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Optimization of Ultrasound Contrast Agents with Computational Models to Improve Selection of Ligands and Binding Strength

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Abstract

Diagnosis of cardiovascular disease is currently limited by the testing modality. Serum tests for biomarkers can provide quantification of severity but lack the ability to localize the source of the cardiovascular disease, while imaging technology such as angiography and ultrasound can only determine areas of reduced flow but not the severity of tissue ischemia. Targeted imaging with ultrasound contrast agents offers the ability to locally image as well as determine the degree of ischemia by utilizing agents that will cause the contrast agent to home to the affected tissue. Ultrasound molecular imaging via targeted microbubbles (MB) is currently limited by its sensitivity to molecular markers of disease relative to other techniques (e.g. radiolabeling). We hypothesize that computational modeling may provide a useful first approach to maximize microbubble binding by defining key parameters governing adhesion. Adhesive Dynamics was used to simulate the fluid dynamic and stochastic molecular binding of microbubbles to inflamed endothelial cells. Sialyl Lewis^x (sLe^x), P-selectin aptamer (PSA), and ICAM-1 antibody (abICAM) were modeled as the targeting receptors on the microbubble surface in both single- and dual-targeted arrangements. Microbubble properties (radius [R_c], kinetics [k_f , k_r] and densities of targeting receptors) and the physical environment (shear rate and target ligand densities) were modeled. The kinetics for sLe^x and PSA were measured with surface plasmon resonance. R_c , shear rate, and densities of sLe^x, PSA or abICAM were varied independently to assess model sensitivity. Firm adhesion was defined as MB velocity < 2% of the free stream velocity. Adhesive Dynamics simulations revealed an optimal microbubble radius of 1–2 μm and thresholds for k_f^{in} ($>10^2 \text{ sec}^{-1}$) and k_r^0 ($<10^{-3} \text{ sec}^{-1}$) for firm adhesion in a multi-targeted system. State diagrams for multi-targeted microbubbles suggest sLe^x and abICAM microbubbles may require 10-fold more ligand to achieve firm adhesion at higher shear rates than sLe^x and PSA microbubbles. The Adhesive Dynamics model gives useful insight into the key parameters for stable microbubble binding, and may allow flexible, prospective design and optimization of microbubbles to enhance clinical translation of ultrasound molecular imaging.

Keywords

Targeted Ultrasound Contrast; Computational Modeling; Adhesive Dynamics; Surface Plasmon Resonance; Stochastic Model; Microbubbles

Introduction

Cardiovascular inflammation is associated with diseases such as atherosclerosis, occlusive stroke, and pulmonary hypertension, as well as ischemia-reperfusion injury following reconstructive surgery and cardiac transplant rejection (Hansson 2009; Hassoun et al. 2009; Ma et al. 2008). Endothelial cell (EC) dysfunction and inflammation following ischemia, and the associated increases in leukocyte adhesion molecules such as P-selectin, E-selectin, and intracellular adhesion molecule-1 (ICAM-1), are potential targets for diagnosis and therapy (Villanueva et al. 2007). Currently, clinical testing for cardiovascular inflammation such as myocardial ischemia/reperfusion or heart transplant rejection relies on the detection of circulating serum biomarkers which lack the ability to localize affected areas, or targeted tissue biopsies which are necessarily invasive and can be associated with co-morbidities.

Non-invasive methods for detecting and anatomically locating EC dysfunction could improve the diagnosis and treatment of cardiovascular inflammation, particularly by the ability to make an earlier or more accurate diagnosis. Molecular imaging with ultrasound contrast has been proposed to identify a number of disease states, including post-ischemia reperfusion injury, organ rejection and tumor angiogenesis. Ultrasound contrast agents, usually gas encapsulated microspheres (microbubbles), are clinically utilized as red blood cell tracers to opacify the blood pool for visualization of endocardial borders during echocardiography, and delineate regional myocardial perfusion (Hundley et al. 1998; Villanueva et al. 2008).

Molecular imaging with ultrasound has been developed by decorating microbubbles with molecules that bind to disease-specific epitopes present on the vascular wall, causing local microbubble binding and a persistent tissue contrast effect during ultrasound imaging. Several studies employing different targeting moieties (e.g., antibodies, natural receptors, and minimal binding peptide sequences) have demonstrated the ability of this method to detect organ transplant rejection, angiogenesis, atherosclerotic plaque, and myocardial ischemic memory (Bachmann et al. 2006; Burns 2002; Ellegala et al. 2003; Kaufmann et al. 2007; Klibanov et al. 2006; Lindner et al. 2001; Miller et al. 2004; Rychak et al. 2006a; Takalkar et al. 2004; Villanueva et al. 1998; Villanueva et al. 2007; Weller et al. 2003; Weller et al. 2005a).

Despite these advances, ultrasound contrast remains limited by a relatively low signal to noise ratio that could be improved by optimization of the adhesive properties of the ultrasound contrast agent (Villanueva et al. 2008). Coordinated studies of the affinities of targeting molecules coupled with computational simulation of the stochastic processes involved in intelligently designed microbubbles may permit researchers to quickly examine multiple combinations of ligand densities in the context of the expected physical environment and determine how each contributes to the desired outcome – namely firm adhesion under flow. Currently, there exist stochastic models of dynamic adhesion developed by Hammer and coworkers (Chang et al. 2000a; Hammer et al. 1987; Krasik et al. 2008) to model leukocyte adhesion. These models have also been utilized to optimize nanoparticle drug delivery systems (Haun et al. 2008), and may be suitable for optimization of ultrasound contrast agents. Their utility lies in integration of the stochastic molecular

processes occurring on the nano-scale with geometric and physical interactions that occur on a macro-scale with a particle engaged in fluid flow.

Using models of particle adhesion under flow as a platform, we may identify important parameters which are under our control. For example, microbubble size impacts both the drag forces as well as the number of bonds which are possible to oppose the drag forces. Reducing the microbubble size will decrease the drag forces, but will also allow fewer bonds to form which may not be able to withstand the smaller drag forces. Another important aspect of the optimization process is the choice of the number and type of targeting ligands to employ. While earlier studies on targeted contrast agents have focused on the use of single antibodies, peptide sequences and protein analogues (Bachmann et al. 2006; Ellegala et al. 2003; Kaufmann et al. 2007; Klibanov et al. 1999; Lindner et al. 2001; Rychak et al. 2007; Rychak et al. 2006b; Takalkar et al. 2004; Villanueva et al. 1998; Weller et al. 2003), recent work has begun to explore the utility of dual targeting agents to improve binding specificity and stability (Ferrante et al. 2009; Klibanov et al. 2006; Weller et al. 2005a).

In addition to the use of antibodies and peptide-based targeting molecules, another class of molecules, oligonucleotides, may provide an attractive alternative to address the immunogenicity and moderate to poor affinity of previous molecular targeting agents. These oligonucleotides, also referred to as aptamers, are developed through directed evolution of random nucleotide sequences selected for their overall affinity to a particular protein target (Eulberg et al. 2005; Keefe et al. 2008; Nimjee et al. 2005). One such aptamer developed against P-selectin has previously been developed with a highly attractive affinity ($K_D=14$ pM) (Jenison et al. 1998).

Based on these complex considerations, we hypothesized that accurate measurement of the individual components of the affinity k_f and k_r , coupled with the computational models, could reveal a narrowed starting point for optimization of targeted ultrasound contrast agents. This work describes the measurement of the kinetics between P-selectin and a P-selectin targeted aptamer (PSA) and the kinetics between E-selectin and sialyl Lewis^X (sLe^X) using surface plasmon resonance technology as well as the results of several computational models utilizing these agents to create a design space for optimizing an ultrasound contrast agent targeted to ECs via inflammatory marker expression.

Methods

Surface plasmon resonance for sLe^X and PSA

Surface plasmon resonance (SPR) was used to obtain the kinetic rates under no physical load for the receptor-ligand pairs in the adhesive dynamics (AD) model (see following section). Recombinant Fc chimeras for human E-selectin and P-selectin were purchased from R&D Systems. Biotinylated sLe^X was purchased from Glycotect for testing the kinetic rates of binding to E-selectin. The P-selectin aptamer (PSA) was synthesized (Dharmacon) according to the sequence published by Jenison et al. (Jenison et al. 1998), but with biotin replacing the fluorophore at the 5' end.

SPR experiments were performed on a Biacore T100 (GE Healthcare) biosensor system with streptavidin (SA) coated chips as the substrate. All SA biosensor surfaces were conditioned with 1 M NaCl 50 mM NaOH to remove incompletely bound streptavidin and to establish a stable baseline for subsequent measurements.

For determination of E-Selectin:sLe^X binding kinetics, the running buffer was 10 mM HEPES, 150 mM NaCl, 1 mM CaCl₂, 0.05% p20 at pH 7.4. 20 nM biotinylated sLe^X in running buffer was captured at a flow rate of 5 μ L/min for ~600 seconds to reach sufficient

capture levels (~100 RU). E-selectin kinetics were determined by a dilution series of 0, 228, 455, 910, and 1820 nM run in single-cycle kinetics mode at a flow rate of 30 $\mu\text{L}/\text{min}$ with a 90 sec contact time and 1200 sec final dissociation time. Due to single-cycle kinetics acquisition, regeneration of the chip surface was not necessary.

For the determination of P-Selectin:PSA kinetics, the running buffer was 20 mM HEPES, 1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM KCl, 120 mM NaCl, 1:25 dilution of RNaseSecure (Ambion), pH adjusted to 7.4, sterile filtered, and any present RNase was deactivated by a 10 min 60°C incubation (per RNaseSecure manufacturer instructions). 20 nM biotinylated PSA was captured for 40 sec at 10 $\mu\text{L}/\text{min}$ flow rate. P-selectin kinetics were determined by a dilution series of 0, 2, 6, 18, and 54 nM run in single-cycle kinetics mode with 60 sec contact times at 30 $\mu\text{L}/\text{min}$ flow rate with a 1200 sec final dissociation time.

All data were doubly-referenced to a control flow cell and buffer blank injection. Due to dimerization of E-selectin (inherent to the use of a recombinant Fc chimera protein), data were fit to a bivalent analyte model including terms for mass transport using Biacore T100 Evaluation Software version 2.0 (GE Healthcare) such that $A + B = AB$ and $AB + B = AB_2$ (Muller et al. 1998). For the P-selectin-PSA kinetics, the tracing was fit to a monovalent analyte model such that $A + B = AB$. DTT present in the RNaseSecure was demonstrated to break the disulfide bridge connecting the monomeric components of P-selectin dimer by non-reducing SDS-PAGE (Data not shown). After determination of the forward kinetic rate (k_f), the Bell assumption for conversion of 3D kinetic rates to 2D kinetic rate was applied (see **Appendix I**) to create the intrinsic forward reaction rate used in the Adhesive Dynamics model (see below).

Adhesive Dynamics model

Adhesive Dynamics (AD) models, developed by Hammer et al., were used to simulate the stochastic and physical processes governing microbubble adhesion (Bhatia et al. 2003; Hammer et al. 1992). The goal for utilizing AD was to determine the optimal properties of a dual-targeted microbubble, and how each design parameter impacts microbubble adhesion behavior.

The AD model (summarized in Chang et al. 1996 and Bhatia et al. 2003) integrates the numerical solutions to Stokes flow of a sphere near a rigid wall (Goldman et al. 1967a; Goldman et al. 1967b) with the Bell model for molecular ligand interactions (Bell 1978). Our detailed methods are presented in the supplementary material. A rigid sphere with a radius, R , and a random distribution of the targeting receptors on its surface was used to approximate the microbubble (see Figure 1). The molecular parameters found in Table 1 were used for the model parameters. At the start of the simulation, the sphere was released into the flow field with the centroid positioned at $1.0032 \cdot R$ (approximately three times the distance of the encounter radius for the receptor-ligand interaction to occur). This allowed the sphere to reach its steady state velocity before the receptor-ligand interactions were applied. The microbubble was modeled for 30 seconds with a time step of 1×10^{-6} sec. The microbubble position and time were exported to MATLAB for calculation of the normalized average velocity, which is given by:

$$V_{\text{norm}} = \frac{\langle V \rangle}{V_H} \quad (1)$$

where $\langle V \rangle$ is the average velocity over the 30 sec simulation time, and V_H is the hydrodynamic velocity. Firm adhesion was defined as V_x/V_H of less than 0.02 (Bhatia et al. 2003).

Size optimization

The radius of the microbubble is controlled by the conditions under which it is synthesized, and therefore provided the most logical first-approach for the use of AD. Size plays an important role in the dynamics of adhesion. Both a single- and dual-targeted microbubbles were modeled at radii ranging from 0.5 μm through 5 μm in increments of 0.5 μm . The purpose of performing both single- and dual-targeted models was to test whether the potentially cooperative effect between the two target receptors would affect the balance of the two opposing properties of size. The endothelium was modeled at the fully inflamed state of 2,700 molecules/ μm^2 for one or both of the targeted ligands. This ligand density was derived from measurements of ICAM-1 expression and the expected surface area of an endothelial cell (Weller et al. 2002). For the single-targeted microbubble, the microbubble receptor was sialyl Lewis X (sLe^x) and the ligand target was E-selectin. For the dual-targeted microbubble, the microbubble receptors were sLe^x and a P-selectin aptamer (PSA) while the ligand targets were E-selectin and P-selectin, respectively. Each simulation was run under the parameters given in Table 1. Four complete, independent sets of simulations were analyzed to determine the variance as a result of the stochastic nature of the simulation.

Sensitivity to on (k^{in}_f) and off (k^{o}_r) rates of targeting receptors

In addition to size of the carrier, the targeting receptor(s) that decorate the surface of the microbubble can also be optimized. To date, there have been several classes of molecules utilized for targeted contrast agents or cell-free models of leukocyte adhesion. These can range from natural receptors to small peptide sequences which confer binding capabilities (Beauharnois et al. 2005; Ellegala et al. 2003; Rychak et al. 2006a; Weller et al. 2005b), to antibodies raised against the targeted ligand (Eniola et al. 2003; Klibanov et al. 2006). Recently, a newer class of molecules suitable for therapeutic applications has been identified. These molecules, known as aptamers, are small oligonucleotide sequences (either RNA or DNA) that have protein binding capabilities and are selected from a large, random library using directed evolution on binding affinity (Keefe et al. 2008; Nimjee et al. 2005; Sun 2000). Given that more and more control over molecular design is not only possible, but becoming increasingly practical, we modeled the independent effects of the unstressed kinetic on and off rates of the receptor ligand interactions as they relate to microbubble binding, as measured by the relative average velocity over the simulation time. The goal here was to determine minimum and/or maximum kinetic rates that would provide selection parameters for targeting receptors of interest in the design process. We utilized the physical parameters for sLe^x outlined in Table 1, and the σ , λ , γ_0 , and D for the abICAM:ICAM-1 pair. The k^{in}_f and k^{o}_r for the abICAM:ICAM-1 pair were independently altered by 8 orders of magnitude around the known kinetic rates of the interaction as reported by Eniola et al. (Eniola et al. 2003). The simulation utilized a microbubble with a radius of 2 μm , a wall shear rate of 500 s^{-1} , and a target ligand density of 2,700 molecules/ μm^2 for both E-selectin and ICAM-1. The normalized average velocity was computed for each simulation.

State diagrams

State diagrams were generated with AD simulations by independently varying the surface concentration of each targeting receptor (using the physical parameters in Table 1) as well as the shear rate. The resulting normalized velocities for each condition were analyzed in MATLAB (Version 7R14, Mathworks) by linearly interpolating the surface density of a targeting receptor between two simulations whose velocities spanned the criteria for an adhesion state. Each pair of targeting receptor densities calculated to achieve the desired adhesion criteria was plotted on a log-log plot to create a state diagram for a given shear rate. Areas to the left of the isoline gave targeting receptor concentrations which were insufficient to achieve the desired adhesion state. Areas to the right of the isoline indicated combinations of targeting receptor densities which would achieve the desired adhesion state.

Similar state diagrams were generated by altering the surface expression of the targeted ligand at a specific shear rate to identify the sensitivity of the microbubble to a given inflammatory state.

Results

Surface plasmon resonance

SPR was used to measure the forward (k_f) and reverse kinetic rates (k_r^o) for the pairs of molecules simulated in the AD model. These values form the basis for the intrinsic reaction rates independent of the forces applied to the bonds and represent k_r^o and k_f^{in} (see Eqns 1 and 5, Appendix I).

The association and dissociation constants, and therefore the overall affinity, for the E-selectin interaction with sLe^x and P-selectin with PSA were determined from single-cycle kinetics experiments (Figure 2). The representative tracings in Figure 2 demonstrate sufficient curvature to determine association and dissociation constants. For the E-selectin-sLe^x kinetics, the bivalent analyte fit resulted in an overall affinity of 2.3 μ M with a χ^2 value of 0.842 RU². For the P-selectin-PSA kinetics, the monovalent analyte fit resulted in a K_D of 59.6 pM with a χ^2 value of 0.5563 RU², somewhat larger than the published value of $\sim$ 10 pM (Jenison et al. 1998), but consistent with the known parameters of this experiment. The fast association constant is evident in the large slope during association (550 – 580 sec), and the very slow dissociation rate is evident in the terminal dissociation (580 sec to end). There are some differences between the fit line and the experimental data in the intermediate dissociation steps, but these are most likely due to the curve-fitting algorithm having difficulty determining a proper fit for bulk refractive index changes due to differences between sample and buffer. Furthermore, since these intermediate dissociation steps are short the fit is naturally weighted to the terminal dissociation step.

Size optimization

The normalized velocities for AD simulations examining the effects of single and multi-targeting and microbubble size on adhesiveness are depicted in Figure 3. Although the magnitudes of the normalized average velocity for the two separate systems are quite different, we found common minima that occurred between radii of 0.5 -1.0 μ m that was shear rate independent. The minima extended to 3–5 μ m for lower shear (<500 s⁻¹) depending on the targeting receptors. However, what was striking was that the radius of the microbubble below 0.5 μ m was affected almost equivalently by shear rates as low as 100 s⁻¹ and as high as 1300 s⁻¹. Clearly, there is a threshold whereby the reduction in size (to reduce drag force) was overcome by the relatively few number of bonds that could form in the smaller binding area. It is also important to note the substantial reduction in relative velocity when adding a second targeting receptor (PSA) to the microbubble. With the dual targeted microbubble, firm adhesion was achieved ($\langle V_x \rangle / V_H < 0.02$) for a specific size range for all shear rates, whereas firm adhesion was only achieved for microbubbles with a radius of >1 μ m for 100 s⁻¹ shear rate, and 0.5–1.5 μ m for 500 s⁻¹ shear rate in the microbubble targeted only to sLe^x. The lack of firm adhesion was affected by the receptor density of sLe^x on the microbubble, which for these simulations was set to 100 molecules/ μ m². Later analysis in the form of state diagrams for these microbubbles indicated that sLe^x was required to be in the range of 100 to 1,000 molecules/ μ m² even with just 0.1 molecules/ μ m² of PSA as the second targeting receptor. As a lone targeting receptor, sLe^x would be required to be in excess of 400 molecules/ μ m² even for a shear rate of 100 s⁻¹.

Sensitivity to on and off rates of targeting receptors

The results of simulations which altered the kinetic on and off rates (k_f^{in} and k_r^{o}) for the second receptor for a multi-targeted system for a given microbubble size, targeting receptor concentration, shear rate, and endothelial surface density are shown in Figure 4. There was a threshold forward rate which was required for firm adhesion, irrespective of the reverse rate. There also was a threshold for the reverse rate required for firm adhesion irrespective of the forward rate. These threshold values are approximately $k_f^{\text{in}} > 113 \text{ s}^{-1}$ and $k_r^{\text{o}} < 1 \times 10^{-3} \text{ s}^{-1}$. Furthermore, there was a strong cooperative effect between the forward and reverse rates up to a plateau value for k_r^{o} of approximately $1 \times 10^{-5} \text{ sec}^{-1}$. This cooperative effect was demonstrated by at least a 25-fold decreases in the normalized velocity for a 10-fold change in the reverse rate for a given forward rate. Once a reverse rate of $1 \times 10^{-5} \text{ s}^{-1}$ was reached, no further decreases in normalized velocity were seen. There also appeared to be a plateau region for the forward rate of approximately $11,300 \text{ s}^{-1}$, above which minimal reduction in the normalized velocity was achieved.

State Diagrams

State diagrams (Figure 5 and Figure 6) were generated from the AD simulations by linearly interpolating the surface densities between two simulations to obtain a normalized velocity equal to 0.02, and would thus be considered firmly adhered. This generated an isoline along the surface densities of both targeting receptors on the microbubble surface that would denote the line between firm adhesion and transient or rolling adhesion. The area of the curve to the left of the isoline (shaded in both figures for the 100 s^{-1} shear rate) indicates surface receptor densities that were insufficient to achieve firm adhesion, while the receptor densities in the area to the right of the isoline would be sufficient to achieve firm adhesion. The state diagrams were generated for dual targeted microbubbles coated with either sLe^x and PSA (Figure 5A) or sLe^x and abICAM (Figure 5B) at five shear rates and for microbubble radii of $1 \mu\text{m}$ (dashed lines in Figure 5) or $2 \mu\text{m}$ (solid lines in Figure 5). Both state diagrams depict a system where sufficient densities of either targeting receptor would be capable of inducing a firm adhesion state in the microbubble (horizontal and vertical isolines), and there are cooperative regions where lower concentrations of both targeting receptors would result in firm adhesion (shoulder regions of the isolines). In comparing the state diagrams for the PSA and abICAM microbubbles, it is evident that for almost any shear rate, the abICAM microbubble would require a 10-fold increase in the microbubble receptor density ($\text{molecules}/\mu\text{m}^2$) relative to the PSA microbubble in order to achieve firm adhesion. The abICAM microbubbles also demonstrated more shear rate dependence as evidenced by the separation between the isolines at different shear rates for both microbubble sizes. Consistent with the findings on the effect of microbubble size from Figure 3, smaller microbubbles (dashed lines) required a lower surface concentration of both targeting receptors for wall shear rates greater than 100 s^{-1} . The change in the required surface concentration required to achieve firm adhesion between the two sizes also appeared to be a function of the affinity of the targeting receptor (PSA vs. abICAM). The better the affinity (smaller K_D values), the less of an increase in the concentration of the targeting receptor on the microbubble surface was required for firm adhesion. The size of the cooperative region (shoulder) of the isolines also appeared to be a function of both the size of the microbubble and the affinity of the secondary targeting receptor. The smaller microbubbles in general had a broader shoulder region, and this was accentuated slightly when comparing the abICAM vs. the PSA microbubble.

In addition to state diagrams corresponding to the wall shear rates, state diagrams were created by changing the concentration of the targeted ligand (P-selectin for PSA and ICAM-1 for abICAM) on the simulated EC surface for a wall shear rate of 500 s^{-1} . These state diagrams demonstrate the sensitivity of the microbubbles to the state of inflammation

in the ECs (Figure 6). For these simulations, the concentration of E-selectin (the target of sLe^x) was kept constant at 2,700 molecules/ μm^2 . The goal here was to determine how the change in affinity of the secondary targeting receptor affected the sensitivity of the microbubble to various states of inflammation. From the results of these state diagrams, it was evident that using the lower affinity abICAM would make the microbubble less sensitive to the difference between 2.7 and 27 molecules/ μm^2 as well as the difference between 2,700 and 270 molecules/ μm^2 . The abICAM microbubbles would be only able to distinguish the difference between 27 and 270 molecules/ μm^2 , as indicated by the separation in the isolines along the abICAM axis. Contrasted with this, the higher affinity PSA microbubble appeared to distinguish between 2.7 and 27 molecules/ μm^2 as well as between 270 and 2,700 molecules/ μm^2 , as evidenced by the large gaps between these isolines on the PSA axis.

Discussion

This study was undertaken to use *in silico* analysis to optimize the design of adhesive contrast agents for ultrasound imaging. We explored those factors of targeted microbubble design – bubble radius, number and type of targeting receptors, and kinetic properties of the targeting receptors – that are under the direct (or nearly direct) control of the designer, and have identified several key characteristics which could aid in focused development for this promising technology. Depending upon the desired targeting location, the size of the microbubble may be optimally controlled. Since drag force and binding potential from increased receptor numbers work in an opposing manner as microbubble size increases, there should be an optimum point, for a given shear rate, where these two factors balance and create the ideal conditions for binding. For example, microbubbles designed to adhere to a post-capillary venule (lower shear rate) might be designed to be larger than those which would adhere to endothelial cells in the arterial circulation (higher shear rate) to prevent unwanted binding to these areas. This conclusion was previously suggested by Tees et al. (Tees et al. 2002), who examined the effects of microparticle size and applied shear rate on particle targeting to different targeting receptors (selectin-like, antibody-like, or streptavidin-like). Their results demonstrated that the types of adhesion that can be expected (rolling, transient, and firm) are a function of the type of targeting receptor (each of which has specific kinetic rate, bond compliance, and bond stiffness), particle radius, and shear rate. However, their results stopped short of identifying optimal sizes and conditions for utilizing a particular microparticle, which we have addressed more explicitly here. Our results have demonstrated that there may in fact be a common minima that can be found for a single targeted system which utilized a selectin-like targeting receptor (sLe^x) and a dual targeted system utilizing both a selectin-like and an aptamer bond type (PSA). While the ultimate magnitude of the normalized velocity appears to be affected by the use of a single versus dual targeted system, both have similar characteristic shapes and may point to an underlying size that is optimal for achieving the best possible binding conditions regardless of the targeting receptors. There are subtle differences in the slopes of these curves as they approach and exit their nadir, but the general result is the same when placed in the context of achieving firm adhesion (i.e., $\langle V_x \rangle / V_H = 0.02$). Currently used gaseous microbubbles typically have radii of 0.5–1.5 μm (Chadderdon et al.), which spans the shear-independent range of 0.5–1 μm predicted by the models, and may indicate that a subset of the injected microbubbles will arrest in a particular vascular target area depending on the applied shear rate.

In addition to optimization of microbubble size, our simulations demonstrate that there may be important transition points within the kinetics rates of the targeting receptors to their ligands when taken in context of an ultimate goal of achieving firm adhesion. Chang et al. (Chang et al. 2000b) in the context of leukocyte adhesion, previously developed state

diagrams for the kinetic rates of bond breakage (k_r^0) and reactive compliance (γ) to explore the effects of the differing values reported in the literature for different bond types with respect to model validation. Their findings that γ and k_r^0 should fall within a narrow range of values in order to achieve the transient adhesion observed by researchers utilizing leukocyte-based and cell-free particle systems (Alon et al. 1997; Chen et al. 1997; Smith et al. 1999) provided evidence that their simulations could capture the characteristic behaviors of similar systems.

Advances in molecular engineering and the advent of applications that utilize leukocyte binding principles have prompted us to ask the question: “what are our design limits?” Technologies to derive peptides or oligonucleotides that are capable of binding specific targets can be utilized to screen candidate receptors that have specific kinetic properties for their intended use. We chose our design space to independently sample the kinetic rates based on the assumption that these types of molecules will likely have a narrow range of bond stiffness (σ) and compliance (γ). This assumption is not outside of convention as reported values for γ and σ have been within an order of magnitude independent of the bond type (Chang et al. 2000b; Smith et al. 1999; Tees et al. 2002). To further validate this premise, we have performed a series of simulations where these parameters were varied independently of each other as well as the targeting receptor density on the surface of the microbubble (see Supplemental Data) and found that their effects are below the firm adhesion threshold for the receptor densities and ligand densities relevant to their clinical application.

In addition to the exploration of the design space around particle size and the receptor kinetic rates, we explored the potential advantage that improved kinetic rates for one receptor would have in a multi-targeting system. Previous work using AD with multi-targeting was performed by Bhatia et al. (Bhatia et al. 2003) and laid the foundations for this work by demonstrating the importance of the kinetic and shear rates on the cooperation between the two targeting receptors. A key difference was the use here of existing targeting receptors and physiologically relevant ligand densities to generate a firmly adherent microbubble, while Bhatia et al. focused on the interaction between the kinetic association rate and shear rate for the integrin-ICAM interaction of a leukocyte. Both results confirm there should exist a cooperative effect between the two targeting receptors and that this effect is dependent on both the availability and kinetic properties of the receptor. For example, in order to achieve firm adhesion with sLe^x as the primary targeting receptor in the model system explored in this paper, a secondary receptor should have an intrinsic on-rate of at least $1,130 \text{ s}^{-1}$ and a reverse rate less than $1 \times 10^{-5} \text{ sec}^{-1}$. These requirements are ultimately a function of the physical system (e.g. shear rate, target ligand densities, and targeting receptor kinetics), which plays a complex roll in the stochastic processes of molecular binding, and should be modeled to identify a specific recipe for optimized adhesion. Evidence for cooperation in adhesion has been demonstrated *in vitro* through the use of cell-free multi-targeting systems (Eniola et al. 2003; Weller et al. 2005a) and provides evidence that the model predictions are reasonable.

Currently, targeted microbubbles utilize antibodies and small peptides, for which some kinetic information is known. The models used here employed these same agents and their measured kinetic rates (abICAM and sLe^x). Current experimental practice is to saturate the surface of the bubbles, with the targeting receptor being present at several thousand molecules per square micron (Chadderdon et al.). The models employed here predict that firm adhesion should result from 1,000 molecules/ μm^2 in a shear-independent fashion for radii of 1 μm and a full expression level for the targeted ligand. This fits with the existing data for aggressively inflamed tissues or tumor growth (Bachmann et al. 2006; Ferrante et al. 2009; Rychak et al. 2007; Weller et al. 2005a; Weller et al. 2005b). However,

optimization of many aspects of the ultrasound contrast agent (i.e., kinetics, size, distribution) may be necessary for early stage detection, or for specific ligands that may not be expressed at the densities modeled here.

Another interesting result from the simulations was the predicted 10-fold reduction in the concentration of required PSA on the surface of the microbubble relative to the concentration of abICAM. As indicated in Table 1, identical model parameters were used to run the model apart from the forward and reverse kinetic rates. Although the overall affinity of abICAM for its target was almost 20 fold lower than for PSA to its target, the reverse rates were just 1/3 of the PSA reverse rate, and the reverse rate has been suggested to play a larger role in achieving firm adhesion under a given flow condition (Chang et al. 2000b). Therefore, the nonlinear difference in the predicted density to achieve firm adhesion arises from both the nonlinearity in the model parameters and the stochastic nature of the process. A similar finding was reported by Bhatia et al. (Bhatia et al. 2003), who showed 100-fold changes in k_{on}^0 significantly changed the effects of reactive compliance (γ) on the state diagrams for a two-receptor system.

The main limitation of our approach in this process was our inability to quantify several parameters that were included in the model. As a result, we were forced to estimate the spring constant (σ), reactive compliance (γ) and equilibrium bond length (λ). However, previous work has shown that the spring constant plays a smaller role relative to the other constants (Chang et al. 2000b). Therefore, the estimates we have used, which are based on measurements and model fitting experiments for similar molecules, may not adversely affect the interpretation of our results. Similarly, while the reactive compliance has previously been shown to have more of an effect on firm adhesion in previous models (Bhatia et al. 2003), the conditions with which we applied our models appears to limit this effect (see Supplemental Results). Additionally, the models employed were single particle simulations, which may underestimate the binding capabilities when accounting for the effects of a currently bound contrast agent on future binding events by passing contrast agents (King et al. 2001). However, the focus of the optimization scheme here is mainly to narrow the design space and should be verified by experimental validation of the design that may result from the model.

Conclusions

Molecular targeting for contrast ultrasound may hold future clinical significance for the diagnosis and treatment of inflammation related to ischemic reperfusion injury or other markers of cardiovascular disease. Further extensions of these applications could include non-invasive imaging of tumors as well as the targeted delivery of stem cells and pharmaceutical therapy to specific areas of the vasculature (Ellegala et al. 2003; Villanueva et al. 2007; Weller et al. 2005b). The use of computational models of leukocyte adhesion (Hammer et al. 1987; Krasik et al. 2008), can be adapted to aid in the optimization of targeted ultrasound contrast agents. Exploration of a multi-targeted system *in silico* may allow the identification of ideal binding characteristics for the *in vivo* system of interest in a controlled and reproducible manner. Such an approach may permit the definition of appropriate size restrictions and receptor densities for the manufacturing process, and also yield insight into the desired kinetic properties of the targeting receptors. By providing an initial framework for rational design, we can thus attempt to identify and optimize new binding receptors for molecules of interest in a high throughput manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Alon R, Chen S, Puri KD, Finger EB, Springer TA. The kinetics of L-selectin tethers and the mechanics of selectin-mediated rolling. *J Cell Biol.* 1997; 138(5):1169–80. [PubMed: 9281593]
- Bachmann C, Klibanov AL, Olson TS, Sonnenschein JR, Rivera-Nieves J, Cominelli F, Ley KF, Lindner JR, Pizarro TT. Targeting mucosal addressin cellular adhesion molecule (MAdCAM)-1 to noninvasively image experimental Crohn's disease. *Gastroenterology.* 2006; 130(1):8–16. [PubMed: 16401463]
- Beauharnois ME, Lindquist KC, Marathe D, Vanderslice P, Xia J, Matta KL, Neelamegham S. Affinity and kinetics of sialyl Lewis-X and core-2 based oligosaccharides binding to L- and P-selectin. *Biochemistry (Mosc).* 2005; 44(27):9507–19.
- Bell GI. Models for the specific adhesion of cells to cells. *Science.* 1978; 200(4342):618–27. [PubMed: 347575]
- Bhatia SK, King MR, Hammer DA. The state diagram for cell adhesion mediated by two receptors. *Biophys J.* 2003; 84(4):2671–90. [PubMed: 12668476]
- Burns PN. Instrumentation for contrast echocardiography. *Echocardiography.* 2002; 19(3):241–58. [PubMed: 12022934]
- Chadderdon SM, Kaul S. Molecular imaging with contrast enhanced ultrasound. *J Nucl Cardiol.*
- Chang KC, Hammer DA. Adhesive dynamics simulations of sialyl-Lewis(x)/E-selectin-mediated rolling in a cell-free system. *Biophys J.* 2000a; 79(4):1891–902. [PubMed: 11023895]
- Chang KC, Tees DF, Hammer DA. The state diagram for cell adhesion under flow: leukocyte rolling and firm adhesion. *Proc Natl Acad Sci U S A.* 2000b; 97(21):11262–7. [PubMed: 11005837]
- Chen S, Alon R, Fuhlbrigge RC, Springer TA. Rolling and transient tethering of leukocytes on antibodies reveal specializations of selectins. *Proc Natl Acad Sci U S A.* 1997; 94(7):3172–7. [PubMed: 9096365]
- Ellegala DB, Leong-Poi H, Carpenter JE, Klibanov AL, Kaul S, Shaffrey ME, Sklenar J, Lindner JR. Imaging tumor angiogenesis with contrast ultrasound and microbubbles targeted to alpha(v)beta3. *Circulation.* 2003; 108(3):336–41. [PubMed: 12835208]
- Eniola AO, Willcox PJ, Hammer DA. Interplay between rolling and firm adhesion elucidated with a cell-free system engineered with two distinct receptor-ligand pairs. *Biophys J.* 2003; 85(4):2720–31. [PubMed: 14507735]
- Eulberg D, Buchner K, Maasch C, Klussmann S. Development of an automated in vitro selection protocol to obtain RNA-based aptamers: identification of a biostable substance P antagonist. *Nucl Acids Res.* 2005; 33(4):e45. [PubMed: 15745995]
- Ferrante EA, Pickard JE, Rychak J, Klibanov A, Ley K. Dual targeting improves microbubble contrast agent adhesion to VCAM-1 and P-selectin under flow. *J Controlled Release.* 2009 In Press, Uncorrected Proof.
- Goldman AJ, Cox RG, Brenner H. Slow viscous motion of a sphere parallel to a plane wall--I Motion through a quiescent fluid. *Chem Eng Sci.* 1967a; 22(4):637–651.
- Goldman AJ, Cox RG, Brenner H. Slow viscous motion of a sphere parallel to a plane wall--II Couette flow. *Chem Eng Sci.* 1967b; 22(4):653–660.
- Hammer DA, Apte SM. Simulation of cell rolling and adhesion on surfaces in shear flow: general results and analysis of selectin-mediated neutrophil adhesion. *Biophys J.* 1992; 63(1):35–57. [PubMed: 1384734]
- Hammer DA, Lauffenburger DA. A dynamical model for receptor-mediated cell adhesion to surfaces. *Biophys J.* 1987; 52(3):475–87. [PubMed: 2820521]
- Hansson GK. Inflammatory mechanisms in atherosclerosis. *J Thromb Haemost.* 2009; 7(Suppl 1):328–31. [PubMed: 19630827]

- Hassoun PM, Mouthon L, Barbera JA, Eddahibi S, Flores SC, Grimminger F, Jones PL, Maitland ML, Michelakis ED, Morrell NW, et al. Inflammation, growth factors, and pulmonary vascular remodeling. *J Am Coll Cardiol*. 2009; 54(1 Suppl):S10–9. [PubMed: 19555853]
- Haun JB, Hammer DA. Quantifying Nanoparticle Adhesion Mediated by Specific Molecular Interactions. *Langmuir*. 2008
- Hundley WG, Kizilbash AM, Afridi I, Franco F, Peshock RM, Grayburn PA. Administration of an intravenous perfluorocarbon contrast agent improves echocardiographic determination of left ventricular volumes and ejection fraction: comparison with cine magnetic resonance imaging. *J Am Coll Cardiol*. 1998; 32(5):1426–32. [PubMed: 9809958]
- Jenison RD, Jennings SD, Walker DW, Bargatze RF, Parma D. Oligonucleotide inhibitors of P-selectin-dependent neutrophil-platelet adhesion. *Antisense Nucleic Acid Drug Dev*. 1998; 8(4):265–79. [PubMed: 9743465]
- Kaufmann BA, Sanders JM, Davis C, Xie A, Aldred P, Sarembock IJ, Lindner JR. Molecular imaging of inflammation in atherosclerosis with targeted ultrasound detection of vascular cell adhesion molecule-1. *Circulation*. 2007; 116(3):276–84. [PubMed: 17592078]
- Keefe AD, Schaub RG. Aptamers as candidate therapeutics for cardiovascular indications. *Curr Opin Pharmacol*. 2008; 8(2):147–152. [PubMed: 18222730]
- King MR, Hammer DA. Multiparticle adhesive dynamics: hydrodynamic recruitment of rolling leukocytes. *Proc Natl Acad Sci U S A*. 2001; 98(26):14919–24. [PubMed: 11752440]
- Klibanov AL, Hughes MS, Villanueva FS, Jankowski RJ, Wagner WR, Wojdyla JK, Wible JH, Brandenburger GH. Targeting and ultrasound imaging of microbubble-based contrast agents. *MAGMA*. 1999; 8(3):177–84. [PubMed: 10504045]
- Klibanov AL, Rychak JJ, Yang WC, Alikhani S, Li B, Acton S, Lindner JR, Ley K, Kaul S. Targeted ultrasound contrast agent for molecular imaging of inflammation in high-shear flow. *Contrast Media Mol Imaging*. 2006; 1(6):259–66. [PubMed: 17191766]
- Krasik EF, Caputo KE, Hammer DA. Adhesive Dynamics Simulation of Neutrophil Arrest with Stochastic Activation. *Biophys J*. 2008; 95(4):1716–1728. [PubMed: 18487296]
- 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(17):2107–12. [PubMed: 11673354]
- Ma A, Qi S, Chen H. Antioxidant therapy for prevention of inflammation, ischemic reperfusion injuries and allograft rejection. *Cardiovasc Hematol Agents Med Chem*. 2008; 6(1):20–43. [PubMed: 18220719]
- Miller JC, Thrall JH. Clinical molecular imaging. *J Am Coll Radiol*. 2004; 1(1 Suppl):4–23. [PubMed: 17411749]
- Muller KM, Arndt KM, Pluckthun A. Model and simulation of multivalent binding to fixed ligands. *Anal Biochem*. 1998; 261(2):149–58. [PubMed: 9716417]
- Nimjee SM, Rusconi CP, Sullenger BA. Aptamers: an emerging class of therapeutics. *Annu Rev Med*. 2005; 56:555–83. [PubMed: 15660527]
- Rychak JJ, Graba J, Cheung AM, Mystry BS, Lindner JR, Kerbel RS, Foster FS. Microultrasound molecular imaging of vascular endothelial growth factor receptor 2 in a mouse model of tumor angiogenesis. *Mol Imaging*. 2007; 6(5):289–96. [PubMed: 18092513]
- Rychak JJ, Li B, Acton ST, Leppanen A, Cummings RD, Ley K, Klibanov AL. Selectin ligands promote ultrasound contrast agent adhesion under shear flow. *Mol Pharm*. 2006a; 3(5):516–24. [PubMed: 17009850]
- Rychak JJ, Lindner JR, Ley K, Klibanov AL. Deformable gas-filled microbubbles targeted to P-selectin. *J Control Release*. 2006b; 114(3):288–99. [PubMed: 16887229]
- Smith MJ, Berg EL, Lawrence MB. A direct comparison of selectin-mediated transient, adhesive events using high temporal resolution. *Biophys J*. 1999; 77(6):3371–83. [PubMed: 10585960]
- Sun S. Technology evaluation: SELEX, Gilead Sciences Inc. *Curr Opin Mol Ther*. 2000; 2(1):100–5. [PubMed: 11249646]
- 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(3):473–82. [PubMed: 15120903]

- Tees DFJ, Chang KC, Rodgers SD, Hammer DA. Simulation of cell adhesion to bioreactive surfaces in shear: The effect of cell size. *Ind Eng Chem Res.* 2002; 41(3):486–493.
- Villanueva FS, Jankowski RJ, Klibanov S, Pina ML, Alber SM, Watkins SC, Brandenburger GH, Wagner WR. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells. *Circulation.* 1998; 98(1):1–5. [PubMed: 9665051]
- Villanueva FS, Lu E, Bowry S, Kilic S, Tom E, Wang J, Gretton J, Pacella JJ, Wagner WR. Myocardial ischemic memory imaging with molecular echocardiography. *Circulation.* 2007; 115(3):345–52. [PubMed: 17210843]
- Villanueva FS, Wagner WR. Ultrasound molecular imaging of cardiovascular disease. *Nat Clin Pract Cardiovasc Med.* 2008; 5(Suppl 2):S26–32. [PubMed: 18641604]
- Weller GE, Lu E, Csikari MM, Klibanov AL, Fischer D, Wagner WR, Villanueva FS. Ultrasound imaging of acute cardiac transplant rejection with microbubbles targeted to intercellular adhesion molecule-1. *Circulation.* 2003; 108(2):218–24. [PubMed: 12835214]
- Weller GE, Villanueva FS, Klibanov AL, Wagner WR. Modulating targeted adhesion of an ultrasound contrast agent to dysfunctional endothelium. *Ann Biomed Eng.* 2002; 30(8):1012–9. [PubMed: 12449762]
- Weller GE, Villanueva FS, Tom EM, Wagner WR. Targeted ultrasound contrast agents: in vitro assessment of endothelial dysfunction and multi-targeting to ICAM-1 and sialyl Lewisx. *Biotechnol Bioeng.* 2005a; 92(6):780–8. [PubMed: 16121392]
- Weller GE, Wong MK, Modzelewski RA, Lu E, Klibanov AL, Wagner WR, Villanueva FS. Ultrasonic imaging of tumor angiogenesis using contrast microbubbles targeted via the tumor-binding peptide arginine-arginine-leucine. *Cancer Res.* 2005b; 65(2):533–9. [PubMed: 15695396]

Figure 1.

Model representing a dual targeted microbubble of radius, R , in a constant shear field with receptors distributed evenly on the surface. Receptor-ligand interactions were modeled according to the Bell model (Bell 1978). The microbubble had rotational (Ω) and translational (u) velocity components and moved at a distance, h , from the wall, which had a uniform distribution of the target ligands. Adapted from Bhatia et al. (Bhatia et al. 2003).

Figure 2.

Representative plots of the response unit (RU) versus time for association and dissociation of A) sLe^x and E-selectin and B) PSA and P-selectin. The kinetic rates were calculated from single cycle runs.

Figure 3.

Effects of microbubble size on adhesion A) Dual targeted microbubble coated with 100 molecules/ μm^2 sLe^x and PSA; B) Single targeted microbubble coated with 100 molecules/ μm^2 sLe^x. Error bars represent the standard deviation of independent simulations.

Figure 4.

The simulated reverse rates (k_r^0) are plotted on the x-axis, and the normalized velocity ($\langle V_x \rangle / V_H$) is plotted in log-scale in the y-axis. The individual lines represent the simulated forward rates (k_f^0). A dashed line represents the firm adhesion cutoff criteria of $\langle V_x \rangle / V_H < 0.02$.

Figure 5.

State diagrams for 1 μm radius (dashed lines) and 2 μm radius (solid lines) microbubbles coated with (A) sLe^x and PSA or (B) sLe^x and abICAM. The isolines corresponding to the indicated shear rates mark the boundary between firm adhesion (to the right of the line) and transient/rolling adhesion (left of the line).

Figure 6.

State diagrams to determine the sensitivity of (A) sLe^x and PSA microbubbles or (B) sLe^x and abICAM microbubbles to the surface concentration of the P-selectin or ICAM-1 on the simulated EC surface.

Table 1

Physical parameters used in AD models.

	Ligand Pair		
	sLe ^x :E-sel	PSA:P-sel	abICAM:ICAM-1
K _D (M) (affinity)	2.3×10 ⁻⁶ [†]	59.6×10 ⁻¹² [†]	1×10 ⁻⁹ (Eniola et al. 2003)
k _f ^o (sec ⁻¹) (intrinsic forward rate)	2.4 x10 ⁴ [†]	2.33×10 ⁵ [†]	1.15×10 ⁵ (Eniola et al. 2003)
k _r ^o (sec ⁻¹) (intrinsic reverse rate)	0.06 [†]	3.4×10 ⁻⁵ [†]	1.13×10 ⁻⁴ (Eniola et al. 2003)
σ (dynes/cm) (spring constant)	10 (Caputo et al. 2007)	50 (Chang et al. 2000a)	
λ (nm) (equilibrium bond length)	29 (Caputo et al. 2007)	50 (Chang et al. 2000a)	
γ _o (Å) (reactive compliance)	0.39 (Caputo et al. 2007)	0.21 (Chang et al. 2000a)	
D (μm ² /s) (diffusivity)	1×10 ⁻⁶ (Caputo et al. 2007)	1×10 ⁻³ (Chang et al. 2000a)	
k _B (μg*μm ² /sec ²) (Boltzmann's constant)	1.38×10 ⁻²		
T (K) (Temperature)	310		
Selectin/ICAM-1 density (mol/μm ²)	3.5 – 2,700 (Weller et al. 2002)		
SR (sec ⁻¹) (Shear rate)	100 – 1700		
R (μM) (Microbubble radius)	0.25 – 4		
Δt (sec) (Time step)	1×10 ⁻⁶		

[†] denotes experimental findings of this study.