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● *Original Contribution*

DYNAMICS OF TARGETED MICROBUBBLE ADHESION UNDER PULSATILE COMPARED WITH STEADY FLOW

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Abstract—Hemodynamic flow variations at low fluid shear stress are thought to play a critical role in local atherosclerotic plaque initiation and development and to affect plaque instability. Targeted microbubbles are being developed as intravascular agents for identifying atherosclerotic lesions using ultrasound. How variations in local hydrodynamic flow influence the adhesiveness of targeted microbubbles is not well understood. We postulated that rates of targeted microbubble binding and accumulation differ when subjected to steady flow (SF) as compared with oscillatory or pulsatile flow (PF), because PF imposes non-uniform blood rheology and periodic acceleration and deceleration of blood velocity, when compared with SF. We assessed the binding rates of targeted microbubbles in seven randomly assigned PF and seven matched SF replicate runs at low (<1 Pa) and intermediate (≥1 and <2.5 Pa) wall shear stress (WSS) by drawing 4.8 × 10^6 microbubbles mL^{-1} over streptavidin-coated substrates, immobilized within a parallel plate flow chamber at a calculated density of 81 binding sites μm^{-2}. Selective binding and accumulation of targeted microbubbles was recorded in a single field of view using real-time video microscopy. Microbubble accumulation was modeled to obtain flow-mediated microbubble binding kinetics (amplitude, *A*, and rate constant, *k*). PF elicited higher microbubble accumulation rates, in comparison to SF. The rates of microbubble accumulation differed significantly between PF and SF ($p < 0.05$) at intermediate WSS but not at low WSS ($p > 0.05$). The rate of microbubble accumulation decreased as WSS increased. (E-mail: c.sennoga@imperial.ac.uk)

Key Words: **Pulsatile flow, Steady flow, Wall shear stress, Targeted microbubbles, Molecular imaging.**

INTRODUCTION

Methods to non-invasively identify vascular disorders are clinically prized and needed. Existing methods for non-invasively visualizing blood flow *in situ* (*e.g.*, angiography) do not provide sufficient spatial resolution for the localization of early non-stenosing atherosclerotic lesions. Molecular imaging using site-targeted microbubbles and ultrasound is presently being investigated as a method for detecting asymptomatic endothelial expression of inflammatory molecules that modulate atherosclerotic plaque initiation, development and instability (Kaufmann et al. 2007). Accordingly, *in vitro* recruitment of site-targeted microbubbles to activated endothelial cells (Takalkar et al. 2004; Weller et al. 2002), ligand-coated substrates, platelets and leukocytes has been assessed under shear-controlled hydrodynamic steady flow (SF) conditions. Despite its physiologic relevance in atherosclerotic plaque initiation and development (Cheng et al. 2006), the question of how non-steady low-shear oscillatory flow influences the kinetics of substrate-targeted microbubble recruitment has not been investigated.

Microbubbles are aqueous dispersions of gaseous spheres (diameter ≤8 μm) stabilized by lipid, protein or polymeric shell at the gas-liquid interface. Microbubbles are currently employed in medical imaging as ultrasound contrast agents, offering, as they do, images with enhanced blood pool to tissue definition and quantification of tissue perfusion (Becher and Burns 2000), for improved diagnostic confidence.

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The development of site-targeted microbubbles (Klibanov 1999, 2002; Lindner 2004) has opened up a whole new area of molecular imaging using ultrasound, with the potential to revolutionize the role that ultrasound imaging plays in various clinical applications (Blomley et al. 2001). In ultrasound molecular imaging, microbubbles are coupled to ligands with binding selectivity to molecular disease biomarker(s) that are sequentially expressed on activated endothelial cells bordering the walls of blood vessels as disease progresses (*e.g.*, Demos et al. 1997; Lanza et al. 1996; Lindner et al. 2000). Because of their binding selectivity, these site-targeted microbubbles, usually administered intravenously, site-selectively localize on walls of pathologic blood vessels where molecular disease biomarkers are expressed. Targeted microbubbles adherent on vessel walls are, in turn, non-invasively monitored using ultrasound. Indeed, site-targeted microbubbles employing antibodies, antibody fragments, peptides and/or glycoproteins as ligands have already demonstrated selective targeting capabilities in a number of preclinical ultrasound diagnostic evaluations, including the imaging of atherosclerosis, myocardial infarction, transplant rejection, tumor angiogenesis (see Lindner 2004 for a review) and, most recently, cancer of the prostate in a human phase 0 (first in human) clinical study (Wijkstra 2012).

Although ultrasound detection of site-targeted microbubbles has potential as a non-invasive diagnostic tool, little is known about the role of hydrodynamic flow variability in the interactions between the target receptors and site-targeted microbubbles. *In vitro* studies examining the adhesion of site-targeted microbubbles (Klibanov et al. 2006; Takalkar et al. 2004; Weller et al. 2002) in shear-controlled flow chambers have yielded conflicting results. These studies indicate that the rates of targeted microbubble attachment and accumulation depend both on the imposed wall shear stress (WSS) and on the density of target molecules on the substrate. Other studies indicate that successful recruitment of microbubbles conjugated with antibodies as ligands to activated endothelial cells or reconstituted ligand-coated substrates is achievable at WSS $<$ 0.15 Pa *in vitro*, whereas *in vivo* recruitment of similar microbubbles is achievable at WSS 10-fold higher, that is, 1.5 Pa. On the basis that red blood cell (RBCs) have been found to augment leukocyte binding, Unnikrishnan et al. (2011) examined the role of RBCs in the interactions of site-targeted microbubbles and concluded that an increase in the frequency of collisions between the flowing microbubbles and the vessel wall caused by near-wall hydrodynamic interactions with RBCs is probably responsible for the observed *in vitro* and *in vivo* mismatch in site-targeted microbubble recruitment. Interested readers are referred to previous reports (Goldsmith and Marlow 1979; Ley 1996; Tokarev et al. 2011) for detailed discussions of the influence of hydrodynamic flow on blood cell collisions, rolling and binding interactions.

Moreover, existing *in vitro* flow studies have, to our knowledge, explored only SF (*e.g.*, Barreiro et al. 2009; Edgeworth et al. 2010; Ferrante et al. 2009; Guenther et al. 2010; Klibanov 1999; Takalkar et al. 2004; Weller et al. 2002) and quasi-SF (Kaufmann et al. 2007), and have not examined the role of flow disturbances, oscillatory or pulsatile flow (PF), which is relevant for atherosclerotic plaque initiation and development. It is worth noting that *in vitro* flow assays conducted under SF conditions are probably not representative of blood flow in the aorta and large arteries, as well as arterioles, capillaries and venules, for which significant pulsatile flow is well documented (see, *e.g.*, Intaglietta et al. 1971). The suggestion that vascular rheology might influence adhesion of blood cells was discussed some 30 years ago (see, *e.g.*, Karino and Goldsmith 1979), and that differences in flow might affect intravascular adhesion of platelets was clearly recognized by Karino and Goldsmith (1984). This article pursues that idea, not for the adhesion of bloodborne cell capsules, but for biocompatible targeted microbubbles.

We postulated that PF would have increased rates of site-targeted microbubble accumulation, because PF is characterized by periodic deceleration in flow velocities, as compared with SF, and that a comparative study of the adhesion behavior of site-targeted microbubbles under SF and PF might help explain the apparent mismatch in adhesion behavior observed *in vitro* as compared with *in vivo*. However, a number of confounding factors associated with ultrasound imaging (Tang et al. 2011) make it difficult to obtain accurate quantitative data on targeted microbubble accumulation *in vivo*. A direct and high-resolution quantitative method is required to assess the differential effects of hydrodynamic flow conditions on adhesion rates of site-targeted microbubbles. One such method is video microscopy used in combination with a parallel plate flow chamber at controlled WSS. In this report, we describe a modular flow apparatus developed for hydrodynamic flow studies. The flow system described here is a modification of a similar apparatus (Lawrence and Springer 1991), previously reported to provide detailed information on the kinetics of leukocyte/bead binding to reconstituted ligand-coated substrates, activated endothelial cells, platelets and other leukocytes and the rheology of site-targeted microbubbles, including the quantification of microbubble adhesion (Weller et al. 2002) and rolling (Klibanov et al. 2006; Takalkar et al. 2004).

In the present study, we first assessed the interaction and selective binding of biotinylated microbubbles to reconstituted streptavidin-coated substrates with similar