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Circulating IgSF Proteins Inhibit Adhesion of Antibody Targeted Microspheres to Endothelial Inflammatory Ligands

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Abstract Proposed methods for detecting circulatory system disease include targeting ultrasound contrast agents to inflammatory markers on vascular endothelial cells. For antibody-based therapies, soluble forms of the targeted adhesion proteins of the immunoglobulin superfamily (IgSF) reduce adhesion yet were left unaccounted in prior reports. Microspheres labeled simply with a maximum level of antibodies can reduce the diagnostic sensitivity by adhering to proteins expressed normally at a low level, while sparsely coated particles may be rendered ineffective by circulating soluble forms of the targeted proteins. A new microdevice technique is applied to simultaneously measure the adhesion profile to a series of IgSF-protein-coated surfaces. In this investigation, we quantify the in vitro binding characteristics of 5-μm microspheres to oriented intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) protein-coated surfaces in the presence of human serum at physiological concentrations. Defined regions of a slide were coated with recombinant chimeric Fc-human ICAM-1 and VCAM-1 in variable ratios but constant total concentration. Monoclonal human anti-ICAM-1 or anti-VCAM-1 antibodies in competition with non-binding mouse anti-rabbit antibodies coat the microsphere surface at a constant surface density with variable yet controlled surface activities. Using multiple slide surface IgSF protein and microsphere antibody concentrations, an adhesion profile was developed for the microspheres with and without IgSF proteins from human serum, which demonstrated that exposure to serum reduced microsphere binding, on average, more than 50% compared to the no-serum condition.. The serum effects were limited to antibodies on the microsphere, since binding inhibition was reversed after rinsing serum from the system and fresh antibody-coated microspheres were introduced. This analysis quantifies the binding effects of soluble IgSF proteins from human serum on antibody-based targeted ultrasound detection and drug delivery methods.

Keywords Immunoglobulin superfamily · IgSF · Human serum · Contrast agents · ICAM-1 · VCAM-1 · Adhesion · Microparticle

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Introduction

The molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), belong to the immunoglobulin superfamily (IgSF) of proteins for cell–cell adhesion, which are upregulated during an inflammatory response and precursor signals to vascular disease including atherosclerosis [1]. The IgSF chemical structure, expression profiles in vivo, and basis in pathology are well described in several comprehensive reviews [2]. Non-invasive detection of inflamed vasculature is possible using molecular imaging techniques, where contrast agents bind specifically to upregulated cell surface markers [3–6]. Furthermore, if the adherent targeted agent is quantified, it may be possible to measure the severity of inflammation in the circulatory system and plan appropriate courses of therapy [7]. While investigators have sought to qualitatively measure the binding of targeted agents in vivo, quantitative analysis of inflammation is needed in a controlled setting to maximize the diagnostic potential, which in vitro experiments provide [8]. Studies have evaluated the adhesion characteristics of solid microspheres [9] as well as air-filled particles as contrast agents [10–13]. Ultrasound contrast microspheres have diameters in the 3- to 5-μm range, which are designed to pass through capillary beds. In vitro testing of microsphere adhesion has been used as a tool to investigate antibody–ligand interaction to the endothelial cell surface [3]. Surprisingly, to our knowledge, no quantitative studies have sought to describe the effects of soluble adhesion IgSF proteins contained in human serum on the adhesion profile of these constructs in an in vivo or in vitro system. Prior studies have focused on the most commonly upregulated ligands, such as ICAM-1, VCAM-1, P-Selectin, E-Selectin, and the chemokine cascades that occur during endothelial cell inflammation [14–18]. The human adhesion molecules, ICAM-1 and VCAM-1, which are expressed at low density on normal endothelial cells, are upregulated with cytokines and therefore have provided a good model of inflammation in other investigations with cell-based or in vivo systems. Several factors such as ligand-binding affinity, surface geometries, and competing soluble proteins may modify the binding profile of antibody-coated microspheres to a ligand-coated surface. Some groups have characterized ligand–antibody pairings [19–21], while others have quantified the in vivo surface concentrations of these proteins, or analyzed the binding kinetics under varying conditions of shear stress [22, 23]. These investigations have relevance when applied to detection or therapy for arterial disease or blockage [3].

The effects of the soluble IgSF adhesion molecules, sICAM-1 and sVCAM-1 [24], present in human serum on adhesion were studied here using a parallel plate flow cell in a controlled experimental setting not available easily in vivo. Of the in vitro studies of microsphere adhesion to these ligands, none have measured microsphere adhesion in the presence of human serum. We present the static binding characteristics of microspheres to VCAM-1- and ICAM-1-functionalized surfaces and an analysis of differential impact on *microsphere adhesion profiles* in the presence of soluble forms of these same IgSF proteins contained in human serum.

Methods and Materials

Attachment of Chimeric Target Proteins to a Polystyrene Slide

A target protein coating of recombinant human ICAM-1 and human VCAM-1 were applied to the surface of a 1″×3″ polystyrene cell culture slide (Nalge Nunc International,

Naperville, IL). A silicone isolation gasket (Sigma-Aldrich) was used to functionalize four separate 8.5-mm circular regions on single microscope slides. The press-on silicone gaskets were scrubbed with 0.25% FL-70 detergent, rinsed well with DI water then ethanol, and dried with compressed nitrogen prior to use. After affixing the four-well pattern gasket to the center of the slide, a permanent marker was used to define the boundaries on the backside of the slide (Fig. 1). To prevent leakage between wells, binder clips were used to sandwich the gasket between the slide and a slide drilled with 8.5-mm access holes. Each well was incubated with 10 μg/mL of staphylococcal protein A (SpA; Pierce Biotechnology, Rockford, IL) in phosphate-buffered saline (PBS) for 1 h at room temperature then rinsed with PBS. The well-characterized SpA, which has five binding domains for the Fc region of IgG antibodies [25], was used to orient the recombinant chimeric human-Fc proteins upward [13] to improve binding with the microspheres, as shown in Fig. 2. The wells were blocked with 2% bovine serum albumin (BSA) for 1 h to reduce non-specific binding interactions. Recombinant human ICAM-1 Fc chimera (720-IC-200) and VCAM-1 Fc chimera (862-VC-100; R&D systems, Minneapolis, MN) were applied to four locations on the slide at concentrations ranging from 0% to 100%. These Fc-end-labeled proteins are bound to the SpA adsorbed to the polystyrene slide. Based on enzyme-linked immunosorbent assay (ELISA) results [13], the total protein concentration in the well was maintained at a surface saturating excess of 5 ug/ml while changing the intended ligand target through a ratio of ICAM-1 and VCAM-1 concentrations. For example, to measure the affect of VCAM-1 concentration on binding, ICAM-1 is used as a non-target diluent, which is expected to maintain a constant total protein surface density due to their similar chemical structure [2]. Protein dilutions were made in a separate 200-uL polypropylene microtubes in PBS with 0.2% BSA prior to addition to the wells on the slide, which then underwent overnight incubation at 4 °C. After rinsing with PBS plus 0.2% BSA, the slide was incubated for 1 h with rat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) to block unoccupied SpA sites. This anti-rabbit antibody is specifically affinity-purified to minimize reactivity to mouse and human proteins. After final rinse of PBS and 0.2% BSA, the binder clips were removed along with the gasket. The slide was stored in PBS+0.2% BSA at room temperature until starting the adhesion testing with the microspheres.

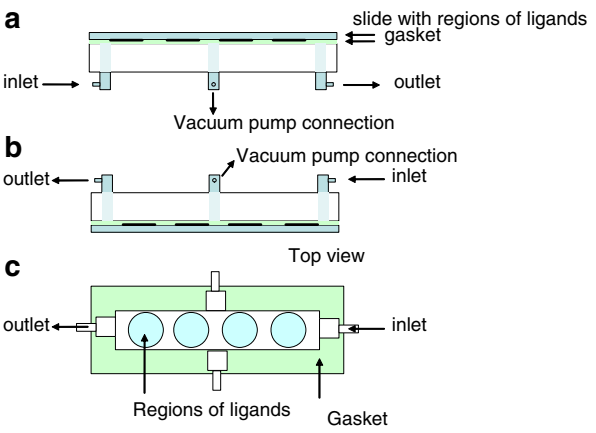

Fig. 1 Diagram showing the inversion steps as part of the adhesion and imaging process. First, the microparticles flow in via syringe pump with the active surface on top so as to avoid adhesion during inflow. Then, the microdevice is inverted, and static binding takes place. Finally, the device is re-inverted, and the remaining unbound microparticles flow out via syringe pump

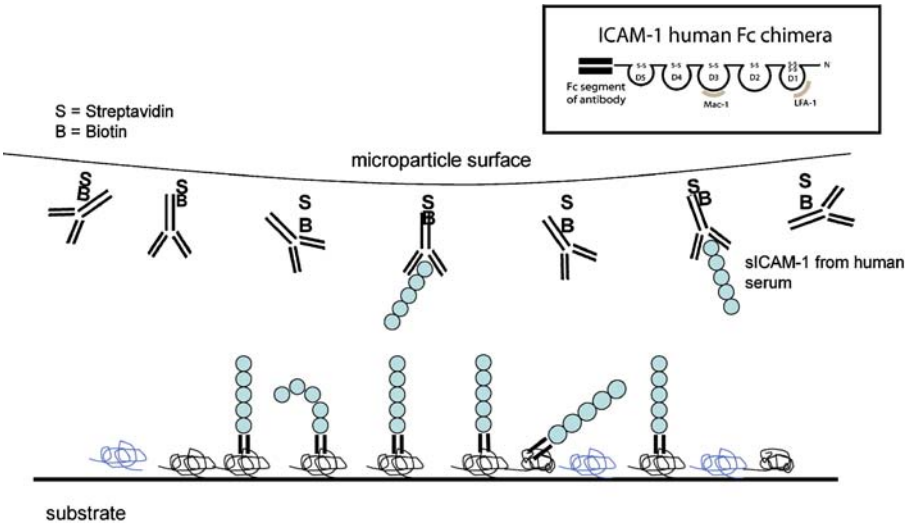

Fig. 2 Diagram of adhesion between microsphere and surface proteins in the presence of soluble IgSF proteins derived from human serum. Polystyrene slides exhibited a consistently lower background microsphere binding compared to glass. Normal adhesion would not have these proteins adhered to the microparticle surface antibodies. For a more detailed diagram of ICAM-1 Chimera, see figure inset

Attachment of Antibodies to Microspheres

Streptavidin-functionalized poly(methyl methacrylate) microspheres (Bang's Laboratories, Fishers, IN, lot 6648, 5.48 μm OD, 94 μm^2 with a binding capacity of 0.06 μg biotin-fluorescein isothiocyanate/mg microspheres) were coated with biotinylated antibodies. The particles were prepared by centrifuging at 4,000×*g* for 5 min, removing the supernatant, and rinsing with PBS and 0.2% BSA three times. Biotinylated mouse anti-human ICAM-1 domain 1 IgG_1 (clone BBIG), biotinylated mouse anti-human VCAM-1 IgG_1 antibodies (Ancell, Bayport, MN), and biotinylated mouse anti-rabbit IgG_1 (Jackson ImmunoResearch Laboratories) were affinity-purified and isotype-matched by the supplier to minimize cross-reactivity. These antibodies in combination with the chimeric proteins used were previously shown to have a high specificity only for their intended target proteins [8]. The total antibody coating concentration (μL biotin-Ab/mg microspheres) was held constant, while the ratio of the active monoclonal antibody (MAb), ICAM-1, or VCAM-1 to the diluting antibody (non-binding mouse IgG) was proportionally adjusted to change the microsphere functionality. Antibodies were premixed at 2.5× molar excess of the manufacturer's reported binding capacity then incubated with the microspheres while shaking overnight at 4 °C, rinsed and stored at 4 °C until needed. Particles were functionalized with VCAM-1 from 3% to 100% using mouse anti-rabbit PAb to compete for microsphere binding sites. Microsphere blanks, coated only with mouse anti-rabbit PAb, were functionalized at 15× molar excess.

Separately, chimeric ICAM-1 and VCAM-1 (1 mg/mL in PBS) were covalently tagged in an environment of 5× molar excess NHS-fluorescein (Biotium, Hayward, CA) in dimethylsulfoxide and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce Biochemical) in PBS for 1 h at 4 °C. Unbound fluorescein was removed via cartridge dialysis using a 3,000-kDa MWCO membrane (Pierce) in PBS overnight at 4 °C. The functionalized surface density of antibodies on the microsphere were quantified by flow cytometry (BD FACSscan) and calibrated fluorescent standards (Quantum Simply

Cellular anti-mouse IgG, Bang's Labs, Fishers, IN) with 'QuickCal' software. Calibration beads, coated with anti-mouse IgG, were exposed to 2× molar excess of mouse anti-human ICAM-1 or VCAM-1 antibodies for 1 h while shaking at room temperature. These beads were rinsed as described previously. The fluoresceinated chimeric human-Fc proteins were then mixed with the beads, the calibrated standards, and our constructs for 30 min according to the manufacturer's directions and quantified via florescence-activated cell sorting (FACS) to quantify the functional binding sites.

Human Serum Characterization

Human serum, (Seracare Life Sciences, Massachusetts; lot number 050-02-006) certified free of HIV, HBV, and HCV was aliquoted to 2 mL and stored at −80 °C until needed. Handling was performed according to Brown University approved protocols. Aliquots were thawed at 37 °C, centrifuged at 10,000×*g* for 10 min and filtered to 0.45 μm with a low-protein binding syringe filter to remove remaining particulates. The sICAM-1 content was assayed kindly by the General Clinical Research Center (GCRC) at Case Western Reserve using the R&D Systems ELISA method (cat. No. BBE1B). The sVCAM-1 concentration was assayed using the flow cytometer-based R&D systems Fluorokine MAP assay for sVCAM-1 (LAD809) according to the manufacturer's instructions. While designed for multiplexed analytes using special cytometers, this kit was used exclusively for sVCAM-1 on a BD FACScan in red wavelengths for phycoerythyrin (PE). Samples were run in triplicate and quantified using the supplied calibration standards.

Microsphere Adhesion Test Procedure

All buffers were warmed to room temperature (25 °C) prior to starting to prevent degassing while inside the test apparatus. The chimeric- protein-coated slide was removed from the buffer and placed on a 256-μm-thick gasket, which seals the slide to the flow cell (Glycotech) when vacuum is applied. Air was flushed from the flow cell and connecting tubes with degassed buffer. A 0.1% suspension of antibody functionalized microspheres were created by dilution of the master particle stock 10:1 with either PBS+0.2% BSA or filtered human serum. The serum was diluted with PBS in a ratio slightly greater than 1:1 (55% to 45%). The microspheres are loaded into a 1-mL syringe, which is purged of all air before connecting to a leur lock input port. While the cell is inverted, the microspheres are injected through a 500-μm ID tube into the cell via syringe pump into the system at a constant 0.5 ml/min. Following injection, the flow is stopped, and the outflow tube is capped. The flow cell is then inverted for 10 min, which allows the microspheres to settle fully onto the ICAM/VCAM-coated slide surface. The slide was viewed through a Nikon TE2000-U inverted microscope using a Nikon L Plan SLWD 20×/0.35NA microscope objective then digitally photographed using a Micropublisher 5.0 camera (QImaging, Surrey, BC, Canada). Non-protein-coated slide regions, which serve as control areas between the four differentially coated protein regions, are photographed as well to measure non-specific binding. The first set of data is collected over a 10-min interval. The flow cell is re-inverted. The syringe pump is re-engaged using PBS+0.2% BSA for 10 min in order to clear out the unbound microspheres at a slow flow rate of 0.5 ml/min, which produces a very limited shear stress of 0.2 dynes/cm^2. This shear rate is at the extreme low end of shear forces experienced within the circulatory system, which ranges from 0 to 100 dynes/cm^2 [26]. After the allotted 10 min, the flow cell is then digitally photographed again in all ligand and control regions. The microspheres adhering to the slide in the "before" and

"after" images (see Fig. 3) are counted using ImagePro Plus version 5.0 (Media Cybernetics, Bethesda, MD). The percentage of bound microspheres is computed for each of the four ligand-coated slide locations. This inflow, inversion, and imaging process is repeated for different surface and bead concentrations to create a binding profile to human ICAM-1 and VCAM-1.

For the human serum experiments, the method is repeated, but the microspheres are added via a solution of PBS and human serum in a biologically relevant concentration and compared to the same system without the human serum added. Again, the microspheres are digitally photographed, and the percentage of adhesion profile is established for both systems.

Results and Discussion

Microsphere adhesion in the presence of circulating IgSF proteins in human serum were measured as a function of ligand density on the targeted surface and as a function of active antibody density coating the microspheres. The concentrations on both the microsphere and the slide surface were carefully controlled using calibrated standards and flow cytometry. The density of human VCAM-1 Fc chimera on the targeted surface is balanced by competition with human ICAM-1 Fc chimera protein, which has a similar size and structure [27]. A quantitative analysis of the attachment and detachment properties of targeted microspheres calculates the effect of soluble IgSF proteins on the binding profile. Experiments were designed to isolate the effect on the chimeric-coated surface and the microspheres by measuring adhesion on a slide surface before and after exposure to human serum.

Under static conditions, the force of adhesion between ICAM-1 or VCAM-1 and anti-ICAM-1 or anti-VCAM-1 is reported to about 200 pN [28], whereas the buoyancy force on the microspheres pulling against adhered ligand–antibody coupling was only about $\Delta\rho\frac{4}{3}\pi r^3 g = 0.135$ pN. This indicates three orders of magnitude in difference between the two forces. Hence, we expect the unstressed dissociation rate k_r^0 of the VCAM-1 bond to be important in the static adhesion. In other words, if a microsphere is able to properly align with the surface and the ligand–antibody bond is properly made, the microsphere is not able to be disrupted by the buoyancy or gravitational forces. Furthermore, the air-filled microbubbles (density, 0.1 gm/cc, $\Delta\rho\frac{4}{3}\pi r^3 g = 0.759$ pN) of the same size would exhibit a

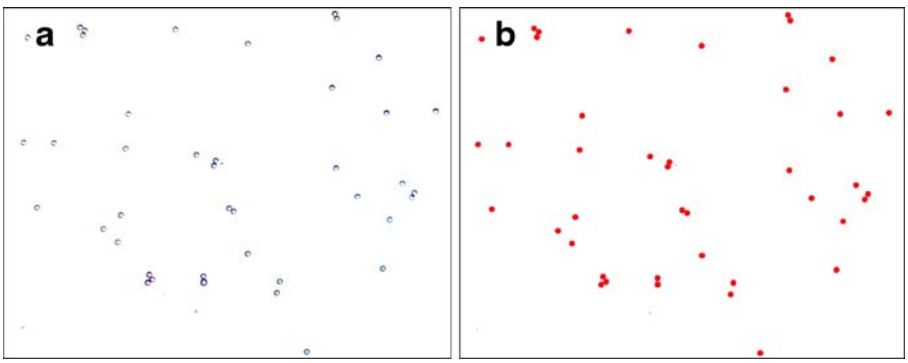

Fig. 3 **a** Image captured of microspheres on slide surface, **b** same image, with ImagePro Plus counting mechanism running. *Red dots* indicate counted microspheres

static adhesion profile with similar behavior to solid microspheres. Similarly, the shear forces, experienced during the bead loading procedure, were computed to be significantly lower than the forces of adhesion.

Figure 4a–d shows adhesion profiles of the microspheres on VCAM-1 target surfaces using 25%, 33%, 50%, and 100% anti-VCAM-1 antibody-coated microspheres. The target surfaces were coated with 25%, 50%, 75%, and 100% saturation of VCAM-1 Fc chimera. The figures show considerable differences in adhesion profiles with and without human serum proteins in buffer. The adhesion profiles of the microspheres without serum are consistently higher than the adhesion profile for those in the presence of serum. Different VCAM-1 slide surface saturations appear to mediate different "states" of adhesion response. For 25% and 33% anti-VCAM-1-coated microspheres, the serum increasingly suppresses (suppression state) the adhesion on targeted surfaces with less than 70% VCAM-1 coverage. The introduction of serum in buffer reduces the adhesion by 50% for targeted surfaces with 70% coverage. Beyond 70% coverage, the effect of serum on adhesion continues to decrease. For 50% anti-VCAM-1-coated microspheres (Fig. 4c), the effect of serum suppression is significant up to 35% target coverage. The 100% anti-VCAM-1-coated beads (Fig. 4d) do not enter a "suppression state" induced by human serum. The microspheres appear to be completely saturated with antibodies, and thus the change in adhesion is much less than the differences seen in the microspheres with lower antibody concentrations. The targeted adhesion is suppressed less than 20% by human

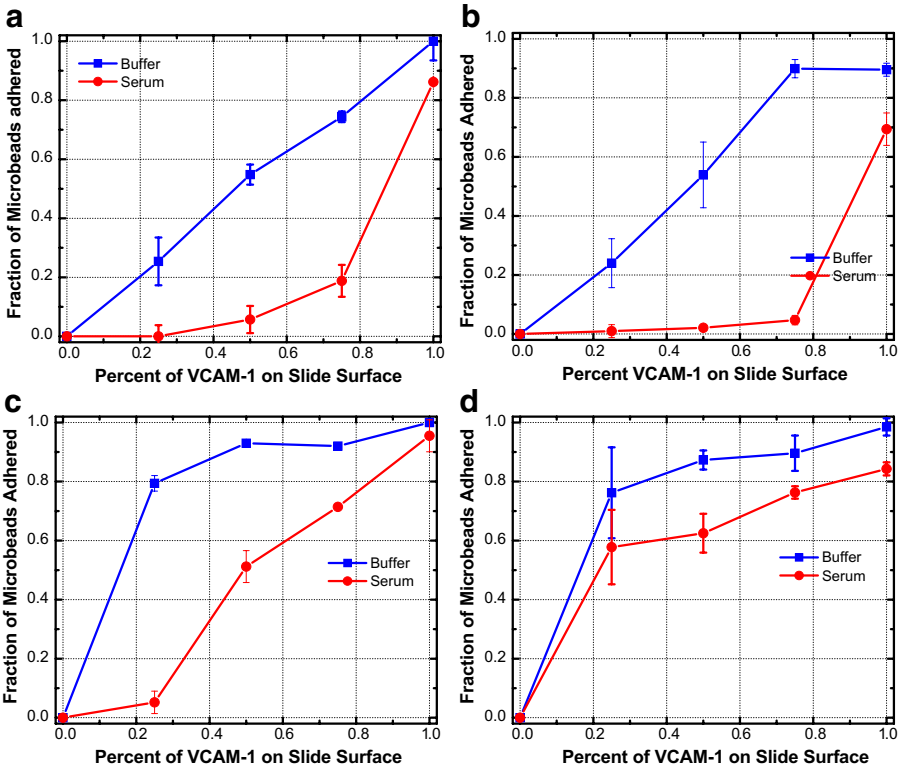

Fig. 4 Adhesion profile of microspheres with buffer and human serum present using **a** 25%, **b** 33%, **c** 50%, and **d** 100% saturated VCAM-1 beads

serum. Due to the high microsphere surface concentration, the microsphere still has available antibody sites, even when put in the presence of sVCAM-1 in the human serum.

Figure 5a–d shows adhesion profiles of microspheres on ICAM-1 target surfaces using 25%, 33%, 75%, and 100% anti-ICAM-1 antibody-coated microspheres. The target surfaces were coated with 25%, 50%, 75%, and 100% saturation of ICAM-1. The figure shows dramatic differences in adhesion profiles with and without human serum proteins in buffer. For 25%, 33%, and 75% anti-ICAM-1-coated microspheres, the serum almost completely suppresses (100% suppression state) the adhesion on all targeted surface coverage. Evidently, for 100% anti-ICAM-1-coated microspheres (Fig. 5d), the effect of serum on adhesion "suppression" decreases for the surfaces with ICAM-1 coverage higher than 50%. The targeted adhesion is suppressed 50% by human serum on 100% ICAM-1 slide surfaces.

The human serum experiments give more insight into the factors that affect the adhesion of the microspheres in vivo, as there are soluble ICAM-1 and VCAM-1 proteins in human serum that occupy available anti-ICAM-1 or anti-VCAM-1 sites [24] on the microspheres. Specifically, the normal range for healthy individuals of soluble ICAM-1 in serum is 100–200 ng/ml [24]. The serum used in our experiment had concentrations of ICAM-1 of 135.9± 20 ng/ml. For VCAM-1, the serum used in our experiment was measured to be 29.4±4 ng/ml, although the average VCAM-1 in human serum was reported to be higher [29]. Studies also show that human serum contains higher levels of sICAM-1 and sVCAM-1 in patients with

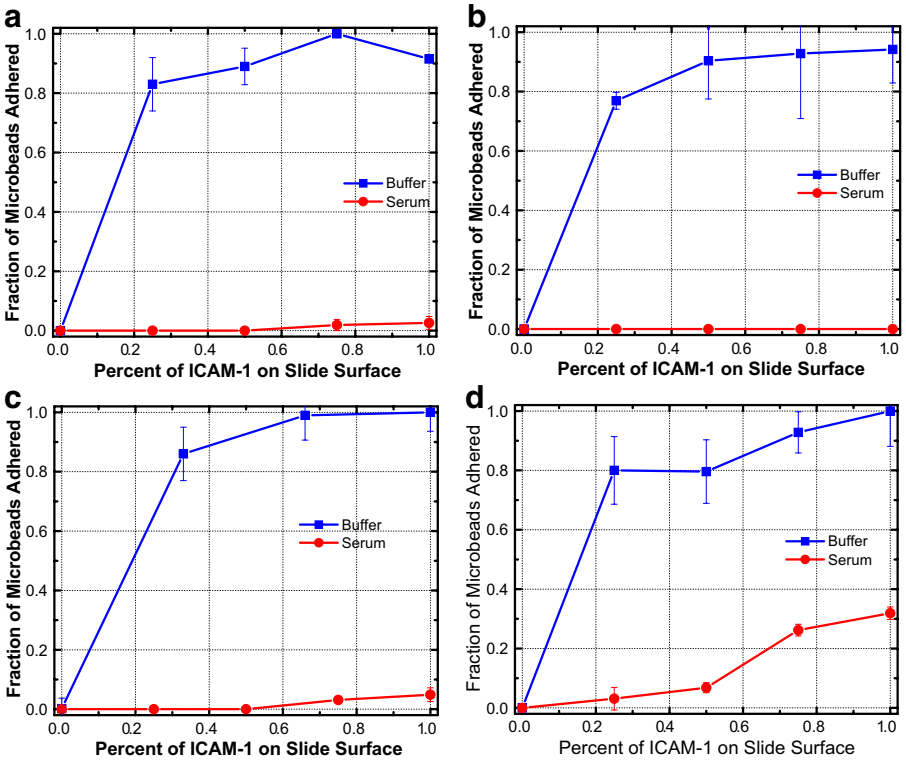

Fig. 5 Adhesion profile of microspheres with buffer and human serum present using **a** 25%, **b** 33%, **c** 75%, and **d** 100% saturated ICAM-1 beads

heart-related conditions and atherosclerosis, which is especially relevant in using these microspheres in vivo to detect atherosclerosis and other inflammation in the vasculature [24, 29].

The maximum microsphere surface-binding capacity for anti-ICAM-1 and anti-VCAM-1 was measured via flow cytometry using fluorescently tagged ICAM-1 and VCAM-1 chimeric proteins in free solution. Since these proteins are unbound, this free solution measurement may overestimate the binding capacity of our microspheres to solid surfaces due to the additional degrees of freedom, which allow the proteins to freely rotate and bind greater numbers of antibodies. These fluorescent calibrating proteins have three additional degrees of freedom, which are full motion in the X, Y, and Z directions. Adhered proteins in the assay have X, Y, and Z motion limited by the stiffness of the protein molecule and steric hindrance of the neighboring molecules. The bound chimeric CAM molecule, however, is located on a tethered extension, which is likely highly available to binding as illustrated by the successful quantitation experiments. While the effect cannot be quantified with the available data, we expect the error to be small. The calibration microspheres act by first binding the anti-ICAM-1 or anti-VCAM-1 mouse antibodies to anti-mouse IgG sites. The additional layer of specific antibodies, which then bind fluorescent ICAM/VCAM, may underestimate the true number of active sites in the calibration values since steric inhibition may prevent the anti IgSF antibodies from binding to the available sites on the calibration microspheres. The data are presented as percentages of maximum binding rather than raw binding site counts to highlight the change in binding state when soluble IgSF proteins are introduced into the system.

The salient findings include a monotonic increase of the rate of adhesion with increase in the VCAM-1 site density on the targeted surface. The adhesion profiles for the microspheres without human serum proteins are shown for VCAM-1 in Fig. 6a. There is an observable relation between increased antibody sites on the microspheres and increased overall adhesion. However, the anti-VCAM-1 densities on microsphere surface and VCAM-1 coverage on the targeted surface give rise to different states of adhesion. A change in the "state" of adhesion appears to exist where the adhesion profile undergoes a transition. The adhesion profiles are almost linear for 25%- and 33%-coated anti-VCAM-1 microspheres, while the 50%- and 100%-coated microspheres show a sudden initial jump followed by a gradual increase in adhesion. Hence, the microspheres appear to undergo a

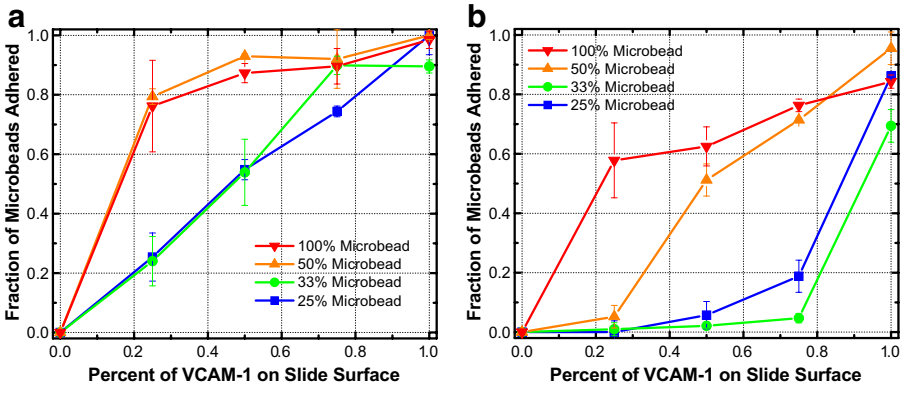

Fig. 6 Composite of all four types of beads used in the buffer–serum comparison, **a** in buffer and (**b**) in serum, using VCAM-1

transition between a "linear adhesion" state to a "sigmoidal adhesion" state somewhere between 33% and 50% saturation of anti-VCAM-1 on the microspheres. These microspheres show nearly 80% adhesion at even 33% VCAM-1 coverage of targeted surface. Higher surface concentrations do not produce substantial increased levels of microsphere adhesion to the surface and impart only 20% increase in adhesion.

The adhesion profiles for the microspheres in the absence of human serum proteins are shown for anti-VCAM-1 in Fig. 6b. Again, there is an observable relation between increased antibody sites on the microsphere and increased overall adhesion. However, in contrast to adhesion of microspheres in buffer, the presence of human serum proteins gives rise to different states of adhesion. The adhesion profiles exponentially grow for 25%- and 33%-coated anti-VCAM-1 microspheres, while 50%- and 100%-coated microspheres show a sudden initial jump followed by a gradual increase in adhesion. Hence, microspheres undergo a transition between a "exponential adhesion" state to a "sigmoidal adhesion" state somewhere between 50% and 100% saturation of anti-VCAM-1 on the microspheres. The 25% and 33% microspheres show only about 20% adhesion to a 60% VCAM-1 chimera-coated target surface. An additional 80% adhesion is picked up only at higher VCAM-1 coverage of the targeted surface. The 100% microspheres show nearly 60% adhesion at 33% VCAM-1 coverage of targeted surface and subsequent gradual increase in microsphere adhesion. It is apparent that the presence of serum modifies the mechanism of adhesion. The total number of anti-VCAM-1 sites on the beads introduced to the flow cell were approximately 4.2×10^{11}, 2.1×10^{11}, 1.4×10^{11}, and 1.0×10^{11} for 100%, 50%, 33%, and 25% microspheres, respectively. In contrast, the number of molecules of sVCAM-1 present in serum available in the flow cell was about 4.4×10^{10}. Hence, on average, 10–42% of anti-VCAM-1 molecules on bead surface were pre-bound, and there were 58–90% of anti-VCAM-1 antibodies available on the bead surface to bind with the targeted surface. The target surface provided approximately 5.7×10^{12} molecules of VCAM. Hence, the presence of soluble VCAM-1 in the serum instigated some of reduction in adhesion. Note that, perhaps, some non-specific interactions between with the serum protein blocked the interaction between anti-VCAM-1 antibody-coated microsphere also affected the adhesion profiles.

The adhesion profiles for the microspheres in the absence of human serum proteins are shown for ICAM-1 in Fig. 7a. The adhesion profiles for 100% anti-ICAM-1 microspheres show a sudden initial jump followed by a gradual increase in adhesion. Hence, the

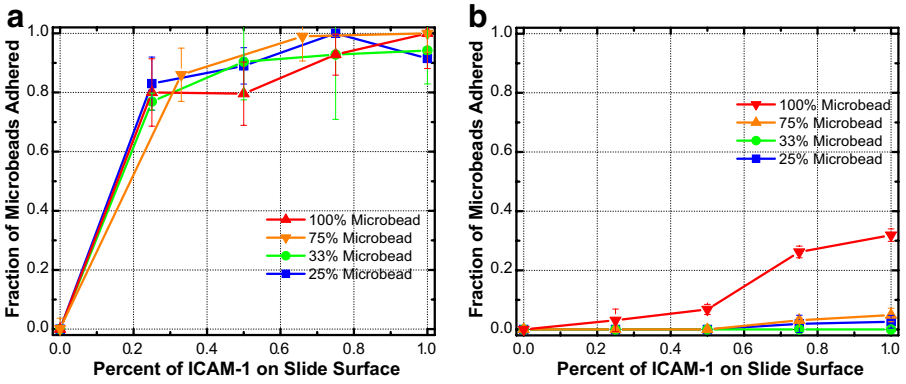

Fig. 7 Composite of all four types of beads used in the buffer–serum comparison, (**a**) in buffer and **b** in serum, using ICAM-1

microspheres stay in the same "sigmoidal adhesion" state. These microspheres show nearly 80% adhesion at even 33% ICAM-1 coverage of targeted surface. Higher surface concentrations impart only a 20% increase in adhesion.

The adhesion profiles for the microspheres in the presence of human serum proteins are shown for ICAM-1 in Fig. 7b. In contrast to adhesion of microspheres in buffer, the presence of human serum proteins severely suppresses the adhesion. The adhesion profiles show only a 5% increase for 25%-, 33%-, and 50%-coated anti-ICAM-1 microspheres, while the 100%-coated microspheres show a 30% increase in adhesion for target surfaces with ICAM coverage higher than 70%. In this case, the microspheres stay in a "suppression adhesion" state. It is clear that the presence of serum severely alters the adhesion rate. The total number of ICAM-1 binding sites on the beads available in the flow cell was approximately 4.2×10^{11}, 3.2×10^{11}, 1.4×10^{11}, and 1.0×10^{11} for 100%, 75%, 33%, and 25% microspheres, respectively. The number of molecules of ICAM-1 present in serum available in the flow cell was about 2.5×10^{11}. Hence, 100% of anti-ICAM-1 sites were pre-bound for 25% and 33% surface-coated beads, and consequently, 100% suppression was observed. On average, 59–78% of anti-ICAM-1 molecules were pre-bound for 75% and 100% beads. There were 20–40% of anti-ICAM-1 molecules available on the bead surface to bind with targeted surface, which lessened the effect of serum suppression for these beads. Note that the target surface provided approximately 5.7×10^{12} molecules of ICAM.

The presence of soluble adhesion molecules and the resulting reduction in adhesion is significant in the design and manufacture of microspheres for targeted adhesion in vivo. The adhesion profile of the microspheres was determined solely by the exposure history of the beads but not the protein-coated slide. Control microsphere adhesion profiles in buffer without serum were repeatable, even after exposure of the slide to human serum. The serum effect on adhesion targets only the beads but not the surface proteins. Although antibodies to self molecules would not be expected in serum in normal individuals, the results indicate that soluble ICAM-1 and VCAM-1 ligands in the human serum are the active agent rather than soluble antibodies, which would have bound to the slide surface and reduced any further adhesion.

Overall, on average, the presence of human serum created a 37±11.6% reduction of adhesion of the VCAM-1 microspheres and a 90±11.6% reduction of adhesion of ICAM-1 microspheres. This phenomenon can possibly be explained by examining the relative content of sICAM and sVCAM in the serum. Since there was significantly more sICAM than sVCAM, or if sICAM has a greater binding affinity for the microspheres than sVCAM, then the relative reduction in binding is in agreement with the data. While these two adhesion molecules are structurally similar [2], the monoclonal antibodies likely target different loop regions of the molecule. Depending on steric hindrance, the adhesion profiles are expected to differ. The small off-rate of these antibodies to their targets essentially insures that soluble molecules are retained. sICAM and sVCAM have been shown to increase in humans who have atherosclerosis or other vasculatory inflammatory problems. This result is significant, since the patient population for targeted particle or imaging applications likely will have elevated sICAM and sVCAM in their bloodstream.

Based on these results, human serum is a significant factor in microsphere adhesion for in vivo applications. As sICAM and sVCAM concentrations in human serum are directly correlated to and increase in inflammation in the vasculature, the effect of human serum is only magnified in patients who would have the greatest need to have ultrasound detection or drug delivery applications using this system. In light of these results, ideal microsphere adhesion molecules would dissociate from their target molecule in the absence of shear and thus be available to bind with surface proteins regardless of soluble protein levels. Specially

designed peptoids or known adhesion molecules, such as P-selectin or fractalkine, which bind firmly under shear stress, would provide a serum insensitive alternate.

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