Ultrasound in Med. & Biol., Vol. 44, No. 2, pp. 447–457, 2018

0301-5629/$ - see front matter

https://doi.org/10.1016/j.ultrasmedbio.2017.10.011

● *Original Contribution*

ACOUSTIC CHARACTERIZATION OF ECHOGENIC POLYMERSOMES PREPARED FROM AMPHIPHILIC BLOCK COPOLYMERS

Lang Xia,* Fataneh Karandish,† Krishna Nandan Kumar,* James Froberg,‡ Prajakta Kulkarni,† Kara N. Gange,§ Yongki Choi,‡ Sanku Mallik,† and Kausik Sarkar*

* Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, DC; † Department of Pharmaceutical Sciences, North Dakota State University, Fargo, North Dakota; ‡ Department of Physics, North Dakota State University, Fargo, North Dakota; and § Department of Health, Exercise, and Nutrition Sciences, North Dakota State University, Fargo, North Dakota

(*Received* 22 *May* 2017; *revised* 6 *October* 2017; *in final form* 24 *October* 2017)

Abstract—Polymersomes are a class of artificial vesicles prepared from amphiphilic polymers. Like lipid vesicles (liposomes), they too can encapsulate hydrophilic and hydrophobic drug molecules in the aqueous core and the hydrophobic bilayer respectively, but are more stable than liposomes. Although echogenic liposomes have been widely investigated for simultaneous ultrasound imaging and controlled drug delivery, the potential of the polymersomes remains unexplored. We prepared two different echogenic polymersomes from the amphiphilic co-polymers polyethylene glycol–poly-DL-lactic acid (PEG-PLA) and polyethylene glycol–poly-L-lactic acid (PEG-PLLA), incorporating multiple freeze-dry cycles in the synthesis protocol to ensure their echogenicity. We investigated acoustic behavior with potential applications in biomedical imaging. We characterized the polymeric vesicles acoustically with three different excitation frequencies of 2.25, 5 and 10 MHz at 500 kPa. The polymersomes exhibited strong echogenicity at all three excitation frequencies (about 50- and 25-dB enhancements in fundamental and subharmonic, respectively, at 5-MHz excitation from 20 μg/mL polymers in solution). Unlike echogenic liposomes, they emitted strong subharmonic responses. The scattering results indicated their potential as contrast agents, which was also confirmed by clinical ultrasound imaging. (E-mail: sarkar@gwu.edu)

Key Words: **Ultrasound imaging, Contrast agent, Microbubble, Polymersomes, Echogenic, Drug delivery.**

INTRODUCTION

Ultrasound imaging is one of the most widely employed diagnostic tools in the medical field. However, it suffers from poor contrast. To improve the contrast of ultrasound imaging, coated microbubbles are used as contrast agents (deJong et al. 1991; Goldberg et al. 2001; Paul et al. 2014). They are also being investigated as potential drug delivery agents (Bull 2007; Eisenbrey et al. 2010; Klibanov 2006; Paul et al. 2014). These microbubbles (diameter: 0.5–5 μm), although ideal as vascular agents, cannot extravasate through pores of blood vessels. The leaky vasculature of a solid tumor with pores typically 100–700 nm offers great potential for targeted delivery into tumors through enhanced permeability and retention (EPR) (Maeda et al. 2000).

Address correspondence to: Kausik Sarkar, Department of Mechanical and Aerospace Engineering, The George Washington University, 800 22nd Street NW, Suite 3000, Washington, DC 20052. E-mail: sarkar@gwu.edu

Nanometer-size lipid bilayer vesicles or liposomes are ideal extravascular drug delivery agents for tumors. Recently, we and other groups have reported that when prepared in the presence of mannitol as an excipient, liposomes can be made echogenic (echogenic liposomes [ELIPs]) (Coussios et al. 2004; Huang 2008; Huang et al. 2001; Kee et al. 2007; Kopechek et al. 2011; Nahire et al. 2012, 2013, 2014b; Paul et al. 2012, 2014). Here we report on a novel echogenic vesicle, which, unlike liposomes, is enclosed by an amphiphilic polymer bilayer.

As noted above, ELIPs have been widely studied. Huang and co-workers optimized the protocol for their preparation (Hamilton et al. 2002; Huang et al. 2001, 2002a, 2002b). The first acoustical characterization of ELIPs was performed employing a 20-MHz frequency catheter for both *in vitro* and *in vivo* experiments (Alkan-Onyuksel et al. 1996). Videodensitometry analysis was used to measure the echogenicity of liposomes (Buchanan et al. 2008; Huang 2008). Coussios et al. (2004) reported an *in vitro* characterization of ELIPs, in which they found backscattering

signals from these liposomes to be comparable to those of the microbubble-based contrast agent Optison. The echogenicity of liposomes was detected with a Philips L12-5 linear array transducer system using B-mode pulses (Smith et al. 2007). Although the exact mechanism underlying the echogenicity of ELIPs remains unknown—specifically the exact location of the gas body in the small vesicles responsible for generating the reflected signals—careful *in vitro* characterization has convincingly indicated its echogenicity for both fundamental and second harmonic signals. (Paul et al. 2012). Modeling of ELIP acoustic behaviors has also been attempted by assuming gas pockets of comparable size (Raymond et al. 2015).

Polymersomes (Discher et al. 1999) are 100- to 600-nm vesicles enclosed by bilayer membranes that are made of amphiphilic block copolymers, instead of lipids. The vesicles carry hydrophobic drugs in the polymer bilayer and hydrophilic molecules in the aqueous core. They provide unique advantages in biomedical applications (Zhang and Zhang 2017). For example, the membrane of the polymersomes is almost an order of magnitude tougher, sustains far greater areal strain before rupture, and is at least 10 times less permeable to water than phospholipid bilayers (Discher et al. 1999). Incorporation of the biocompatible polyethylene glycol (PEG) as the hydrophilic block renders the vesicles long circulating (Mohammadi et al. 2017). A targeting peptide or ligand actively transports the polymersomes to the cancer tissues and inside the cytosol (Simon-Gracia et al. 2016). We recently reported that the presence of small organic molecules can successfully target the polymeric vesicles to cancer cell nuclei (Anajafi et al. 2016) or mitochondria (Kulkarni et al. 2016b). Because of their stability, after targeting, the polymersome requires a trigger to release the encapsulated drugs (Hu et al. 2017; Thambi et al. 2016). We have used enhanced concentrations of reducing agents and hypoxia (internal triggers) of solid tumors to deliver anticancer drugs from polymersomes successfully (Anajafi et al. 2016; Karandish et al. 2016; Kulkarni et al. 2016a).

Although ultrasound is known primarily for its imaging capabilities, it is also widely used as an external trigger to release encapsulated drugs from liposomes (Moussa et al. 2015; Zhang et al. 2016). However, to date, ultrasound-sensitive polymersomes have hardly been studied. In a preliminary investigation of polymersomes prepared with PEG–poly-DL-lactic acid (PEG-PLA) block co-polymers in the presence of mannitol, Zhou et al. (2006) reported acoustic activity in a diagnostic imaging system. We recently reported the preparation of echogenic polymersomes from the reduction-sensitive, amphiphilic block copolymer PEG–S–S–PLA with 10-dB enhancement in the scattering signal for a concentration of 10 μg/mL (Nahire et al. 2014a). To our knowledge, there has not been another report on interactions between ultrasound and polymersomes. Moreover, detailed acoustic characterization of such polymersomes is lacking.

The meager literature on echogenic polymersomes and, at the same time, their potential for dual drug delivery and imaging capabilities warrant further careful investigation. Also, polymersome (as well as liposome) suspensions, although of an average size of 100–600 nm, have significant polydispersity (Nahire et al. 2014a), indicating the possibility of excitation frequency-dependent responses. The resonant frequency of an entrapped air pocket responsible for echogenicity depends on its size. As noted earlier, the exact location and size of the air pocket responsible for the echogenicity of ELIPs or echogenic polymersomes remain uncertain. It is possible that the vesicles act as cavitation nuclei that are responsible for generating air bubbles, which then determine the echogenicity. In any event, investigation of the acoustic response of echogenic polymersomes as a function of frequency is key to their desired clinical use. Such an investigation, in turn, could shed light on the mechanism of echogenicity of ELIPs or polymersomes. In the work described here, we investigated the scattered responses of echogenic polymersomes) to ultrasound waves as a function of frequency (2.25, 5 and 10 MHz. We also studied polymersomes made of two different amphiphilic polymers, PEG-PLA and PEG–poly-L-lactic acid (PEG-PLLA) (PLA and PLLA are stereoisomers) to investigate the effects of stereochemistry and how they might affect the packing of the bilayer and thereby the mechanical properties and scattered response.

METHODS

Preparation of polymersomes

Echogenic polymersomes were prepared by the solvent exchange method (Lee and Feijen 2012) using the synthesized PEG(2000)-PLA(5000) and commercially available PEG(2000)-PLLA(5000) (Akina, West Lafayette, IN, USA). Polymers (6 mg/mL) were dissolved in tetrahydrofuran (THF). Note that the choice of molecular weight of the polymers is determined by the ability to form bilayers and polymersomes. In the future, we plan to explore the effects of molecular weight variation with these or other polymersomes. Each polymersome batch was prepared by adding 2.5 mg of polymer dropwise to 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution (pH 7.4) containing 0.32 M mannitol. The solutions were stirred for 20 min at room temperature, and then air was passed through the mixture for 45 min to remove the organic solvent. The polymersomes formed were sonicated at 25 °C for 60 min (Symphony 117 V, 60 Hz, power level: 9). Subsequently, the polymersomes were freeze-thawed for 3 cycles (–80 °C and 65 °C) and then freeze-dried using a Labconco instrument for 4 d. The