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Bilayer Alteration from Ultrasound-Induced Microbubble Cavitation

A Thesis

Submitted to the Faculty

of

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Dedications

I dedicate this work to my parents, family, friends, and God who have been with me every step of the way.

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Abstract

Bilayer Alteration from Ultrasound-Induced Microbubble Cavitation

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Advisor: Steven P. Wrenn, Ph.D.

The ability of ultrasound-induced cavitation of microbubbles to impact cells is firmly established, but the mechanism by which the acoustic phenomena affects the phospholipid bilayers are not fully understood. Here, we examine the interactions of acoustically driven microbubbles by themselves and in two other different architectures: microbubbles mixed with liposomes and microbubbles tethered to liposomes. Using a combination of ultrasound acoustic spectra and the Wrenn modified RPNNP colloidal model, we observe the effects of microbubble size distribution, radius, and shell chemistry, along with ultrasound frequency and peak negative pressure on the cavitation behavior of the microbubble. We identify the ultrasound intensities corresponding to stable and inertial cavitation and concomitant acoustic microstreaming and shockwave to reversible and irreversible pore formation, respectively, for each architecture.

The size distribution of microbubbles are similar between the two different chemistries, but with the use of size isolation by differential centrifugation, different size distribution and polydispersity were observed. The decrease in the polydispersity of the microbubbles increased the growth rate of microbubbles destroyed, while the microbubbles with a higher concentration of microbubbles above a micron had a higher amount of acoustic activity. The increase in the frequency slowed the growth rate of microbubbles destroyed from inertial cavitation, while shifting the onset of inertial cavitation to a higher pressure

and as the acoustic activity decreased. The addition of polyethylene glycol increased the shell's area expansion modulus which had a similar effect as an increase in frequency.

The effect of the proximity of the liposome to the microbubble was examined with the use of the two architectures with a variety of acoustic parameters, including driving frequency, peak negative pressure (PNP), and duty cycle. The addition of positively charged phospholipid to the microbubble monolayer was used here to establish sufficiently close proximity between the microbubbles and liposomes, which are negatively charged and interact with the microbubbles through electrostatic attractive forces. On the one hand, the electrostatic tethering of a liposome to a microbubble dampens the magnitude of the ultrasound-induced oscillation as compared with freely-floating microbubble; the liposome effectively serves to stiffen the system. On the other hand, the close proximity established by electrostatic tethering is necessary for the microbubble oscillations – which proceeds with sufficient magnitude despite the dampening to impact the liposomal bilayer. FRET measurements made both before and after insonation establish unequivocally the proximity requirement, revealing that energy transfer changes only when the microbubbles are tethered to the liposomes. Additionally, this result indicates that the fluorescent probes are effectively diluted due to phospholipid mixing between the microbubble and liposomes. The reduction in energy transfer becomes more significant with increasing insonation pressure within a stable cavitation regime. Specifically, at an insonation driving frequency of 3.3 MHz, the microbubble underwent stable cavitation at peak negative pressures of 0.24, 0.39, and 0.47 MPa. At the lower driving frequency, 1 MHz, the possibility that microbubbles experienced

inertial cavitation could not be ruled out. The phospholipid mixing from microstreaming produced by the stable cavitation of microbubbles was independent of pressure and led to a constant mixing value of 19% of the microbubble lipids mixing within the liposome. This is from a mixture of pore formation from microstreaming and lipid shedding from the stable cavitation of the microbubble. In the inertial cavitation regime, the phospholipid mixing increases from approximately 18% to 50% as more microbubbles are predicted to exhibit inertial cavitation. Findings in this study will potentially be useful in designing a new method of fresh produce decontamination based on ultrasound-induced cavitation of microbubbles that are electrostatically tethered to microorganisms.

Inertial and stable cavitation both showed to affect the liposome bilayer, but only when in close proximity through the tethering of the microbubble to the liposome. A further look into if these cavitation behavior could destroy bacteria was done with *Escherichia coli* K12 (*E. coli* K12). The microbubble cavitation showed to not affect the bacteria. This is believed to be because of the gram-negative bacteria having both an outer and inner membrane, therefore being more resilient as compared to the liposome bilayer.

The microbubble cavitation was found to have no effect on the destruction of gram-negative bacteria, therefore further investigation is still needed. However, the findings in this study have shown how ultrasound-induced cavitation of microbubbles electrostatically tethered to liposomes can alter the bilayer of liposomes by both stable cavitation with microstreaming and inertial cavitation shockwaves. This information will be useful for designing a new method for fresh produce decontamination and drug

delivery for microorganism with a single bilayer; more research is need for those microorganisms complex in nature, such as gram-negative bacteria.

Chapter 1: Food Decontamination and Ultrasound Background

1.1 Sanitation Techniques and Food Contamination

Common pathogens found on fresh produce from contamination led to seventy-two foodborne illness outbreaks between 1996 and 2006 [1]. Foodborne illness outbreaks were estimated each year to cause 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths [2]. Along with hospitalizations and deaths, foodborne illnesses cost the U.S. Department of Agriculture more than $15.6 billion each year [3]. The 'how' and 'where' fresh produce is contaminated is important information to in turn determine the best ways in which to avoid future outbreaks.

The contamination of fresh produce can occur during the cultivation, harvest, and processing/preparation of it [4]. *Salmonella* in the United States in 2008 was linked to contamination at jalapeno farms in Mexico, the norovirus outbreak in Denmark in 2010 was caused by infected lettuce from a producer in France, and *Listeria monocytogenes* in the United States in 2011 was from infected cantaloupe in packing facilities in Colorado [5, 6, 7, 8]. These four instances all show how fresh produce can be infected at any stage from cultivation to processing. Increases in the consumption of fresh produce have in turn led to larger scale production and distribution, which incidentally, can increase the chances of outbreaks [9]. Fresh produce can be contaminated by bacterial, parasite, and viral pathogens which can not only harm us but harm the shelf life of the produce. The main bacterial pathogens that contaminate fruits and vegetables are *Salmonella spp.,*

Escherichia Coli O157:H7, Staphylococcus aureus, Campylobacter spp., and Listeria monocytogenes [10]. Even with current decontamination methods to clean fresh produce from these pathogens, numerous outbreaks still occur.

1.1.1 Existing Sanitation Technologies

The main method to remove dirt, microorganisms, and other contamination from fresh produce is washing it with water. This washing method can be done with or without an antimicrobial to help reduce contamination. However, these methods can be ineffective and not fully eliminate all the pathogens due to the increase in bacterial resistance, cross-contamination from wash water, minimal processing, or pathogen uptake into the produce [11, 12, 13, 14, 15, 16]. The wash water's purpose, if contaminated, will have a reverse effect and increase the contamination on the produce [16]. The addition of antimicrobials aim to decrease the contamination in the wash water as well as contamination on the fresh produce. However, an antimicrobial has to be tested and approved to conclude it can decontaminate the produce while not affecting its shelf-life or flavor; testing is also done to ensure that these antimicrobials do not produce harmful byproducts.

Chlorine is widely used antimicrobial that is low cost and effective, though it can produce carcinogenic and mutagenic compounds and be corrosive [11, 17]. Chlorine's effectiveness is dependent on the pH, which needs to be between 6.0 to 7.5 for the optimal antimicrobial activity of hypochlorous acid and hypochlorite [18]. However, chlorine has shown, in the presence of organic material in water, to become inactive and

produce byproducts that can have possible carcinogenic effects like chloramines and trihalomethanes [19, 20, 21]. Because of chlorine's pH sensitivity, potential to produce a carcinogenic, and corrosive abilities, many other methods for decontamination are being researched.

Other antimicrobials that have been researched are chlorine dioxide gas, acidified sodium chlorite, organic peroxides, hydrogen peroxide, ozone, and organic acids [18]. Chlorine dioxide gas has shown to decrease the total yeast and mold count on raspberries postharvest [22]. It is also more water soluble and doesn't react to organic material to create carcinogens like chlorine. However, at high concentrations, chlorine dioxide gas can be explosive [18]. Peroxyacetic acid is environmentally friendly and has shown to reduce yeast and mold counts when used with octanoic acid [23]. Hydrogen peroxide, ozone, and some organic acids have shown to be less effective as compared to chlorine [18]. These alternative antimicrobials show potential to be new methods with each having its own advantages and disadvantages. One downfall is that most of these chemicals have to be monitored to be under a certain ppm to follow FDA guidelines [18, 24]. A wide range of alternative methods are being investigated including biological methods, physical technologies, mild-heat treatment and combinations of methods [11, 18]. An upcoming method of decontaminating fresh produce that is being researched is the use of ultrasound or ultrasound combined with an antimicrobial or microbubbles.