Duquesne University

Annual Progress Report: 2010 Formula Grant

Reporting Period

January 1, 2011 – June 30, 2011

Formula Grant Overview

The Duquesne University received $116,091 in formula funds for the grant award period January 1, 2011 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

From Insoluble Perfluorocarbon Oils to Multifunctional Nanoparticles for Breast Cancer Imaging and Treatment - The purpose of this project is the development of novel multifunctional perfluorocarbon (PFC) based magnetic resonance (MR) detectable drug delivery vehicles. Specifically, nanoemulsions, microemulsions and gels for localized delivery of anti-inflammatory agents to breast tumors will be prepared. The PFC based drug delivery vehicle localization, accumulation and distribution can ultimately be quantitatively and qualitatively assessed *in vivo* by ^{19}F MRI. Fluorine-19 has very low biological abundance in tissues and ^{19}F MR directly detects the density of ^{19}F spins contained in the PFC molecules without background. We hope to develop true theranostic agents, therapeutic and diagnostic for breast tumor imaging and treatment. Recent epidemiological studies demonstrated that treatment with nonsteroidal anti-inflammatory agents (NSAIDs), such as COX2 inhibitors, can reduce the risk of developing breast cancer, with aspirin and celecoxib showing the most significant effects. Clinical and experimental evidence strongly suggest COX2 inhibitors as new adjuvant breast cancer treatments. The purpose of this project is to incorporate a COX2 inhibitor into a ^{19}F MRI visible nanoreagent for anti-inflammatory adjuvant treatment in breast cancer.

Anticipated Duration of Project

1/1/2011 - 12/31/2012

Project Overview

The focus of this project is the design, synthesis and *in vitro* evaluation of novel multifunctional perfluorocarbon (PFC) based magnetic resonance (MR) detectable drug delivery vehicles. We will examine synthetic and formulation aspects of several carefully selected PFCs and assess their viability for theranostic, therapeutic and diagnostic reagent development.

Hypothesis: Perfluorocarbons are a highly viable platform for engineering multifunctional nanoreagents for simultaneous breast cancer treatment and imaging. The hypothesis will be

tested by the following specific aims. Specific Aim 1: Design and synthesis of perfluorinated amphiphiles, perfluorocarbon-hydrocarbon conjugates and perfluorinated crosslinkers. Specific Aim 2: Formulation of chemically modified PFCs into nanoemulsions, microemulsions and gels for localized drug delivery to breast tumors.

Methods: Perfluorinated conjugates will be designed and synthesized to incorporate fluorocarbon polymers, simple perfluorocarbon chains $(CF_2)_n$ or perfluoropolyethers (CF_2CF_2O), and either a lipophilic or hydrophilic short chain hydrocarbon or polymer. Synthetic protocols will include, but are not limited to, Mitsunobu reactions, click chemistry and activated fluorinated ester conjugations. Trifluoromethyl group(s) will be introduced into lipids, alcohols and surfactants. Perfluoropolyethers will be conjugated to hydrocarbons or crosslinked into gels. Nanoemulsions and microemulsions will be prepared by sonication, microfluidization and low energy emulsification methods. Droplet size, shape and surface properties will be measured by dynamic light scattering (DLS), zeta potential and microscopy. Perfluorinated gels will be synthesized by chemical crosslinking of ionic and non-ionic polymers with perfluorocarbon crosslinking reagents. Gels will be evaluated for rheological properties (e.g., viscosity, elasticity), spreading properties and physical strength. The effects of pH, temperature and ionic strength on drug incorporation and release will be tested in all formulations. ^{19}F NMR will be used to measure ^{19}F content, evaluate fluorocarbon-hydrocarbon interactions and assess the potential "imageability" of each vehicle by ^{19}F MRI.

The focus of our efforts is on the synthesis and formulation of PFC reagents and assessing their viability as drug delivery vehicles, using celecoxib as a model drug, *in vitro*. The attractive PFC drug delivery candidates will be subjected to simple cell culture tests. *In vivo* biological evaluation is beyond the scope of this project.

Principal Investigator

Jelena M. Janjic, PharmD, PhD
Assistant Professor of Pharmaceutics
Duquesne University
415 Mellon Hall
600 Forbes Avenue
Pittsburgh PA 15282

Other Participating Researchers

Claire E. O'Hanlon, BS, Robert K. Tunney Jr., Sravan Patel, MS - employed by Duquesne University

Expected Research Outcomes and Benefits

Each year, 465,000 people die of breast cancer and 1.3 million new cases are diagnosed. One third of women diagnosed with early stage breast cancer will eventually develop metastatic breast cancer. Many women do not seek treatment during the early stage of the disease and are first diagnosed in stage II or III. The goal of this work is to improve breast cancer therapy by

targeting primary tumor-associated inflammation with the hope to decrease its metastatic potential and improve chemotherapy response. Recent epidemiological studies demonstrated that treatment with nonsteroidal anti-inflammatory agents (NSAIDs), such as COX2 inhibitors, can reduce the risk of developing breast cancer, with aspirin and celecoxib showing the most significant effects. Clinical and experimental evidence strongly suggests COX2 inhibitors as new adjuvant breast cancer treatment. However, when applied systemically, COX2 inhibitors can cause potentially life-threatening side effects, such as cardiovascular toxicity and gastrointestinal disturbances. Targeted delivery of COX2 inhibitors to breast tumors and inflammatory peritumoral areas directly may overcome these problems. Celecoxib, a selective COX2 inhibitor, is insoluble in water and has low oral bioavailability (20-60%).

Drug delivery vehicles for poorly soluble anti-inflammatory drugs will be prepared. The PFC component will serve two purposes: 1) provide a biologically inert platform for delivering an anti-inflammatory agent, and 2) serve as a MRI tracer for *in vivo* monitoring of the drug delivery system efficiency. The PFC based nanoreagent development in this project has the potential to open new avenues for drug delivery and development, potentially leading to new breast cancer treatment approaches. The project is highly collaborative and will strengthen partnerships between Duquesne University, Carnegie Mellon University and Celsense Inc. (Pittsburgh, PA), a biotechnology company, the current leader in ^{19}F MRI tracer development. During the course of this project we will generate critical proof of principle data for further extramural funding, peer review publications and patent applications.

Summary of Research Completed

Significant early progress towards synthesis and formulation of new PFC based nanoemulsions, gels and micelles is presented. The focus of the supported work is on ^{19}F MRI detectable celecoxib formulations for breast cancer treatment. Key assays for *in vitro* evaluation of drug delivery vehicle have been established and preliminary cell culture work was used for early assessment of the reagents behavior in cells. A new imaging modality (near infrared, NIR fluorescent dye) was introduced to achieve greater selectivity and sensitivity of future *in vivo* imaging.

Several characteristics of PFC nanoemulsions make them desirable as imaging agents (^{19}F MRI) and drug delivery vehicles. The carbon-fluorine bond is highly stable chemically; there are no known enzymes that degrade fluorocarbons *in vivo* and PFCs are inert and nontoxic. However, PFCs are both lipophobic and hydrophobic and do not incorporate into cells on their own. Formulation of PFCs into imaging capable drug delivery vehicles is challenging. In the PFC drug delivery vehicles, the key is to balance high level of MRI visible ^{19}F nuclei necessary for imaging sensitivity and ability to incorporate drugs which would not mix or integrate directly into the perfluorocarbon.

<u>Progress towards Aim 1: Design and synthesis of perfluorinated amphiphiles, perfluorocarbon-hydrocarbon conjugates and perfluorinated crosslinkers:</u> To facilitate PFPE-tyramide formulation optimizations, it was necessary to synthesize large scale of the polymer conjugate in a single batch. Up to 10 g of PFPE-tyramide product can be obtained with high purity (>99% by ^{1}H and ^{19}F NMR) as a waxy solid. The product is analyzed by FT-IR and DSC, Figure 1.

Optimization of PFPE-tyramide for drug delivery and imaging: In preliminary formulation studies, PFPE-tyramide self-assembled into an emulsion with droplet size around 150 nm and polydispersity index (PDI) around 0.3 in the presence of a Pluronic® (BASF) surfactant. We report dramatic improvements in the PDI and droplet size and increased capacity for PFPE-tyramide emulsions to carry a water insoluble drug (e.g. celecoxib). Several non-ionic surfactants with varied HLB values were tested at a fixed ratio to PFPE-tyramide (1:0.32). When a hydrophilic surfactant (HLB = 29) was used, it appeared that sonication and solvents produced no significant effect on the final size and polydispersity, Figure 2A. However, when a more lipophilic surfactant (HLB = 15) was used, we observed significantly different results, Figure 2B. Preparing PFPE-tyramide emulsions with a thin film method followed by a gentle hydration with the surfactant present in the water phase yielded the smallest ever reported PFPE nanoparticles (less than 110nm). This is a significant finding, because it can facilitate tumor tissue penetration via enhanced permeability and retention effect (EPR) and avoid clearance by reticuloendothelial system (RES). The high hydrophobicity and substantial lipophobicity of perfluoropolyether polymer in PFPE-tyramide is unlikely to be able to carry water-insoluble and lipophilic drugs. To overcome this, hydrocarbon oils were introduced. However, it was found that the amount of incorporated PFPE was too low to produce sufficient ^{19}F signal in future animal experiments, while significant increase in size and PDI were observed (data not shown). To facilitate hydrocarbon oil incorporation, while maintaining a desired size, additional surfactant was introduced. Additional surfactant decreased the droplet size to around 200 nm in the presence of hydrocarbon oil. As the total amount of surfactant increased, the nanoemulsion droplet size decreased, Figure 3A, and the overall PFPE incorporation increased as measured by ^{19}F NMR, Figure 3B, reaching levels necessary for successful *in vivo* imaging. Now, we have a formulation prepared with PFPE-tyramide (^{19}F MRI reagent) combined with an oil and two surfactants able to carry a water insoluble drug. This is a first report of such a reagent and a relevant publication (O'Hanlon *et al*, 2011) is in preparation. Overall HLB was tuned to 12-15 and olive oil was chosen as a vehicle for celecoxib, a COX-2 inhibitor with poor water solubility. Olive oil was incorporated into the formulation and PFPE-tyramide/olive oil ratio varied. Droplet size slightly increased, yet remained under required 200nm, Figure 4. Olive oil could not carry larger doses of celecoxib due to limited solubility of the drug (< 10 mg/mL of oil). A synthetic hydrocarbon oil was then introduced to mitigate this problem (detailed description of this formulation is planned to be patented and will not be disclosed in detail, however relevant data is presented). The microfluidized emulsions with and without celecoxib had particle diameters around 180 nm and PDI less than 0.2. Figure 5 shows DLS measurements of PFPE-tyramide nanoemulsions before and after microfluidization. Presence of celecoxib showed no significant effects on droplet size and PDI after high sheer processing, ($p>0.05$, paired t-Test, Graph Pad Prism statistical software).

Biological testing of PFPE-tyramide emulsions *in vitro*: PFPE tyramide emulsions with and without the drug were tested for their effect on cell proliferation upon 24 h exposure by MTS colorimetric assay (Promega). Cells were plated in 96 well plates and exposed to increasing concentrations of PFPE-tyramide emulsion without celecoxib, PFPE-tyramide emulsion with celecoxib, the drug alone at the same concentration levels as in the emulsion, and DMSO alone (the solvent used to add free drug). After 24 h, absorbance was measured at 490 nm on a plate reader, Figure 6. We have observed certain toxicity at higher doses (>1mg/mL) and studies are underway for further improvements in the formulation. To facilitate future *in vivo* biological

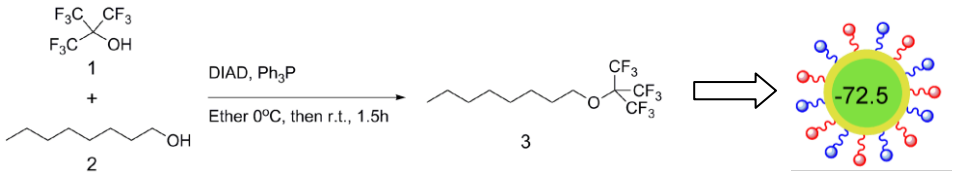
studies, we prepared a PFPE-tyramide vehicle with an NIR dye (CelVue, NIR815nm, gift from MTTI Inc). Preliminary confocal experiments were performed at 0.5 mg/mL concentration and cells were imaged 3 h later. No morphological changes were observed and the celecoxib-carrying PFPE-tyramide nanoemulsion accumulated in the cytoplasm, Figure 7. This was an encouraging finding. Further studies are underway to assess COX-2 inhibition in immune cells and cancer cells.

Optimization of PFTE nanoemulsions for 'two color' ^{19}F MRI of drug delivery: ^{19}F MRI detects only organic fluorine present in the nanoreagent, while the ^{1}H MRI gives anatomical structure. PFCs resonance would depend on its structure. Celecoxib loaded PFC nanoemulsions are formulated with perfluoro-*tert*-butyl-ethers (PFTE) or PFTE conjugates with a single ^{19}F NMR peak (-72.5 ppm). Simultaneously, inflammation can be visualized by the commercial perfluorocabon nanoemulsion V-Sense (gift from Celsense Inc), which specifically labels macrophages and monocytes, accumulates at sites of inflammation and shows a ^{19}F NMR chemical shift at -92.5 ppm. Multispectral ^{19}F NMR and MRI provide a viable platform to visualize and assess co-localization of the accumulated celecoxib nanoemulsion and tumor associated inflammation. Sufficient spectral separation (>20 ppm) in the ^{19}F NMR spectra of PFTE and PFPE nanodroplets allows for simultaneous imaging of multiple nanocarriers in cells and /or extracellular tissues. A simple aliphatic PFTE, 1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl octyl ether, **3**, with a lipophilic tail (n-octyl) and three trifluoromethyl groups, was synthesized using a modified Mitsunobu protocol, Scheme 1.

Highly fluorinated PFCs exhibit both lipophobic and hydrophobic character. The n-octyl lipophilic chain on compound **3** was introduced to facilitate nanoemulsion formation by promoting interaction with surfactants via decreasing its lipophobicity. It is common to use two surfactants to form a mixed surfactant system; therefore, PFTE was dispersed in water by sonication, and the dispersion stabilized by a pre-made non-ionic surfactant mixture. A variety of Pluronic® surfactant combinations were screened to formulate stable o/w nanoemulsions with PFTEs. The lipophilic nature of PFTE was exploited to form nanodroplets stabilized in water by soluble amphiphilic surfactant(s). However, use of surfactants alone was not effective to produce a stable nanoemulsion of PFTE. The stability of the PFTE nanoemulsion can be enhanced by adding a small amount of a second component (e.g., hydrocarbon oil) which is insoluble in water, but miscible with PFTE. Association of PFTE with a low diffusive component, such as hydrocarbon oil, increases the residence time of PFTE inside the nanoemulsion droplet as the diffusion effort is counterbalanced by an osmotic pressure that acts oppositely. To evaluate the use of hydrocarbon as a nanoemulsion stabilization agent, a PFTE nanoemulsion was formulated using olive oil as the second hydrophobic component. The presence of olive oil would also aid in the incorporation of celecoxib as PFTE alone cannot solubilize the drug. The PFTE nanoemulsion was prepared by dispersion of PFTE and olive oil in water by sonication and the dispersion stabilized by surfactant mixture. Subsequent droplet size reduction was performed on a Microfluidizer M110S (Microfluidics, Inc., Newton, MA). The average droplet size was 180-190 nm and PDI ~0.18, as determined by DLS (Figure 8A). Stability was monitored for 144 days at 4°C and 25°C and displayed no change in droplet size or PDI (Figure 8B). The cell loading was high, reaching 1×10^{12} F/cell (Figure 8C), and a dose-dependent uptake was observed. Cell viability assays showed no apparent toxicity with this formulation (Figure 8D). Out of all the surfactants used, P105/Cremophor EL produced a stable formulation (up to 5 months at 4°C and

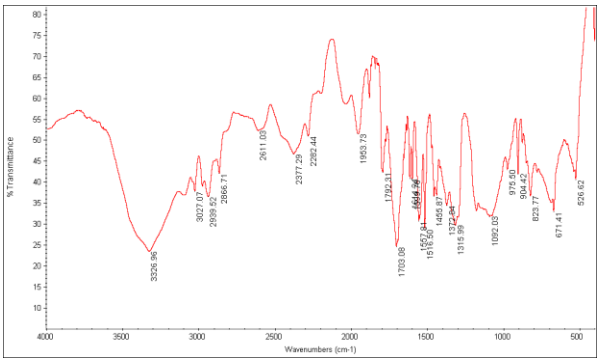
room temperature) that can incorporate olive oil (Figure 8).

First multimodal nanogels for drug delivery and imaging by both NIR and ^{19}F MRI: Kabanov and co-workers first introduced the term "nanogels" to describe nanoparticles derived from swollen chemically crosslinked networks of cationic and neutral polymers such as the branched PEG-cl-PEI made from polyethylenimine (PEI) and poly(ethylene glycol) (PEG). Nanogels are very promising as drug delivery carriers because of their high loading capacity, high stability, and responsiveness to environmental factors that are unprecedented for common pharmaceutical nanocarriers, such as ionic strength, pH, and temperature. PFC based macro and nanogels, reported here, as opposed to classical hydrogels, add ^{19}F MRI tracer capabilities, NIR optical imaging and potential to carry targeting agent and small molecule drugs. The initial results are presented below. The development was focused first on preparing highly homogenous macrogel loaded with NIR dye, Figure 9. The macrogel was further processed to make it amenable for drug incorporation and size reduced to below 100 nm. Figure 10 shows DLS results of a PFC/NIR815 nanogel dispersion in water. Nanogel particulates show average size of only 90 nm and low polydispersity (<0.18). These findings put Janjic lab at the forefront of perfluorocarbon based drug delivery research and these are so far the smallest ever reported dual mode NIR/perfluorocarbon reagents.



Scheme 1. Synthesis of PFTE-tyramide (3) and incorportation into a nanodroplet with ^{19}F NMR single peak at -72.5 ppm.

A



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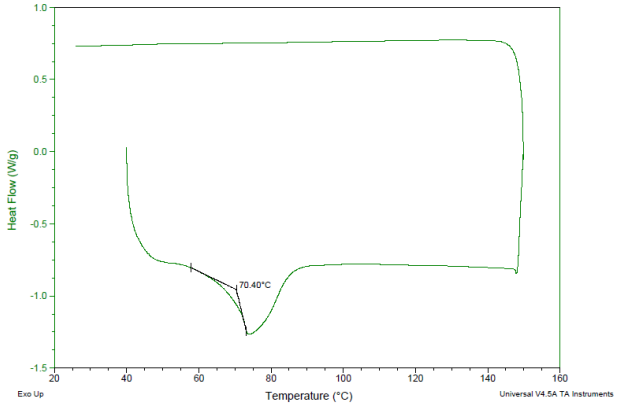

Figure 1. Characterization of PFPE-tyramide.
A. FT-IR and **B.** Differential Scanning Calorimetry (DSC) analysis.

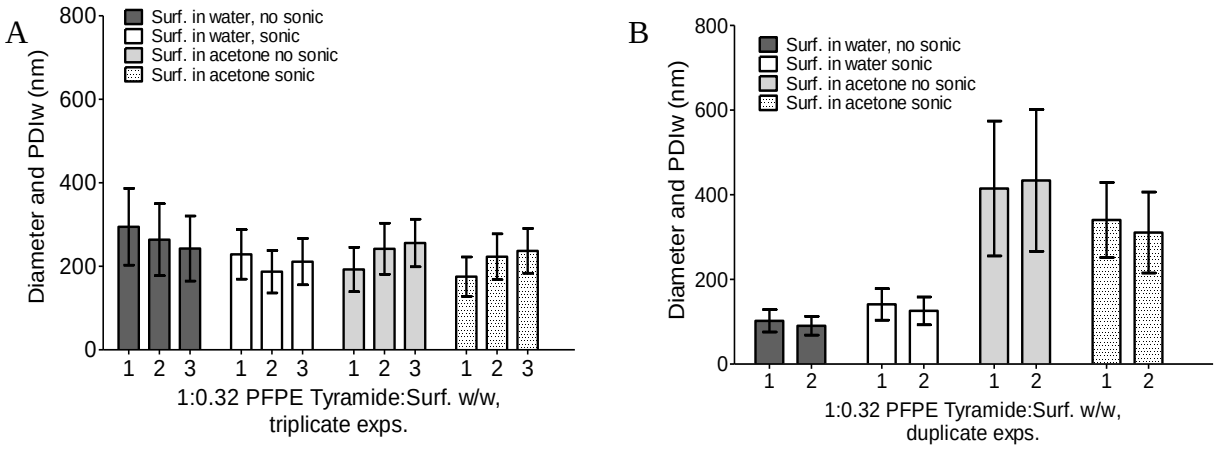

Figure 2. **A.** Diameter and PDI of small scale emulsions containing PFPE-tyramide and a surfactant (HLB = 29) under different experimental conditions. **B.** Diameter and PDI of small scale emulsions containing PFPE-tyramide and a surfactant under the same varied conditions as 2A, but with a different surfactant (HLB = 15).

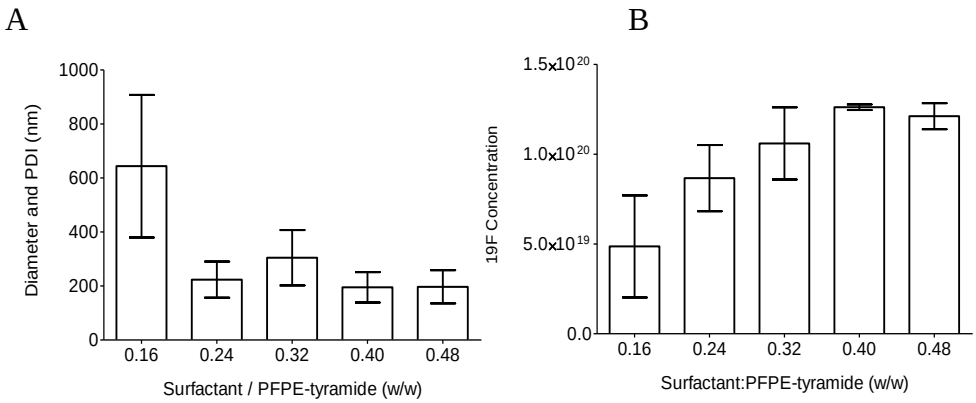

Figure 3. **A.** Diameter and PDI of small scale emulsions containing PFPE-tyramide and surfactants at various ratios. **B.** ^{19}F concentration in emulsions shown in 3A, measured by ^{19}F NMR using TFA at 0.02% as an internal standard.

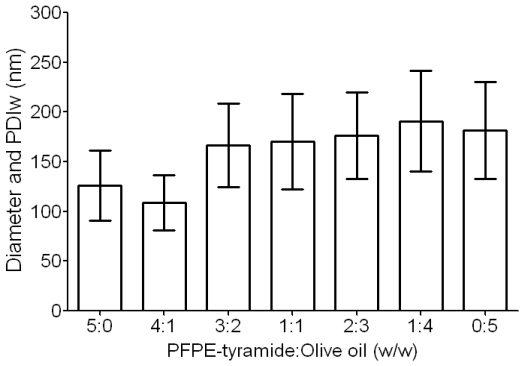

Figure 4. Diameter and PDI of small scale emulsions containing PFPE-tyramide, olive oil, and surfactants at various ratios.

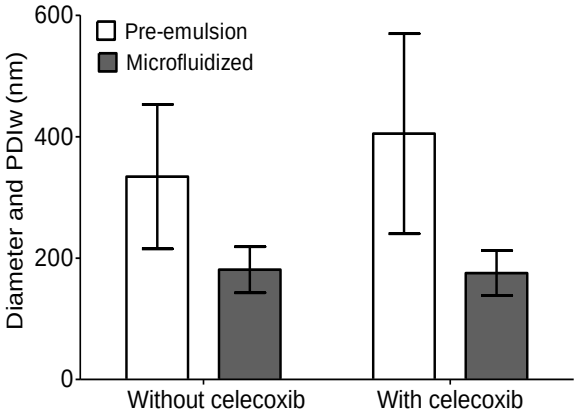

Figure 5. Diameter and PDI of large-scale pre-emulsion and microfluidized emulsion containing PFPE-tyramide, a hydrocarbon oil, and surfactants. White bars represent the coarse emulsion and gray bars represent the emulsion after microfluidization.

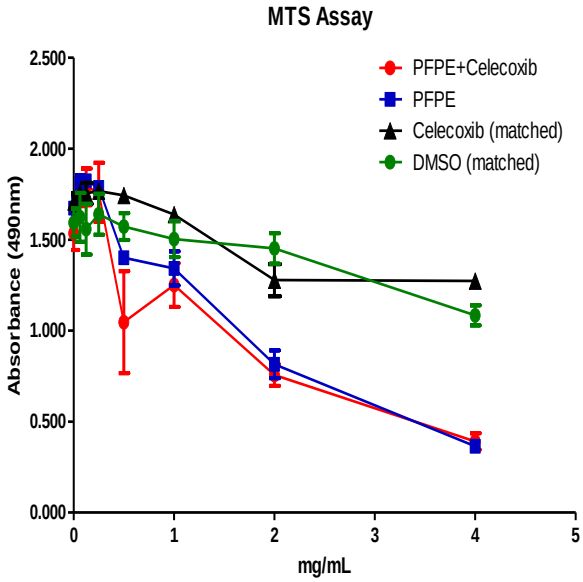

Figure 6. MTS Assay (Promega, WI) of cell proliferation. Cells were exposed to increasing doses of PFPE-tyramide emulsion w/o celecoxib for 24 h and absorbance measured at 490 nm on a spectrophotometer. Data represents means of one experiment performed in triplicate.

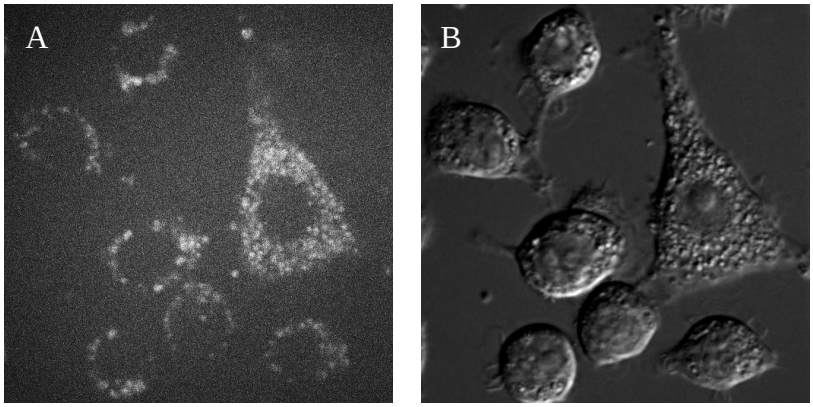

Figure 7. Mouse dendritic cell line was exposed to PFPE-tyramide emulsion labeled with an NIR dye. Cells were washed and imaged on an Andor Revolution XD Spinning Disk Confocal microscope (M. Patrick, CMU)

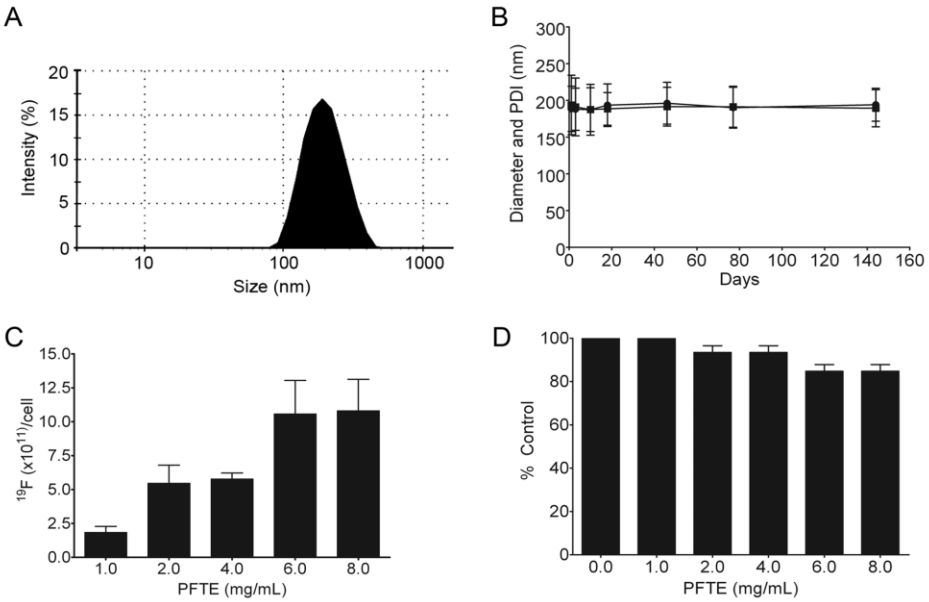

Figure 8**.** Characterization of PFTE nanoemulsion. **A.** Nanoemulsion droplet size distribution by intensity. **B.** Stability of microfluidized PFTE nanoemulsion containing olive oil at 4°C and 25°C as determined by DLS. **C.** Dose-dependent uptake of PFTE in FSDCs. **D.** FSDC viability post-labeling. Each data point in Panels C and D represent the average of three independent measurements, where the error bars are the standard deviation of the mean.

A

B

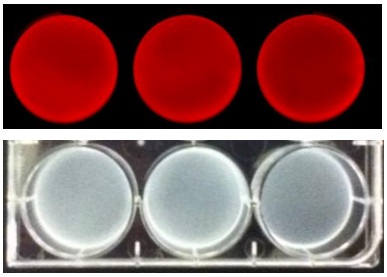

Figure 9. **A.**NIR image of a PFC/NIR815 Macrogels obtained on an Odyssey NIR imager (LI-COR, Lincoln, Nebraska). **B.** Photograph of the 6-well plate with distributed gels.

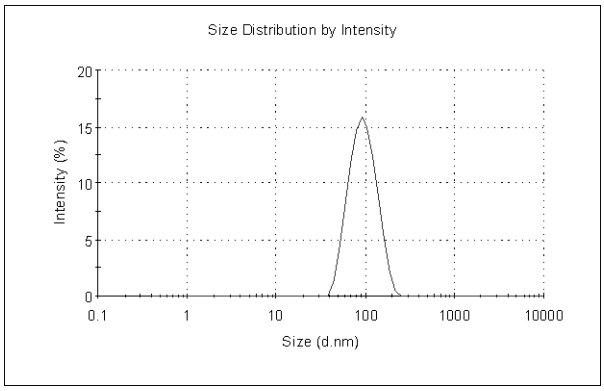

Figure 10. Dynamic Light Scattering (DLS) measurement of the PFPE/NIR815 nanogel size and polydispersity on a Zetasizer Nano (Malvern, U.K.)

Research Project 2: Project Title and Purpose

Promoting Health and Health Care Access in the African Refugee and Immigrant Community: A Participatory Action Research Study - The purpose of this Participatory Action Research project is to understand specific culturally shared knowledge about health and to develop strategies to promote health and health care access in the African immigrant and refugee community. The overall goal is to engage the African immigrant and refugee community in identifying, planning, prioritizing and evaluating strategies to promote health from the unique cultural view and to empower people to create their own destiny regarding the reduction of health disparities in this community.

Anticipated Duration of Project

1/1/2011 - 12/31/2012

Project Overview

The broad research objective for this project is to gain an understanding of specific cultural beliefs, values and strategies to promote health and health care access in the African immigrant and refugee community. The specific aims of the research are to: 1) explore the health promoting needs of African immigrants and refugees; 2) describe the health care access needs of African immigrants and refugees; 3) understand the culturally congruent process of developing strategies within the specific immigrant and refugee community to address the health promoting and health access needs of the community; and 4) compare the perceptions of self-reported health status for African immigrants and refugees at the beginning and end of the study.

Method and design: This Participatory Action Research (PAR) project will utilize a mixed methods approach, including focused ethnography and the Short Form Health Survey Instrument (SF-12), to gather data at the beginning and end of the study from the informants. Four common characteristics of PAR are: 1) uncovering solutions to health problems; 2) collaboration between

researchers and the community; 3) implementation of change during the process; 4) and development of a theory. The design includes a cycle, which includes the plan, action, observation, reflection, and plan or revised plan, followed by acting, reflecting, and evaluating.

Informants and setting: Informants for this study will be recruited from the Pittsburgh and Allegheny County area and include any adult members (>18-90 years of age) from the African immigrant and refugee community who are willing to participate in the study. The researchers will seek out approximately 50 to 60 adults for the core and focus groups for this study. In addition, the groups will include a researcher, nursing faculty, and the Executive Director of Acculturation for Justice, Access and Peace Outreach (AJAPO). Informants will be purposefully sought via word of mouth and snowball method by AJAPO from cultures (Somali, Burundi, Sudanese, Liberian, Zambian) representing the African immigrant and refugee population in this city.

Instruments: A researcher-generated, semi-structured interview guide and demographic form will be used to understand specific cultural knowledge and strategies to promote health and health care access in the African immigrant and refugee community. In addition, the SF-12 will be used to measure (pre and post-intervention) eight domains of health: physical functioning, role limitations due to physical health, bodily pain, general health perceptions, vitality, social functioning, role limitations due to emotional problems, and mental health.

Principal Investigator

Rick D. Zoucha, PhD, RN, PMHCNS-BC
Associate Professor
Duquesne University
600 Forbes Avenue
Pittsburgh, PA 15282
526 Fisher Hall

Other Participating Researchers

Shirley Smith, PhD, RN, Kathy Mayle, MNEd, MBA, RN - employed by Duquesne University
Yinka Aganga-Williams, PhD – employed by Acculturation for Justice, Access and Peace Outreach

Expected Research Outcomes and Benefits

The primary expected outcomes and benefit are that the community can immediately use the findings from this project to promote health and health care access for this population. Through the process of Participatory Action Research, members of the community are part of the research process in identifying problems and solutions and can control their own destiny regarding health and health care access based on their unique cultural values. It is significant that care and health care needs be articulated and understood and treated in the cultural context of the people being served. The immigrant population in this community experiences health disparities due to their immigrant or refugee status. Research with outcomes that result in immediate benefit to the

community is imperative.

The African immigrant and refugee community, which utilizes the services of AJAPO, is plagued with problems related to economic issues, social concerns, violence, political impact, and other health-related issues. Nurses, collaborative health care professionals, and community members can and will work together to form partnerships that promote healthy communities. The goal of empowering individuals, families, and communities to create their own destiny regarding their health and health care access is the ultimate benefit of this research. The expected outcomes and benefits of this project include a community-academic partnership with the continued goal of promoting quality health care to this underserved community.

Summary of Research Completed

Project personnel have been hired. The study has progressed as planned with the exception of the focused ethnography (focus group) for the core group, which includes representative leaders of the five cultures (Zambian, Somali, Burundi, Sudanese, Liberian). It was imperative that the researchers and research partners meet with the community leaders prior to the focused ethnography in order to elicit a commitment from the leaders regarding the study. The focused ethnography has therefore been delayed and is planned for August 13, 2011.

The first meeting with the community leaders was held on June 18, 2011. The leaders included two representatives from the Burundi community and one representative from the Zambian, Somali, Sudanese and Liberian communities living in the Pittsburgh area.

Acculturation for Justice, Access and Peace (AJAPO) provided interpreters for the meeting in the following languages: English, French, Arabic and Swahili. The team elicited the support of the community leaders as community partners who would then recruit at least eight to ten participants from their communities to identify health needs and access issues specific to each culture and community, and who would be willing to be part of a focus group for an ethnography and self-perceived health status. The consent procedure, informed consent, demographic forms and the project time-line and the next steps of the project were discussed in detail. Three of the six community leaders agreed to participate and the other three agreed to further consider their participation. The next meeting for the ethnography and focus group with the core group was set for July 9, 2011.

In preparation for recruiting community participants, the team discussed travel reimbursement and token gifts for participants. The decision regarding gifts is an important one in the individual cultures and communities because the participants have many varying needs. For this reason, much time was spent regarding this decision. It was decided that each person who participated in the study would receive a gift card from the local grocery store, as well as a specified amount of money to assist with travel expenses to the research site.