A Real-Time *in Vitro* Assay for Studying Functional Characteristics of Target-Specific Ultrasound Contrast Agents

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Purpose. To develop an *in vitro* assay for studying the feasibility of specific targeting of ultrasound contrast agents (USCAs) for ultrasound diagnostics by employing the parallel plate flow chamber, which provides an environment that mimics some aspects of the *in vivo* conditions like shear rate and flow effects.

Methods. USCAs based on air-filled microparticles (MP) were functionalized with specific antibodies using carbodiimide coupling chemistry and characterized by fluorescence activated cell sorter (FACS). The binding experiments were done by subjecting the MP to shear stress as they interact with the target-coated surface of the flow chamber.

Results. A successive modification of MP with antibody and the glass surface with antigen was achieved and quantified. The binding studies showed specific attachment of targeted MP to EDB-FN (EDB domain of fibronectin) surface. The binding of MP via nonspecific interactions was minimal. The binding efficiency of antibody-loaded MP is dependent on the applied shear stress. An increase in the wall shear stress resulted in a decrease in binding efficiency. Binding efficiency was found to be correlated with the antibody density and antigen density on the interacting surfaces.

Conclusions. The results indicate that the test system developed is reliable for characterizing targeted MP without any additional labeling and can be used as a functionality assay for studying the binding characteristic of USCA with respect to different parameters like density of targeting antibodies on the microparticle surface and of target protein. In addition, the microparticles can be studied in detail under different shear rates and flow conditions. Further studies concerning the *in vitro-in vivo* correlation will be necessary to further increase the value of this *in vitro* method.

KEY WORDS: flow chamber assay; microparticles; specific targeting; tumor marker; ultrasound diagnostics.

INTRODUCTION

The diagnostic applications of ultrasound imaging have expanded enormously in the past decades. Ultrasound contrast agents (USCAs) have widely been recognized not only to improve the quality of imaging, but also to create a new perspective in ultrasound diagnostics by allowing the imaging of disease processes on the molecular level (molecular imaging). USCAs are mostly micrometer-sized and consist of a gas core encapsulated by shell-forming materials like polymers, lipids, or proteins. Once injected into the bloodstream, they can reach even the smallest capillaries. When exposed to an ultrasound beam, these agents return signals that are thousands of times more reflective than blood. The improvement of the imaging quality relies on this characteristic of the contrast agents. Studies of different research groups have shown that air-filled microparticles (MP) may have the potential to be used as targeted USCA (1–3). USCA labeled with antibodies against a specific marker can significantly enhance the content of information of ultrasound images in the desired body areas. Due to the high spatial resolution (micrometer range) and a pronounced sensitivity (single bubbles can be imaged and quantified), it possesses clear advantages compared to other imaging modalities like MR, CT, and optical imaging in the field of molecular imaging (4).

Because the USCAs are in the micrometer range, they cannot leave the circulation and therefore cannot be used for targeting and imaging of extravascular structures, but specific targeting to targets exposed to the bloodstream can be done by simply administering the target-specific MP to the bloodstream.

As a first step to evaluate the feasibility of this approach, *in vitro* assays have to be developed on the basis of molecular recognition interactions. We have carried out *in vitro* targeting studies using a model system consisting of a target-ligand pair. We have prepared target-specific USCA based on polybutylcyanoacrylate (PBCA) that selectively bind to the model target EDB-fibronectin (EDB-FN), by attaching AP39, a single-chain antibody fragment (scFv), on their surface. AP39 has been derived from the scFv L19 by extending the C-terminus with Gly-Gly-Gly-Cys-Ala. It is a covalent dimer and is equal to L19 specific for the EDB domain of fibronectin (5). Fibronectin is the "universal adhesion molecule" and represents the most widely distributed extracellular matrix protein of all. EDB-FN was discovered by Zardi and coworkers (6). His group reported the strict association of EDB fibronectin to cancer cells and fetal tissues.

In the past several years, fundamental engineering concepts have been used to design devices that simulate flow conditions encountered in the vasculature (7–9). These *in vitro* experimental systems are capable of generating controlled mechanical forces similar to those induced by hemodynamic forces *in vivo*. We have developed a novel *in vitro* binding assay that examines interactions between particles and target molecules immobilized on a rigid surface under flow conditions. In contrast to others, who used cells carrying the antigen on their surface for studying the binding behavior of target-specific MP (10,11), we decided to develop a matrix-based flow chamber assay that allows the reproducible immobilization of target protein and the visual interpretation of the binding efficiency of MP without any further labeling. Flow chamber binding assays are uniquely suited for the investigation of adhesive processes, which can be visualized and then quantified by selective image acquisition and subsequent image processing (12). The most common model of flow chamber systems is the parallel-plate type (Fig. 1), which provides a well-defined hydrodynamic environment to study dynamic adhesion processes; for example, often used for the study of leukocyte rolling and binding (13,14). It consists of (a) base plate with an entrance and exit port through which analyte and media are perfused, (b) a glass or plastic slide plate on which the target is immobilized, (c) a gasket that controls the chamber dimensions, and (d) a vacuum outlet so that the

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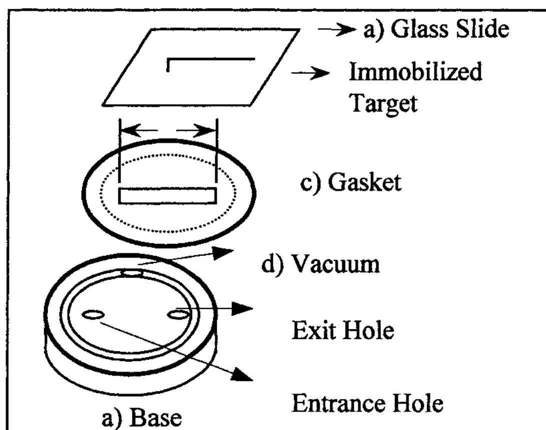

Fig. 1. Schematic of parallel plate flow chamber configuration. The components are (a) base plate, (b) glass slide plate, (c) gasket, (d) vacuum outlet.

apparatus can be held in place. By controlling the volumetric flow rate through the parallel plates, it is possible to change the wall shear stress acting on the slides according to the following expression (15):

$$\tau_{wall} = \frac{6 \times \mu \times Q}{w \times h^2}$$

where τ is shear stress, μ is fluid viscosity, Q is volumetric flow rate, w is chamber width, and h is the chamber height.

MATERIALS AND METHODS

Materials

Organic compounds and biochemicals were purchased from Sigma (Diesenhofen, Germany). The antigen EDB-FN (biotinylated and non-biotinylated) and the antibody AP39 (biotinylated and non-biotinylated) were developed by Schering research laboratories (Berlin, Germany). The K_D value of AP39 was determined to be about 5×10^{-10} mol using the SPR technique (Biacore 2000, Freiburg, Germany).

Preparation of Target-Specific USCA

The preparation of the USCA based on air-filled poly-(butylcyanoacrylate-co-cyanoacrylic acid) (PBCA-co-CA) MP is described elsewhere (16). In short, the particles were prepared as follows: Butylcyanoacrylate (BCA) (Sichel Werke, Hannover, Germany) was added in drops to a solution of Triton X-100 (Octoxynol 9) (Fluka, Diesenhofen, Germany) at pH 2 (hydrochloric acid, Fluka) under intensive stirring (UT T50, Jahnke/Kunkel, Stauffen/Breisgau, Germany). Air-filled PBCA microparticles were harvested after flotation, and by incubation in a diluteted sodium hydroxide solution, the butyl-ester groups were hydrolyzed (releasing butanol), and cyanoacrylic acid residues were obtained allowing carbodiimide coupling chemistry. Anti–EDB-FN (AP39) was coupled to the MP by using the streptavidin-biotin interaction. For this, streptavidin was at first coupled to the MP. Carbodiimide chemistry was used to link the amino groups of streptavidin to the activated carboxylate groups on the surface of MP. One hundred microliters of MP (1×10^{10} MP/ml) were diluted in 15 ml of sodium acetate buffer, pH 4.5, and incubated with 7.5 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 300 μg streptavidin for 1 h at room temperature (RT) under stirring. After the incubation overnight at 4°C, the coupled MP were washed two times with HEPES buffer (pH = 7.4) containing 0.01% Triton X-100. The presence of streptavidin on the surface of the MP was determined by FACS using biotin-FITC as marker. Streptavidin-loaded MP (1×10^6) were further incubated with 4 μg biotin-labeled AP39 for 30 min at room temperature. The coupling medium used was PBS (pH 7.4). To remove the unreacted antibody from the system, the mixture was centrifuged for 2 min at 2000 rpm, and the floating MP were separated from the system and resuspended in PBS containing 0.01% Triton. To vary the site densities of AP39 on the particle surface, 1×10^6 MP were incubated with 1 μg, 0.1 μg, and 0.01 μg biotinylated AP39, respectively. The presence and the site density of AP39 on the MP was measured by flow cytometry using a conjugate of biotinylated EDB-FN and streptavidin-coupled R-phycoerythrin as marker system. For the quantification of coupled antibody fragments, the Quantibrite PE (Beckton Dickinson, Heidelberg, Germany) kit containing standard beads with different loadings of R-phycoerythrin was used. Streptavidin-loaded MP were also coupled with isotype IgG for negative binding studies. For this, 1×10^6 streptavidin-loaded MP were incubated with 2 μg biotinylated IgG for 30 min. The coupled MP were washed with PBS buffer to remove unbound IgG from the surface.

Characterization of Targeted Contrast Agent

For the determination of the presence of streptavidin on MP, 100 μl of streptavidin-loaded MP (10^7 MP) were incubated with 2.5 μg bio-FITC in 200 μl PBS buffer containing 1% BSA and 0.01% Triton X-100 for 20 min. For the removal of unreacted biotinlyted FITC, the mixture was diluted with the same buffer and centrifuged for 2 min at 2000 rpm. The washed MP were resuspended in FACS flow buffer and analyzed. The antibody-coupled MP were labeled with R-phycoerythrin for the FACS detection. To achieve this, the streptavidin-loaded MP were incubated in a PBS buffer containing the conjugate of EDB-FN and R-phycoerythrin at room temperature for 30 min. The conjugate was made by mixing biotinylated EDB-FN and streptavidin-labeled R-phycoerythrin in 1:1 ratio. Before the FACS analysis, the MP were washed with buffer to remove the unbound R-phycoerythrin. Uncoupled MP were also incubated with streptavidin-labeled R-phycoerythrin and used as control.

Immobilization of Target

Carboxy-terminated pegylated glass plates (17) were used as the cover glasses for the flow chamber. For the functionalization of cover glass plates with the target EDB-FN, the COOH groups on their surface were activated with NHS (*N*-hydroxy succinimide)/EDC (0.05 M/0.2 M) for 30 min. After washing with HEPES buffer (10 mM, pH 7.4) containing 150 mM of NaCl and 3 mM EDTA, the glasses were incubated with 20 μl of EDB-FN (260 μg/ml) for 30 min. The unbound EDB-fibronectin was removed from the surface by washing with PBS buffer. In order to deactivate the remaining free activated COOH groups, the glasses were treated with 50 mM ethanol amine (pH 8.5) for 15 min after the washing

steps. Glasses were washed thoroughly with Milli-Q water and dried under N_2.

Enzyme-linked immunosorbent assay (ELISA) was used to quantify the amount of immobilized EDB-FN. The coated surface was incubated with 20 μl of biotinylated AP39 (260 μg/ml) for 15 min. After washing, 20 μl of avidin peroxidase conjugate (280 μg/ml) was dropped over the coated surface. Excess of free conjugate was removed by washing after an incubation period of 15 min. After that, the flow chamber was assembled using the modified glass plate. A 100-μl solution of 3,3′,5,5′-tetra-methyl benzidine (TMB) containing H_2O_2 was added into the flow chamber. After 5 min, the reaction was stopped by the addition of 50 μl of 0.5 M H_2SO_4, and the product was transferred to a microtiter plate. The absorbance was measured at 450 nm (OD_{450}) on an ELISA micro well plate reader. A standard curve in a concentration range of 10–500 ng was created, and the amount of immobilized antigen was determined with reference to this standard curve.

Flow Chamber Assay

Functionality Test of Site-Targeted Contrast Agent and Its Reproducibility

The flow chamber (GlycoTech, Rockville, MD, USA) was assembled with the modified glass plate using a silicon gasket building a flow channel of 0.25 × 2.5 × 0.025 cm. After that, the chamber was placed on the stage of an inverted microscope and attached to a pulse-free peristaltic pump (Perimax 16, Spetec, Erding, Germany). The chamber and the tubing were equilibrated with running buffer PBS (pH 7.4, 0.01% Triton X-100) for 10–15 min, and care was taken to ensure that no air was trapped in the lines. A suspension of 2×10^7/ml MP carrying AP39 in running buffer was injected into the flow system over a 100-μl loop [Rheodyne (Abimed), Langenfeld, Germany] at a shear stress of 5.7 dyne/cm^2 by adjusting the corresponding flow rate. The wall shear stress was calculated assuming a viscosity of assay buffer equal to the viscosity of water at room temperature (1 centipoise, 25°C). After the complete passage of MP, the flow of running buffer through the chamber was allowed for a further 5 min. For the quantification of bound MP, 10 defined fields of view on the glass plate were recorded photographically. The control experiment was carried out in the same manner using a suspension of 2×10^7/ml unmodified MP and 2×10^7/ml isotype loaded MP, respectively. For the reproducibility test, the binding assays were repeated 3 times at a shear stress of 5.7 dyne/cm^2 by perfusing 1×10^7/ml targeted, isotype loaded, and nontargeted MP, respectively. For the blocking experiment of the EDB-FN surface, the following procedure was used. After the binding experiment using AP39 modified MP, all attached MP were removed by the injection of air into the chamber. After that, the surface in the flow chamber was pretreated with free AP39 (260 μg/ml), and after the elution of unbound AP 39, AP39-carrying MP were injected as described before. The number of particles adhering to the surface was determined after 5 min of flowing medium.

Influence of Target and Ligand Concentration

In order to study how the binding characteristics were influenced by the surface concentration of EDB-FN, adhesion experiments were carried out with glass plates coated with different concentrations of antigen. Three plates were prepared with an antigen loading of 5.63 ng/cm^2, 1.97 ng/ cm^2, and 1 ng/cm^2, respectively. One plate was coated with fibronectin, which does not contain the EDB-segment. Binding studies were carried out using the chamber assembled with respective slides at a predetermined shear stress of 5.7 dyne/cm^2 by perfusing the antibody-coated MP (1×10^7/ml) through the chamber. For studying the influence of antibody densities on the MP, binding assays were conducted using MP with various antibody site densities (7650 molecules AP39/MP, 19830 molecules AP39/MP, 34600 molecules AP39/MP) while keeping the antigen site density constant at 5.6 ng/cm^2.

Influence of Shear Stress

The hydrodynamic shear stress is considered to play an important role in driving the cell forward while the specific adhesion force holds them to the surface. To determine the effect of shear stress on the binding, 2×10^7/ml antibody-loaded MP were perfused through the chamber at different defined flow rates between 5.7 dyne/cm^2 and 14 dyne/cm^2. This range of shear stress is similar to those seen in venules and veins (18). After the passage of MP, running buffer was

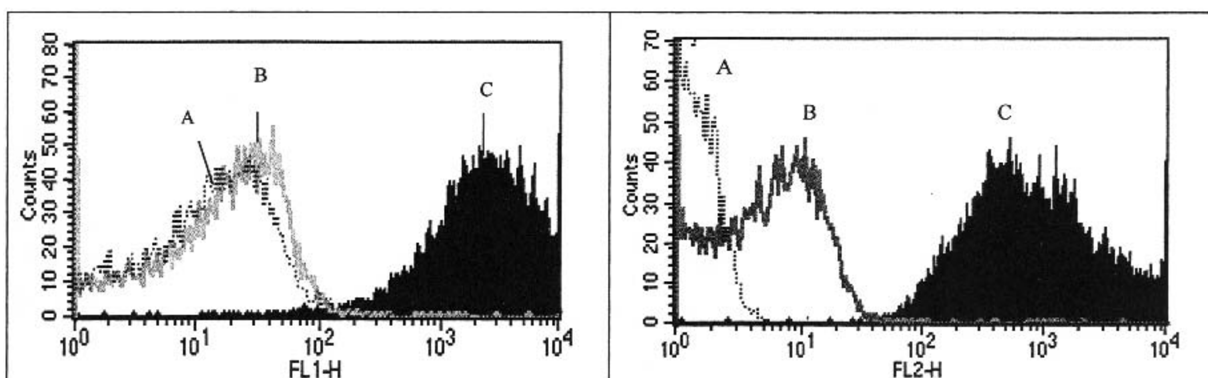

Fig. 2. Characterization of coupled MP. Left: flow cytometry comparison of (A) uncoupled/FITC-labeled, (B) streptavidin-coupled/nonlabeled, and (C) streptavidin-coupled/FITC-labeled MP. Right: FACS analysis of (A) uncoupled/phycoerythrin-labeled, (B) AP39-coupled/nonlabeled, and (C) AP39-coupled/phycoerythrin-labeled MP. Experimental conditions are described in "Materials and Methods."

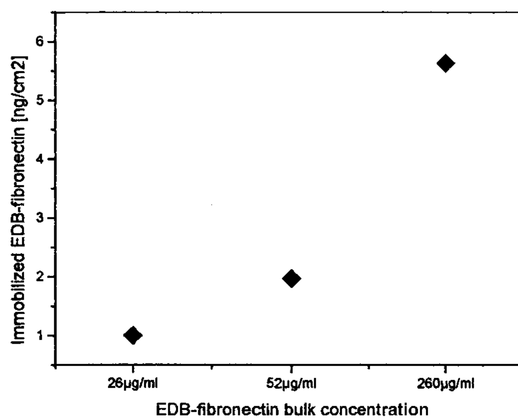

Fig. 3. Amount of immobilized EDB-fibronectin on pegylated glass slides as a function of bulk incubation concentration.

allowed to flow for 5 min, and the images were collected at 10 fields on the glass plate.

RESULTS

Characterization of the Targeted MP

The anti–EDB-FN antibody AP39 was coupled to PBCA MP using streptavidin-biotin interaction. The successful coupling of streptavidin on the surface of MP was confirmed by FACS analysis (Fig. 2; left). Flow cytometry experiments with AP39 coupled and uncoupled MP also confirm the presence of AP39 on the MP surface. Figure 2, right, shows the fluorescence histogram obtained by flow cytometry for labeled/nonlabeled AP39 coupled MP and uncoupled MP. According to this histogram, AP39-coupled MP show about a 100-fold increase in fluorescence intensity over the control.

Quantification of the Target

The amount of immobilized EDB-FN on the glass surface is measured using an ELISA-like assay by comparing the level of color change during the peroxidase reaction with the created calibration curve. Figure 3 shows the amount of immobilized EDB-FN as a function of EDB-FN concentration in the incubation medium. The measured optical density increased with the increase in the amount of immobilized EDB-FN, but saturation was not seen in the tested range. The estimation of immobilized EDB-FN is based on the assumption that there is at least a 1:2 binding between the extravidin peroxidase and biotinylated antibody and a 1:1 binding between antibody and EDB-FN.

Flow Chamber Assay

Functionality Test

The specific attachment of the MP on the target surface of EDB-FN was studied under the controlled shear conditions using the parallel plate flow chamber. For this, AP39 targeted, isotype loaded, and unmodified MP were perfused over the immobilized EDB-FN. As seen in Fig. 4, the number of isotype loaded and unmodified MP attached to the EDB-FN surface was much lower than that of AP39-loaded MP. During the perfusion, AP39-loaded MP rolled on the EDB-fibronectin surface and were finally firmly attached. The binding was relatively stable and could withstand high shear stress (Fig. 5). The injected particle dispersions were aggregate-free (studied using a Multisizer 3), and the aggregates visible on the images were results of the *in vitro* situation (one microparticle is attached, and this attached microparticle is the reason for other particles being attached there).

For the quantification, the recorded images (10 for each experiment) were analyzed using the Scion image analysis program. Data obtained from this analysis were used to evaluate the average number of adsorbed MP. The data analysis showed that the attachment of targeted MP on EDB-FN surface was reasonably efficient, and the binding of nontargeted MP was minimal. The binding experiments at 5.7 dyne/cm^2 wall shear stress using 2×10^7 MP (targeted, isotype loaded, or unmodified) showed that whereas about 105 antibody-loaded microbubbles per area were bound to the EDB-FN coated glass, only 5 unmodified MP and 19 isotype loaded MP per area were attached to the EDB-FN surface (Fig. 5, left). The reproducibility of the binding assay was found to be satisfactory (Fig. 5 right). The blocking of binding sites on the EDB-FN surface by free AP39 antibody fragments led to a significant reduction in the number of bound MP (Fig. 6). When free AP39 was administered prior to the target-specific MP, the number of attached MP is reduced by about 76%. This finding also confirms that the binding occurred due to specific interaction between the ligand on the MP and EDB-FN.

Influence of Target and Ligand Concentration

In order to determine the influence of receptor and ligand site density on the binding characteristics, both receptor

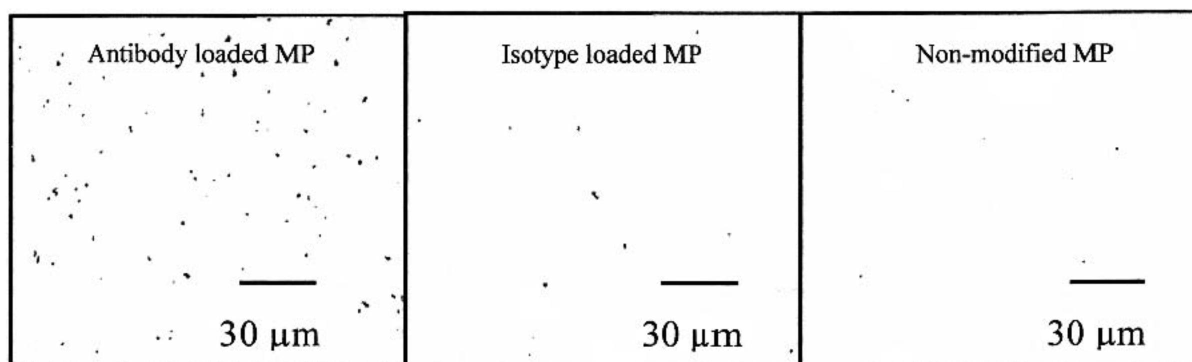

Fig. 4. Microscopic images of MP bound to the glass plate.

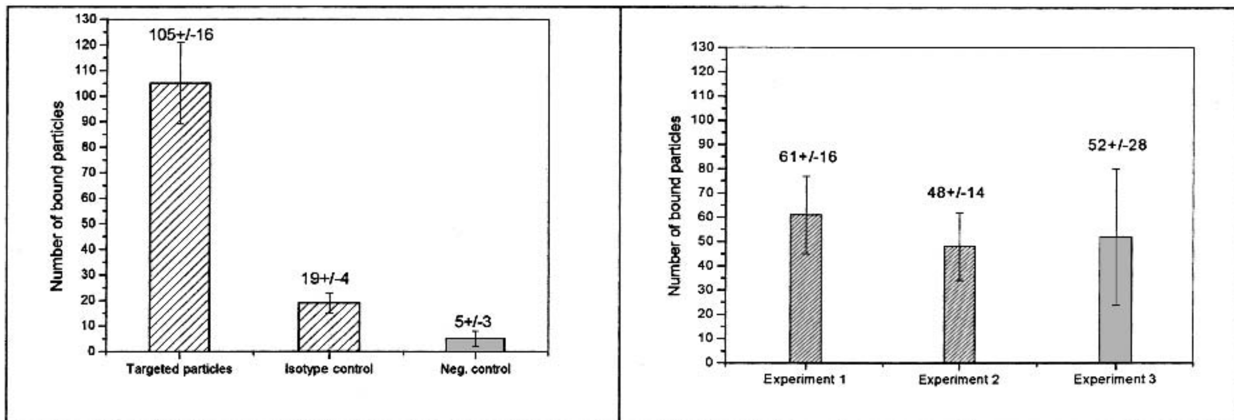

Fig. 5. The quantification of bound MP (targeted, isotype loaded, and unmodified) on the EDB-FN–coated glass plate (left) and the reproducibility of the binding assay (right). For the reproducibility assay, only 1×10^7 MP were used. The error bars represent the standard deviations between the images (10 images per each experiment) of independent experiments.

and ligand site densities were systematically varied. Binding studies were carried out at different site densities of EDB-fibronectin on the glass surfaces, varying from 1 ng/cm^2 to 5.63 ng/cm^2 by keeping a constant concentration of AP39 on the MP surface (16,200 antibody fragments (ab)/microparticle). Binding of targeted MP was also studied using a cover glass coated with only fibronectin (without EDB-segment) as control. The dependence of antibody loading on the binding efficiency was examined by conducting the experiment at a constant site density of antigen on the cover glass by changing the antibody concentration on the microparticle surface

Figure 7 shows the influence of antigen site density on the binding efficiency of targeted MP. Binding efficiency increases with antigen site density. It means that the degree of expression of antigen on the tumor surface plays an important role for the success of specific targeting. The binding of targeted MP on the cover glass coated only with fibronectin was very low (data not shown). The degree of antibody loading also shows an effect on binding efficiency. A 2-fold increase was found when the antibody loading changes from about 7650 AP39 molecules/MP to 19830 AP39 molecules/MP. No remarkable change in efficiency was observed with AP39 densities higher than 19,830 antibody molecules/MP. In order to make a reliable statement on the influence of target and antibody site density on binding efficiency, these binding studies must be repeated under different shear conditions.

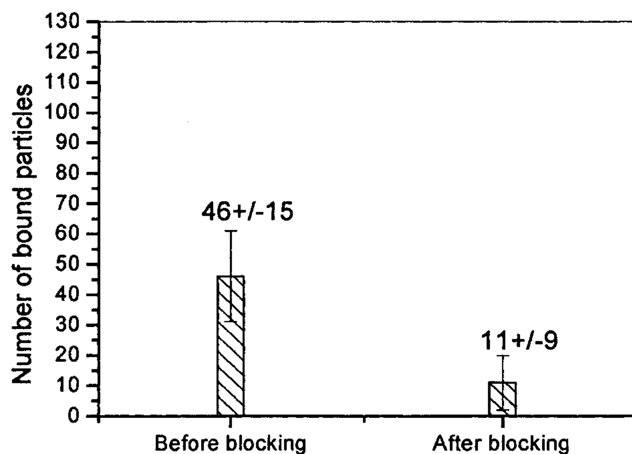

Fig. 6. Blocking experiment. The number of attached targeted microparticles was determined after the pretreatment of the EDB-FN carrying surface in the flow chamber with free AP39 (260 μg/ml).

Influence of Shear Stress

The data demonstrate the dependence of binding efficiency on shear stress. A high shear produces significant reduction in the binding efficiency of targeted microparticles (Fig. 8). At low shear rates, the binding of targeted MP on the EDB-FN surface was reasonably efficient.

DISCUSSION AND CONCLUSIONS

In this work, a feasibility study was conducted using the parallel plate flow chamber to examine the interaction between a targeted USCA and its molecular target under flow conditions that mimic the situation in venules and veins. Flow chamber assays were used to characterize the interaction between AP39-coupled MP and EDB-FN. In this model, we could directly observe the attachment of the targeted MP to the target-coated surface from the flow. The results showed that AP39-coated MP specifically interact with EDB-fibronectin–coated surfaces as quantified by counting of attached MP. Furthermore, the binding observed between the AP39-coated MP and EDB-FN surfaces is very firm, and the binding studies showed good reproducibility. Unspecific binding originating from nonmodified microparticles and isotype modified microparticles was very low, demonstrating the ability of measuring specific interactions of target-specific microparticles and the immobilized corresponding antigen. To investigate the effect of wall shear stress on binding, assays were carried out at various shear stress by changing the volumetric flow rate of the microparticle suspension through the flow chamber, and the number of firmly bound MP was plotted vs. shear stress. The binding efficiency of antibody-loaded MP is dependent on the shear stress. An increase in the wall shear stress resulted in a decrease in binding efficiency. Increasing the microparticle flow rate can be intuitively expected to decrease the residence/contact times between the EDB-FN and the antibody on the surface of the MP and thereby decrease the specific binding. This could be observed as seen in Fig. 8. The reduction of binding efficiency could also be due to the

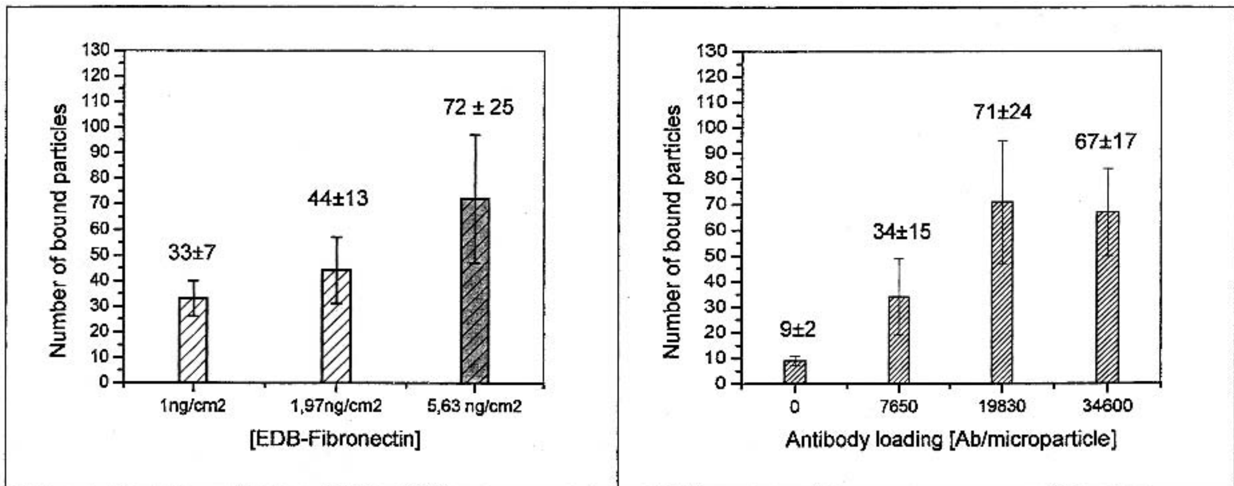

Fig. 7. The number of bound target-specific MP as a function of EDB-fibronectin site density on the cover glass (left) and AP39 site density on the MP (right).

increased mechanical force exerted on the MP. But when the MP are selectively bound to the target surface (at a low flow rate), they can tolerate high shear stress, and the binding is strong enough to resist high dynamic forces of flowing medium (data not shown). Furthermore, the experiments also indicate that the binding efficiency is correlated to the ligand density on the MP and receptor density on the glass surface. The reduction of the antigen density on the glass surface resulted in significant decrease in binding efficiency of targeted contrast agent.

One limitation of this model is that the flow does not adequately mimic the capillary blood flow. Another drawback of this model is that the ligands and receptors are exposed to nonphysiological medium, because blood cannot be used due to its opacity. Despite these limitations, we assess the reliability of the flow chamber model to simulate *in vivo* conditions more closely than any static models. It also provides an inexpensive means of studying bimolecular interactions of targeted particular agents with entities in the size range of optical microscopes (>500 nm).

Furthermore, one aim of our study was to set up a routine method for characterizing our targeted contrast agent with respect to its ability to interact with specific target molecules. The results indicate that the test system developed is reliable to characterize the targeted MP without any further labeling and can be used as a functionality assay for studying the binding characteristic of USCA. Further work is necessary to use this method for the accurate and reliable quantification of the ligand loading on the MP and more studies are necessary to evaluate the influence of ligand loading under different shear conditions, representing different dimensions of blood vessels, on binding. In addition to this, it is important to know about the influence of different K_D values of ligands on the binding behavior of targeted MP for the development of an efficient targeted agent, and, of course, studies concerning the correlation of *in vitro* results with the situation *in vivo* will be necessary.

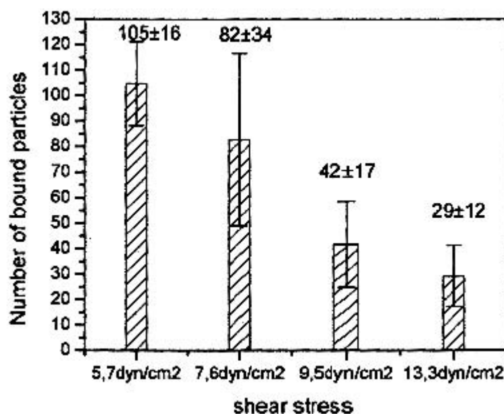

Fig. 8. Effect of shear stress on the binding of antibody-loaded MP to the antigen-coated surface.

REFERENCES

1. A. L. Klibanov, M. S. Hughes, F. S. Villanueva, R. J. Jankowski, W. R. Wagner, J. K. Wojdyla, J. H. Wible, and G. H. Brandenburger. Targeting and ultrasound imaging of microbubble-based contrast agents, magnetic resonance materials in physics. *Biol. Med.* **8**:177–184 (1999).
2. H. Leong-Poi, A. L. Klibanov, J. P. Christiansen, Y. Huo, and J. R. Lindner. Development of an angiogenesis-targeted microbubble ultrasound contrast agent. *J. Am. Coll. Cardiol.* **39**: 360–364 (2002).
3. R. J. Lindner. Detection of inflamed plaques with contrast ultrasound. *Am. J. Cardiol.* **90**:L32–L35 (2002).
4. M. Rudin and R. Weissleder. Molecular imaging in drug discovery and development. *Nat. Rev. Drug Discov.* **2**:123–131 (2003).
5. S. Melkko, C. Halin, L. Borsi, L. Zardi, and D. Neri. An antibody-calmodulin fusion protein reveals a functional dependence between macromolecular isoelectric point and tumor targeting performance. *Int. J. Radiat. Oncol. Biol. Phys.* **54**:1485–1490 (2002).
6. L. Zardi, B. Carnemolla, A. Siri, T. E. Petersen, G. Paolella, G. Sebastio, and F. E. Baralle. Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J.* **6**:2337–2342 (1987).
7. G. Theilmeier, T. Lenaerts, C. Remacle, D. Collen, J. Vermylen, and M. Hoylaerts. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. *Blood* **94**:2725–2734 (1999).
8. Z. J. Li, N. Mohamed, and J. M. Ross. Shear stress affects the kinetics of Staphylococcus aureus adhesion to collagen. *Biotechnol. Prog.* **16**:1086–1090 (2000).

9. S. K. Bhatia and D. A. Hammer. Influence of receptor and ligand density on the shear threshold effect for carbohydrate-coated particles on L-selectin. *Langmuir* **18**:5881–5885 (2002).
10. L. Klibanov. Targeted delivery of gas-filled microspheres contrast agents for ultrasound imaging. *Adv. Drug Del. Rev.* **37**:139–157 (1999).
11. G. E. R. Weller, E. Lu, M. M. Csikari, A. L. Klibanov, D. Fischer, W. R. Wagner, and F. S. Villanueva. Ultrasound imaging of acute cardiac transplant rejection with microbubbles targeted to intercellular adhesion molecule-1. *Circulation* **108**:218–224 (2003).
12. D. A. Jones, C. W. Smith, and L. V. McIntire. Flow effects on leukocyte adhesion to vascular endothelium. In P. Richardson and M. Steiner (eds.), *Principles of Cell Adhesion*, CRC Press, Boca Raton, FL, 1995, pp. 143–160.
13. G. Thoma, W. Kinzy, L. Bruns, J. T. Patton, J. L. Magnani, and R. Banteli. Synthesis and biological evaluation of a potent E-Selectin antagonist. *Med. Chem.* **42**:4909–4913 (1999).
14. U. Bakowsky, G. Schumacher, C. Gege, R. R. Schmid, U. Rothe, and G. Bendas. Cooperation between lateral ligand mobility and accessibility for receptor recognition in selectin-induced cell rolling. *Biochemistry* **41**:4704–4712 (2002).
15. L. D. McIntire and S. G. Eskin. Mechanical and biochemical aspects of leucocyte interactions with model vessel wall. In H. J. Meiselman, M. A. Lichtman, and P. L. La Celle (eds.), *White Cell Mechanics: Basic Science and Clinical Aspects*, Wiley Publishers, New York, 1984, pp. 209.
16. U. Budde, A. Briel, G. Roessling, K. Lovis, W. Schmidt, M. Gottfried, and J. P. Ingwersen. Multi-stage method for producing gas-filled microcapsules, EP1180043, 2002.
17. J. Piehler, A. Brecht, R. Valiokas, B. Liedberg, and G. Gauglitz. A high-density Poly (ethylene glycol) polymer brush for immobilization of glass-type surfaces. *Biosens. Bioelectron.* **15**:473–481 (2000).
18. M. B. Lawrence and T. A. Springer. Neutrophils roll on E-selectin. *J. Immunol.* **151**:6338–6346 (1993).