposomes with mesoporous silica nanoparticles\textsuperscript{98}. Silica nanoparticles are typically used as carrier systems, in particular, mesoporous silica nanoparticles have unique structural features beneficial for controlled delivery of cargo such as tunable nanometer pore sizes and a large surface area that make them attractive scaffolds for imaging and therapeutic applications\textsuperscript{99,100}. Combining mesoporous silica nanoparticles with liposomes into nanocarriers (referred to as protocells) was shown to improve selectivity and stability, enhance targeting specificity, increase loading capacity of multicomponent cargo and control release of these cargos, due to the support and large surface area of the silica core, compared with traditional liposomes\textsuperscript{98}. Protocells were loaded with drug cocktails, siRNA and nanoparticles, such as quantum dots, to demonstrate the multifunctionality of these nanoparticles. Protocells had 1000x higher loading capacity than regular liposomes and remained stable in simulated bodily fluids, becoming unstable and releasing their cargo at pH 5.0 (mimicking an endosomal environment).

Protocells were modified with histidine-rich fusogenic peptides, which promoted endosomal escape. Uptake in Hep3B cells demonstrated that after 15 min of incubation, the protocells and
their contents remained in the endosomes whereas after 4 hr, the lipid, silica and cargo entered the cytosol (Figure 1.10). Other synergistic effects as a result of combinatory materials have also been reported including amplified PA and photothermal effects observed with carbon nanotubes coated with a layer of gold over their single counterparts.

The combination of materials in a nanoparticle may also introduce new imaging modalities not previously possible with the single nanomaterial components, such as coating iron oxide with a gold shell introduced magnetomotive PA imaging which has shown greater PA enhancement over PA imaging with conventional contrast agents. Therefore, despite the many hurdles in developing multifunctional nanoparticles using an all-in-one approach such as multi-step synthesis and potential heterogeneities in formulation, the combination of components may provide advantages to overcome hurdles associated with therapeutic and imaging agent delivery, including stability, loading capacity, controlled release for drugs or enhanced contrast in the case of imaging agents.
Figure 1.9. Multimodal CT/MR liposomal contrast agent. a) Schematic diagram of liposome loaded iohexol and gadoteridol. Courtesy of Dr. Jinzi Zheng. b) Pharmacokinetics of free iohexol (filled square) and gadoteridol (filled circle) and liposomal encapsulated iohexol (empty square) and gadoteridol (empty circle) in mice. c) Contrast enhanced in vivo CT and MR images of white rabbits before and after injection of CT/MR liposomes with observed changes in the heart (H), aorta (A), vena cava (V), carotid artery (C), kidney (K) and spleen (S). Reprinted with permission from reference96 with kind permission from Springer Science and Business Media.
Figure 1.10. Silica supported lipid bilayer nanoparticles (‘protocells’). a) Schematic diagram protocells for targeted delivery of multicomponent cargo. b) Cryogenic transmission electron microscopy image of protocells. Arrows indicate a 4nm thick lipid bilayer. 25nm scale bar. c) Hyper spectral confocal fluorescence microscopy of Hep3B cells 15min (c) and 4hr (d) after incubation with multicomponent loaded protocells. Punctate signal at 15min indicates that all components of the protocell are within the endosomes (c) whereas after 4hr, the cargo and all components of the protocell have been released into the cytosol. Reprinted by permission from Macmillan Publishers Ltd: Nature Materials (Ashley et al. Nat Mater 2011, 10:389-397.), Copyright 2011.
1.3.3 One-for-all multifunctional agents

Many of the multifunctional particles reported have followed an all-in-one approach; however, as several materials possess intrinsic multifunctional capabilities, these properties have been exploited through a one-for-all design, where the building blocks of the particle possess multiple functionalities. Many inorganic nanoparticles possess both imaging and therapeutic potential such as the use of IONPs for MRI and thermal therapy\textsuperscript{102}, gold nanoparticles for CT and radiation therapy\textsuperscript{103} and gold nanorods for both CT contrast enhancement and photothermal therapy (PTT)\textsuperscript{104}. Gold nanocages have been used for both PA imaging\textsuperscript{105} and PTT\textsuperscript{106} (Figure 1.11). Song et al. showed that gold nanocages could detect the sentinel lymph node 33mm below the surface of the skin in rats using PA imaging\textsuperscript{105}. Chen et al. investigated the application of gold nanocages for PTT\textsuperscript{106}. Mice with tumors on both flanks were injected with PEGylated gold nanocages and the tumor on the right flank was irradiated with a laser. The temperature of the tumor heated beyond 55°C. The metabolic activity in the tumor was measured before and after treatment using $[^{18}\text{F}]$ fluorodexoyglucose ($^{18}$F-FDG) PET. Tumors treated with laser irradiation and gold nanocages showed significant decrease in the FDG uptake 24 hr after treatment, however those tumors that did not receive gold nanocages or laser irradiation or both (saline controls) showed no significant difference. This study illustrated the potential of gold nanocages for PTT. These intrinsically multifunctional materials hold promise for potential clinical translation as they simplify the composition, yet maintain their multifunctionality. However, the majority of these multifunctional materials are inorganic which poses concerns regarding potential long-term toxicity and \textit{in vivo} clearance as they are not biodegradable.

Porphysomes, introduced in Section 1.2.2.4, fall under this category of a one-for-all approach. Porphysomes are formed from a single building block, a porphyrin-phospholipid, synthesized by an alkylation reaction between a porphyrin and lyso phospholipid. Through self-assembly processes mimicking liposome formulations, the high packing density of porphyrins within each porphysome resulted in a multidimensional synergistic effect enabling porphysomes to be a powerful tool for multimodal imaging and therapy.

Porphysomes have been applied for a number of biophotonic applications including PTT, PA imaging and activatable fluorescence imaging. PTT relies on the use of an optical absorber to efficiently absorb light and release it as heat to generate temperature increases of 30-35°C,
causing irreversible cell damage. While some chromophores have been applied for PTT\textsuperscript{107 108 109}, they are limited due to their low extinction coefficients, and therefore, inorganic nanoparticles have typically been used\textsuperscript{110 111 112}. Porphysomes enhanced photothermal therapy by selectively accumulating in the tumor due to the enhanced permeability and retention effect\textsuperscript{113} and generated temperatures up to 60°C upon laser irradiation (Figure 1.12a). This increase in temperature resulted in complete eradication of the tumor compared to laser alone and porphysome alone controls (Figure 1.12b, c). Furthermore, the nanostructure assembly of porphysomes provided a unique opportunity to study the ablation of hypoxic and well-oxygenated tumors, comparing PTT and PDT using a single nanoparticle \textit{in vivo}. Jin et al. showed that porphysomes were able to ablate tumors in mice in both hypoxic and hyperoxic environments, identifying that the mechanism of porphosome phototherapy is primarily thermally induced and not photodynamic, based on an intact nanostructure\textsuperscript{114}.

PA imaging is an emerging imaging technique that has received widespread attention due to its ability to provide excellent contrast and good imaging depth. Ideally, PA uses absorbers with high optical extinction coefficients\textsuperscript{115}. PA is based on efficient conversion of light to heat, causing transient thermo-elastic expansion and the production of ultrasound waves, which are then detected to reconstruct the image. Porphysomes are also excellent PA contrast agents and were able to identify the sentinel lymph node and surrounding lymph vessels upon intradermal injection in a rat (Figure 1.13). The mechanism that facilitates porphysomes to be excellent PTT and PA agents is based on the nanostructure assembly of the vesicle. The close packing density of the porphyrins within porphysomes causes the self-quenching required for nonradiative decay (heat) and therefore, disruption of their nanoscale structure results in a decrease in these thermal properties, restoring its original fluorescence and singlet oxygen generation. When detergent was added to porphysomes, a 6-fold decrease was observed in the PA signal, indicating a less efficient conversion from light to heat. In addition, when porphysomes were injected into mice bearing xenografts, after 48 hr, fluorescence was observed in the tumor, demonstrating that upon accumulation and uptake in the tumor, the nanostructure was disrupted and fluorescence restored. This structure dependent property of porphysomes enables activatable fluorescence imaging, which could serve as a potential sensor in future applications.
Figure 1.11. Intrinsically multifunctional gold nanocages for PA imaging and photothermal therapy. a) Transmission electron microscopy image of pegylated gold nanocages. b) In vivo PA images of gold nanocages for sentinel lymph node mapping before (i) and 194min after (ii) injection; BV: blood vessel, SNL: sentinel lymph node. Reprinted (adapted) with permission from Song et al. Nano Lett 2009, 9:183-188. Copyright 2009 American Chemical Society. c) (i) Photograph showing a subcutaneous tumor on the right flank of a mouse after intravenous injection of gold nanocages. Mice were injected with either gold nanocages (ii, iv) or saline (iii, v). Thermal images of the tumor on the right flank during laser irradiation at 1min (ii, iii) and 5 min (iv, v). d) $^{18}$F-FDG PET/CT co-registered images of mice with saline injection before (i) and after (iii) laser irradiation and mice with gold nanocage injection before (ii) and after (iv) laser irradiation. T: tumor. Reprinted with permission from reference$^{106}$. 

**Figure 1.12.** Porphysomes for photothermal therapy (PTT). a) Thermal image of mice bearing tumor xenografts after being intravenously administered porphysomes or PBS and irradiated with a laser for PTT. b) Resulting tumor response after PTT treatment. c) Survival curve of mice receiving PTT. Reprinted with permission from \(^7\). Copyright 2011 Nature Publishing Group.

**Figure 1.13.** Porphysomes for photoacoustic (PA) imaging. PA images of rat lymphatic system before (left) and after (right) the administration of porphysomes (red – lymph node, cyan – secondary lymph vessel, yellow – inflowing lymph vessel). Reprinted with permission from \(^7\). Copyright 2011 Nature Publishing Group.
Beyond photonics, the metal chelating property of porphyrins provides multimodal imaging capabilities for porphysomes without requiring exogenous chelating agents or other modifications. $^{64}\text{Cu}$, a radioisotope used for PET, was inserted into porphysomes in a simple, fast and robust synthesis procedure (Figure 1.14a)$^{116}$. $^{64}\text{Cu}$ porphysomes were able to identify a tumor in a clinically relevant orthotopic prostate cancer model and small micrometastases in the bone (Figure 1.14b)$^{117}$. Other metals may also be inserted into the porphyrin such as manganese for MRI expanding the future potential applications of multifunctional porphosome nanovesicles (Figure 1.14c, d)$^{118}$.

In addition to the multimodal imaging and therapy applications of porphysomes, the most notable characteristic is their organic nature and thus, the biocompatibility and biodegradation of porphysomes. Porphysomes are composed of porphyrin-lipid in which the porphyrin was derived from *Spirulina Pacifica* algae. Intravenous injection of porphysomes at high doses (1000 mg/kg) did not induce any acute toxicity in mice, demonstrating their biocompatibility as a result of their organic nature. Mice were healthy without any changes in weight and behavior, and no significant changes were observed in liver function, red blood cell counts, white blood cell counts and histology of the liver and kidneys two weeks after administration$^{71}$. Furthermore, porphysomes were enzymatically degraded *in vivo* into their porphyrin conjugates in the liver and spleen which are both part of the mononuclear phagocyte system$^{119}$. The biocompatibility and biodegradation of porphysomes suggests that the future of nanoparticles for biophotonic applications resides in organic nanoparticles, which maintain the strong light interaction property of inorganic materials yet overcomes their biodegradation concerns. Furthermore, porphysomes maintain the capacity to load large amounts of payload, reminiscent of liposomes as drug carriers. Porphysomes merge the niches of both organic and inorganic nanoparticles into a single nanoparticle, overcoming the common limitations of both materials.
Figure 1.14. Metallic porphysomes. a) Schematic of a $^{64}$Cu porphosome radiolabelling. b) PET image of radiolabelled $^{64}$Cu porphysomes injected intravenously in an orthotopic prostate cancer model, with the ability to I) delineate the prostate tumor (white arrow) (Reprinted with permission from$^{116}$. Copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim), and II) boney metastases (arrows). Reprinted with permission from$^{117}$. Copyright 2013 American Chemical Society. c) Schematic of inserting porphyrin-lipid with manganese. d) Magnetic resonance imaging of manganese porphysomes (MnPS) and controls. Reprinted with permission from$^{118}$. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.
1.3.4 All-in-one versus one-for-all

Both all-in-one and one-for-all design approaches have advantages and disadvantages (Table 1.1). However, the factors most prominently distinguishing the two approaches are related to potential clinical translation – manufacturing methodologies and potential toxicity. In an all-in-one design, the synthesis of multifunctional agents requires the addition of single functional moduli in a step-by-step manner and therefore also requires multiple purification processes. Such lengthy and multistep synthesis are fairly time consuming and purification at multiple steps often decreases yield, which may result in high production costs and potentially limit widespread clinical use. Furthermore, there is also the potential of heterogeneous formulations as a result of multiple cargos loaded into a single nanoparticle or microparticle. While a step-by-step procedure may limit the amount of heterogeneity and increase reproducibility, it comes at the cost of increased synthesis time and expenses. Combining multiple steps may decrease the number of synthesis steps, but may decrease reproducibility and increase heterogeneity amongst agents in the same production batch.

Furthermore, biodistribution, biocompatibility, toxicity and biodegradation will also need to be considered for the individual components of the formulation, as many multifunctional nanoparticles and microparticles are composed of both organic as well as inorganic components, which are not cleared from the body or degraded in the same manner. Furthermore, particles of different size are cleared from the body via different routes (liver or renal filtration\(^{120}\)) and therefore, a multifunctional agent with smaller particles packaged within it may have several routes of clearance if it is degraded \textit{in vivo}. Alternatively, an one-for-all approach can minimize these hurdles as a single building block will not require multiple synthesis steps, but will ensure a homogenous formulation and only require biodistribution, biocompatibility, toxicity and biodegradation studies for the single component.

The main advantage associated with an all-in-one approach is the readily available components, many based on materials already used in the clinic, which may decrease challenges faced for regulatory approval. Furthermore, the characteristics of each individual component are well known; therefore, by compiling each component into a single particle, one can predict the multifunctional characteristics of that particle. Several of the current nano- and micromaterials possessing intrinsic multifunctional capabilities used in a one-for-all approach are predominantly
inorganic nanoparticles. These materials are often limited in their ability to carry high concentrations of a drug or are restricted to the use of specific drugs. Furthermore, inorganic nanomaterials are often specific for an individual imaging modality. As a result, these materials may be limited in flexibility for multiple desired therapeutic and/or imaging functionalities. Therefore, a one-for-all approach may require the synthesis and discovery of new materials that are intrinsically multifunctional, which will need to undergo their own lengthy process to acquire their own regulatory approval. Furthermore, it may be unknown how the new compound may behave in vivo and its associated toxicity. Both all-in-one and one-for-all approaches have a potential advantage of discovering new imaging platforms and synergistic effects associated with either the combining of multiple materials (as in all-in-one approaches) or the formation of new materials to produce unprecedented properties (as in one-for-all).

The pros and cons of using multifunctional nanoparticles has been previously discussed, with the disadvantages predominantly focussing on the increasing complexity of nanoparticles with the addition of multiple functionalities largely amongst studies using an all-in-one approach. While the majority of the discussions thus far have focussed on nanoparticles, these concepts and approaches are also applicable to microparticles. In this work, we sought to apply the concept of one-for-all to microparticles, where the ultimate goal of this approach is to simplify the composition of the multifunctional microparticle while maintaining the desired properties.
Table 1.1 Comparison of All-in-One and One-for-All multifunctional designs

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<th>All-in-One</th>
<th>One-for-All</th>
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<td>Multiple Components Combined</td>
<td>Intrinsically Multifunctional Building Blocks</td>
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<td>Components readily available</td>
<td>Lowers clinical translation hurdles</td>
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<td>Some components may be already approved for use in humans</td>
<td>- Simplified synthesis (‘one pot synthesis’)</td>
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<td>Combinations of materials may provide synergistic effects in enhancing properties</td>
<td>- Easy reproducibility and scale-up; homogenous formulations</td>
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<td>- Simple toxicity studies</td>
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<td>- High yield</td>
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<td>Discover unprecedented enhancement in properties</td>
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<td>Cons</td>
<td>Multi-step synthesis and purification</td>
<td>Current multifunctional materials are limited in high payload delivery of drugs and are specific to a single imaging modality</td>
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<td>Potential heterogeneous formulations</td>
<td>Synthesize new inherently multifunctional building blocks which may require a lengthy regulatory approval process for the new compound</td>
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<td>Difficulty scaling-up and reproducibility due to multiple steps</td>
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1.4 Porphyrin-lipid microstructures

The studies thus far utilizing porphyrin-lipid have introduced a platform for organic biophotonic nanoparticles. The aim of the present work was to expand the utility of porphyrin-lipid to micrometer-scale supramolecular structures using the information gathered about porphyrin-lipid through its nanostructure investigations.

Two distinct structures were developed and studied: 1) porphyrin shell microbubbles composed of a monolayer of porphyrin-lipid encapsulating a gas (Figure 1.15a) and 2) giant porphyrin vesicles formed from a bilayer of porphyrin-lipid with a large aqueous core (Figure 1.15b). While similar in size scale and composition, these two porphyrin microstructures vary in their properties and utility.

In Chapter 2, porphyrin shell microbubbles (pMBs) were developed as intrinsic multimodality US-based contrast agents based on a one-for-all approach. The multifunctionality of porphyrins rendered them an optimal building block for the generation of multimodal agents. First, the development of pMBs and the physical, acoustic and optical properties of microbubbles generated with porphyrin-lipid were reported. These pMBs were monodisperse, generated a greater shell stiffness compared to clinical lipid microbubbles and had intrinsic US and PA properties. Secondly, by increasing the porphyrin-lipid content of pMBs, ordered aggregation was observed, generating fluorescence. Therefore, Chapter 2 describes the development and demonstration of both a bimodal (US and PA) and trimodal (US, PA and fluorescence) contrast agent for multimodality imaging.

In Chapter 3, the response of pMBs to low frequency US was investigated. pMBs were found to convert into nanobubbles and maintain their PA and fluorescence imaging properties, while decreasing their ultrasound scattering behavior. This property was utilized for the delivery of porphyrins to a solid tumor without the reliance on the enhanced permeability and retention effect, in which optical imaging could be used to verify successful delivery of the porphyrins. The generation of multimodality ultrasound and porphyrin-based contrast agents has potential biomedical applications in imaging and therapy of cancer or other diseases.

In Chapter 4, the generation of giant porphyrin vesicles (GPVs) are described, in which confocal microscopy was used to investigate the bilayer porphyrin-lipid response to high intensity laser
irradiation. GPVs responded to laser irradiation by forming large pores in the membrane, allowing the trafficking of biomolecules across the porphyrin-lipid membrane. Furthermore, these pores resealed over time, permitting robust and repeated pore formation upon laser irradiation. Changes in these properties were investigated by varying the laser fluence, which enabled size-selective release of cargo. Furthermore, deoxyribonucleic acid (DNA) hybridization and avidin-biotin binding were also conducted as demonstrations of reactions within the GPVs. GPVs have potential applications in protocell development and as low-volume microreactors. These microscale structures are the topic of the following chapters.
Figure 1.15. Schematics of microstructures generated with porphyrin-lipid. a) Porphyrin shell microbubbles (pMBs). b) Giant porphyrin vesicles (GPVs).
Chapter 2
Intrinsically Multimodal Ultrasound-Based Contrast Agents

2.1 Acknowledgements
The bulk of this chapter is a modified and reformatted merge of two separate manuscripts entitled “Porphyrin shell microbubbles with intrinsic ultrasound and photoacoustic properties”, published in the *Journal of the American Chemical Society*\textsuperscript{121}, and “Aggregate enhanced trimodal porphyrin shell microbubbles for ultrasound, photoacoustic and fluorescence imaging”, published in *Bioconjugate Chemistry*\textsuperscript{122}. Under the supervision of Dr. Gang Zheng, my contribution to this work included designing and carrying out most of the experiments, analyzing and interpreting the data and writing the manuscript. Thanks to Brandon Helfield and Ben Leung for the acoustic measurements, Drs. Mansik Jeon and Chulhong Kim for the photoacoustic imaging, Cheng Jin for help with the *in vivo* experiments and Drs. Jonathan Lovell, David E. Goertz and Brian C. Wilson for insightful discussions and editing of the manuscript.

2.2 Abstract
Microbubbles are currently used as ultrasound (US) contrast agents and as delivery vehicles for site-specific US-triggered drug and gene delivery. Multimodal US-based imaging methods have been applied preclinically to assess and validate the effectiveness and fate of microbubbles in imaging and therapy. Porphyrin-phospholipid conjugates were used to create photonic microbubbles comprising a porphyrin shell, termed porphyrin shell microbubbles or pMBs, and their acoustic and photoacoustic (PA) properties were investigated. The inclusion of porphyrin-lipid in the microbubble shell increased the yield, improved the serum stability and generated a narrow volumetric size distribution with a peak size of $2.7 \pm 0.2 \mu$m. Using an acoustic model, we calculated the pMB shell stiffness to be 3-5 times greater than clinically used lipid microbubbles. pMBs were intrinsically suitable for both US and PA imaging with a resonance frequency between 9-10 MHz. Furthermore, by inclusion of a greater porphyrin-lipid content, we presented the first intrinsically trimodal microbubbles with US, PA and fluorescence properties. They also have potential to be expanded to other imaging modalities such as magnetic resonance
imaging and nuclear imaging. The distinctive properties of pMBs make them potentially advantageous for a broad range of biomedical imaging and therapeutic applications.

2.3 Introduction

2.3.1 Ultrasound imaging

US is a widely used imaging modality in clinical practice. One application is to use US to image soft tissue in the body using a pulse-echo approach and brightness mode (B-mode) display. US pulses are transmitted from a transducer that propagate through the body and interact with tissues. The US pulse can be transmitted through the tissue, reflected or absorbed by the tissue. Different tissues have different acoustic impedances that are intrinsic properties of the tissue, dependent on the density and speed of sound in the tissue. Two materials with large differences in acoustic impedance will result in significant reflection of the US pulse back to the transducer, known as an echo. An US pulse may undergo several of these interactive processes with tissue, in which some of the pulse may be reflected, some may be absorbed and some may continue to propagate through the tissue to interact with structures deeper in the body. The US transducer behaves as both the transmitter of the US pulses and receiver of the US echoes. The detected US echoes are linearly related to the transmitted US pulse. These echoes from many US pulses are processed and combined to generate an image.

US transducers typically operate in the 1-20 MHz range. The frequency of US is inversely proportional to depth penetration and proportional to image resolution. Low frequency US has greater depth penetration, however has lower image resolution than high frequency US. High frequency US has been used clinically for superficial structures and preclinically for small animal imaging as deep penetration depth is not necessary.

2.3.2 Contrast enhanced ultrasound imaging

Microbubble contrast agents display a large acoustic impedance mismatch between blood vessels and surrounding tissue as a result of their highly scattering gas core. Commercially available US microbubbles, which are typically 1-10 µm in diameter, are composed of fluorinated gases encapsulated within a biocompatible shell comprised of lipids, albumin or polymers. The shell minimizes the surface tension at the gas-liquid interface, while the fluorinated gases have low solubility in blood. Both components stabilize the gas core and prolong the in vivo life-time.
Due to their size, microbubbles have optimal acoustic responses in the megahertz range for US imaging. Furthermore, microbubbles have nonlinear properties as a result of asymmetrical oscillations in an US field, in which the microbubble expansion is greater than its contraction, giving rise to an US echo that contains nonlinear components\textsuperscript{125}.

Nonlinear US imaging techniques have been developed to specifically image microbubble contrast agent. Several nonlinear US imaging techniques have been proposed\textsuperscript{126}, however, in the interest of the current studies, contrast imaging mode in the US imaging instrument used in the majority of these studies (Vevo 2100) used an amplitude modulation technique which will be described as follows. Amplitude modulation uses multiple US pulses to differentiate linear and nonlinear signals. For example, if two incident pulses are transmitted, where the first pulse is scaled by a factor $n$ compared to the second pulse, after reflection by a scatterer, the two pulses can be subtracted after scaling the second pulse by a factor $n$. If the pulses interact with a linear scatterer, the two pulses will cancel. However, if the pulses interact with a nonlinear scatter such as a microbubble, which will respond differently to different amplitudes of incident US, subtraction of the two pulses after scaling, will not cancel. These differences in response have been implemented in US contrast imaging modes. It is important to note that tissue may also respond nonlinearly to US, although this typically occurs at higher acoustic pressures than necessary for microbubble nonlinear responses, however, this may generate background noise in the contrast image.

Microbubbles are routinely used in the clinic for low frequency applications ($f < 5$ MHz) to observe blood flow in the heart, liver and kidneys\textsuperscript{35} for diagnosis and treatment guidance of focal lesions\textsuperscript{127-129}. More recently, microbubble applications that exploit conventional diagnostic imaging platforms with higher frequency transducers ($f = 5$-15 MHz) are beginning to gain interest, including imaging of the breast\textsuperscript{130} and carotid neovascularization detection\textsuperscript{131}. In addition, the use of microbubbles for molecular imaging\textsuperscript{132,133}, US-triggered drug and gene delivery\textsuperscript{28,134} and vasculature remodelling\textsuperscript{135} have been investigated preclinically for various diseases. In these preclinical studies, fluorescent molecules are often incorporated into the microbubble shell to verify targeting\textsuperscript{136} and/or US-mediated microbubble disruption\textsuperscript{137,138}. However, the influence of these fluorophore molecules on the properties of microbubbles has not been widely investigated, and normally the fluorophore fraction within the shell is very small.
2.3.3 Photoacoustic imaging

PA imaging is a technology that images optical absorbers and relies on the conversion of photons into ultrasonic waves. It is based on the local absorption of short (~ns) pulsed laser light in tissue, resulting in transient thermoelastic expansion and the generation of pressure waves, which are then detected by a conventional ultrasound transducer. In PA imaging, the US transducer only behaves as a receiver of US waves. PA imaging provides high-resolution imaging with significantly greater penetration depth than other optical imaging modalities since acoustic scattering in tissue is about three magnitudes less than optical scattering. PA imaging can provide functional and metabolic information using endogenous optical absorbers such as melanin and hemoglobin, in addition to molecular and genetic imaging with the use of exogenous contrast agents\textsuperscript{139}.

2.3.4 Contrast enhanced photoacoustic imaging

With PA imaging, exciting different molecules at different wavelengths can detect a wide variety of molecules. In addition to endogenous absorbers to provide PA contrast, exogenous absorbers have also been developed. Exogenous absorbers are most ideally developed to absorb in the near-infrared window (700 nm-1000 nm) where optical attenuation in tissue is low\textsuperscript{140}. This criterion serves two purposes: to enable deep penetration of light and to avoid absorption spectral overlap with blood, one of the most prominent endogenous absorbers in the near-infrared window.

Metallic contrast agents, such as gold nanoparticles, have dominated the field of contrast agents for PA imaging due to their long wavelength absorption, large absorption cross section and high thermal conversion efficiency. Furthermore, gold nanoparticles in particular allow tuning of the optical absorption peak wavelength by controlling the physical dimensions of the metallic layer\textsuperscript{141}. The field of PA contrast agents is beginning to see a shift from metallic contrast agents to organic contrast agents to provide similar PA contrast properties but with improved biocompatibility and biodegradation.

There has been a recent growing interest in combined US and PA (US/PA) imaging to produce simultaneous morphological and physiological information through the mechanical contrast generated from US and optical contrast from PA\textsuperscript{142-146}. Despite the complementary information
provided by this dual-modality approach, limitations on US contrast and interference by endogenous optical absorbers in PA persist without the use of a contrast agent. Only a few dual-modality US/PA contrast agents have been proposed, many of which involve complicated encapsulation or tethering of the optical absorber to an ultrasound-compatible micro- or nano-bubble[^147-150]. Here we report the first intrinsically dual-modality US/PA agent and trimodality US/PA/fluorescence agent with a synthesis based upon a one-for-all design approach, through the formation of a microbubble shell with porphyrin-lipid conjugates.

### 2.4 Materials and methods

#### 2.4.1 Dual modal pyropheophorbide porphyrin shell microbubbles

##### 2.4.1.1 Formation and agent handling

Lipid films were prepared in 12 mm x 35 mm clear glass threaded vials (Fisher Scientific) by combining 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids) dissolved in chloroform with porphyrin-lipid (Sn2 pyropheophorbide-lipid as described previously with the modification of using 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine as the starting lipid instead of 1-palmitoyl-2hydroxy-sn-glycero-3-phosphocholine[^119]).

Films were dried under a stream of nitrogen gas and further dried under vacuum for 30 min. Lipid films were stored under argon gas at -20°C until hydration with 0.01 M filtered phosphate buffered saline (PBS) (150 mM NaCl, 10m M phosphate, pH 7.4) and polyoxyethylene-40 stearate (PEG40S, Sigma-Aldrich) dissolved in deionized water. The total lipid concentration was either 0.5 mg/ml or 1 mg/ml in a 0.5 ml or 1 ml volume. The headspace of each vile was filled with the indicated gas. Each vial was placed in a 65°C heated water bath to raise the solution temperature above the transition temperature of the incorporated lipids and then sonicated for 30 s (Bransonic Model 2510) until a homogeneous mixture was achieved in order to ensure dispersion of the lipid film into solution. The headspace was filled with gas once again and stored at 4°C until activation. Prior to activation, agent vials were equilibrated to room temperature. Microbubbles were formed with a Vialmix agent activator (Lantheus Medical Imaging). This activates the agent through mechanical agitation process for 45 s. After activation, agent vials were left to rest for 15 min in order to cool to room temperature before use.
and used within 3 hr. For all measurements, microbubbles were gently mixed by hand for 10 s and then decanted for 1 min before extracting a sample from the bottom of the vial.

2.4.1.2 Characterization of concentration and size distribution

pMB and unimodal microbubbles were formulated using porphyrin-lipid at the indicated molar %, 10 mol% PEG40S and the remaining DSPC at a total lipid concentration of 0.5 mg/ml in 1 ml volumes as previously described. The headspace of the vial was filled with sulfur hexafluoride (SF₆, Sigma-Aldrich), perfluorobutane (C₄F₁₀, PFB, Fluoromed L.P) or perfluoropropane (C₃F₈, PFP, Fluoromed L.P) gas. The size distribution and concentration (number of microbubbles/ml) of each formulation was measured with a Coulter Counter Multisizer Z3 (Beckman Coulter Inc.). Varying volumes (5–50 µl) of microbubbles were extracted and added to 10 ml of Isoton-II electrolyte solution (Beckman Coulter Inc.) to obtain a microbubble count in the range of 100 000–300 000. A background count of buffer was taken prior to measurement and subtracted. Dilution was accounted for in the calculation of the microbubble concentration. The number and size distribution were measured using a 30 µm aperture, which detected microbubbles with diameters in the range 0.76-18 µm. For each microbubble formulation, three samples were measured and averaged.

To determine the approximate porphyrin-lipid content per microbubble, microbubbles were separated from submicrometer particles using a centrifugation method¹⁵¹. Two vials of pMBs containing 15 mol% porphyrin-lipid, 75 mol% DSPC, 10 mol% PEG40S at a total lipid concentration of 0.5 mg/ml in a 1 ml volume with the headspace filled with perfluoropropane gas, was activated and cooled to room temperature. After decantation for 1 min, 600 µl of the total pMB volume was extracted from the bottom of each vial. The microbubble suspension was collected in 3 mL syringes that were used as flotation columns and placed in a bench-top centrifuge (Sorvall Legend RT Plus, ThermoFisher Scientific, model #75004373). The initial size distribution was measured using a Coulter Counter. Centrifugation (8 min, 50 G) was performed to separate submicrometer particles from the pMBs. The microbubble cake resting against the syringe plunger contained microbubbles > 1.5 µm. The remaining solution, containing submicrometer particles formed from lipids that did not form a microbubble shell, was discarded. The microbubble cake was resuspended with 1 ml of 0.01 M PBS and repeatedly washed with 7 cycles of centrifugation and resuspension in PBS. After the final wash, pMBs
were resuspended in 200 µl of 0.01 M PBS and the size distribution and concentration was measured with a Coulter Counter. To determine the porphyrin-lipid concentration, a standard curve was generated using ultraviolet spectroscopy (CARY 50 UV/VIS Spectrophotometer, Varian Inc.) and fluorescence (Fluoromax fluorometer, Horiba Jobin Yvon) to determine the concentration of porphyrin-lipid conjugates suspended in methanol with an extinction coefficient of 97 000 M⁻¹ cm⁻¹ at 410 nm and the corresponding fluorescence emission intensity at 670 nm using 2 nm slit widths and 410 nm excitation. 10-20 µl of the washed pMB suspension was added to methanol for a total volume of 1 ml in order to disrupt the microbubble structure and the concentration of porphyrin-lipid was determined. The amount of porphyrin-lipid per microbubble was calculated by dividing the porphyrin-lipid concentration by the microbubble concentration.

2.4.1.3 Microbubble stability in serum

Microbubble stability was compared between unimodal microbubbles (90 mol% DSPC, 10 mol% PEG40S) and pMBs (15 mol% porphyrin-lipid, 75 mol% DSPC, 10 mol% PEG40S) in fetal bovine serum (FBS) at 37°C. Microbubbles were incubated with FBS at a concentration of 4.7 x 10⁸ microbubbles/ml in a 96-well plate. The absorbance was recorded at 580 nm with a SpectraMax Plus384 plate reader (Molecular Devices) at 0, 1, 5, 10 and 15 min and then in 15 min intervals up to 4 hr after beginning incubation at 37°C. The plate was mixed for 5 s prior to each measurement. The stability of three samples was measured for each formulation and averaged.

2.4.1.4 Acoustic characterization

For acoustic characterization, pMB samples were composed of 15 mol% porphyrin-lipid, 10 mol% PEG40S, 75 mol% DSPC in a 0.5 mg/ml total lipid concentration with PFP gas. After 1 min decantation, the total volume extracted from the bottom of each vial was 0.5 ml using an 18-gauge needle. Following extraction, the agent was placed within a new 1.5 ml vial, topped with PFP gas and loosely sealed with parafilm (Fisher Scientific). The vial was then gently mixed by hand for 10 s prior to Coulter Counter and attenuation measurements. All measurements were carried out at room temperature.
Frequency-dependent attenuation measurements were performed to assess the acoustic response using a method initially described by de Jong et al. For this, a narrowband pulse-echo approach was employed, similar to the configuration reported in Goertz et al. Two transducers (Model #595396, 5 MHz, 76 mm focus, 12.7 mm diameter, Olympus NDT Canada Inc.; Model #ISO2002HR, 20 MHz, 38 mm focus, 6.35 mm diameter, Valpey-Fisher) were used to cover the frequency range 1.5-27 MHz. The transducers were situated within a water bath and their beams passed through a sample chamber containing diluted agent through a mylar window and were focused upon aluminum reflectors. An arbitrary waveform generator (Model AWG5002C, Tektronix) was used to generate pulses with center frequencies spaced at 0.5 MHz intervals. The input waveforms were then amplified by 53 dB (Model A-150, ENI) and then sent to one of the two transducers. On receipt, the echoes were amplified by 35 dB (Model AU1583, Miteq), band-pass filtered and then digitized (400 MHz sampling frequency, Agilent Technologies Inc.) for offline analysis. The peak negative pressure at the focus for all waveforms was calibrated to be 25 kPa using a 0.075 mm diameter needle tip hydrophone (Model 1544, Precision Acoustics).

Experiments were conducted with agent diluted (1:7500) in saline (0.9% NaCl) and acoustic measurements commenced at the 1 min following dilution. Experiments were conducted once per sample for a total of 3 samples. Size measurements were performed immediately after having extracted the agent from the vial, and attenuation experiments were performed following the Coulter counter measurements using samples from the same vial extraction. Microbubble shell properties were estimated using the general approach initially described in de Jong et al. The specific details of the approach employed here are discussed elsewhere.

2.4.1.5 In vivo ultrasound imaging

A NIH swiss female athymic nude mouse bearing a subcutaneous MDA-MB-231 tumor in the left flank was injected via the tail vein using a 26 gauge indwelling catheter with a 50 µl bolus of \(~7.5\times10^8\) pMBs/ml followed by a 150 µl saline flush. The mouse was age 12-14 weeks at the time of injection. The tail was warmed using a heated saline pouch to dilate the tail vein prior to catheterization. The tumor was imaged using the Philips iU22 xMATRIX ultrasound system (Philips Medical Systems) and the L12-5 probe with an operating frequency of 5-12 MHz and a mechanical index of 0.07 during simultaneous contrast mode and B-mode images.
2.4.1.6 Optical, photoacoustic, and ultrasonic characterization

Absorption spectra of pMBs and porphyrin-lipid conjugates were measured using a CARY 50 UV/VIS Spectrophotometer (Varian, Inc.) in the range of 300 nm to 800 nm and normalized to the maximum absorbance. Unimodal microbubbles and pMBs were measured in 0.01 M PBS, while the absorption spectrum of the porphyrin-lipid conjugate was determined in methanol.

PA and US characterization was completed using a custom made set-up. A wavelength-tunable-optical parametric oscillator laser (Surelite OPO PLUS; Continuum; wavelength tunability: 650 nm to 1060 nm) pumped by a Q-switched Nd:YAG laser was employed to provide laser pulses with a width of 5 ns and a repetition rate of 10 Hz. When the collimated laser pulse traveled through a homemade axicon lens, a ring-shaped light beam was formed. This was focused through an optical condenser and coaxially aligned with the ultrasound focal zone in the water tank. Generated PA waves were detected by a single-element 10 MHz transducer (V315; Panametrics-NDT), with axial and transverse resolutions of 125 µm and 140 µm, respectively. A sample container was positioned underneath the water tank with an optically and acoustically transparent window covered by a thin transparent membrane coupled with ultrasound gel. Aqueous samples filled the wells that were 9 mm diameter and 3 mm deep. The detected PA signals were first amplified by a low-noise amplifier (5072PR, Panametrics-NDT) and recorded by a digital oscilloscope (TDS 5054, Tektronix). For US imaging, we utilized the same transducer and raster scanning system as for PA imaging. In the US mode, the low-noise amplifier served as both an US pulse transmitter and receiver. The PA and US generation were measured and imaged from three samples: unimodal microbubbles (0 mol% porphyrin-lipid), pMBs (15 mol% porphyrin-lipid) and unimodal microbubbles co-incubated with the same molar concentration of pyro acid as present in pMBs.

2.4.2 Trimodal pyropheophorbide porphyrin shell microbubbles

2.4.2.1 Fabrication and characterization

Lipid films were prepared in 12 mm x 35 mm clear glass threaded vials (Fisher Scientific) by combining 0.5 mg of 50 mol% porphyrin-lipid together with 40 mol% 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 10 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol) - 2000] (DSPE-mPEG2000) in chloroform, and dried by nitrogen gas and vacuum. Unimodal microbubbles were generated by replacing the porphyrin-lipid with
DSPC. Lipid films were then rehydrated with 1 ml of 10 vol% propylene glycol, 10 vol% glycerol and 80 vol% PBS. Samples were then briefly heated and sonicated to disperse the lipid film, topped with PFP gas and then mechanically agitated using a VialMix shaker for 45 s. After activation, vials containing the agent were passively cooled to room temperature over 15 min before use and then used within 3 hr. For use, the microbubbles were gently mixed by hand for 10 s and then decanted for 2 min before extracting a sample from the bottom of the vial. The resulting micrbubbles were separated from sub-micrometer particles by differential centrifugation\textsuperscript{154} at 50 G for 8 min. The resulting microbubble cake was re-suspended with 10 vol% propylene glycol, 10 vol% glycerol and 80 vol% PBS.

The size distribution and concentration of each formulation was measured with a Coulter Counter Multisizer Z3 (Beckman Coulter Inc.). For this, 20 µl of microbubbles were extracted and added to 10 ml of Isoton-II electrolyte solution (Beckman Coulter Inc.) to obtain a microbubble count in the range of 100 000-300 000. A background count of buffer was taken immediately prior to this measurement and subtracted. Dilution was accounted for in calculating the microbubble concentration. The number and size distribution were measured using a 30 µm aperture which detected microbubbles with diameters in the range 0.76 µm - 18 µm. Three samples were measured and averaged for each microbubble formulation.

2.4.2.2 Optical characterization

Absorption spectra were determined by UV spectroscopy (CARY 50 UV/VIS S3 Spectrophotometer, Varian Inc.) in PBS for the pMB spectrum and in 1% Triton X-100 for the pyro-lipid spectrum. Circular dichroism spectra were determined in PBS for unimodal microbubbles and trimodal pMBs and in 1% Triton X-100 for pyro-lipid using a J-815 Circular Dichroism Spectrometer (JASCO Inc.). Fluorescence spectra were determined in PBS using a Fluoromax fluorometer (Horiba Jobin Yvon) with an excitation wavelength of 410 nm and a 5 nm slit width, collecting the fluorescence emission from 600 nm to 800 nm using a 5 nm slit width.

2.4.2.3 Trimodal imaging in solution

PBS, unimodal microbubbles and pMB solutions were placed in an US-transparent holder made of 20% gelatin at a concentration of 120 µl of $10^9$ microbubbles/ml. US and PA imaging were
conducted using a commercial PA imaging system (Vevo LAZR: FUJIFILM VisualSonics, Inc.), operating with a 21 MHz transducer in both US and PA modes. PA spectra were obtained from 680 nm to 800 nm. Fluorescence images were obtained using a whole-body small animal imager (CRi Maestro: Caliper Life Science Inc.) under blue-light excitation (435 nm – 480 nm), 515 nm long pass detection and an integration time of 600 ms.

2.4.2.4  

In vivo trimodal imaging

Animal experiments were performed in compliance with institutional animal care approval (University Health Network, Toronto) using nude mice bearing KB xenografts. The model was generated by subcutaneous inoculation of 2x10^{6} KB cells in PBS in the flank of adult female Nu/nu mice. Imaging was performed when the tumors achieved a surface diameter of 4-5 mm (~10 days). For the imaging, the mice were anesthetized with 2% (v/v) isoflurane inhalation and 280 µl of 2x10^{9} microbubbles/ml unimodal microbubbles or trimodal pMBs was injected via the tail vein. Acoustic coupling gel was applied over the tumor and the mice were then imaged over the tumor region in both US and PA mode. Final PA images were obtained by subtracting images acquired at 704 nm and 750 nm where the pMBs do not absorb significantly in order to remove background signal as a result of blood. At 4 hr after microbubble injection the mice were sacrificed by cervical dislocation under general anesthetic and the tumors were resected, placed in PBS and imaged in fluorescence mode (under blue-light excitation (435 nm – 480 nm), 515 nm long pass detection, 4000 ms integration time).

2.5  

Results and discussion

2.5.1  

Dual modal pyropheophorbide porphyrin shell microbubbles

Previously, new paradigm for forming phospholipid bilayer nanovesicles entirely from porphyrin-lipid conjugates that have structure-dependent photonic properties was introduced^{119,155}. Here we describe microbubbles formed from a porphyrin-lipid shell encapsulating a fluorinated gas, termed ‘porphyrin shell microbubbles’ or pMBs (Figure 2.1). These were synthesized using a mechanical agitation method, as follows. Lipid films of porphyrin-lipid (1-stearoyl 2-pyropheophorbide sn-glycero-3-phosphocholine) and DSPC were rehydrated with a solution of PEG40S and PBS, and the headspace of the vial was filled with one of various fluorinated gases. After dispersion of the lipid film, microbubbles were formed using a
Vialmix™ (Lantheus Medical Imaging) agent activator, which is used for activation of a commercial microbubble (Definity™, Lantheus Medical Imaging). With this approach, high-shear gas dispersion in the aqueous solution occurs and results in the formation of lipid encapsulated microbubbles.

To investigate the effects of porphyrin-lipid on the microbubble shell, increasing molar concentrations of porphyrin-lipid were titrated into a microbubble formulation. Unexpectedly, with 5-20 mol% porphyrin-lipid incorporation, the microbubbles were formed in significantly higher yield than without porphyrin-lipid, for a variety of fluorinated gases (Figure 2.2a). We hypothesize that the porphyrin components stabilize the shell and further prevent gas dissolution, resulting in greater yield; however, some regular phospholipids were necessary to maintain the high yield. A 15 mol% porphyrin-lipid formulation encapsulating perfluoropropane gas was chosen for further studies, as these were formed in high yield with a relatively large amount of incorporated porphyrin-lipid.

The properties of pMBs were compared to microbubbles formed without porphyrin-lipid and only DSPC and PEG40S in PBS. Extensive efforts have been invested in optimizing clinical formulations for improved acoustic microbubble stability and size distribution, using a mixture of phospholipids and additives to the buffer. Although the microbubbles used here as a control (formed without porphyrin-lipid) are not equivalent to any clinically-used agents, a similar simplified formulation has also been used in preclinical studies and in this study, serves to elucidate the effect of porphyrin-lipid on the microbubble shell, without the complications of additional factors. To evaluate the influence of the porphyrin-lipid on the microbubble stability in solution, microbubbles formed without porphyrin-lipid and pMBs were incubated in FBS at 37°C. Optical absorbance was used as a measure of microbubble concentration due to light scattering and monitored over a period of 4hr. pMBs remained nearly twice as long than microbubbles without porphyrin-lipid in serum (Figure 2.2b). This could potentially elude to increased microbubble stability due to the presence of porphyrin-lipid, however, this would require further studies to be validated, directly comparing the two formulations according to concentration, in vivo and acoustically.
**Figure 2.1.** Porphyrin shell microbubbles (pMBs). a) Schematic of pMBs microbubbles formed from a monolayer of porphyrin-lipid mixed with regular phospholipid encapsulating a fluorinated gas. b) Image of microbubbles formed with (right) and without (left) 15 molar % porphyrin-lipid (pyropheophorbide-lipid).

**Figure 2.2.** pMB yield and serum stability. a) Number of microbubbles as a function of molar % of porphyrin-lipid included in a control formulation for different filling gases: perfluoropropane (PFP), perfluorobutane (PFB) and sulfur hexafluoride (SF6). The porphyrin concentration was calculated from the optical absorbance at 410 nm and the known extinction coefficient of pyropheophorbide (97000 M$^{-1}$ cm$^{-1}$). Each data point represents the mean +/- SD for 3 separate microbubble preparations. b) pMB concentration in fetal bovine serum at 37°C compared with microbubbles formed without porphyrin-lipid. Optical absorbance at 580 nm is proportional to the microbubble concentration due to light scattering as porphyrin (pyropheophorbide) does not absorb at this wavelength. Experiments were conducted in triplicate (mean +/- SD).
pMBs had a narrow volumetric size distribution that peaked at 2.7 ± 0.2 µm. Microbubbles smaller than 7 µm accounted for > 95% of the population by volume and > 99% by number (Figure 2.3). Gas volume is often used as a more accurate indicator of ultrasound efficacy over number distribution, since larger bubbles dominate the echogenicity of a microbubble population\textsuperscript{159}. Under the same preparation conditions, microbubbles formed without porphyrin-lipid were more polydispersed (Figure 2.3a). Monodispersed microbubbles are of particular interest because of their high acoustic response at the resonant frequency, especially for targeted microbubbles in molecular imaging where only a small fraction of the administered dose remains at the target site\textsuperscript{158}.

To estimate the average porphyrin-lipid content of each microbubble, pMBs were separated from submicrometer particles that consisted of residual lipids forming vesicles and possible aggregates using a centrifugation method\textsuperscript{160} (Figure 2.4). As porphyrin-lipid is naturally fluorescent, we determined the porphyrin-lipid concentration in the microbubble population using fluorescence and UV spectroscopy. A single microbubble formed from 15 mol% porphyrin-lipid with an average diameter of 1.5 µm was determined to contain approximately (1.4 ± 0.2) x 10\textsuperscript{7} porphyrins.

The narrow volumetric size distribution of pMBs produced a sharp rise in US attenuation with increasing frequency, exhibiting a peak between 9-10 MHz and gradually tapering off by 27.5 MHz (Figure 2.5). Attenuation measurements reported for clinical lipid encapsulated agents typically show resonant frequencies below 6 MHz\textsuperscript{159,161} except for Definity\textsuperscript{TM}\textsuperscript{153} which has an attenuation peak within a similar region as pMBs. Thus, having a higher resonance frequency than some commercial lipid-based microbubbles, pMBs may be well suited for imaging applications exploiting the 5-15 MHz frequency range, including imaging of the breast, carotid neovascularization, superficial tumors.
Figure 2.3. Size distribution of microbubble populations formed without porphyrin-lipid and with porphyrin-lipid (pMBs). Microbubbles < 7 µm account for > 99% of the population number. a,c) Microbubbles formed without porphyrin-lipid using mechanical agitation are polydispersed (grey). b,d) pMBs have a narrow volumetric distribution (black) with a peak size of 2.7 ± 0.2 µm (mean ± SD from 5 measurements).

Figure 2.4. Size distribution before (a) and after (b) centrifugation washing. After repeated washing cycles, the large population of submicrometer particles was eliminated and pMBs microbubbles remained with a peak in the number distribution of 1.51 ± 0.09 µm (mean ± SD from 4 samples). No significant differences were observed in the volume distribution before and after washing.
Figure 2.5. Acoustic attenuation as a function of frequency for pMBs. Grey: 1.5-8.5 MHz transducer. Black: 7-27.5 MHz transducer. pMBs exhibit a resonance frequency between 9–10 MHz.
In order to gain insight into pMB mechanical properties, we applied a theoretical approach initially described by de Jong et al\textsuperscript{152} to determine the microbubble shell parameters, shell stiffness and shell friction. Shell stiffness $S_p$ is a measure of the microbubble elasticity, while the shell friction $S_f$ accounts for viscous effects. Briefly, using a linearized encapsulated microbubble model, and the frequency dependent attenuation and size distribution of pMBs, $S_p$ and $S_f$ were determined using Equation (1):

$$f_{res} = \frac{1}{2\pi} \sqrt{\left(\frac{3g p_o}{\rho r^2} + \frac{2S_p}{\rho r^2}\right)} \left(1 - \frac{\delta_t}{2}\right)$$

where $f_{res}$ is the resonance frequency, $r$ is the microbubble radius, $g$ is the polytropic gas index, $p_o$ is the ambient pressure, $\rho$ is surrounding medium density (e.g. water), and $\delta_t$ is the total damping. The pMB shell stiffness and friction were determined to be 5.32 ± 0.43 N/m and (0.19±0.14) x 10\textsuperscript{-6} kg/s, respectively. Shell stiffness estimates have been previously reported for several commercial lipid microbubbles and the pMBs were 3-5 times stiffer than these agents (Table 2.1). This corresponds to the increase in resonance frequency for a given microbubble size for pMBs over other lipid microbubbles.

Since the microbubble stability is related to the properties of the shell and encapsulated gas, it has been suggested by Borden et al\textsuperscript{162} that a possible mechanism for increased microbubble stability is an increase in shell stiffness and decreased gas permeability. Increased shell stiffness may result in a greater resistance to collapse. Thus, we hypothesize that the increase in shell stiffness due to the porphyrin-lipid may contribute to the increased yield and serum stability over microbubbles formed without porphyrin-lipid. However, further investigation is required to elucidate the influence of porphyrin-lipid on acoustic dissolution in solution and in vivo. With respect to the shell friction, the determined value of 0.19x10\textsuperscript{-6} kg/s between 6-13 MHz is on the lower end of the range derived at lower frequencies ($f < 8$ MHz) (Table 2.1). In comparison with Definity\textsuperscript{TM} characteristics, pMBs are less polydisperse and Definity\textsuperscript{TM} contains both smaller microbubbles and larger microbubbles, which may potentially possess a larger shell friction.

Despite having greater shell stiffness than clinical microbubbles, pMBs maintained the nonlinear properties associated with lipid microbubbles. pMBs were injected intravenously into mice bearing human breast cancer (MDA-MB-231) xenografts and imaged using a 5 MHz US clinical scanner (Figure 2.6). The subcutaneous tumor was imaged in B-mode and contrast mode
simultaneously. The B-mode image shows the soft-tissue contrast and outlines a cross section of the entire subcutaneous tumor on the mouse hind leg. The contrast mode image depicts the nonlinear signal generated from pMBs. Shortly after pMB injection, the blood vessels of the tumor became clearly visible, appearing as bright spots, and reached maximum signal intensity within 10 s, which allowed the identification of the skin and necrotic core of the tumor. Therefore, this initial feasibility study investigated the use of pMBs as US contrast agents, and presented promising results.
Table 2.1. Summary of microbubble parameters and shell properties for pMBs and commercial lipid microbubbles (literature values)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Vol. Peak Size (µm)</th>
<th>Resonance Frequency Range (MHz)</th>
<th>Shell Stiffness $S_p$ (N/m)$^b$</th>
<th>Shell Friction $S_f$ ($10^{-6}$ kg/s)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definity$^153$</td>
<td>1 – 2 and 7$^a$</td>
<td>9 – 12</td>
<td>$1.71 \pm 0.24$</td>
<td>$0.015 \pm 0.015$</td>
</tr>
<tr>
<td>Sonovue$^{159}$</td>
<td>5 – 6</td>
<td>1.5 – 2</td>
<td>1.1</td>
<td>0.27</td>
</tr>
<tr>
<td>Sonazoid$^{161,163}$</td>
<td>3.2 ± 0.2</td>
<td>3 – 5</td>
<td>$1.20 \pm 0.07$</td>
<td>$0.48 \pm 0.06$</td>
</tr>
<tr>
<td>pMB</td>
<td>2.7 ± 0.2</td>
<td>9 - 10</td>
<td>$5.32 \pm 0.43$</td>
<td>$0.19 \pm 0.14$</td>
</tr>
</tbody>
</table>

$^a$ Definity$^\text{TM}$ contains a polydispersed size population exhibiting 2 peaks

$^b$ See Table 2.2 for the frequency range over which shell parameters were estimated

Table 2.2. Frequency ranges over which shell parameters were estimated

<table>
<thead>
<tr>
<th>Agent</th>
<th>Frequency Range (MHz)</th>
<th>Shell Stiffness $S_p$ (N/m)</th>
<th>Shell Friction $S_f$ ($10^{-6}$ kg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definity$^{153}$</td>
<td>12 - 28</td>
<td>$1.71 \pm 0.24$</td>
<td>$0.015 \pm 0.015$</td>
</tr>
<tr>
<td>Sonovue$^{159}$</td>
<td>0.8 - 3</td>
<td>1.1</td>
<td>0.45</td>
</tr>
<tr>
<td>Sonazoid$^{161,163}$</td>
<td>1.5 – 8</td>
<td>$1.20 \pm 0.07$</td>
<td>$0.48 \pm 0.06$</td>
</tr>
<tr>
<td>pMB</td>
<td>6 - 13</td>
<td>$5.32 \pm 0.43$</td>
<td>$0.19 \pm 0.14$</td>
</tr>
</tbody>
</table>
Figure 2.6. *In vivo* ultrasound imaging of pMBs in a MDA-MB-231 xenograft bearing mouse. Contrast mode images showing the nonlinear contrast from pMBs in a cross section of the entire subcutaneous tumor on the mouse hind leg. Microbubble signal is shown as bright spots within the tumor after i.v. injection which enabled identification of tumor vasculature using a 5-12 MHz ultrasound transducer. The bright rod-shaped object in the contrast mode image is an artefact of the system. Inset image: B-mode images showing the soft-tissue contrast of the same cross section as contrast mode. B-mode and contrast mode images were acquired simultaneously. Scale bar: 1 cm.
We previously demonstrated that, in a bilayer nanoscale structure, the normally fluorescent porphyrin component of pyropheophorbide-lipid undergoes extreme self-quenching due to the high packing density, making porphysomes a useful PA contrast agent with a high optical cross section. Similarly, the presence of porphyrin-lipid enables pMBs to generate a PA signal that peaks at 700 nm (Figure 2.7) corresponding to the Q band peak in optical absorbance, which is slightly red-shifted from the Q band of pyropheophorbide in solution (Figure 2.8). This red-shifted peak is further evidence of the porphyrin-porphyrin interactions at a high packing density within the monolayer lipid shell of pMBs.

Microbubbles formed without porphyrin-lipid (unimodal microbubbles), pMBs and unimodal microbubbles co-incubated with equimolar free porphyrin (pyropheophorbide) were imaged using PA with a 700 nm 5 ns pulsed laser and 10 MHz single-element US transducer, in sample wells (Figure 2.7b). pMBs generated 10-fold greater PA signal over unimodal microbubbles and 6-fold greater PA signal over unimodal microbubbles mixed with free porphyrin (Figure 2.7c). This demonstrates that not only must the porphyrin be present in order for the microbubbles to generate a PA signal, but the porphyrin must also be conjugated to the lipid and present within the microbubble shell. The organized and high packing density of the porphyrin-conjugates within the microbubble shell is requisite for a significant PA signal generation. All microbubble populations generated strong ultrasound signals (Figure 2.7b, c bottom), confirming that pMBs have the capacity to serve as a dual-modality US/PA contrast agent.

In summary, pMBs were developed as a dual modality US/PA contrast agent that shows unexpected physical properties arising from the inclusion of porphyrin-lipid in the shell. These include increased microbubble yield and increased shell stiffness, resulting in enhanced stability over microbubbles without porphyrin-lipid. pMBs possess acoustic properties compatible with clinical and higher frequency ultrasound imaging applications, together with intrinsic PA capabilities. They should have potential both as combined US/PA imaging agents as well as for emerging microbubble applications such as drug or gene delivery.
Figure 2.7. Photoacoustic (PA) and ultrasound (US) measurements of pMBs. a) PA spectrum of pMBs from 3 measurements +/- SD. b) PA (top) and US (bottom) images of microbubbles formed without porphyrin-lipid, pMBs and microbubbles without porphyrin-lipid incubated with equimolar pyropheophorbide-α (pyro) in 9 mm diameter wells within a plastic phantom. PA images were acquired using a 700 nm 5 ns pulsed laser and 10 MHz single element transducer. c) Quantitative PA amplitude and US intensity for microbubbles formed without porphyrin-lipid, microbubbles formed with porphyrin-lipid (pMBs) and microbubbles formed without porphyrin-lipid co-incubated with free porphyrin (mean of 100 pixel points +/- SD).

Figure 2.8. Normalized absorbance spectra of pMBs (grey) measured in PBS and porphyrin-lipid conjugates (black) measured in methanol.
2.5.2 Trimodal pyropheophorbide porphyrin shell microbubbles

In formulating these dual modality US/PA pMBs, a distinct shift in the Q-band absorbance from that of the monomeric porphyrin was observed, which is a characteristic of ordered aggregation. Although ordered aggregation can result in the generation of fluorescence, very little fluorescence was observed in practice due to the relatively low porphyrin content. A significantly improved pMB construct was developed in which the porphyrin content was increased to 50 mol% porphyrin-lipid. Ordered aggregation was also observed and fluorescence generation was detectable. Thus, pMBs were investigated as a trimodal contrast agent for US, PA and fluorescence imaging, further expanding their potential clinical utility and applications.

Unimodal microbubbles and trimodal pMBs were formed by lipid film rehydration and mechanical agitation. Unimodal microbubbles were composed of 90 mol% DSPC and 10 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)2000] (DSPE-mPEG2000) encapsulating perfluoropropane gas (Figure 2.9a). Trimodal pMBs were prepared in a similar manner but with the substitution of 50 mol% DSPC by porphyrin-lipid (pyro-lipid) (Figure 2.18d). The resulting microbubbles were separated from submicrometer particles by differential centrifugation, giving a size distribution between 2 and 6 µm at a concentration of 2 x 10^9 microbubbles/ml (Figures 2.9b, c, e, f). The absorbance spectrum of the pMBs in PBS displayed a Q-band peak at 704 nm, which is 37 nm red-shifted from the pyro-lipid monomeric peak wavelength (Figure 2.10a).

This red-shifted peak is an indication of ordered aggregation of the porphyrins within the microbubble shell, which was further confirmed by the presence of circularly polarized light in the circular dichroism spectrum of only the trimodal pMBs at 704 nm (Figure 2.10b). The optical absorption spectrum dictates the PA spectrum. The measured PA spectrum corresponded well with the absorption spectrum, with a peak PA signal at 704 nm. By comparison, since regular phospholipids are weakly light absorbing in the visible/near infrared range, unimodal microbubbles did not show any distinct peaks in the PA spectrum (Figure 2.10c). Ordered aggregation is known to result in the generation of fluorescence, which was exhibited by pMBs, which had a Stokes shift of 4 nm with the fluorescence emission peak at 708 nm (Figure 2.10d).
Figure 2.9. Unimodal microbubbles and trimodal pMBs. Schematic representations of a) microbubbles formed from regular phospholipids (unimodal microbubbles), and d) microbubbles including porphyrin-phospholipids (pyro-lipid). Corresponding size distributions (b, e) and light microscopy images (c, f). Scale bar 10 µm.
Figure 2.10. Optical characteristics of pMBs. a) Absorbance spectra of trimodal pMBs in PBS and monomeric pyro-lipid in 1% Triton-X (detergent). The difference in the background is due to light scattering of the microbubbles. b) Circular dichroism spectra of pyro-lipid in detergent (I), unimodal microbubbles (II) and trimodal pMBs (III) in PBS. c) PA spectra of unimodal microbubbles and trimodal pMBs in PBS. d) Corresponding fluorescence emission spectra under 410 nm excitation.
To further investigate the imaging properties of the trimodal pMBs, an US transparent gelatin holder was formed with three empty cavities, which were used to immobilize the sample solutions (Figure 2.11a). Unimodal microbubbles and trimodal pMBs in PBS were inserted into the cavities in the gelatin holder at equal microbubble concentrations (10^9 microbubbles/ml) and imaged using US, PA and fluorescence (Figures 2.11b, c, d; top). Both microbubble samples generated nearly equal US signals, but the trimodal pMBs generated approximately 40 times higher PA signal and 17 times higher fluorescence signal (Figures 2.11b, c, d; bottom).
Figure 2.11. Trimodal imaging of pMBs in solution. White light image (a) of the gelatin holder containing three samples: 1) PBS only, 2) unimodal microbubbles, and 3) trimodal pMBs. The microbubbles were matched in concentration (10^9 microbubbles/ml). (b) US (21 MHz), (c) PA (704 nm, 21 MHz) and (d) fluorescence (435 nm - 480 nm excitation, >515 nm detection) images and corresponding signal intensities (mean ± 1 SD from 3 measurements).
In order to demonstrate the applicability of the pMBs as trimodal contrast agents, mice bearing KB tumor xenografts were administered either unimodal microbubbles or trimodal pMBs via tail vein injection and imaged *in vivo* sequentially by US, PA and fluorescence. B-mode US images were captured to exhibit the soft tissue contrast from the tumor (Figure 2.12, top). Increase in the US contrast signal was observed for both unimodal microbubbles and trimodal pMBs, however, the microbubbles did not distribute homogenously within the tumor, potentially indicating the presence of a necrotic core (Figure 2.12, middle).

PA images were captured pre- and post-injection of both microbubble formulations, and only the trimodal pMBs showed detectable increase in PA signal after injection (Figure 2.12, bottom). Differences between the acquired US and PA images after trimodal pMB injection may be attributed to sensitivity of PA imaging which is highly absorber concentration dependent. The tumor vasculature is most dense around the periphery of the tumor, therefore, upon injection of pMB, there is a greater concentration of pMBs around the periphery of the tumor compared to the center. US contrast imaging methods are designed to be sensitive to microbubble contrast agents, even to single microbubble detection, which allows contrast to be observed within the tumor where a low concentration of microbubbles may be found. However, the same sensitivity is not exhibited by PA imaging for a single absorbing microsphere, and therefore does not show the same contrast from within the tumor.

Furthermore, the porphyrin (pyro) naturally accumulates in tumor tissue 2-3 fold higher than in normal tissue\(^{165}\) so that *ex vivo* fluorescence imaging of the resected tumors confirmed the tumor uptake of the trimodal pMBs and generation of significant fluorescence signal compared with the unimodal microbubbles (Figure 2.13). This fluorescence property of trimodal pMBs could be used as an *ex vivo* validation tool to confirm tumor binding and uptake and biodistribution of pMB fragments post-injection or potentially also *in vivo*, either by external, endoscopic or intraoperative imaging, depending on the tumor location and clinical application. These images collectively confirm the ability of trimodal pMBs to function as a trimodality contrast agent.

In addition to the ability to behave as a trimodal contrast agent for US, PA and fluorescence imaging, the generation of microbubbles with optical properties presents a unique opportunity to apply US-specific properties to an optical-contrast agent and *vice versa*. For example, microbubbles have been investigated for drug delivery or for crossing the blood-brain barrier...
through spatially-localized ultrasonic destruction of the bubbles\textsuperscript{166,167,168}. This does not allow real-time tracking of the resulting fragmented microbubble, since the acoustic signal is lost upon destruction. However, with the intrinsic optical properties imparted by the porphyrin-lipid in the pMBs, real-time tracking may be possible with optical imaging. This option is under investigation and the topic of the following chapter. In addition to the trimodal properties of these pMBs, the increase in porphyrin content opens a window of opportunity for combined US and optical imaging with, for example, MRI using manganese-conjugated porphyrins or therapy.
**Figure 2.12.** *In vivo* US and PA imaging of microbubbles in a KB tumor xenograft at 10 s - 30 s post intravenous injection of 280 µL of pMB solution at a concentration of $2 \times 10^9$ microbubbles/ml. The US B-mode images (top) show the soft tissue contrast of the tumor. The US contrast mode images (middle) and the PA images (bottom) show the infusion of microbubbles by an increase in signal. Scale bar 1 mm.

**Figure 2.13.** *Ex vivo* fluorescence imaging of pMBs in a KB tumor at 4 hr after i.v. administration of unimodal (a) or trimodal pMBs (b). 435 nm – 480 nm excitation, > 515 nm detection, overlaid with grey-scale white light images.
2.6 Conclusion

pMBs were generated with a 15 mol% porphyrin content, resulting in a dual modal US and PA contrast agent. When the porphyrin content was increased by 3-fold from 15 to 50 mol%, fluorescence was produced from ordered aggregation and high optical absorption, thereby generating an intrinsically trimodal agent for US, PA and fluorescence imaging based on a single-component porphyrin-lipid structure. Importantly, this multimodal capability is achieved without the complexity and potential errors of combining multiple separate imaging components. The increased porphyrin content has been shown to enable PA and fluorescence imaging in addition to the previously reported US properties of regular phospholipid microbubbles\textsuperscript{169}, expanding the multimodality imaging applications.
Chapter 3
In situ conversion of microbubbles to nanoparticles

3.1 Acknowledgements

The bulk of this chapter is a modified and reformatted version of a manuscript entitled “In situ conversion of porphyrin microbubbles to nanoparticles for multimodality imaging”, currently in press in Nature Nanotechnology. Under the supervision of Dr. Gang Zheng, my contribution to this work included designing and carrying out most of the experiments, analyzing and interpreting the data and writing the manuscript. Thanks to Brandon Helffield and Ben Leung for the acoustic measurements, Mojdeh Shakiba for help with the transmission electron microscopy images, Cheng Jin for assistance with the in vivo experiments and Drs. David E. Goertz and Brian C. Wilson for insightful discussions and editing of the manuscript.

3.2 Abstract

Triggered conversions of supramolecular structures have been of growing interest to image and treat disease. These materials originate as monomeric compounds or nanoparticles, which are elicited by endogenous or external stimuli to self-assemble, aggregate or expand in vivo, forming larger structures. Here we investigate the micro-to-nano conversion of microbubbles, identifying, for the first time, a utility for this conversion using a trimodality microbubble with US, PA and fluorescence properties. Based upon a porphyrin-lipid building block, porphyrin shell microbubbles (pMBs) transition to form porphyrin shell nanoparticles (pNPs) upon sonication with low frequency US. We investigated the effects of this pMB transition on the structure and optical properties. pMBs had an acoustic resonance peak at 4.5 MHz and PA absorption and fluorescence emission in the near infrared region. Upon application of US, the concentration of pMBs significantly decreased with an increase in pNP concentration, confirming the conversion from microbubble to nanoparticle. Furthermore, the pMB US imaging contrast significantly decreased while the PA and fluorescence imaging intensities were maintained. In situ micro-to-nano conversion of pMBs to pNPs was demonstrated in vivo to deliver porphyrins to a tumor xenograft. Circulation and conversion of pMBs was monitored using US imaging, while PA imaging validated successful delivery of porphyrins to the tumor.
These findings expand the utility of conventional microbubbles to agents that can simultaneously deliver, image and treat disease utilizing the conversion to pNPs to overcome biological barriers.

3.3 Introduction

3.3.1 Microbubble destruction response to ultrasound

Interaction of a lipid microbubble with US pulses of sufficient amplitude (appropriate frequency and sufficient pressure) can result in its fragmentation into a number of smaller microbubbles or shrinkage, which may be accompanied by the ‘shedding’ of shell material\(^{162,173,174}\). These processes lead to the formation of structures that have significantly decreased US imaging contrast relative to the initial microbubble\(^{175,176}\).

This property of microbubbles has been utilized for various biomedical purposes. For example, destruction-replenishment methods apply a higher pressure US pulse to destroy the microbubbles within the field of view. Lower pressure non-destructive US pulses are used to monitor the influx of microbubbles in circulation into the specified region\(^{177}\). This technique has been used to investigate blood flow in the myocardium\(^{177-179}\), as well as in the liver and kidneys\(^{180,181}\). Oscillation and/or destruction of microbubbles with low and high acoustic intensities have been used as a method of delivering cargo into cells/tissue. The detailed mechanism by which cargo are delivered intracellularly is highly dependent on the acoustic parameters, microbubble composition and cargo size and remains a topic of investigation by many groups\(^ {182}\), however, many have proposed that uptake may be due to pore formation and/or endocytosis. A stably oscillating microbubble close to a cell membrane generates microstreams that create shear stress on the membrane. This may result in deformation of the cell membrane, cytoskeletal rearrangement and changes in membrane tension\(^ {183-187}\). These effects can be detected by mechanosensors in the cell membrane, which can initiate processes that influence endocytosis\(^ {188,189}\). Furthermore, during microbubble oscillation, reactive oxygen species can also be generated\(^ {190,191}\), which can modulate ion channels\(^ {192}\) or disrupt the membrane\(^ {193}\). Modulation of ion channels can result in an influx of calcium and stimulate endocytosis\(^ {194}\). For an imploding microbubble, pore formation may due to the production of shock waves and microjets\(^ {195,196,197}\), which allow the passive diffusion of drugs and calcium ions into the cell\(^ {198,199}\). These techniques have been used for delivery of drugs and genes\(^ {27,30,200,201}\), either loaded into/on to the surface of the microbubble\(^ {202-205}\) or co-injected\(^ {206,207}\).
3.3.2 Conversion of ultrasound-based agents

The intrinsic conversion of supramolecular structures involves the activation of shape transitions. Of growing interest in the biomedical field, is the conversion of perfluorocarbon nanodroplets as activatable US contrast agents. These nanometer-sized droplets expand to form micrometer-sized bubbles upon heating by US\textsuperscript{208,209}. They have been applied as both US contrast agents\textsuperscript{210,211} and drug delivery vehicles\textsuperscript{212}. More recently, nanodroplets encapsulating gold nanoparticles have been studied, using light to trigger the conversion as another mechanism for PA signal generation\textsuperscript{148}. Despite a growing number of investigations utilizing the conversion from nano-to-micrometer sized structures, there have not been any studies exploiting the advantages of a micro-to-nano conversion. The most conventional micrometer-sized structure investigated for biomedical applications is the microbubble.

Recently, we discussed the concept of intrinsically multimodal contrast agents\textsuperscript{2} and developed multimodal US contrast agents, in which the building blocks of the microbubble had optical and metal chelation properties. We used a porphyrin-lipid to form a shell around a perfluorocarbon gas forming pMBs. The encapsulated gas provided US imaging contrast and the high density of porphyrins enabled PA and fluorescence contrast\textsuperscript{121,213}. Here we utilize these properties to investigate the response of pMBs to low frequency US. The unique characteristic of forming the multimodality microbubble with building blocks that possess intrinsic optical properties ensures that the responses observed are properties of the pMB after destruction/deflation and forming pNPs from the pMB (Figure 3.1), and not simply the release of imaging agents from the microbubble as others have investigated\textsuperscript{214,215}. 
Figure 3.1. Schematics of a) porphyrin shell microbubbles and b) micro-to-nano conversion via sonication with low frequency, high duty cycle US (conversion US).
3.4 Materials and methods

3.4.1 Fabrication and size characterization

To form pMBs, a 1mg lipid mixture consisting of 40 mol% porphyrin-lipid (bacteriochlorophyll (BChl)-lipid as previously described\textsuperscript{155}), 42 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 10 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA) and 8 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (DPPE-mPEG5000) were combined in chloroform in 12 mm x 35 mm glass threaded vials (Fisher Scientific). For experiments using regular microbubbles without porphyrin-lipid, the porphyrin-lipid in the mentioned formulation was replaced with DPPC. DPPC, DPPA and DPPE-mPEG5000 lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The chloroform was dried by nitrogen gas and vacuumed for 1 hr to form a lipid film. Lipid films were then rehydrated with 1 ml of 10 vol% glycerol, 10 vol% propylene glycol and 80 vol% PBS. Sample vials were topped off with perfluoropropane gas (C\textsubscript{3}F\textsubscript{8}, PFP, Fluoromed L.P), briefly heated with a heated water bath and sonicated (Bransonic Model 2510) to disperse the lipid film. Samples were then topped off again with perfluoropropane gas and mechanically agitated for 45 s using a VialMix\textsuperscript{®} shaker. Samples were allowed to cool for 15 min before use and were used within 30 min of opening the vial to mitigate potential temporal effects on pMB properties.

pMBs were gently shaken for 10 s and decanted for 2 min before 500 µl was extracted into a 3 ml syringe. Using the differential centrifugation method\textsuperscript{151}, pMBs were separated from residual nanoparticles by centrifugation for 8 min at 50 G twice, washing with 10 vol% propylene glycol, 10 vol% glycerol and 80 vol% PBS. For conversion US pulsing experiments, samples were diluted 6 x in PBS and placed within 1 ml eppendorf tubes. The samples were then situated in a water tank and sonicated with 1 MHz ultrasound (Vevo\textsuperscript{®} SoniGene\textsuperscript{TM}, FUJIFILM VisualSonics, Inc.) using the 10 W setting (2 W/cm\textsuperscript{2} intensity, 50% duty cycle, 2 s long pulses). The exposures consisted of the application of 0, 1, 3 or 10 pulses (separated by 5 s). These settings were selected based on pilot experiments that were found to progressively decrease pMB concentrations with each pulse and are similar to those previously used by others in MB drug delivery\textsuperscript{216-219}.

pMB concentration was determined using a Coulter Counter Multisizer Z3 (Beckman Coulter Inc.), in which pMB samples were diluted in Isoton-II electrolyte solution (Beckman Coulter
Inc.) to obtain a count within 100 000 – 300 000 in a 50 µl volumetric sample. A background count of buffer was taken and subtracted from the final count. Dilution was accounted for in calculating the pMB concentration. The number and size distribution were measured using a 30µm aperture which detected pMBs with diameters in the range 0.8 – 18 µm.

pNP size distribution and concentration was measured using a NanoSight LM10 and Nanoparticle Tracking Analysis Software (Malvern Instruments Ltd, Malvern, UK). Pulsed samples were centrifuged using a benchtop ultracentrifuge after conversion US pulses were applied and 200 µl of the infranant was removed from the bottom of the sample, collecting only pNPs as the remaining pMBs formed a cake on the top. Samples were then diluted with PBS 2x before measuring the size distribution and concentration.

3.4.2 Acoustic characterization and ultrasound imaging in solution

The acoustic attenuation spectrum of pMBs was measured using a narrowband pulse-echo substitution method similar to that used by Goertz et al. Using two transducers (Model #595396, 5 MHz, 76 mm focus, 12.7 mm diameter, Olympus NDT Canada Inc.; Model #IS2002HR, 20 MHz, 38 mm focus, 6.35 mm diameter, Valpey-Fisher), the attenuation was measured in the frequency range between 1.5 - 27.5 MHz in 0.5 MHz increments. US pulses were generated by an arbitrary waveform generator (model WW2572A, Tabor Electronics Ltd., Tel Hanan, Israel) and amplified (model A-150; ENI, Rochester, NY) before reaching each transducer; voltages were calibrated for each transducer to deliver 25 kPa peak negative pressure at its geometric focus for each frequency. An aluminum rod was placed at each transducer's focus to serve as a reflector to generate echoes that were detected by the same transducer. Echoes were then amplified (model AU1579; Miteq, Hauppauge, NY) and bandpass filtered before being digitized (400 MHz sampling frequency; Agilent Technologies Inc., Palo Alto, CA) for post-process analysis. Acquisitions occurred prior to and after introducing pMBs (1:15000 contrast agent to saline dilution ratio) in a chamber with an acoustically transparent window between the transducer and aluminum reflector. The attenuation per unit length was calculated at each frequency by comparing the echo amplitudes and knowing the distance through which US interacted with the pMBs.

Linear and non-linear US imaging was performed in a tissue-mimicking flow phantom similar to that described by Helfield et al. and imaged using a Phillips iU22 clinical scanner with a L9-3
probe (3-9 MHz) (Phillips Medical Systems, Seattle, WA) operating in B-mode for linear imaging and contrast mode for non-linear imaging. The tissue-mimicking phantom was composed of a 2% agar and 3% graphite medium, the latter acting as an acoustic scatterer, with an acoustic attenuation of 0.3 dB/mm/MHz as reported by Burlew et al. A wall-less vessel was created by placing a 6.3 mm diameter rod within the phantom prior to casting; after the gel solidified the rod was removed. pMBs were diluted with gas-equilibrated saline (0.9% (w/v) NaCl) at a ratio of 1:5000 in a reservoir and mixed with a magnetic stirrer to ensure a representative population of agent was being used. After 30 s of mixing, the diluted agent was allowed to flow through the phantom using a gravity feed approach. The flow rate was maintained around 8-9 mm/s in order to minimize decorrelation effects in contrast mode. The agent required ~1 min to reach the imaging plane (with the vessel located 4.5 cm below the surface) from the reservoir. US images were captured 2 min and 20 s post agent dilution.

### 3.4.3 Microscopy

pMBs were diluted 50x for imaging with confocal microscopy (Olympus FluoView FV1000) using a 633 nm laser and 40x water objective lens. pNPs were imaged with transmission electron microscopy (TEM) (Hitachi H-7000 electron microscope) with an acceleration voltage of 75 kV. Samples that were pulsed 10 or 20 times and were prepared for TEM by applying 10 µl of pNPs to a glow-discharged 200-mesh copper-coated grid, washed once with deionized water and stained with 2% (w/v) uranyl acetate.

Vacuum pressure experiments were performed on pNP samples after the pMBs had 10 conversion US pulses applied, using conditions similar to those described by Huang et al. The pNP samples were transferred to a 2 ml glass vial with a screw cap and syringe seal closure. A 5 ml syringe with a 25-guage needle was introduced through the seal and the plunger of the syringe was withdrawn to obtain a pressure of 0.5 atm, determined from the vapor phase volume in the vial and the syringe according to Boyle’s law. The pressure was maintained for 3 min and then the samples were imaged using TEM.

### 3.4.4 Optical characterization

To measure the UV/Visible absorption spectra of pMBs and pNPs, samples were diluted 8x in PBS or 50 vol% FBS and measured using a Cary 50 UV/Vis spectrophotometer (Agilent,
Mississauga, ON), scanning from 300 nm – 900 nm. Circular dichroism spectra were determined in PBS for pMBs and pNPs using a J-815 Circular Dichroism Spectrometer (JASCO Inc.) scanning from 300 nm – 900 nm. The fluorescence spectra of 8x diluted pMBs and pNPs samples were measured in PBS using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, NJ). Fluorescence emission was collected 700 nm – 900 nm using an excitation of 520 nm and 5 nm slit widths. Measurements of BChl-lipid monomeric samples were obtained by adding Triton X-100 to pNPs to disrupt the nanoparticle structure and BChl ordered aggregates (final detergent concentration: 1vol%).

### 3.4.5 Stability measurements

pMB stability in solution was measured over time using the Coulter Counter Multisizer Z3 as previously described. Samples were measured immediately after opening the pMB sample vial, 0.5 hr and 1 hr. pMBs were converted into pNPs in PBS and pNP stability was measured in solution using the NanoSight LM10 up to 22 days post-formation.

pNP stability in FBS was determined by absorbance of the 824 nm aggregation peak. pMBs were diluted in FBS to a final serum concentration of 80 vol%. Samples were then subjected to 10 conversion US pulses to generate pNPs in serum. Absorbance was measured using a plate reader at 824 nm (BChl J-aggregation peak) and 750 nm (monomer BChl peak) over time up to 12 hr.

### 3.4.6 Imaging in a gel phantom

pMBs and pNPs were immobilized in a polyacrylamide hydrogel phantom that was prepared using a similar method described by Choi et al. \(^{223}\) and Ng et al. \(^{224}\). Briefly, 59 mL of ddH\(_2\)O, 30 mL of 30% (w/v) 19:1 acrylamide (Biorad, Mississauga, ON) and 10 mL of 1 M Tris buffer (pH 8) were degassed for 20 min. Ammonium persulfate (APS; 10% w/v) and N,N,N′,N′-tetramethylethlenediamine (TEMED) were added to the monomer mixture to a final concentration of 0.84% and 0.2%, respectively, mixed, poured into a gel mold with a comb and allowed to polymerize for 1 hr. After 1 hr, the comb was removed and the same mixture with the pMBs or pNPs (5x diluted) in place of a volume of ddH\(_2\)O was poured into the 2 mm x 2 mm x 20 mm empty cavities and allowed to polymerize.

US and PA imaging were performed with a commercial PA imaging system (Vevo LAZR: FUJIFILM VisualSonics, Inc.), operating with a 21 MHz transducer in both US and PA modes.
US and PA images were obtained with 25 dB and 40 dB gains, respectively. PA spectra were obtained from 680 nm to 900 nm with 2 nm steps. Fluorescence images were obtained using a whole-body small animal imager (CRi Maestro: Caliper Life Science Inc.) under green-light excitation (503 nm – 548 nm), 560 nm long pass detection, an integration time of 700 ms and then spectrally unmixed. Comparisons between 0 pulses and pulsed samples were made using the two-sample homoscedastic Student’s t-Test, with the level of significance was set at $p < 0.05$.

### 3.4.7 In vivo imaging and blood collection

Animal experiments were conducted in compliance with institutional animal care approval (University Health Network). The model was generated by subcutaneous inoculation of $2 \times 10^6$ KB cells in the right flank of 20 g female athymic nude mice. Experiments were performed when tumors reached a surface diameter of 5-7 mm. Mice were anesthetized with 2% (v/v) isoflurane inhalation to insert a 26-gauge indwelling catheter into the tail vein. Mice were then injected with 100 mg/kg of ketamine and 10 mg/kg of xylazine via intraperitoneal injection and removed from isoflurane inhalation.

pMB in vivo stability was determined via the in vivo circulation time as observed by US imaging by injecting a 150 µl bolus of $(8.4 \pm 0.4) \times 10^7$ pMB/ml followed by a 100 µl saline flush. The tumor was then imaged using the Vevo 2100 US system (FUJIFILM VisualSonics, Inc.) and the MS250 probe operating at a frequency of 13-24 MHz. Region of interest analysis was conducted by selecting a region inside of the tumor, and normalizing it to the maximum US signal determined after injecting the pMBs. Trendlines were plotted using a 10 period moving average using Microsoft Excel.

pMB to pNP conversion in vivo experiments were also performed on female athymic nude mice bearing subcutaneous KB tumors in the right flank. For “no conversion US applied” control animals, mice were injected with a 150 µl bolus of $(8.4 \pm 0.4) \times 10^7$ pMB/ml followed by a 100 µl saline flush. Mice were monitored with PA imaging over time (assessed at 5 min and 15 min post-injection and then at 15 min intervals up to 2 hr) using a 21 MHz transducer and at wavelengths of 824 nm and 850 nm for PAs.

For “conversion US applied” animals, mice were harnessed to a custom animal holder shown in Figure 3.2, and immersed in a 35°C heated water tank. Conversion US and imaging US
transducers were aligned to both irradiate the tumor region with conversion US and simultaneously image the pMB influx and externally triggered destruction using US imaging as shown in Figure 3.2. The conversion US transducer was aligned to a distance of 1 cm from the surface of the center of the tumor using a custom-made detachable alignment tool cap for the conversion US transducer (Figure 3.3). After removal of the alignment tool and repositioning of the conversion US transducer, the imaging US transducer (Vevo 2100, 13-24 MHz functioning in B-mode and contrast mode) was placed over the tumor in order to image the pMB influx and destruction. Mice were injected with a 150 µl bolus of (8.4±0.4) x 10^7 pMB/ml followed by a 100 µl saline flush. 20 s after pMB injection, circulation of pMB was visible via US imaging, and the tumor was then sonicated with 60 s of 10 W (2 W/cm²) and 50% duty cycle conversion US. Mice were then removed from the heated water bath and placed in the Vevo LAZR system for PA imaging. PA imaging was performed at 5min and 15min post-injection and then in 15min intervals up to 2 hr. PA images were processed by subtracting the data acquired at 850 nm from that at 824 nm to remove any background signal. Region of interest analysis was performed by selecting a region in the tumor at 824 nm and subtracting the PA value at 850 nm in the same region.

To assess the fluorescence spectra of pMBs after circulation in vivo, mice were injected with a 150 µl bolus of (8.4±0.4) x 10^7 pMB/ml followed by a 100 µl saline flush. After 5 or 15 min post-injection, 500 µl of blood was collected via intracardiac puncture using a syringe rinsed with 5% heparin saline. Mice were sacrificed immediately after blood collection. Whole blood was imaged in eppendorf tubes by fluorescence imaging (CRi Maestro: Caliper Life Science Inc.) under green-light excitation (503 nm – 548 nm), 560 nm long pass detection, an integration time of 5000 ms and then spectrally unmixed.
Figure 3.2. Experimental set-up for pMB to pNP in vivo experiments. Mice bearing KB xenografts were harnessed in a custom-made animal holder with an indwelling 26 G catheter and submerged in a 35°C heated-water bath. A conversion US transducer was placed perpendicular to the tumor and an imaging US transducer was used to observe the inflow of pMBs and confirm a decrease in US signal upon application of conversion US.
**Figure 3.3.** Alignment tool designed for *in vivo* experiments to position the conversion US transducer in the center of the tumor after harnessing the mouse in the waterbath. a) Photograph of the custom-made alignment tool with a 1cm distance between the tip and the face of the transducer. b) Photographs demonstrating the positioning of the transducer using the alignment tool and removal of the tool after repositioning.
3.5 Results and discussion

3.5.1 Development and characterization of bacteriochlorophyll porphyrin shell microbubbles

While previously reported pMBs were formed using pyropheophorbide-lipid, the current pMBs were formed using a bacteriochlorophyll-lipid (BChl-lipid) (Figure 3.1), which possessed more favorable optical properties in the near-infrared window for PA and fluorescence imaging. pMBs composed with BChl-lipid had a final concentration of \((8.4\pm0.4)\times10^7\) MB/ml and were acoustically responsive with a resonance attenuation peak at 4.5 MHz (Figure 3.4a). pMBs were able to generate both a linear and non-linear responses to US. A tissue mimicking flow phantom composed of agar and graphite was used with a wall-less vessel in which pMBs were allowed to flow through and imaged with a clinical US scanner. The phantom produced linear backscatter shown in the B-mode image, similar to tissue, but did not possess non-linear properties, shown by a lack of contrast in the contrast mode image (Figure 3.4b). When the vessel was filled with saline, a lack of US signal was observed in both B-mode and contrast mode. However, when pMBs filled the vessel, they were able to produce both linear backscatter and non-linear response (Figure 3.4b), characteristic of microbubble US contrast agents. The pMB peak size distribution was between 2-8 µm by volume (Figure 3.5a) with 99.9% < 10 µm by number (Figure 3.6a). After activation, pMBs were stable in solution for up to 1 hr after opening the sample vial, maintaining its peak size distribution (Figure 3.6b). However, after 1 hr the pMB concentration decreased to <80% of its initial concentration (Figure 3.6c); therefore, pMBs were used immediately after opening the sample vial or within 30 min, a common practice with microbubble handling.\(^{225}\) pMBs had an \textit{in vivo} circulation time of ~2 min (Figure 3.6d), determined by US imaging, which is similar to other coated microbubbles\(^{31,226,227}\).
Figure 3.4. US characterization of pMBs. a) Acoustic attenuation measurement of pMBs with a resonance frequency of 4.5 MHz using two transducers (transducer 1: 1.5 – 12 MHz, black; transducer 2: 7 – 27.5 MHz, grey). Mean ± 1 SD with n=3. b) B-mode (linear) and contrast mode (non-linear) US imaging of saline (top) and pMBs (bottom) in a vessel within an US tissue mimicking phantom. 5 mm scale bar shown.

Figure 3.5. Conversion of pMBs to pNPs. a) Size distribution of pMBs prior and after application of conversion US pulses according to volume distribution. b) concentration of pMBs and pNPs before and after application of conversion US pulses. Mean ± 1 SD with n=3. c) size distribution of pNPs.
3.5.2 Microbubble to nanoparticle conversion

pMB samples were exposed to 1 MHz, high duty cycle (50%) US (2 W/cm²), hereafter referred to as conversion US, in 2 s pulses and were characterized after sonication with 0, 1, 3 or 10 pulses of conversion US. A decrease in the overall pMB population volume was observed even after 1 pulse of conversion US; further decrease was observed by increasing the number of pulses (Figure 3.5a, Figure 3.7). After the application of 10 pulses, the majority of the pMBs had undergone conversion. Although the size distribution by volume of the pMBs provides greater insight into the acoustic efficacy of pMBs¹⁵⁹, this trend in the size distribution after applying US was also observed by concentration. The number of pMBs significantly decreased with a shift in the size distribution to smaller diameters with increasing number of US pulses (Figure 3.6a). The pMB concentration decreased as the pNP concentration increased after each conversion US pulse (Figure 3.5b). Furthermore, the resulting pNPs were polydisperse in size between 5 nm – 500 nm in which 99% of pNPs in all samples were < 500 nm (Figure 3.5c, Figure 3.8). Although there were some residual pNPs present even without any US pulses applied due to the washing and nanoparticle isolation procedures, an increase in pNP concentration was observed after applying conversion US pulses. pNPs were stable in solution for at least 3 weeks, maintaining similar concentrations and size distributions with 99% of pNPs < 500 nm in diameter (Figure 3.9).
Figure 3.6. Size distribution and stability of pMBs. a) Size distribution of pMBs according to concentration before and after sonication with conversion US. b) Temporal stability of pMBs. Size distribution of pMBs over time. Average of 3 samples for each time point. c) Change in pMB concentration over time expressed as a percentage of the initial pMB concentration. Mean ± 1 SD with n=3. d) In vivo circulation time of pMBs in a mouse bearing a KB xenograft determined by high frequency US imaging (13-24 MHz). Solid line: 10 period moving average trend line.
Figure 3.7. Size distribution of pMBs showing the mean distribution ± 1 SD from 3 measurements for samples with 0 pulses (a), 1 pulse (b), 3 pulses (c) and 10 pulses (d).
Figure 3.8. Size distribution of pNPs showing the mean distribution ± 1 SD from 3 measurements for samples with 0 pulses (a), 1 pulse (b), 3 pulses (c) and 10 pulses (d).
Figure 3.9. pNP temporal stability in phosphate buffered saline. a) Size distribution of pNPs in solution over time. Average of 3 samples for each time point. b) pNP concentration over time. Mean ± 1 SD with n=3.
Light microscopy confirmed the formation of spherical micrometer-sized pMBs after activation (Figure 3.10a), which converted into spherical nanoparticles after interaction with conversion US, seen by electron microscopy (Figure 3.10b). Applying a greater number of conversion US pulses (e.g. 20 pulses) did not further induce any changes in morphology in the pNPs (Figure 3.10c).

We hypothesized that the porphyrin-lipid shell and perfluorocarbon comprising the pMBs were the components forming the pNPs after conversion with US. To investigate the contents of the pNP nanostructure, pMBs were converted into pNPs and then were introduced into vacuum conditions. Electron microscopy revealed that the resulting nanostructures from the pNP samples, after exposure to vacuum conditions, had generated liposome-like lipid bilayer vesicles (Figure 3.10d), confirming that after conversion from pMB to pNP, the pNPs contained the perfluorocarbons from the pMBs.

While the specific mechanism of conversion of pMBs to pNPs remains to be investigated, the increased number density of pNPs relative to pMBs is consistent with the occurrence of pMB fragmentation. As noted earlier, the fragmentation and lipid shedding response of encapsulated microbubbles has been previously investigated, however, these studies have been conducted in the context of individual optical microbubble experiments. Therefore, to investigate whether this micro-to-nano conversion was specific to pMBs, “regular” unimodal microbubbles were generated in the same manner as pMBs, except with the substitution of porphyrin-lipid with DPPC, a phospholipid without a porphyrin group attached. After sonication with conversion US, these regular microbubbles also formed nanostructures similar to the pNPs (Figure 3.10e).
Figure 3.10. Microscopy images of pMBs and pNPs. a) Light microscopy image of pMBs. b) transmission electron microscopy (TEM) image of pNPs formed from pMBs after 10 US pulses. c) TEM image of pNPs after 20 pulses of conversion US were applied. d) TEM image of liposome-like structures formed from pNPs after placing pNPs in a vacuum. e) TEM image of regular microbubbles without BChl-lipid after 10 pulses of conversion US were applied.
We investigated the effects of the conversion from microbubble to nanoparticle on the optical properties of pMBs. pMBs exhibited a peak absorbance at 824 nm, which is red-shifted from the 750 nm absorption peak characteristic of monomeric BChl (Figure 3.11a). This 74 nm bathochromic shift and increase in intensity are signature characteristics of ordered aggregation (J-aggregation)\textsuperscript{232, 224} of the porphyrins in the monolayer shell. The presence of ordered aggregates in pMBs was further confirmed using circular dichroism spectroscopy, which displayed peaks corresponding to the absorption spectra of pMBs and was void of detectable peaks with the BChl monomeric sample in which the aggregates had been disrupted using detergent (1 vol% Triton X-100) (Figure 3.11b). Upon application of conversion US pulses, the background scattering decreased, yet the peak absorbance and ordered aggregates at 824 nm was maintained (Figure 3.11a,b). This behavior was also confirmed in 50 vol% FBS, ensuring the optical functionality of this conversion in the presence of serum proteins (Figure 3.12). The 824 nm peak absorbance of the pNPs was maintained even when incubated in FBS at 37°C, gradually decreasing over time to 75% of the initial absorbance after 3 hr (Figure 3.13). This was observed with an increase in the monomeric 750 nm peak absorbance, indicating a disruption of the ordered aggregates in the pNPs.

PA imaging is an emerging technique that combines the depth penetration of US imaging with the high contrast of optical imaging and is based on the absorption of light, transient thermoelastic expansion and detection of the generated US waves\textsuperscript{115}. The PA spectra of pMBs before and after sonication corresponded well with the absorption spectra, maintaining a peak signal at 824 nm (Figure 3.11c). Furthermore, pMBs were also able to generate fluorescence as a result of their ordered aggregation\textsuperscript{232}, with a peak emission at 830 nm, red shifted from the BChl monomeric fluorescence emission peak at 765 nm (Figure 3.14). The ordered aggregation peak at 830 nm was also maintained even with the conversion to pNPs (Figure 3.11d). An increase in fluorescence emission intensity was observed after application of conversion US. This has been previously observed with other groups while detecting the release of drugs from microbubbles, and has been attributed to scattering of the excitation and emission light by the gas in microbubbles\textsuperscript{233}.  


Figure 3.11. Optical spectra of pMBs and resulting pNPs after interaction with conversion US. a) Absorption spectra and b) circular dichroism spectra of pMBs after several bursts compared with BChl-lipid in its monomeric form. c) PA and d) fluorescence spectra of pMBs before and after applying conversion US.

Figure 3.12. Absorption spectra of pMBs before and after application of conversion US pulses in 50 vol% FBS.
Figure 3.13. Stability of pNPs in FBS measured by optical absorption. pMBs were sonicated in 80 vol% serum to obtain pNPs. The stability of pNPs were determined via their 824 nm absorption peak and 750 nm monomeric BChl absorption peak. Instability was assessed by a decrease in the 824 nm absorption and an increase in the 750 nm absorption. Mean ± 1 SD with n=3.

Figure 3.14. Fluorescence spectra of pNPs intact (black) and disrupted (grey) using detergent. After disruption of the pNPs, the red-shifted aggregation induced fluorescence emission peak at 830 nm is restored to the fluorescence emission peak characteristic of monomeric BChl at 765 nm.
To demonstrate the imaging capabilities of pMBs and pNPs, samples were embedded in an US and PA transparent polyacrylamide gel and imaged using US, PA and fluorescence imaging. US imaging showed a distinct decrease in contrast with a greater number of conversion US pulses applied (Figure 3.15a I), which was further verified with a relative comparison in contrast mean power between samples (Figure 3.15b). The PA and fluorescence intensities remained consistent in pMBs and pNPs after conversion and were not statistically significant (p > 0.05) between pulsed samples (Figure 3.15a II and III, c, d). Differences observed in fluorescence intensity between the phantom imaging and spectra in Figure 3.11d may be due to detector sensitivity, as the detector used to obtain the fluorescence spectra is more sensitive than the camera used for imaging. These phantom images confirm the applicability of trimodal imaging of pMBs, applying the unique US property of microbubbles, their ability to burst, shrink or fragment in response to US, and optically image the resulting nanoparticles formed.

Porphyrin-based nanoparticles have been of increasing interest for imaging and treatment of disease, in particular, cancer, due to their intrinsically multifunctional nature. They have been applied as fluorescence imaging\(^\text{155,234}\), PET\(^\text{116,117}\), MRI\(^\text{235,236}\) and PA imaging\(^\text{155,224}\) contrast agents, as well as for PDT\(^\text{234,237,238}\) and PTT\(^\text{114}\). As with the majority of nanoparticles intended for tumor targeting, the delivery of these porphyrin-based nanoparticles relies on the enhanced permeability and retention (EPR) effect, requiring on the order of hours to days for maximum accumulation. However, the clinical relevance of the EPR effect has undergone significant debate due to difficulty in predicting nanoparticle uptake \textit{in vivo} as a result of a number of factors affecting EPR, such as heterogeneity in the vascular bed, tumor growth microenvironment and infiltrating macrophages. Furthermore, the EPR effect has also not been well understood and correlated between preclinical and clinical patient solid tumors\(^\text{239}\). Alternatively, microbubbles have been investigated for drug and gene delivery, in which a drug-loaded microbubble may be sonicated with US causing the microbubble to interact with the endothelial cells of blood vessels, and result in extravasation or uptake into surrounding tissue\(^\text{28,240-242}\). Therefore, we sought to utilize the micro-to-nano conversion of pMBs to bypass the EPR effect, using an external US trigger, to deliver pNPs to a solid tumor.
Figure 3.15. Multimodal imaging of pMBs and resulting pNP upon US induced conversion. a) pMBs and pNPs embedded in an acrylamide gel phantom were imaged using I) US (13-24 MHz transducer in contrast mode), II) PA (21 MHz transducer, 824 nm) and III) fluorescence (green exc. 503 nm - 548 nm; 560 nm long pass detection). Samples: 1) PBS, 2) pMBs without applying conversion US pulses – 0 pulses, 3) 1 pulse, 4) 3 pulses, 5) 10 pulses. b-d) Quantified signals from I) US, II) PA and III) fluorescence imaging. Data are expressed as mean values (n=3) ± 1 SD; *p < 0.05; **p > 0.05.
pMBs were intravenously injected into KB xenograft bearing mice and the mice were divided into two groups in which the tumor was either subjected to conversion US (“conversion US applied” group) or did not have conversion US applied (“no conversion US applied” group). US imaging was used to monitor the influx of pMBs into the tumor (Figure 3.16a, b). When conversion US was not applied, the pMBs circulated for approximately 2 min before returning to baseline (Figure 3.16aI, bI). In contrast, in the conversion US applied group, after 20 s post injection, in which the pMBs could be observed circulating in the tumor, conversion US was applied and a drastic decrease in US contrast was observed within a few seconds, quickly returning back to baseline (Figure 3.16aII, bII), indicating a conversion from pMB to pNP.

PA imaging was used to verify the conversion to pNP and validate successful delivery and retention of porphyrins in the tumor. When conversion US sonicated the tumor after injection of pMBs, the PA signal was maintained within the tumor for at least 2 hr. However, when conversion US was not applied, the PA signal decreased within 30 min post-injection. (Figure 3.17a,b). Fluorescence imaging of whole blood was used to investigate the cause of this decrease in PA signal. The US contrast from pMBs is generated from the presence of gas within the microbubble. After 2 min of circulation, this gas diffuses out of the pMB, however, the lipid shell may still form some remnant structure, which remains in circulation and will also be cleared by the reticuloendothelial system.
Figure 3.16. US images of conversion of pMBs to pNPs in vivo. a) pMBs were intravenously administered into KB xenograft bearing mice and imaged using high frequency US. I) After injection, the pMBs circulated into the tumor, reaching a peak in circulation at 20 s and could continuously be observed in circulation beyond 40 s. 2 mm scale bar shown. II) When conversion US was applied after the 20 s time point, a decrease in contrast mode US signal was observed, for example at 40 s. 3 mm scale bar shown. b) Region of interest analysis of the contrast mean power expressed as a percentage of the mean maximum signal in a mouse I) without conversion US applied and II) with conversion US applied after the 20 s time point. Black line indicates a 10 period moving average trend line.
Figure 3.17. PA imaging of pMB to pNP conversion in vivo. a) PA images of mice I) without or II) with conversion US applied over time. 2 mm scale bars shown. b) Normalized PA signal over time for mice in which conversion US was not applied (blue) or was applied (green). PA values normalized to the peak PA value. Mean ± 1 SD with n=3.
Fluorescence imaging of whole blood extracted 5 min and 30 min after pMB injection revealed that after 5 min of circulation, the ordered aggregation peak remained intact with the fluorescence emission peak at 830 nm. However, by 30 min, the ordered aggregation had been disrupted, shifting back to its monomeric fluorescence emission peak at 765 nm (Figure 3.18). This corresponded to the observed decrease in PA signal without application of conversion US within 30 min. Therefore, the presence of PA signal generation in tumors in which conversion US had been applied indicates the successful delivery of porphyrins, in the supramolecular structure of pNPs, to the tumor. Thus, we envision the use of PAs or another imaging modality to confirm successful delivery of pNPs via the \textit{in situ} micro-to-nano conversion within minutes of administration, which can then be used for therapy (e.g. PDT or PTT).

The intrinsic properties of pMBs and their \textit{in situ} conversion to pNPs presented here are a mere representation of the many therapeutic and imaging applications of pMBs, expanding the utility of conventional microbubbles. For example, as conventional microbubbles are used to permeabilize the blood brain barrier (BBB)\textsuperscript{243} in order to enhance drug and gene delivery\textsuperscript{28}, the use of pMBs in these instances could enable an additional intrinsic imaging and/or therapeutic property along side delivering drugs. The application of pMBs would therefore allow imaging of the delivery of these drugs by tracking the pNPs, whether by optical imaging methods, or whole-body techniques such as PET or MRI, which could provide assurance of successful drug delivery. Furthermore, the porphyrin in pMBs is also a photosensitizer for PDT\textsuperscript{244,245}. Therefore, pMBs could also be used to homogeneously deliver a high payload of photosensitizer to a tumor region or across the BBB for therapy.
Figure 3.18. Fluorescence imaging of whole blood extracted from mice without pMB injections (grey), 5 min post-injection (red) and 30 min post-injection (green). a) Fluorescence spectra of whole blood without pMB injection, 5 min post-injection and 30 min post-injection. b) Corresponding fluorescence images.
3.6 Conclusion

pMBs serve as trimodality contrast agents for US, PA and fluorescence. Furthermore, they also possess the same ability as conventional microbubbles to respond to low frequency, high duty cycle US, thus forming nanoparticles. Just as conventional microbubbles lose their US contrast, pMBs also lose their US contrast, however, they maintain their PA and fluorescence properties. We utilized this property to deliver porphyrin nanoparticles to a solid tumor and used PA imaging to validate the successful delivery. These porphyrins could also potentially be applied for therapeutic purposes. As a result, the presence of the porphyrin in pMBs has transformed a conventional microbubble from a unimodal US contrast agent and simple drug delivery vehicle, without any imaging and therapeutic properties after destruction, to a trimodality contrast agent in which the resultant pNP has imaging and therapeutic properties and can be delivered to tumors without relying on the EPR effect. Therefore, pMBs introduce new imaging and therapeutic applications for microbubbles, harnessing the advantages of US-based methods.
Chapter 4
Giant Porphyrin Vesicles

4.1 Acknowledgements

This chapter is in large a reformatted version of the manuscript entitled “Optically controlled pore formation in self-sealing giant porphyrin vesicles”, published in *Small*\(^\text{246}\). Under the supervision of Dr. Gang Zheng, my contribution to this work included designing and carrying out the experiments, analyzing and interpreting the data and writing most of the manuscript text. I would like to thank Dr. Jonathan Lovell for his valuable contributions and insightful discussions, and Ryan Fobel for developing the electrical circuit used in this study.

4.2 Abstract

Efforts to develop self-contained microreactors and artificial cells have been limited by difficulty in generating membranes that can be robustly and repeatedly manipulated to load and release cargo from phospholipid compartments. Here we describe a purely optical method to form pores in a membrane generated from porphyrin-phospholipid conjugates electro-assembled into microscale giant porphyrin vesicles and manipulated using confocal microscopy. The pores in the membrane resealed within a minute allowing for repeated pore formation with spatial and temporal control, and optical gating properties allowing selective diffusion of biomolecules into the vesicle compartment. Temporal control of pore formation was illustrated by performing sequential DNA hybridization reactions. Optical gating was demonstrated by the selective loading and/or release of dextrans based on their size by varying the laser fluence irradiating the porphyrin-phospholipid membrane. A biotin-avidin based strategy was developed to selectively attach enzymes to the interior of the vesicle, demonstrating spatial control and the potential of giant porphyrin vesicles as versatile microreactors.
4.3 Introduction

A wide range of protein-based transport systems has evolved in organisms to permit the movement of molecules through bilayers without destroying the overall membrane integrity. However, these transport systems are typically specific for certain cargo and are not suitable as general-purpose gateways to the interior of natural or synthetic phospholipid-enclosed compartments. Thus, disruptive techniques such as, electroporation and heat shock have been developed to permit the passage of biomolecules through cell membranes. For example, electroporation involves the application of electrical pulses with intensity on the order of kilovolts per centimeter and duration of microseconds to milliseconds. This causes a transient increase in the permeability of the cell membrane, thereby allowing the leakage of ions and metabolites or the uptake of drugs, molecular probes and DNA. These techniques are generally applied to a bulk population of cells. While highly practical for some applications, the positioning and timing of pore formation for an individual cell or giant vesicle using these methods are not easy to control.

More precise control of bilayer permeability has been achieved using novel approaches such as local electroporation, proximal heating of gold nanoparticles, electroinjection and photoporation, many of which involve the addition of an exogenous agent or highly sophisticated instruments. The pore formation and resealing of swollen, giant vesicles have been well characterized, but again, the process is not readily controllable and has traditionally made use of highly viscous solvents that preclude many application.

Here we describe a simple optical technique to robustly control membrane permeability in a cell-sized vesicle based on the unique properties of a porphyrin-lipid bilayer, referred to as giant porphyrin vesicles (GPVs) and confocal microscopy. The porphyrin-lipid bilayer responds to light by forming a transient pore upon laser irradiation, which then proceeded to self-seal.

4.4 Materials and methods

4.4.1 Giant porphyrin vesicle formation

All chemical materials were obtained from Sigma-Aldrich and electronic materials were obtained from Mouser, unless otherwise indicated. GPVs were formed using a modified electroformation method. Pyropheophorbide-lipid (pyro-lipid; prepared as previously
described\textsuperscript{155}, but with a modified protocol to generate an isomerically pure conjugate\textsuperscript{119} in combination with egg phosphatidylcholine (egg PC) and cholesterol (chol) (3:2 molar ratio egg PC: chol) (Avanti Polar Lipids), was dispersed in chloroform to form 0.1 mg/ml - 0.5 mg/ml stock solutions. Two 0.5 mm diameter platinum wires (#267228, Sigma) were positioned in parallel separated by a distance of 2 mm through a small polytetrafluoroethylene O-ring (#9559K208, McMaster-Carr) adhered to a cover slide using vacuum grease. 6-10 equally spaced 1 µl droplets of stock solution were deposited on the two platinum wires. Unless otherwise noted, 70 mol% pyro-lipid was used. Residual chloroform was evaporated by placing the O-ring apparatus in a vacuum for 20 min. A 0.6 mL water solution with 2 mM Tris pH 8 was then used to hydrate the lipids on the wire. The apparatus was connected to a 3 V, 10 Hz alternating current in order to induce electroformation of the vesicles. A low cost, open source Arduino microcontroller was used to generate the electric field. Vesicles formed on the wire were visible 15 min after turning on the electric field. To visualize vesicles detached from the wire, the lipids were hydrated with a 200 mOsM sucrose solution. After applying the electric field for 45 min, 25 µl of the vesicle solution was diluted in 100 µl of a 200 mOsM glucose solution on a cover slide where the vesicles sunk to the bottom of the solution and could be visualized by microscopy.

4.4.2 Microscopy and pore formation

Confocal microscopy (Olympus FluoView FV1000) was used to inspect the vesicles using a 633 nm laser and 40 x water objective lens. Pore formation was induced using a 405 nm laser pulse with a power of 660 µW. Laser irradiation spot size and irradiation time were chosen based on the noted laser fluence in the text. Z-stack slices were obtained using a 0.4 µm step size and reconstructed into a 3D image using ImageJ (National Institutes of Health). For all experiments requiring the loading or release of molecules, GPVs were formed for 2 hr prior to addition of fluorophores or manipulation of GPVs. For fluorophore diffusion into the GPVs, carboxyfluorescein (81002, AnaSpec Inc.), Texas Red dextran (MW 10 kDa, D-1828, Invitrogen) and tetramethylrhodamine (TRITC) dextran (MW 155 kDa,T1287, Sigma-Aldrich) were added to the medium and observed using a 488 nm laser for carboxyfluorescein and 543 nm for both Texas Red dextran and TRITC dextran. For release of cargo from GPVs, lipids were rehydrated in the presence of TRITC dextran (155 kDa) and carboxyfluorescein (0.4 kDa). GPVs were washed with 2 ml of 2 mM Tris pH8 using a syringe pump to replace the buffer containing fluorescent molecules exterior to the GPVs. A low flow rate (6 ml/hr) was used in order to not
disturb the GPVs attached to the wire. GPVs were then irradiated with low or high laser fluence as indicated in the text. Diffusion equilibrium was quantified by normalizing the fluorescence inside the GPV to the initial fluorescence outside of the GPV as a function of time using ImageJ. For physiological conditions, GPVs were formed in phosphate buffered saline under a 5 V, 500 Hz electric field for 2 hr. 155 kDa TRITC dextran was added to the well and GPVs were irradiated using a laser fluence of 40 µJ/µm².

4.4.3 DNA hybridization
For controlled DNA fluorescence and quenching, an oligonucleotide with the sequence GGTGTTTGGTTGTTGTTTTC-Fluorescein (Sigma) was added to the external medium of a GPV at 1 µM concentration with 1 mM sodium chloride (NaCl). After performing light induced loading, the complementary sequence DAB-GAAAAACAACAACAAAAACACCC (Sigma) was added to the external medium in ten-fold excess and GPV pore formation was repeated.

4.4.4 Avidin-biotin binding
For avidin-biotin binding, GPVs were formed with 0.05 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (Avanti Polar Lipids) by depositing eight 1 µl droplets of 0.5 mg/ml on the wires and rehydrating with 2 mM Tris pH8. Once formed, 12 nM avidin (AVD407, BioShop Canada Inc.) dissolved in 2 mMTris pH 8 was added to the external medium to block the biotin binding sites on the outer leaf of the GPV. After 15 min, 24 nM fluorescein conjugated avidin (APA011F, BioShop Canada Inc.) was added to the buffer and the GPV was irradiated several times to observe fluorescent avidin binding to the biotin binding sites on the inner leaflet of the GPV.

4.5 Results and discussion

4.5.1 Giant porphyrin vesicle formation
GPVs were formed using a modified electroformation approach, based on the alternating current method.257 Using a low cost, open-source programmable Arduino microcontroller, a solution of varying fractions of porphyrin-lipid (pyropheophorbide-lipid; pyro-lipid) (Figure 4.1a) and egg phosphatidylcholine with cholesterol in chloroform was coated onto platinum wires, evaporated, rehydrated and subjected to a low-frequency alternating square wave field (Figure 4.1a II). Using this method, micrometer-scale vesicles were readily generated and could be visualized using
confocal microscopy (Figure 4.1a II, inset). Pyrophoephorbide (pyro) was chosen as the porphyrin for the lipid-conjugate as the characteristic optical absorption spectral properties (Figure 4.2) allowed both manipulation by high intensity light (using a 405 nm laser) and imaging of the response to irradiation (using a 633 nm laser) by confocal microscopy since pyro absorbs strongly at both these wavelengths. In the presence of the electric field, vesicles formed spontaneously and slowly detached from the platinum wires, and this process continued repeatedly over time. Removal of the electric field prevented the oscillations necessary for vesicle detachment, leaving a high density of relatively immobilized GPVs proximal to the wires, which greatly facilitated time-series observation by confocal microscopy. Because the porphyrin component of the lipid conjugate is fluorescent, the bilayer could be imaged using fluorescence microscopy without any exogenous label.

We examined how increasing proportions of porphyrin-lipid affected two types of movement-restricted vesicles: detached, sucrose-containing vesicles that sank when transferred to a separate solution of lesser density glucose, and vesicles immobilized on the platinum wire. In both cases, spherical vesicles 10-50 µm in size were formed, and the addition of greater than 1 mol% porphyrin-lipid led to fluorescence self-quenching of the entire vesicle due to the higher porphyrin content (Figure 4.1b). The consistent porphyrin depth within the bilayer held in place by the amphipathic nature of the porphyrin-lipid, combined with the high porphyrin density suggest that the bilayer environment created dynamic face to face porphyrin interactions, leading to fluorescence quenching. However, microvesicle yield and circular geometry quality decreased beyond 70 mol% porphyrin-lipid, demonstrating that some standard phospholipids were helpful to form the GPVs.
Figure 4.1. Generation and pore formation in giant porphyrin vesicles (GPVs). a) I) Schematic of porphyrin-lipid used to form GPVs; II) Experimental setup used for GPV electroformation using a microcontroller to generate an AC field. The apparatus set up is shown on the right. The left insets show a confocal micrograph (left) and a schematic image (right) of GPVs formed on the wire. b) Fluorescence quenching of GPVs formed in two different environments: 1) encapsulating sucrose and dispersed in glucose, or 2) immobilized on the platinum wire. Fluorescence settings were the same in each image. 10µm scale bar is shown.
Figure 4.2. Optical spectra of pyro-lipid. a) Absorption spectrum of pyro-lipid in methanol. b) Fluorescence emission spectrum of pyro-lipid in methanol (excitation: 410 nm).
4.5.2 Response of GPV bilayer to laser irradiation

Each 10 µm porphyrin vesicle formed from 70 mol% porphyrin-lipid was estimated to contain approximately $6 \times 10^8$ porphyrins, all confined to the thin, enclosing porphyrin bilayer. Given the high optical absorption of the porphyrin bilayer, the membrane response to laser irradiation was investigated. Despite the high level of fluorescence self-quenching (75% quenched compared to 1 mol% pyro-lipid giant vesicles), the bilayer retained enough fluorescence to enable clear optical observation of the bilayer response using a 633 nm laser to excite the Q-band of pyro.

Confocal microscopy is commonly used to study the fluorescence recovery after photobleaching (FRAP) of a cell membrane induced by a focused laser incorporated into the microscope set-up. We applied this focused laser pulse with a wavelength of 405 nm to the GPV membrane, which directly excited the more intense pyro Soret band. The laser power was estimated to be 660 µW and was focused into a small area to achieve a laser fluence rate on the order of kWs per cm$^2$. When the bilayer was subjected to a 200 ms pulse confined to a 2 µm diameter spot on the membrane (a high laser fluence of 42 µJ/µm$^2$; intensity: 210 µW/µm$^2$), the bilayer was observed to form a large micrometer sized pore, 10-15 µm in diameter in the membrane of a 30 µm diameter GPV for an extended period of time (Figure 4.3a). After 30 s, the membrane edges of the pore came together, resealed and the vesicle appeared intact again, returning to its original state. Although they displayed less contrast, phase contrast images confirmed that the bilayer was physically forming a large pore and resealing, as opposed to a local fluorescence bleaching of the bilayer. Single slice images were taken through the volume of the GPV and reconstructed to provide a 3D rendering of the vesicle before and after laser irradiation, demonstrating that upon laser irradiation a large micrometer sized pore formed through the bilayer and extended in all directions (Figure 4.3b).
Figure 4.3. Pore formation in GPVs upon laser irradiation. a) GPVs containing 70 mol% pyrolipid formed a pore upon laser irradiation (white dashed circle) and then self-sealed. Arrows show GPV pore. 10µm scale bar is indicated. b) 3D image of a GPV reconstructed from z-stack slices. Schematic representation of a GPV (left) indicating the section of the vesicle imaged (blue dashed box), pore region (red circle) and the point of view (black arrow) corresponding to pre-irradiation, pore formation and after resealing images (right). White arrow indicates pore. 5 µm scale bar is indicated.
Having observed this pore formation and resealing behavior of GPVs formed from 70 mol% porphyrin-lipid, repeated pore formation and self-sealing of GPVs was investigated and compared with the behavior of vesicles formed from 1mol% porphyrin-lipid. As shown in Figure 4.4, a GPV formed from 70 mol% porphyrin-lipid could repeatedly form large pores and self-seal indefinitely. However, when 1 mol% porphyrin-lipid was incorporated into the bilayer, no membrane opening was observed after applying the same high laser fluence (42 μJ/μm²; intensity: 210 μW/μm²). Various GPV responses to laser irradiation were categorized as no response, membrane stretching, opening alone (pore formation), or opening and closing (pore formation and resealing) (see Figure 4.5 for an example of membrane stretching). None of the varying laser powers examined (fluence range: 4 μJ/μm² to 42 μJ/μm²; intensity: 21 μW/μm² to 210 μW/μm² irradiated for 200 ms) could induce pore formation in the highly fluorescent 1 mol% porphyrin-lipid microvesicles (Figure 4.6a). However, pore formation and resealing was observed consistently for the 70 mol% porphyrin-lipid GPVs, with the percentage of opening and closing events increasing with greater laser power. A small subset of GPVs remained open and did not reseal even after several minutes. A similar trend was observed as the irradiation area was increased from 2 μm to 12 μm, effectively decreasing the laser fluence from 42 μJ/μm² (intensity: 210 μW/μm²) to 1 μJ/μm² (intensity: 6 μW/μm²), respectively (Figure 4.6b).
Figure 4.4. GPV repeated pore formation and self-sealing. Repeated pore formation and self-sealing of a GPV. Arrows indicate time of laser pulsing (laser fluence: 40 µJ/µm$^2$). 5 µm scale bar is indicated.

Figure 4.5. Membrane stretching in GPVs following laser irradiation. White dashed circle indicates area of laser irradiation. 5 µm scale bar is shown.
Figure 4.6. GPV response to laser irradiation with varying. a) Laser power and b) irradiation spot diameter using a 200 ms irradiation time. Frequency charts are based on results from 10 separate GPVs irradiations per bar.
A substantial amount of experimental and theoretical work has led to an understanding of pressure-induced opening and closing of conventional giant unilamellar vesicles\textsuperscript{262-265}. In these well-established models, pore formation is initiated and propagated by increased surface tension. Once the pore forms, lipids re-orient themselves to minimize hydrophobic side-chain exposure to the aqueous environment, but this modified packing structure has a free energy cost. Thus, an edge tension force is generated that opposes pore formation and is responsible for pore closure. Pore dynamics are balanced by the opposing forces of edge tension and surface tension. We hypothesize that in the case of GPVs, the porphyrin bilayer may stabilize the pore edge and reduce the edge tension force. As shown in Figure 4.7, a typical GPV opening followed conventional patterns of lipid vesicle opening, with a rapid opening, slow closing and fast closing phase. Based on mathematical models developed by the Brochard-Wyart group\textsuperscript{262} and recently further elucidated by the Dimova group\textsuperscript{264}, it has been demonstrated that the edge tension can be calculated during the slow closure period from the slope of $R^2\ln(r)$ as a function of time, where $R$ is the radius of the vesicle and $r$ is the radius of the pore\textsuperscript{262,264}. The edge tension, $\gamma$, can then be calculated from Equation 1:

$$\gamma = -(3/2) \pi \eta a$$  \hspace{1cm} (1)

$a$ represents the slope of the linear fit of the slow closure phase shown in Figure 4.7 and $\eta$ is the viscosity of the medium, water in this case (8.9x10\textsuperscript{-4} Pa s). Using this technique, we estimate a typical edge tension force during GPV closing of 29±7 fN. This value is noteworthy as it is approximately 3 orders of magnitude smaller than conventional phospholipid bilayers\textsuperscript{264}. Thus, the edge of the porated porphyrin bilayer appears to be significantly stabilized by the porphyrin itself. This may be from the extensive and dynamic face-to-face porphyrin pi-pi electron interactions that occur in the GPV bilayer.
Figure 4.7. Estimation of edge tension force in GPV pores. A typical pore opening is shown, plotted as $R^2 \ln(r)$ as a function of time, where $R$ is the GPV radius and $r$ is the pore radius. The edge tension during the slow close period, based on the slope of the black line ($\text{slope} = -6.75 \pm 1.51, n=3 \pm \text{SD}$), was $29 \pm 7 \, \text{fN}$. Laser fluence: $40 \, \mu\text{J}/\mu\text{m}^2$. 
4.5.3 Diffusion of biomolecules

In order to examine whether small molecules could diffuse through the porated membrane and the effect of varying the laser fluence, carboxyfluorescein was added to the exterior of the GPV. No fluorescence was observed inside the GPVs following addition of the fluorophores into the solution, demonstrating that the porphyrin bilayer was impermeable to these molecules. GPVs were irradiated with high and low laser fluences using 42 µJ/µm² (intensity: 210 µW/µm²) for high laser fluence and 1 µJ/µm² (intensity: 6 µW/µm²) for low laser fluence, unless otherwise indicated. When the GPVs were irradiated with a high laser fluence (40 µJ/µm²), carboxyfluorescein freely diffused into the GPV within seconds and the edges of the GPV membrane was seen to physically come apart and form a micrometer sized pore, verified by the phase contrast image (Figure 4.8a, b). When GPVs were irradiated with a low laser fluence (1 µJ/µm²), carboxyfluorescein also diffused into the GPV but at a much slower rate, indicating the presence of small pores that limited the rate but did not restrict molecule diffusion (Figure 4.8c, d). Furthermore, the membrane of the GPV was not observed to form a large micrometer sized pore as with the higher laser fluence, suggesting that nanopores could form in the membrane under low laser fluence but were not visible due to the resolution limitations of the microscope. Therefore, two types of pores can be formed in the GPV membrane: nanopores using low laser fluence and a micropore using high laser fluence.

The formation of these pores in the GPV membrane were dependent upon two factors: laser fluence and porphyrin-lipid concentration, as GPVs formed from 70 mol% porphyrin-lipid, laser fluences less than 0.5 µJ/µm² did not induce pore formation (Figure 4.8e) and vesicles formed from 1 mol% porphyrin-lipid did not exhibit the ability to form visible pores or allow the diffusion of carboxyfluorescein using either the high or low laser fluence (Figure 4.9). Porphyrin fluorescence self-quenching has been shown to be highly correlated with self-quenching of singlet oxygen quantum yield. Although less quenched, 1 mol% porphyrin-lipid GPVs generated substantially more fluorescence and thus more singlet oxygen (hundreds of fold more, based on the quenching in phorphosomes of similar composition), they did not form pores in response to laser irradiation, indicating that pore formation is not a result of singlet oxygen interaction with the membrane. This is consistent with previous examination of singlet oxygen generation of porphyrins anchored in low molar percentages (1 – 10 mol%) in phospholipid giant unilamellar vesicles, which did not produce visible membrane poration in response to
irradiation\textsuperscript{74}. Furthermore, destabilization of membranes based on singlet oxygen production occurs by reduction of phospholipid tails when unsaturated bonds interact with singlet oxygen\textsuperscript{74,266}. When GPVs were formed with 30 mol\% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), a saturated lipid, in place of egg PC that contains 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), an unsaturated lipid, pore formation was still consistently observed. In addition, when GPVs were formed with different transition temperature lipids, while applying the same laser parameters, increasing the transition temperature of the lipid resulted in a slower efflux of fluorophore out of the GPV (Figure 4.10).
Figure 4.8. Types of pores formed by GPVs. a) Low laser fluences (1 µJ/µm²) produce nanopores (left) whereas high laser fluences (40 µJ/µm²) produce micropores (right), both allowing the diffusion of carboxyfluorescein (0.4 kDa). 5 µm scale bar shown. b) Diffusion equilibrium of carboxyfluorescein into GPVs through different pore types. c, d) Diffusion of carboxyfluorescein through nanopores (c) and a large micropore (d) generated from laser irradiation to the GPV membrane. Carboxyfluorescein diffuses through the large micropore at a faster rate than through nanopores. 5 µm scale bar shown. e) GPVs formed with 70 mol% pyro-lipid and irradiated with a laser fluence of 0.5 µJ/µm² do not allow the diffusion of carboxyfluorescein, indicating the lack of nanopore formation. 5 µm scale bar shown.
Figure 4.9. Laser irradiation of vesicles formed with 1 mol% pyro-lipid in the presence of carboxyfluorescein with a) low laser fluence (1 µJ/µm²) and b) high laser fluence (40 µJ/µm²). 5 µm scale bar shown.
Figure 4.10. Thermally dependent pore formation in GPVs. a) GPVs formed with different transition temperature lipids encapsulating carboxyfluorescein. Successive images show release of carboxyfluorescein after laser irradiation. Transition temperature of lipids shown below lipid name. b) Quantified release of carboxyfluorescein from the interior of the GPVs over time. 10 µm scale bar shown.
We hypothesize that the formation of these pores observed in GPVs are induced by a thermal mechanism. We previously demonstrated that nanoparticles formed from porphyrin-lipid possessed intrinsic biophotonic properties, rivalling gold nanoparticles, due to extreme fluorescence self-quenching resulting in high heat generation. Gold nanoparticles are known to provide efficient conversion of optical energy into thermal energy. Furthermore, under high laser fluence, gold nanoparticles generate vapor bubbles around the nanoparticle due to rapid heating and vaporization of the surrounding medium. The dependence of pore formation in GPVs on high porphyrin content (resulting in fluorescence self-quenching (Figure 4.1b)), and high laser fluence indicate that pore formation may be due to high heat generation. For nanopores, irradiation with low laser fluence causes thermal heating of the membrane, resulting in an increase in membrane fluidity, which allows small molecules to permeate the membrane. However, upon irradiation using high laser fluence, rapid local heating may result in the formation of transient vapor bubbles which rapidly increases the membrane tension near at the pore site, forcing the edges of the membrane apart, resulting in the formation of a micrometer sized pore, much larger than the irradiation spot size (Figure 4.2a,b). Vapor bubbles generated by gold nanoparticles are known to have a life time on the scale of nanoseconds, which is beyond the temporal resolution of the confocal microscope and could not be directly observed in GPVs. Further work is required to fully elucidate and model the mechanism and physics of GPV pore formation and resealing, but it is likely due to a photophysical process which results in the production of thermal energy which increases the membrane tension near the pore site.

The ability to form two types of pores in GPVs permits a number of possible applications as an artificial cell or microreactor. The micropore allows the encapsulation of large particles such as microparticles, cells or bacteria, whereas the nanopores allow for the loading and release of smaller cargo such as chemical reactants. Furthermore, the rate of diffusion of biomolecules through micropores is proportional to the size of the molecule. We quantified the internalization rate of three fluorophores of varying sizes through micropores and observed that the smaller 0.4 kDa carboxyfluorescein diffused in faster than the 10 kDa Texas Red dextran, which in turn diffused faster than the 155 kDa TRITC dextran (Figure 4.11a,b). The time required for half the molecules to diffuse into the GPVs following opening varied from 1 to 8 s based on the size of the cargo (Figure 4.11c) as would be expected for smaller molecules, which diffuse more rapidly.
To demonstrate the release of cargo from GPVs, GPVs were formed in the presence of two fluorophores, carboxyfluorescein (0.4 kDa) and TRITC dextran (155 kDa). The exterior medium was replaced in order to wash away free fluorophore. Using a low laser fluence (2 µJ/µm²; intensity: 10 µW/µm²), low molecular weight cargo (carboxyfluorescein, 0.4 kDa) could be released through nanopores, while retaining larger molecular weight cargo (TRITC dextran, 155 kDa) (Figure 4.9a, b). Applying higher laser fluence (20 µJ/µm²; intensity: 33 µW/µm²) enabled the release of the larger cargo (Figure 4.12a, b), indicating the presence of larger pores. The dependence of pore size on laser fluence, especially with nanopores, provides a means of optical gating with the user having the ability to control the precise loading/release of specific cargo. Nanopores enable user-controlled loading or release of size dependent cargo. This method of optical gating is especially important in microreactor applications, allowing multistep reactions with the loading of specific reactants without releasing products generated from previous steps and without the use of exogenous agents, not previously possible with other injection techniques used for vesicle compartments.
Figure 4.11. Diffusion of biomolecules into GPVs through a micropore. a) Confocal images of GPVs and exogenous fluorophores added to the solution before and after laser irradiation (laser fluence: 40 μJ/μm²). 10 μm scale bar is shown. b) Diffusion equilibrium of various fluorophores into GPVs. c) Half-times to diffusion equilibrium for various sized fluorophores (mean ± SD from 5 separate GPV irradiations per fluorophore).
Figure 4.12. Size dependent optical gating of cargo. Different molecular weight fluorophores, carboxyfluorescein (0.4 kDa) and TRITC dextran (155 kDa), were co-encapsulated in GPVs and external fluorophores were removed by washing. a,b) A GPV was irradiated with a pulse of low laser fluence (laser pulse 1: 2 µJ/µm²) and low molecular weight molecules (carboxyfluorescein) were released; however the larger fluorophores (TRITC dextran) remained trapped inside the GPV. 10 µm scale bar shown. c,d) A GPV was first irradiated with a pulse of low laser fluence (laser pulse 1: 2 µJ/µm²) and carboxyfluorescein was released while TRITC dextran remained inside. After 2 min, a second pulse of greater laser fluence (laser pulse 2: 20 µJ/µm²) was applied and the large TRITC dextran was released. 10 µm scale bar shown.
4.5.4 Temporal and spatial control

As photoporation techniques are frequently used for gene transfection into cells, to demonstrate that a GPV could be repeatedly optically manipulated with precise timing and allow the encapsulation of DNA into an artificial cell-like membrane, laser induced membrane pore formation was used to control the passage of oligonucleotides (Figure 4.13). When a fluorescein-labeled DNA oligonucleotide was incubated with preformed GPVs, it remained outside the vesicles. Upon laser-induced opening (fluence: 42 µJ/µm²; intensity: 210 µW/µm²), the DNA diffused into the GPV, as indicated by an increase in internalized fluorescence. Next, a complementary strand labeled with dabcyl, a dark quencher, was added to the external buffer. The fluorescence of the solution was markedly attenuated. However, the interior of the GPV remained fluorescent, as the quenching oligonucleotide could not pass the porphyrin bilayer to reach the DNA that had been encapsulated. Finally, when the GPV was opened again, the quencher and quenched hybridized DNA could diffuse into the interior and eliminate the fluorescence coming from the GPV. The timing of pore formation in the GPV was precisely controlled between addition of the oligonucleotides and closing of the membrane. This illustrates the temporal control associated with this method, which is essential for sequential reactions, and the ability to transfect GPVs with DNA, a common application of photoporation.
Figure 4.13. Temporal control illustrated using sequential DNA hybridization in GPVs. Fluorescently labeled DNA exterior of the GPVs (1, yellow) was permitted to enter the left GPV following laser opening (2) (laser fluence: 40 µJ/µm²) whereas the right GPV remained empty. A complementary sequence with a quenching moiety was then added to the external medium attenuating the external fluorescence outside the GPVs (3) and again the left GPV was opened (laser fluence: 40 µJ/µm²) to allow the quenching to occur inside the GPV (4). 10 µm scale bar is shown.
The ability to predetermine the location of pore formation and induce a pore at a specific time point is a highly advantageous characteristic that many membrane permeability techniques lack. In addition, a useful enclosed microreactor should also confine the desired reaction to the interior space of the vesicle. We developed a strategy to selectively attach enzymatic molecules of interest to specific locations within the interior of GPVs (Figure 4.14a). By including a small molar percentage of biotinylated lipid in the formulation, GPVs could be formed that were prone to avidin binding, which is essentially an irreversible association with biotin in standard aqueous conditions. The exterior of the GPVs were blocked with a 2-fold molar excess of avidin, ensuring all biotin sites on the exterior leaflet of the GPV bilayer were occupied. A 4-fold excess of fluorescein labeled avidin was then added to the external medium. The single pores were formed in preselected regions of the GPV membrane using laser irradiation (fluence: 42 µJ/µm²; intensity: 210 µW/µm²). The labeled avidin did not freely diffuse into the GPV and bind uniformly around the circumference. Instead, it bound exclusively around the pore site inside the GPV (Figure 4.14b). This may be due to the smaller opening that was induced in the membrane when the biotin and avidin was used. This process was repeated 8 times to illustrate the spatial control of this method and achieve uniform spacing of labeled avidin around the periphery of the GPV interior. In this case, we used fluorescently labeled avidin, but enzyme-avidin conjugates are also available and could be placed in the GPV interior in the same manner. Eventually, following selective enzyme attachment to the GPV interior, substrates could be diffused into the GPVs to become enzymatically transformed into products and then released on demand by porphyrin bilayer pore formation. This approach could prove useful for small volume reactions for enzyme activity optimization, screening approaches or sequential reactions. In addition, as several reactions and biological processes are observed in physiological conditions, GPVs could also be formed in physiological salt conditions and demonstrate the same pore formation behavior by simply increasing the parameters of the electric field used for electroformation and laser irradiation (Figure 4.15).

In photoporation, ultraviolet femtosecond lasers are often used to generate highly localized transient pores in cells in a sterile environment for a number of applications, including gene transfection\textsuperscript{253-255}. The formation of these pores are a result of non-linear absorption in biological molecules such as membrane proteins, that create electron plasmas which induce thermal, chemical and thermomechanical effects\textsuperscript{269}. Photoporation is an attractive option for loading of
cargo as it is a purely optical, repeatable and sterile method with precise control over pore formation, all of which are characteristics that other methods lack. To our knowledge, femtosecond laser photoporation has been restricted to applications directly involving cells and has not been applied with artificial cells composed of phospholipid membranes, as phospholipids do not absorb in the ultraviolet range. Continuous wave lasers have also been employed for photoporation, especially for membrane permeabilization applications in cells\textsuperscript{270}. However, this requires staining of the cell membrane with a dye\textsuperscript{271,272} or the use of high output laser powers (>1 W)\textsuperscript{273} as the use of continuous wave lasers for photoporation is based on linear absorption\textsuperscript{269}. To date, photoporation techniques require instruments not commonly available in biological research settings. Cell-sized phospholipid compartments have been generated in the presence of dyes or membrane-anchored dyes and have observed vesicle bursting\textsuperscript{274} or destabilization\textsuperscript{74} upon laser irradiation. However, they have not demonstrated advantageous spatially selective membrane poration or precise optical gating of cargo.
Figure 4.14. Spatial control and a strategy for selective attachment of enzymes to the interior of the GPV. a) Schematic representation of blocking the external leaflet biotin sites using avidin, followed by addition of an avidin-conjugate, and opening and closing of the GPV to selectively place the avidin conjugate (which could be labeled with a fluorophore or attached to another enzyme) inside the GPV. b) Multiple pore formation and resealing events can evenly distribute the avidin-conjugates of interest inside GPVs. Following exterior blocking, FITC-avidin was placed in the medium and the GPVs were opened and closed multiple times in the order the numbering indicates (laser fluence of 40 µJ/µm²). 10 µm scale bar shown.
Figure 4.15. GPV formation and behavior in physiological conditions. a) GPVs electro-assembled in phosphate buffered saline using an AC field with a voltage of 5 V and frequency of 500 Hz. b) GPVs formed in physiological conditions maintain the ability to form pores and load cargo using a laser irradiation fluence of 40 µJ/µm² to load in 155 kDa TRITC dextran. 10 µm scale bar shown.
4.6 Conclusion

Here we have described a purely optical technique for the formation of pores in a porphyrin-lipid bilayer possessing unique control characteristics over the timing and spatial region of pore formation as well as the ability to selectively choose the cargo to load or release. This extends the advantages of photoporation to microreactor applications without the use of highly sophisticated lasers and equipment but using widely available confocal microscopy. The laser induced pore formation and self-sealing behavior of GPVs overcomes the limitations involving a lack of control and precision with other techniques used to induce membrane permeability currently associated with synthetic phospholipid compartments while maintaining a sterile environment. In addition, the porphyrin bilayer provides an additional cell-mimicking characteristic to giant vesicles in their ability to robustly and repeatedly respond to laser irradiation and proceed to self-seal, and thus, another step forward towards the generation of a true protocell. The ability to robustly form pores that self-seal has broad reaching consequences and porphyrin bilayers enable programmable vesicle opening and closing.
Chapter 5
Discussion and Outlook

5.1 Multimodality porphyrin shell microbubbles

Multimodality ultrasound (US)-based contrast agents are a fairly recent development, especially dual modal US and photoacoustic (PA) contrast agents. The physics behind microbubble response to acoustic waves has, and is continuing to be, thoroughly investigated, with numerous studies reporting the response with varying parameters such as acoustic pressure\textsuperscript{33,202,275-278}, microbubble composition\textsuperscript{279,280,281,162} and environmental influences such as boundary effects\textsuperscript{282,283}, to name a few. Furthermore, the physics of the PA response of small dyes and nanoparticles\textsuperscript{284,285} has been investigated, however, not many studies have investigated the physics behind the generated response of US-based PA agents.

Within the current studies completed in this body of work, the optical and acoustic properties of porphyrin shell microbubbles (pMBs) were reported in Chapter 2. However, the acoustic response triggered by the PA effect was not thoroughly studied. US/PA agents are unique in that the presence of the gas, responsible for the US contrast, must have an influence on the generated US waves triggered from the pulsed laser. It is unknown whether this could potentially either enhance the US signal due to neighbouring resonance effects between microbubbles or hamper the US signal due to US scattering from neighbouring gas microbubbles. Each microbubble would behave as an US source but also as an US scatterer of incoming US waves from adjacent microbubbles. The interaction of these generated acoustic waves with each other, the gas core of the microbubbles and microbubble oscillations may change the amplitude, phase or frequency of the detected US wave in comparison with an acoustic wave generated from irradiating a solid sphere absorber. While this is speculation of what may be occurring, the physics of these interactions are unknown and yet to be investigated.

Varying the pulse repetition rate or pulse duration of the laser may modulate the response of the US/PA contrast agent as the pMB has the ability to significantly expand and contract, more than a conventional nanoparticle. Correlations between the pMB acoustic resonance frequency and the frequency dependence of the US emission triggered by the PA laser and how these vary with
pMB diameter could also be investigated. These investigations could potentially lead to new contrast enhanced PA imaging methods and algorithms that would be specific for US/PA contrast agents and significantly increase the contrast agent signal-to-noise ratio.

In addition to the pMB US/PA physics studies, the biomedical applications of pMBs have yet to be further pursued. In Chapter 1, the multiple imaging and therapeutic functions of porphyrins were described, while in Chapter 3, the delivery of porphyrins using pMBs was demonstrated in a tumor xenograft. Potential applications could include using the resulting porphyrin shell nanoparticle (pNP) for phototherapy after delivery to a tumor site to ablate the tumor. The pMBs could also be loaded with a drug, such as a chemotherapeutic, to be delivered to tumor cells, or with an immunosuppressant to be delivered to a transplanted organ for localized immunosuppressant delivery. The imaging functionality of pMBs would then be used to validate successful delivery without having to wait days, weeks, or months, to observe a response to the drug as validation of successful delivery.

Another potential application of pMBs could be for the treatment of brain tumors. The blood brain barrier (BBB) impedes and often prevents the delivery of imaging and therapeutic agents to brain parenchyma, including brain tumors, and confines them within the vasculature. The BBB has greater permeability in large tumor lesions, however, when tumors reach this size, prognosis is fairly poor. Permeability of the BBB is less significant in smaller metastatic lesions in which the cancer may still be treatable and therefore requires a technique to deliver imaging and therapeutic agents across the BBB to the tumor in high quantities. Focused US in conjunction with microbubbles has been used to allow the passage of molecules across the BBB. However, the BBB remains a barrier for delivery of large quantities of agents for imaging and treatment. Upon destruction of pMBs, they form pNPs that will enter into the extracellular space in the brain. As porphyrins, especially pyropheophorbide, are known to accumulate specifically in tumor tissue 2-3 fold higher than in normal tissue, once pNPs cross the BBB and accumulate in tumor tissue, fluorescence imaging may be used to determine successful accumulation and then photodynamic therapy (PDT) may be used to treat these tumors, providing a new alternative method for treatment of early stage brain tumors. These are a couple of examples of the biomedical potential of pMBs.
As the generation of intrinsically multimodality US-based contrast agents are a recent development, there are numerous avenues, mechanisms and questions that remain to be pursued both in basic physical science and translational applications.

5.2 Giant porphyrin vesicles

Giant vesicles present a unique opportunity to investigate a simple model of a cell and directly observe the dynamics of the system. In Chapter 4, giant porphyrin vesicles (GPVs) were introduced, which would use a purely optical method for incorporation and release of biomolecules into the GPV compartment. Using confocal microscopy, the ability to determine the timing as well as spatial location of pore formation are characteristics unique to the GPV technology. Furthermore, by varying the laser fluence, biomolecules could be loaded or released based on their cargo size. Future experiments could determine a direct mathematical correlation between laser fluence (spot size diameter, irradiation time, laser power) and generated pore size in the membrane. With such a correlation, one could determine the exact parameters required to load and release specific cargo, which would be advantageous for sequential microreactor reactions.

While we believe this to be a promising technology, limitations exist, especially in regards to washing procedures when incorporating different biomolecules. For example, conducting an enzymatic reaction within GPVs requires the sequential loading of enzymes and substrates. Without an efficient washing procedure, once enzymes are loaded into GPVs, there may be residual enzymes exterior to the GPV that may react with substrates before they enter the GPV. To overcome this limitation, microfluidics may be an option. Incorporating GPV technology into a microfluidic device would provide precise control over buffer flow rates in order to efficiently wash the exterior of the GPV without disturbing the GPV integrity or contents. This would enable a diverse range of applications for GPVs in which biomolecules could be loaded, reacted and released without interference from external molecules.

5.3 Porphyrin-lipid supramolecular assemblies

Other opportunities exist for the expansion of porphyrin-lipid supramolecular assemblies. For porphyrin-lipid monolayer systems, the gas core of pMBs may be replaced with an oil to generate an emulsion. Lipid-based nano and micro emulsions have been investigated for drug
delivery (incorporating a hydrophobic drug into the oil core)\textsuperscript{22,290-292} or for imaging (such as using an iodinated oil to provide CT contrast)\textsuperscript{293 294-298}. The presence of the porphyrin-lipid would provide stability to the structure, along side a potential imaging aspect (such as optical, PET, MRI, etc.) or additional therapeutic aspect (PDT or PTT).

Furthermore, porphyrin-lipid could be used to generate porphyrin shell perfluorocarbon nanodroplets consisting of a monolayer of porphyrin-lipid surrounding liquid perfluorocarbon. Conventional perfluorocarbon droplets have been utilized to be activated by thermal heating, usually via US\textsuperscript{212} or by optical absorption when an absorber is encapsulated within the droplet, such as gold\textsuperscript{148}. While reports have just begun investigating expansion of perfluorocarbon nanodroplets using optical absorption, a unique opportunity rests with using porphyrin-lipid, as the porphyrin is confined to the shell of the nanodroplet, instead of embedded within the droplet. These porphyrin nanodroplets may be converted to a porphyrin shell microbubble via an optical trigger.

5.4 New self-assembly frontiers

In this body of work, two porphyrin-lipid supramolecular structures were generated. However, the term “porphyrin-lipid” is a generalization of a compound consisting of a porphyrin conjugated to a phospholipid. Within the structures presented, three different porphyrin-lipids were used. In Chapter 2, pMBs were generated using pyropheophorbide as the porphyrin and an 18-carbon length phospholipid chain (Figure 5.1a). This difference in 2 carbons in the acyl chain resulted in greater yield in pMB formation, attributed to an increase in van der Waal interactions as described by Borden et al.\textsuperscript{162}, and was chosen for the final pMB formulation. In Chapter 3, pMBs were generated using bacteriochlorophyll (BChl) as the porphyrin and a 16-carbon length phospholipid chain (Figure 5.1b), where the change in porphyrin resulted in more optimal optical characteristics for PA imaging. This BChl-lipid had also been used to generate nanovesicles for PA imaging as a thermal nanoswitch\textsuperscript{224}. In Chapter 4, GPVs were formed using a porphyrin-lipid containing pyropheophorbide as the porphyrin and a 16-carbon length phospholipid chain (Figure 5.1c). This was similar to the porphyrin-lipid used in the majority of the studies for porphysomes, introduced in Chapter 1.
Figure 5.1. Porphyrin-lipids with varying lipid chain length and porphyrin. Porphyrin-lipids consisting of a) pyropheophorbide conjugated to an 18-carbon chain length lipid, b) bacteriochlorophyll conjugated to a 16-carbon chain length lipid, c) pyropheophorbide conjugated to a 16-carbon chain length lipid.
Therefore, this work not only demonstrates a development in technology and agents for biological and biomedical applications, but also shows that these porphyrin-lipids may extend beyond nanotechnology. Changing characteristics of the porphyrin-lipid have resulted in differences observed in self-assembly and packing of the porphyrins. For example, in porphysome nanovesicles, ordered aggregation determined by a shift in absorption spectra is not observed using pyropheophorbide-lipid. However, in incorporation into a monolayer microscale supramolecular structure, ordered aggregation was observed which was confirmed by a shift in absorption and then further validated using circular dichroism spectroscopy. In addition, pyropheophorbide-lipid did not exhibit a spectral shift in a nanovesicle confirmation, however, bacteriochlorophyll-lipid exhibits a distinct 74 nm bathochromic shift in a nanovesicle confirmation. Therefore, the supramolecular structure, as well as the individual properties of each lipid, has the potential to influence not only the physical and morphological properties of the self-assembled structures, but also the photophysical properties.

Thus, a whole platform has yet to be investigated in the development of supramolecular structures by systematically varying the porphyrin-lipid structure. Variations could include, changing the porphyrin, lipid chain length, saturated vs. unsaturated lipid chains, inserting a spacer between the porphyrin and head group of the lipid, attaching multiple lipids to each porphyrin, attaching a modified hydrophilic porphyrin to the headgroup of a phospholipid. These are just a few examples of modifications that could be included in the porphyrin-lipid structure, which may affect the self-assembly behavior of these lipids and resulting physical and photophysical behavior and/or expansion of porphyrin-lipid assemblies with other imaging/therapeutic properties. For example, the use of a texaphyrin in place of a porphyrin may be used for the generation of supramolecular structures for radiation therapy, as gadolinium texaphyrin is a radiation sensitizer. Therefore, changes in the porphyrin may also result in the expansion of these supramolecular structures into other biomedical areas that have yet to be investigated.

Porphyrin-lipid supramolecular structures offer an exciting new platform for studying both the physics and biological and biomedical applications with unknown frontiers generated from the interactions of the porphyrins confined within each unique structure. Our hope is that future discoveries and technologies may be generated from these interactions and structures to better understand, image and treat disease.
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