Published in final edited form as:

J Am Soc Echocardiogr. 2012 April ; 25(4): 460–466. doi:10.1016/j.echo.2011.12.016.

Factors Affecting the Endothelial Retention of Targeted Microbubbles: Influence of Microbubble Shell Design and Cell Surface Projection of the Endothelial Target Molecule

Elham Khanicheh, MD, Martina Mitterhuber, MS, Katharina Kinslechner, BSc, Lifan Xu, PhD, Jonathan R. Lindner, MD, and Beat A. Kaufmann, MD

Department of Biomedicine (E.K., M.M., K.K., L.X., B.A.K.) and Cardiology (B.A.K.), University Hospital Basel, Basel, Switzerland; and the Cardiovascular Division, Oregon Health & Science University, Portland, Oregon (J.R.L.)

Abstract

Background—In biologic systems, the arrest of circulating cells is mediated by adhesion molecules projecting their active binding domain above the cell surface to enhance bond formation and tether strength. Similarly, molecular spacers are used for ligands on particle-based molecular imaging agents. The aim of this study was to evaluate the influence of tether length for targeting ligands on ultrasound molecular imaging agents.

Methods—Microbubbles bearing biotin at the end of variable-length polyethylene glycol spacer arms (MB₂₀₀₀ and MB₃₄₀₀) were prepared. To assess in vivo attachment efficiency to endothelial counterligands that vary in their distance from the endothelial cell surface, contrast-enhanced ultrasound (CEU) molecular imaging of tumor necrosis factor- α -induced P-selectin (long distance) or intercellular adhesion molecule-2 (short distance) was performed with each agent in murine hind limbs. To assess the influence of the glycocalyx on microbubble attachment, CEU molecular imaging of intercellular adhesion molecule-2 was performed after degradation of the glycocalyx.

Results—CEU molecular imaging targeted to P-selectin showed signal enhancement above control agent for MB₂₀₀₀ and MB₃₄₀₀, the degree of which was significantly higher for MB₃₄₀₀ compared with MB₂₀₀₀. CEU molecular imaging targeted to intercellular adhesion molecule-2 showed low overall signal for all agents and signal enhancement above control for MB₃₄₀₀ only. Glycocalyx degradation increased signal for MB₃₄₀₀ and MB₂₀₀₀.

Conclusions—Microbubble targeting to endothelial ligands is influenced by (1) the tether length of the ligand, (2) the degree to which the endothelial target is projected from the cell surface, and (3) the status of the glycocalyx. These considerations are important for designing targeted imaging probes and understanding potential obstacles to molecular imaging.

Keywords

Molecular imaging; Microbubbles; Contrast ultrasound; Microbubble design

Contrast-enhanced ultrasound (CEU) molecular imaging has been developed in recent years for the noninvasive assessment of pathophysiologic changes that are present on a molecular level in atherosclerosis, myocardial ischemia, angiogenesis, thrombus formation, and heart

transplant rejection.¹⁻⁴ CEU molecular imaging relies on the selective retention of targeted microbubbles within diseased tissue.⁵ Microbubble contrast agents are gas-filled, lipid-shelled or albumin-shelled structures that range from 1 to 4 μm in diameter and are confined to the vascular compartment, thus restricting the potential targets to disease markers present on the endothelial cell surface. Targeting of lipid shelled microbubbles is achieved by conjugation of antibodies or other specific ligands to the microbubble shell surface via functionalized lipids. Targeted retention of microbubbles in a vessel under any given shear condition depends on a number of factors, including (1) ligand density on the microbubble surface,⁶ (2) ligand-specific bond kinetics, and (3) site density of the molecular target.⁷ Similar to the case with leukocyte and platelet adhesion molecules that have their binding domains projected away from the cell surface to enhance bond formation,⁸ the active ligand on targeted microbubbles is often placed at the end of polyethylene glycol (PEG) multimers that project the ligand away from the shell surface and beyond any PEG brush that has been added to the shell surface to stabilize microbubbles.

The aim of this study was to assess whether the length of the functionalized PEG molecules used to conjugate antibodies to the microbubble shell surface influences targeting efficiency. The importance of this issue is underscored by the desire to target not only long adhesion molecules that project their binding moiety far away from the endothelial cell surface and beyond the glycocalyx but also smaller molecules that reside closer to the endothelial cell surface. We therefore compared the targeting efficiency of microbubbles with different-length functionalized PEG molecules to adhesion molecules with a long-distance (P-selectin) and short-distance (intercellular adhesion molecule [ICAM]-2) projection from the endothelial surface, with and without modification of the glycocalyx.

METHODS

Microbubble Preparation

Lipid-shelled decafluorobutane microbubbles were prepared by sonication of a gas-saturated aqueous suspension of distearoylphosphatidylcholine (2 mg/mL) and polyoxyethylene-40-stearate (1 mg/mL). For ligand conjugation, functionalized PEG-lipid conjugates containing biotin at the PEG N-terminus were added to the aqueous suspension at concentrations corresponding to equal molar ratios of 50:10:1 (distearoylphosphatidylcholine/polyoxyethylene-40-stearate/functionalized PEG). The PEG length was varied by using 1,2-distearoyl-sn-glycero-phosphoethanolamine-N-[biotinyl(PEG)-2000] (Avanti Polar Lipids, Alabaster, AL), 1,2-distearoyl-sn-glycero-phosphoethanolamine-N-[biotinyl(PEG)-3400] (Creative PEG Works, Winston-Salem, NC), or 1,2-distearoyl-sn-glycero-phosphoethanolamine-N-[biotinyl(PEG)-10000] (Nanocs Inc., Boston, MA). Thus, microbubbles with different-sized PEG-biotin moieties (MB_{PEG2000}, MB_{PEG3400}, and MB_{PEG10000}) were prepared with PEG multimers of 45, 80, and 225, respectively.

Monoclonal antibodies were conjugated to the microbubble surface as previously described using a streptavidin-biotin link.⁹ Rat antimouse monoclonal immunoglobulin G1 against either P-selectin (Rb40.34) or ICAM-2 (mLC2/4) were conjugated to the microbubble surface to produce P-selectin-targeted (MB_{PEG2000}-PSEL, MB_{PEG3400}-PSEL, and MB_{PEG10000}-PSEL) or ICAM-2-targeted (MB_{PEG2000}-ICAM, MB_{PEG3400}-ICAM, and MB_{PEG10000}-ICAM) microbubbles, each with different-length PEG-biotin moieties. Isotype control antibodies (R3-34; BD Bioscience, San Jose, CA) were conjugated to the surfaces of microbubbles with randomly selected PEG spacer-length moieties to produce control microbubbles (MB_{Ctrl}). Microbubble concentration, size, and surface area were measured by electrozone sensing (Multisizer III; Beckman Coulter, Brea, CA).

Biotin and Antibody Density on the Microbubble Surface

To quantify the number of PEG spacer moieties and antibodies on the microbubble surface by quantitative fluorescence, aliquots of 1×10^8 for MB_{PEG2000}, MB_{PEG3400}, and MB_{PEG10000} were washed and incubated with 30 μ g fluorescein isothiocyanate (FITC)–labeled streptavidin (Leinco Technologies, St. Louis, MO). Excess FITC–streptavidin was removed with three cycles of flotation centrifugation. Microbubble concentration and mean surface area were measured, and the microbubbles were destroyed by prolonged application of pressure at 100 mm Hg. The concentration of fluorescently labeled streptavidin was measured using a Gemini XPS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) and compared with a concentration-intensity reference standard to derive the density per surface area. For determination of the number of antibodies bound per square micrometer of microbubble surface, a known concentration of biotinylated immunoglobulin G was labeled with FITC. Labeled antibodies were conjugated to the different microbubble preparations, and quantified as described for FITC–streptavidin. All experiments were performed in triplicate.

Animal Preparation

All experiments were performed in accordance with Swiss federal legislation and were approved by the local animal care and use committee. Male wild-type C57BL/6 mice aged 8 to 10 weeks were anaesthetized with 1.5% inhaled isoflurane. Body temperature was maintained at 37°C with a heating pad. A jugular vein was cannulated for administration of microbubbles.

CEU Imaging

Ultrasound imaging (Sequoia Acuson C512; Siemens Medical Systems USA Inc., Mountain View, CA) was performed with a high-frequency linear-array probe (15L8) held in place by a railed gantry system. The adductor muscle of the mouse hind limb was imaged in short axis. Anatomic images of the hind limb were acquired with fundamental imaging at 14 MHz. CEU was performed with power modulation and pulse inversion (Contrast Pulse Sequence) imaging at a centerline frequency of 7 MHz. The gain settings were adjusted just below visible tissue speckle and held constant.

In vivo circulation half-life of microbubble preparations with different length PEG spacer moieties were compared using low-mechanical index ultrasound imaging. Nontargeted MB_{PEG2000}, MB_{PEG3400}, and MB_{PEG10000} (1×10^7 each) were injected intravenously as a bolus in random order. Thirty seconds after the bolus injection, single image frames of the proximal hind limb were obtained at a mechanical index of 0.2 at 15-sec intervals for 10 min. It has previously been shown that these imaging parameters do not result in microbubble destruction when imaging the mouse hind limb.¹⁰ On digitized image sequences, acoustic intensity from a region of interest over the hind limb adductor muscle was derived over time after removal of logarithmic signal compression.

For in vivo assessment of attachment efficiency of microbubbles targeted to P-selectin with different-length PEG spacer moieties, a mouse model of murine hind limb inflammation was used ($n = 16$). CEU molecular imaging of the hind limb was performed 45 min after intramuscular injection of tumor necrosis factor (TNF)- α (250 ng). MB_{PEG2000}-PSel, MB_{PEG3400}-PSel, MB_{PEG10000}-PSel, and MB_{Ctrl} (1×10^7 each) were injected intravenously as a bolus in random order. Ultrasound imaging was paused for 8 min after each injection. Imaging was then resumed at a mechanical index of 0.87. The first acquired image frame was used to derive the total amount of microbubbles present in tissue. The microbubbles in the ultrasound beam were then destroyed with several (>10) image frames. Several image frames at a long pulsing interval (10 sec) acquired after microbubble destruction were then

acquired to measure signal attributable to freely circulating microbubbles. Data were log-linear converted, and frames representing freely circulating microbubbles were digitally subtracted from the first image frame to derive signal from attached microbubbles alone.⁹

For in vivo assessment of the attachment efficiency of microbubbles targeted to ICAM-2, imaging was performed as described above in murine hind limbs ($n = 16$), without induction of inflammation, because ICAM-2 has been shown to be constitutively expressed on endothelial cells, with the level of expression not influenced by cytokines.^{11,12} In an additional 16 mice, molecular imaging was performed 60 min after intravenous injection of 32 IU of *Streptomyces* hyaluronidase (Sigma-Aldrich, St. Louis, MO), which was performed to reduce microvascular endothelial glycocalyx thickness.¹³

Statistical Analysis

Data were analyzed using GraphPad Prism version 5.0d (GraphPad Software, Inc., La Jolla, CA). Data are expressed as mean $\pm$ SEM unless stated otherwise. One-way analysis of variance with Tukey's post hoc test was used for multiple comparisons of normally distributed variables. Friedman's repeated-measures analysis of variance with Dunn's post hoc test was used to compare variables not normally distributed. Two-sided P values $< .05$ were considered statistically significant.

RESULTS

Microbubble Shell Characteristics

Because microbubble acoustic signal is influenced by size,¹⁴ microbubble diameter was assessed for the different agents according to PEG spacer length. The microbubble agents did not differ in terms of their mean diameters or their size distributions (Figure 1), with $10 \pm 6\%$, $8 \pm 4\%$, and $8 \pm 5\%$ of MB_{PEG2000}, MB_{PEG3400}, and MB_{PEG10000}, respectively, having diameters $> 5 \mu\text{m}$ ($P = \text{NS}$ for multiple comparison).

Fluorescent labeling with streptavidin showed similar numbers of biotin molecules on the microbubble surface for MB_{PEG2000} and MB_{PEG3400} but a significantly lower number of biotin molecules for MB_{PEG10000} (Table 1). As a result, surface density for antibody that was coupled via biotin was similar for MB_{PEG2000} and MB_{PEG3400} but was more than an order of magnitude lower for MB_{PEG10000}.

In Vivo Circulation Time

Because the duration of recirculation could potentially affect the extent of agent attachment, microbubble circulation life span was assessed in vivo using nondestructive low-mechanical index ultrasound imaging. The time intensity profile and circulation half-life were similar for MB_{PEG2000}, MB_{PEG3400}, and MB_{PEG10000} (Figure 2).

Influence of PEG-Spacer Length on Attachment

CEU molecular imaging for P-selectin in TNF- α -induced hind limb inflammation showed selective signal enhancement for MB_{PEG2000}-PSel and MB_{PEG3400}-PSel compared with control microbubbles (Figure 3). Signal enhancement for MB_{PEG3400}-PSel was approximately 30% higher than for MB_{PEG2000}-PSel. Signal for MB_{PEG10000}-PSel was not significantly different from control microbubbles, although there was a trend toward selective signal enhancement. The signal from control microbubbles was not dependent on the PEG spacer length that was used (signal for MB_{PEG2000}-Ctr 0.50 ± 0.14 , for MB_{PEG3400}-Ctr 0.53 ± 0.17 , and for MB_{PEG10000}-Ctr 0.35 ± 0.13 ; $P = \text{NS}$).

CEU molecular imaging for ICAM-2, which has a shorter projection distance for the active binding site from the endothelial cell surface, showed very low signals overall for all agents. However, there was selective signal enhancement for MB_{PEG3400}-ICAM but not for MB_{PEG2000}-ICAM and MB_{PEG10000}-ICAM compared with control microbubbles (Figure 4). Signal intensity for MB_{PEG3400}-ICAM was twofold higher than for MB_{PEG2000}-ICAM.

After glycocalyx degradation, the signal increased for both MB_{PEG2000}-ICAM and MB_{PEG3400}-ICAM. Although nonspecific attachment of MB_{Ctrl} increased as well, this signal was significantly lower than that for MB_{PEG2000}-ICAM and MB_{PEG3400}-ICAM.

DISCUSSION

The aim of this study was to assess the influence of different-length functionalized PEG molecules for targeting ligand attachment on in vivo contrast-enhanced molecular imaging signals. We show that the use of longer (PEG₃₄₀₀) functionalized PEG molecules to target adhesion molecules on the endothelial surface results in better attachment efficiency and thus better signal enhancement than the use of shorter (PEG₂₀₀₀) functionalized PEG molecules. Microbubbles with a PEG₁₀₀₀₀ chain, which was not incorporated well into the shell surface, yielded signals that were consistently lower than for MB_{PEG3400} and MB_{PEG2000}, confirming earlier in vitro results that found a significant impact of microbubble surface antibody density on targeted adhesion to endothelial cells.⁶

For ultrasound molecular imaging, targeted microbubble attachment occurs under flow conditions, with only milliseconds available for bond formation between the receptor and the targeting ligand. Once formed, the bonds have to withstand shear forces acting on the microbubble at the bloodstream-endothelial interface. In addition, the glycocalyx constitutes a physical barrier to the interaction of particle-based ligands with endothelial targets. Leukocytes accomplish attachment to endothelial cells in a remarkably efficient and specific manner. The initial tethering of leukocytes is conveyed by the interaction of selectins with cell-surface glycoconjugates. Both selectins and their glycoconjugate ligands are unusually long molecules,⁸ and the lengths of selectins and their ligands have an impact on leukocyte rolling.^{15,16} Thus, targeted microbubbles are designed with the ligands attached to the distal tips of functionalized PEG spacer arms with the goal of emulating leukocytes. Mimicry of such targeting mechanisms on in vitro experiments with microparticles targeted to P-selectin has shown an increased microparticle binding frequency and increased adhesive lifetimes with ligands attached to longer PEG spacer molecules.¹⁷

In the present study, we incorporated different-length PEG spacers functionalized with biotin for ligand attachment into the lipid shell of decafluorobutane microbubbles. Equimolar dissolution of PEG₂₀₀₀ and PEG₃₄₀₀ spacers resulted in equivalent numbers of spacer arms available for streptavidin binding on the microbubble surface. In contrast, the number of PEG₁₀₀₀₀ spacer arms on the microbubble surface available for streptavidin binding was seven to eight times lower at equimolar concentrations of the PEG₁₀₀₀₀ spacer, and the relative reduction in the density of mAb conjugated to the surface was even lower. Incorporation of PEG₁₀₀₀₀ was not increased when using higher molecular ratios (data not shown). A larger extent of polymer chain collapse may have impeded integration of the longer and thus bulkier PEG₁₀₀₀₀ chains into the microbubble surface at sufficient densities because of spatial constraints and the increased repulsion between larger individual molecules, an effect also referred to as steric hindrance.

When polymers are grafted on a surface at low density, the individual polymer chains will curl up and form mushroom-like structures. As the grafting density is increased, the polymer chains start to repel each other and extend away from the grafting surface in a brush-like

fashion. On the basis of the number of functionalized PEG spacer chains per square micrometer derived from fluorescence labeling experiments and the 10:1 molar ratio of PEG-stearate to functionalized PEG spacer, it can safely be assumed that a brush confirmation was obtained for MB_{PEG2000} and MB_{PEG3400}.¹⁸ In this configuration, it has been estimated that true lengths of MB_{PEG2000} and MB_{PEG3400} at the measured site densities would be about 10 and 18 nm, respectively, corresponding to about 70% of the maximal length of the PEG chains.^{18,19}

In vivo ultrasound molecular imaging showed selective signal enhancement for both MB_{PEG3400} and MB_{PEG2000} targeted to P-selectin but only for MB_{PEG3400} targeted to ICAM-2. Overall, signal enhancement was strong for both MB_{PEG3400} and MB_{PEG2000} targeted to P-selectin, which projects its binding domain about 40 nm away from the endothelial cell surface.²⁰ In contrast, and despite a high level of constitutive expression of ICAM-2 on the endothelium that has been reported to be even higher than TNF- α -stimulated expression of P-selectin,¹² signal enhancement was weak for MB_{PEG3400} and MB_{PEG2000} targeted to ICAM-2. We believe this was because the adhesion molecule extends <10 nm from the endothelial cell surface.^{21,22} In both animal models, signal from MB_{PEG3400} was significantly higher than signal from MB_{PEG2000}, indicating advantages of the longer PEG spacer arms for binding efficiency.⁶

The mechanism for increased binding efficiency when using a longer PEG spacer arm may result from better accessibility to the target. In addition, it has been shown that longer PEG spacer arms may also improve targeting efficiency by increasing the bond lifetime of microparticles under flow conditions by preferentially localizing the bond structure at the trailing end of the microparticle, thus reducing torque forces acting on the microparticle under flow conditions.¹⁷

Microbubble interaction with endothelial targets occurs on the endothelial cell surface covered by the glycocalyx. Recent data suggest that the glycocalyx measures up to 500 nm in normal microcirculatory venules.²³ The low signal for both MB_{PEG3400}-ICAM and MB_{PEG2000}-ICAM in vivo model may thus be secondary to steric hindrance and/or charge repulsion caused by the glycocalyx, which lowered accessibility of the microbubbles to the target. Still, differences in PEG spacer length seemed to retain an influence on attachment efficiency, possibly by increasing the chance of a microbubble for deploying the targeting ligands close enough to the endothelial cell surface for bond formation. Degradation of the glycocalyx lead to increased retention of MB_{PEG3400}-ICAM and MB_{PEG2000}-ICAM but also to increased nonspecific attachment of MB_{Ctrl}. This may have been secondary to complement-mediated attachment to either endothelial cells or to adherent leukocytes as a result of hyaluronidase treatment.^{24,25}

Several limitations of this study should be mentioned. We prepared microbubbles with three different PEG spacers carrying a biotin moiety for ligand conjugation and noted a reduced incorporation of the PEG 10000 spacer molecule. However, whether incorporation of intermediate length PEG spacers (eg, PEG 5000) would be possible was not tested.

On in vivo experiments, comparisons of the targeting efficiency of different microbubble species (e.g., MB₂₀₀₀ vs MB₃₄₀₀ vs MB₁₀₀₀₀) were made within identical models and with identical targeting antibodies. Thus, targeted imaging signals can be compared safely among microbubble preparations with different-length PEG spacers. In contrast, comparison between the two in vivo models is not only influenced by targeting efficiency but could also be dependent on the properties of the two different antibodies used for targeting and differences in the site density of the target molecule. However, given the reported high level of constitutive expression of ICAM-2 on endothelial cells in different murine tissues¹² and

the large difference in targeted signals for molecular imaging of P-selectin versus ICAM-2, we still think that it is reasonable to conclude that the overall lower targeting efficiency for ICAM-2 is due to lower accessibility.

Also, in our study, we used monoclonal antibodies as ligands. Whether humanized antibodies or smaller peptide ligands will ultimately be used for translation into clinical trials of ultrasound molecular imaging is uncertain, and the specific properties of smaller peptide ligands conjugated to different length spacers would have to be tested.

Last, we did not measure directly the glycocalyx thickness in our two different mouse models. However, it has been well documented that TNF- α -induced inflammation leads to an almost complete degradation of the glycocalyx²³ and that systemic application of hyaluronidase reduces glycocalyx thickness.¹³

CONCLUSIONS

We have shown that the microbubble architecture, specifically the length of PEG spacer moieties used for conjugation of targeting ligands, has a significant impact on targeting efficiency. These results have an implication for the shell design of targeted microbubbles. In addition, reduced accessibility due to the thickness of the glycocalyx in the vessels of interest may have an important influence on microbubble attachment. This observation could have implications for the selection of potential molecular imaging targets.

Abbreviations

CEU	Contrast-enhanced ultrasound
FITC	Fluorescein isothiocyanate
ICAM	Intercellular adhesion molecule
PEG	Polyethylene glycol
TNF	Tumor necrosis factor

References

1. Kaufmann BA, Carr CL, Belcik JT, Xie A, Yue Q, Chadderdon S, et al. Molecular imaging of the initial inflammatory response in atherosclerosis: implications for early detection of disease. *Arterioscler Thromb Vasc Biol.* 2010; 30:54–9. [PubMed: 19834105]
2. Kaufmann BA, Lindner JR. Molecular imaging with targeted contrast ultrasound. *Curr Opin Biotechnol.* 2007; 18:11–6. [PubMed: 17241779]
3. Kaufmann BA, Lewis C, Xie A, Mirza-Mohd A, Lindner JR. Detection of recent myocardial ischaemia by molecular imaging of P-selectin with targeted contrast echocardiography. *Eur Heart J.* 2007; 28:2011–7. [PubMed: 17526905]
4. Behm CZ, Kaufmann BA, Carr C, Lankford M, Sanders JM, Rose CE, et al. Molecular imaging of endothelial vascular cell adhesion molecule-1 expression and inflammatory cell recruitment during vasculogenesis and ischemia-mediated arteriogenesis. *Circulation.* 2008; 117:2902–11. [PubMed: 18506006]
5. Kaufmann BA. Ultrasound molecular imaging of atherosclerosis. *Cardiovasc Res.* 2009; 83:617–25. [PubMed: 19493955]
6. Weller GE, Villanueva FS, Klivanov AL, Wagner WR. Modulating targeted adhesion of an ultrasound contrast agent to dysfunctional endothelium. *Ann Biomed Eng.* 2002; 30:1012–9. [PubMed: 12449762]

7. Takalkar AM, Klibanov AL, Rychak JJ, Lindner JR, Ley K. Binding and detachment dynamics of microbubbles targeted to P-selectin under controlled shear flow. *J Control Release*. 2004; 96:473–82. [PubMed: 15120903]
8. Li F, Erickson HP, James JA, Moore KL, Cummings RD, McEver RP. Visualization of P-selectin glycoprotein ligand-1 as a highly extended molecule and mapping of protein epitopes for monoclonal antibodies. *J Biol Chem*. 1996; 271:6342–8. [PubMed: 8626430]
9. Lindner JR, Song J, Christiansen J, Klibanov AL, Xu F, Ley K. Ultrasound assessment of inflammation and renal tissue injury with microbubbles targeted to P-selectin. *Circulation*. 2001; 104:2107–12. [PubMed: 11673354]
10. Kaufmann BA, Carr CL, Belcik T, Xie A, Kron B, Yue Q, et al. Effect of acoustic power on in vivo molecular imaging with targeted microbubbles: implications for low-mechanical index real-time imaging. *J Am Soc Echocardiogr*. 2010; 23:79–85. [PubMed: 19910159]
11. Nortamo P, Li R, Renkonen R, Timonen T, Prieto J, Patarroyo M, et al. The expression of human intercellular adhesion molecule-2 is refractory to inflammatory cytokines. *Eur J Immunol*. 1991; 21:2629–32. [PubMed: 1680706]
12. Langley RR, Russell J, Eppihimer MJ, Alexander SJ, Gerritsen M, Specian RD, et al. Quantification of murine endothelial cell adhesion molecules in solid tumors. *Am J Physiol*. 1999; 277:H1156–66. [PubMed: 10484438]
13. Henry CB, Duling BR. Permeation of the luminal capillary glycocalyx is determined by hyaluronan. *Am J Physiol*. 1999; 277:H508–14. [PubMed: 10444475]
14. de Jong N, Bouakaz A, Frinking P. Basic acoustic properties of microbubbles. *Echocardiography*. 2002; 19:229–40. [PubMed: 12022933]
15. Yago T, Leppanen A, Qiu H, Marcus WD, Nollert MU, Zhu C, et al. Distinct molecular and cellular contributions to stabilizing selectin-mediated rolling under flow. *J Cell Biol*. 2002; 158:787–99. [PubMed: 12177042]
16. Patel KD, Nollert MU, McEver RP. P-selectin must extend a sufficient length from the plasma membrane to mediate rolling of neutrophils. *J Cell Biol*. 1995; 131:1893–902. [PubMed: 8557755]
17. Ham AS, Klibanov AL, Lawrence MB. Action at a distance: lengthening adhesion bonds with poly(ethylene glycol) spacers enhances mechanically stressed affinity for improved vascular targeting of microparticles. *Langmuir*. 2009; 25:10038–44. [PubMed: 19621909]
18. Naji A, Seidel C, Netz RR. Theoretical approaches to neutral and charged polymer brushes. *Adv Polym Sci*. 2006; 198:149–83.
19. Borden MA, Zhang H, Gillies RJ, Dayton PA, Ferrara KW. A stimulus-responsive contrast agent for ultrasound molecular imaging. *Biomaterials*. 2008; 29:597–606. [PubMed: 17977595]
20. Bevilacqua MP, Nelson RM. Selectins. *J Clin Invest*. 1993; 91:379–87. [PubMed: 7679406]
21. Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. *Blood*. 1994; 84:2068–101. [PubMed: 7522621]
22. Springer TA. Adhesion receptors of the immune system. *Nature*. 1990; 346:425–34. [PubMed: 1974032]
23. Potter DR, Jiang J, Damiano ER. The recovery time course of the endothelial cell glycocalyx in vivo and its implications in vitro. *Circ Res*. 2009; 104:1318–25. [PubMed: 19443840]
24. Khan AI, Kerfoot SM, Heit B, Liu L, Andonegui G, Ruffell B, et al. Role of cd44 and hyaluronan in neutrophil recruitment. *J Immunol*. 2004; 173:7594–601. [PubMed: 15585887]
25. Anderson DR, Tsutsui JM, Xie F, Radio SJ, Porter TR. The role of complement in the adherence of microbubbles to dysfunctional arterial endothelium and atherosclerotic plaque. *Cardiovasc Res*. 2007; 73:597–606. [PubMed: 17196951]

Figure 1.

(A) Mean $\pm$ SD diameter for microbubble preparations containing PEG spacer molecules with a PEG 2000, PEG 3400, or PEG 10000 spacer length. Data are from 10 microbubble preparations for each PEG spacer length. (B) Histograms illustrating the size distribution of the three microbubble preparations. ANOVA, Analysis of variance.

Figure 2.

CEU data used to evaluate recirculation time for the microbubble agents that varied according to PEG length. **(A)** Time versus mean $\pm$ SEM video intensity plots on CEU used to assess the duration of recirculation for the three microbubble preparations ($n = 5$ mice with injection of the three microbubble preparations in random order). **(B)** Examples of CEU images at incremental intervals after bolus injection of the three microbubble preparations. Time intervals after bolus injection are provided at the bottom of each image. **(C)** Mean $\pm$ SEM circulation half-life for each agent. $P = NS$ between the three agents. ANOVA, Analysis of variance.

Figure 3.

Molecular imaging data for the three microbubble preparations targeted to P-selectin ($n = 16$ mice). (A) Mean $\pm$ SEM of background-subtracted targeted signal intensity for the three microbubble preparations bearing a P-selectin antibody and microbubbles bearing a control antibody. Examples of background-subtracted color-coded images of targeted signal from one animal are illustrated for MB_{PSel} with a PEG 2000 spacer arm (B), MB_{PSel} with a PEG 3400 spacer arm (C), and MB_{Ctr} (D). Color scales at bottom. * $P < .001$ versus MB_{Ctr}; † $P < .01$ versus PEG 2000 and PEG 10000; ‡ $P < .01$ versus MB_{Ctr}.

Figure 4.

Molecular imaging data for microbubble preparations targeted to ICAM-2 in murine hind limb skeletal muscle. **(A)** Mean $\pm$ SEM of background-subtracted targeted signal intensity for the three microbubble preparations bearing an ICAM-2 antibody and microbubbles bearing a control antibody ($n = 16$ mice). Examples of background-subtracted color-coded images of targeted signal from one animal are illustrated for MB_{ICAM} with a PEG 3400 spacer arm **(B)** and MB_{Ctrl} **(C)**. **(D)** Mean $\pm$ SEM of background-subtracted signal intensity for MB_{PEG3400-ICAM}, MB_{PEG2000-ICAM}, and MB_{Ctrl} 60 min after intravenous injection of hyaluronidase ($n = 16$). Examples of background-subtracted color-coded images of targeted signal from one animal are illustrated for MB_{ICAM} with a PEG 2000 spacer arm **(E)** and MB_{Ctrl} **(F)**. * $P < .01$ versus MB_{Ctrl}; $^{\dagger}P < .05$ versus PEG 2000; $^{\ddagger}P < .05$ versus MB_{Ctrl}.

Table 1

PEG-biotin and antibody surface density

PEG spacer length	PEG-biotin surface density (μm^{-2})	Antibody surface density (μm^{-2})
PEG 2000	12,463 $\pm$ 1,834	4,052 $\pm$ 1,480
PEG 3400	14,799 $\pm$ 5,567	3,703 $\pm$ 1,762
PEG 10000	2,173 $\pm$ 2,349	176 $\pm$ 120