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Flow dynamics, binding and detachment of spherical carriers targeted to ICAM-1 on endothelial cells

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Abstract

Vascular drug delivery by administration of carriers targeted to endothelial surface determinants, such as intercellular adhesion molecule (ICAM-1), holds considerable promise to improve disease treatment. As a model to define elusive factors controlling the interplay between carrier motion in the bloodstream and its interactions with molecular targets in the endothelial wall, we used 1 μm beads coated with ICAM-1 monoclonal antibody (Ab) at 370, 1100 or 4100 Ab/ μm^2 . Carriers were perfused at two shear rates over resting or activated endothelial cells, expressing minimum vs. maximum ICAM-1 levels, to determine carrier rolling, binding and detachment. Even at 0.1 Pa and 4100 Ab/ μm^2 , carriers attached only to activated cells (21 fold increase over resting cells), ideal for specific drug targeting to sites of pathology. Binding was increased by raising the Ab surface density on the carrier, e.g., $59.4 \pm 11.1\%$ increase for carriers having 4100 vs. 1100 Ab/ μm^2 , as a consequence of decreased rolling velocity. Carrier binding was stable even under a high shear stress: carriers with 1100 and 4100 Ab/ μm^2 withstand shear stress over 3 Pa without detaching from the cells. This is further supported by theoretical modeling. These results will guide vascular targeting of drug carriers via rational design of experimentally tunable parameters.

Keywords

Drug delivery; endothelium; ICAM targeting; particles; targeted delivery

1. Introduction

Targeted delivery of diagnostic and therapeutic agents to diseased tissues may enhance their efficacy and reduce toxicity [43,52]. The vascular route is a standard and convenient path for targeted drug delivery, and endothelial cells (EC) represent an important target [13,40,43,48, 58]. EC are involved in pathologies including inflammation, acute lung injury, atherosclerosis, thrombosis, hypertension and metabolic diseases, among others [7,9,13,19,29,48]. In most of these conditions, pathological factors, including inflammatory cytokines such as interleukin-1- β and tumor necrosis factor- α (TNF- α), stimulate abnormal endothelial expression of intercellular adhesion molecule 1 (ICAM-1), an endothelial trans-membrane glycoprotein [26,32,34]. Therefore, ICAM-1, exposed in EC to the circulation and implicated in pathogenesis of diverse diseases [34,43,60], is a good target for drug delivery. ICAM-1 monoclonal antibodies can be coupled to drug carriers [43,48,60] for endothelial-targeted delivery. There is a vast literature reporting the targeting of anti-ICAM particles to EC in cell culture and *in vivo* for applications including delivery of drugs, therapeutic enzymes [5,18, 33,35–39,41], and ultrasound and magnetic resonance imaging contrast probes [54,56–58]. Binding of anti-ICAM/carriers to endothelial ICAM-1 may also attenuate inflammation by blocking leukocyte binding [2,22].

For optimal carrier targeting to EC, diverse design parameters have to be defined, including affinity of the targeting vector (e.g., anti-ICAM) and its density on the carrier surface, as well as the carrier size, shape and dose (concentration in blood). By varying these parameters carriers can be designed to stably bind on the endothelial surface [33,36,40,48], facilitating their uptake by cells and transport to intracellular compartments [37,39,41] or even across the endothelium [44,55]. In theory, strong and stable binding of carriers to endothelial cells may not necessarily need a maximal antibody density on the carrier surface. Indeed, a sub-maximal antibody density may be preferred to reduce potential immune reactions and in cases where presentation of the therapeutic agent on the carrier surface (sharing this area with targeting antibodies) is required.

Understanding the parametric dependencies of carrier anchoring with the target in relation to carrier antibody density (a key parameter that controls the valency and affinity of carrier binding) is required for the design of optimal drug targeting.

Importantly, targeting parameters must be characterized under physiological flow conditions, given that hydrodynamic forces due to blood flow govern the collision interactions of carriers with EC. Shear forces can tear carriers free from the EC surface. Flow also affects the dynamic interaction of carriers with EC. Affinity interactions of model polymeric particles with immobilized ICAM-1 and other endothelial adhesion molecules have been studied in the context of both drug targeting and simulating leukocyte adhesion [14–17,48]. Thus, previous researchers have observed that carriers roll over substrates functionalized with cell adhesion molecules, similar to white blood cells [15–17], or move in a biphasic motion with carriers attaching and detaching from functionalized substrates or EC by jumping dynamically with little or no rolling [6,27,48]. Flow parameters may also affect EC targeting due to their effect on EC phenotype [19,24]. For example, flow affects endothelial endocytosis, which might alter intracellular delivery of carriers [25,46,53].

Resident adhesion times have to be sufficiently long for adequate therapeutic effects to occur or for cellular mechanisms to be activated to render adequate uptake of carriers. The surface density of targeting vector molecules is an important parameter in the ability of the carrier to form stable bonds with the receptors in the EC surface. The bonds formed must be sufficiently high in number and bond strength to withstand variations in shear stress experienced in the vasculature. In this context, it is noteworthy that the effect of antibody density on carrier detachment is an important factor that has not been studied thoroughly under flow conditions.

Previous studies have given important insights into carrier adhesive interactions with endothelial cells and immobilized endothelial molecules under flow [15,17,48]. However, there has not been a systematic study that has quantified the real-time binding and detachment of micron-scale drug delivery carriers with varying vector density under different levels of shear stress. The present study fulfills this gap of knowledge by providing experimental data and a modeling approach to describe the dynamics of adhesion and detachment of targeted carriers to resting and activated EC. We used real-time fluorescence microscopy to visualize and quantify endothelial rolling, binding and detachment of anti-ICAM/carriers under flow in EC cultured under resting or pro-inflammatory conditions. We used 1 μm polystyrene model anti-ICAM/carriers at various anti-ICAM surface densities. These particles provide a simple and reliable model to study vascular drug delivery *in vivo* [38] and have the same binding kinetics as ICAM-targeted PLGA or PGA particles [36], which could potentially be used in a clinical setting [36]. In order to understand the dynamic interaction of carrier with EC, we first determined instantaneous rolling velocities and qualitatively observed dynamic interactions of carriers with EC. Binding kinetics was also quantified under physiological flow conditions. Finally, the effect of increased shear stress on carrier detachment was tested. Based on empirical data obtained we offer a general theoretical analysis of how antibody surface density affects endothelial detachment of carriers under flow conditions. This work offers new insight into the interaction, attachment and detachment of targeted carriers to EC by observing with real-time microscopy how carriers behave under flow conditions.

2. Materials and methods

2.1. Antibodies and reagents

Green–yellow fluorescent 1- μm diameter spherical polystyrene latex particles (Polysciences, Warrington, PA, USA) were coated with monoclonal antibody R6.5 [26] against human ICAM-1. Iodogen was purchased from Pierce Biotechnology (Rockford, IL, USA). All other reagents were from Sigma Chemical (St. Louis, MO, USA).

2.2. Preparation of anti-ICAM/carriers

Anti-ICAM/carriers and control IgG/carriers were prepared by absorption of R6.5 or non-specific IgG onto the surface of polystyrene beads. Free non-coated anti-ICAM or IgG were then separated by centrifugation, and the coated carriers were finally resuspended in PBS supplemented with 1% BSA [59]. The amount of antibody on the particle surface was quantified in a gamma counter using carriers prepared with a mixture of 90% R6.5 and 10% ^{125}I -R6.5 [40]. Anti-ICAM/carriers at three surface densities were used: 370, 1100 and 4100 antibody (Ab) molecules per μm^2 , corresponding to 1160, 3460 and 12,900 anti-ICAM molecules per carrier, respectively. Table 3 shows the saturation percentage that anti-ICAM molecules cover on the surface of the carriers. These densities were chosen so that enough space on the surface of carriers was still available in order to add potential therapeutic material. The saturation level was previously measured to be close to 7000 Ab/ μm^2 [36]. The surface concentration of control IgG carriers used was 4100 Ab/ μm^2 . The diameter of the final anti-ICAM and IgG carriers averaged $1.14 \pm 0.21 \mu\text{m}$, as measured by dynamic light scattering [40,59]. Bare carriers without anti-ICAM or IgG were also used for control experiments.

2.3. Cell culture

Pooled human umbilical vein endothelial cells (HUVEC) (Lonza, San Diego, CA, USA) were cultured on gelatin-coated $22 \times 40 \text{ mm}$ glass coverslips at 37°C , 5% CO_2 and 95% relative humidity in M199 media supplemented with 15% fetal bovine serum, 2 mM glutamine, 15 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement, 100 $\mu\text{g}/\text{ml}$ heparin, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin [42,59]. To mimic inflammatory activation of endothelial cells (EC) causing maximal expression of ICAM-1, confluent cells (passages 4 to 5) were treated overnight with

10 ng/ml of TNF- α , vs. control media used in the case of resting cells expressing minimum levels of ICAM-1. The increase in ICAM-1 expression has been measured previously by us [33] and increases 60 to 100 fold from resting cells to activated cells. The total amount of ICAM-1 on the surface of the activated endothelial cell was estimated to be in the order of $\sim 10^6$ molecules/cell [33,36].

To study binding of anti-ICAM/carriers under flow conditions, confluent EC were fixed with cold 2% paraformaldehyde prior to the experiments. Fixed cells were used instead to live cells to avoid the influence of carrier internalization, which may have confounded our results [42]. Verifying the adequacy of this model, binding of 4100 Ab/ μm^2 anti-ICAM/carriers was similar for both activated live and fixed cells (15.2 ± 1.8 vs. 15.9 ± 2.5 carriers per cell, respectively) at incubation times less than 15 min, for which internalization is not significant. This comparison was done for 15 min because after that time the cells will likely begin to internalize the carriers as well as start to remove or recycle ICAM-1 from the cell surface [33,37]. These two factors will strongly influence the dynamics of carrier interactions with cells and alter attachment.

2.4. Flow chamber assays

After cell fixation, the coverslips were mounted in a parallel-plate flow chamber (RC-30HV; Warner Instruments Inc., Hamden, CT, USA). Cell culture media (described above) supplemented with 1% BSA was used to perfuse the chamber from a re-circulating reservoir. Anti-ICAM or IgG coated carriers at three different antibody surface densities were then added to the reservoir at a final carrier concentration of 6.825×10^8 carriers/ml, and were perfused through the flow chamber using a peristaltic pump (Pharmacia Biotech AB, Uppsala, Sweden).

Experiments were done at two wall shear stress levels: 0.1 and 0.5 Pa. Shear stress was calculated using:

$$\tau = \frac{6\mu Q}{h^3 w}, \quad (1)$$

where μ is the fluid viscosity, Q is the average flow rate, and h (375 μm) and w (1.8×10^3 μm) are the flow chamber's height and width, respectively.

We chose a peristaltic pump because it allowed for particle recirculation during the experiments. A non-recycling perfusion approach was simply unrealistic to use in terms of total amount of particles otherwise required. To assure that the oscillatory component of the peristaltic pump flow does not affect carrier binding and detachment, we performed limited additional experiments using a syringe pump to provide continuous flow at a shear stress of 0.1 Pa. Carrier binding was statistically no different for the two systems at 60 min (17.87 ± 1.2 carriers/cell for the peristaltic pump versus 16.31 ± 0.85 carriers/cell for the syringe pump) using activated cells and carriers coated at 4100 Ab/ μm^2 .

To study rolling velocity, binding and detachment of anti-ICAM/carriers, the flow chamber was placed on a fluorescence microscope (Eclipse TE2000-U; Nikon, Melville, NY, USA) equipped with a 40 $\times$ /NA1.4 PlanApo objective (Nikon). Time-lapse fluorescence and phase-contrast images were captured using an Orca-1 charge-coupled device camera (Hamamatsu, Bridgewater, NJ, USA) and analyzed using Image Pro 3.0 software (Media Cybernetics, Silver Spring, MD, USA). Time points from 2 min to 3 h were analyzed for rolling velocities and carrier binding. For each time point, 20 or 30 consecutive images (0.1 or 0.06 s apart) were taken within a definite time interval, and from three different fields of views.

2.5. Rolling velocities and binding data analysis

Carriers were first observed under a live preview to make qualitative observations in real time. Instantaneous rolling velocities of carriers were subsequently calculated by analyzing the fluorescent digital images obtained. For the time points selected, two consecutive images were combined, and the displacement between carriers was measured and divided by the time interval between the captured frames. The mean rolling velocities and standard error were then calculated by averaging at least 50 different instantaneous velocities of carriers for each condition. Carrier rolling was determined to have occurred if a carrier remained in focus on the cell surface while traveling at least 100 μm . We excluded from the analysis those carriers, which only randomly and transiently contacted the cell surface, as in the case of control IgG/carriers and bare carriers. It was crucial to focus on the surface of the cells in order to differentiate between rolling carriers and carriers present in the free stream. Carriers rolling along the surface of the EC distinctly appear as spheres, whereas the carriers located in the free stream appear out of focus and also as streaks (see Fig. 2).

To distinguish between rolling carriers and firmly bound carriers, the average light intensity of all consecutive images was computed for each time point. The carriers that were firmly bound to the EC surface appeared in all or most of the frames, hence yielding a higher average light intensity at the corresponding pixels. The carriers that only rolled over the cells but did not firmly bind, disappeared from the average light-intensity image (see Fig. 2). Kinetics of binding for carriers were calculated as mean $\pm$ standard error of the mean per cell ($n \geq 30$), for each time recorded.

2.6. Detachment experiments

After assessing binding kinetics for 3 hours at 0.1 Pa, the detachment of anti-ICAM/carriers comprising 1100 or 4100 $\text{Ab}/\mu\text{m}^2$, already firmly bound to the cells, was examined by fluorescence microscopy by gradually increasing the shear stress from 0.1 to 13 Pa. In the case of 370 $\text{Ab}/\mu\text{m}^2$ anti-ICAM/carriers, given their very marginal binding to the cells under flow conditions, carriers were first incubated with cells for 3 hours under static conditions and non-bound carriers were then washed, followed by gradually increasing the shear stress to assess detachment of the pre-bound carriers. In all cases, flow rate was increased every 5 minutes (t_0 was considered the start time when a new flow rate was applied), by increments of 0.1 Pa until 3 Pa and then increased by increments between 1.0–2.0 Pa. The number of carriers still bound to the cells was determined after each period (t_f). The ratio of bound carriers at times t_f and t_0 was calculated.

2.7. Theoretical analysis of detachment

To examine the influence of flow on anti-ICAM/carriers bound to EC, a theoretical analysis was used to estimate the number of bonds formed between the carriers and the cells, and the minimum number of bonds needed to prevent detachment. The number of bonds engaged, as well as the size of the carrier and the shear stress to which carriers are exposed, will determine if the carrier remains bound to or detaches from the cell surface, due to the hydrodynamic forces experienced. The number of bonds formed between the carriers and the cells depends mainly on the surface density of antibodies (“ligands”) on the carriers, the target (“receptor”) density on the cell surface, the size and relative curvature of both the cell and the carrier, and the energy and chemical properties between the antibody–target (“ligand–receptor”) bonds.

The more bonds are formed, the stronger the carrier attachment to the cell surface is, and the higher hydrodynamic forces the carriers can withstand. Our interest is to estimate the number of bonds formed once a carrier is bound to a cell. The total number of bonds that can form depending on the geometry of a sphere can be expressed as [30]:

$$B_{\text{Total}} = 2a\pi\delta_b\rho_l, \quad (2)$$

where a is the effective radius of interaction between the cell and the carrier (this value is approximated as the radius of the carrier, since the surface of curvature of EC is significantly larger than that of the carriers), δ_b is the length of the antibody–target bond and ρ_l is the antibody density on the carrier surface [31]. The antibody surface-density is the limiting density between the antibody and the target in activated EC, due to the over expression of ICAM-1 in the cell surface [31].

The values for the different parameters determined are shown in Table 1. For δ_b in Eq. (2) we use an estimated compressed value for R6.5 of 18 nm taken from Agrawal and Radharkrishnan, 2007 [1]. Equation (2) assumes that the carrier and the cell are in contact as in Fig. 1, and that $a \gg \delta_b$, which is appropriate for these carriers and the anti-ICAM–ICAM-1 interaction described here. Equation (2) gives a maximum number of bonds that a carrier can form with the cell, but it does not take into account the random orientation of antibodies in the surface of carriers [1] and other more complicated aspects of the carrier–cell interaction, including energy of bond formation, flexibility of receptors, and potential effects of the cell–glycocalyx layer [1,11,30,31]. Assuming the same probability of binding for any antibody orientation [1] we can use a correction factor (C_f) for the number of antibodies in the correct orientation to form a bond. We incorporate this correction factor as:

$$B_{\text{bound}} = \frac{2a\pi\delta_b\rho_l}{C_f}. \quad (3)$$

This correction provides a fair estimate on the amount of bonds formed due to geometry and the surface density of antibodies on the carriers.

The number of bonds formed should be enough to prevent detachment of carriers from physiological hydrodynamic forces. The hydrodynamic forces that a carrier experiences are mainly a drag force (F_D), and a torque on the stretch bonds (T). For a spherical bound carrier F_D and T can be expressed as [20]:

$$F_D = 10a^2\pi\tau, \quad (4)$$

$$T = 12a^3\pi\tau. \quad (5)$$

The torque is assumed only to affect the stretch bonds, which should be equal or less than the half of the total number of bonds engaged, F_D affects all the bonds. F_D and T will act on the bonds formed on the contact area (A_c) between the carrier and the cell [11]. This contact area can be calculated to be:

$$A_c = \pi a^2 \left[1 - \left(1 - \frac{d\delta_b}{a} \right)^2 \right], \quad (6)$$

where $d\delta_b$ is the difference between the maximum stretch and the maximum compression length of the bond. If the total hydrodynamic force (F_{hf}) experienced by the carriers is higher

than the total force provided by the bonds engaged, carriers will detach from the surface of the cell. The number of bonds to prevent detachment (B_d) of a carrier under shear can be calculated by $B_d = F_{hf}/S_b$, where S_b is the force acting on the anti ICAM–ICAM bond. Equation (3) and Equation (4) are added to calculate F_{hf} which in the final form is [11]:

$$F_{hf} = F_d + \frac{2T}{a[1 - (1 - d\delta_b/a)^2]^{1/2}}. \quad (7)$$

Using these sets of equations we can estimate the number of bonds needed to prevent detachment at different levels of shear stress in terms of carrier size and antibody density. The process of bond formation is stochastic in nature and others have calculated the probabilistic likelihood of bond formation [28] based on kinetic formulations. Piper et al. [45] introduced a probability of adhesion (P_a) which is defined as the probability to form at least one ligand–receptor bond. The larger P_a is the stronger the adhesive strength of a particle will be to the substrate. P_a is defined as:

$$P_a = \rho_r \rho_l K_z^0 A_c \exp\left(-\frac{\lambda S_b}{k_b T}\right), \quad (8)$$

where ρ_r is the receptor density, K_z^0 is the association constant with no load on the bond and has units of length squared and λ is a characteristic length of the bond. It is important to note that P_a varies linearly with both ligand density (ρ_l) and A_c . Given this relationship it is reasonable to use a geometrical formulation to understand adhesion strength in terms of antibody density. It is also important to note that increasing the force acting on the bonds, S_b , will decrease the adhesion strength of the carriers, thus making detachment more likely as hydrodynamic forces increase.

2.8. Statistics

The data were calculated as mean $\pm$ standard error. Statistical significance for differences between groups was determined by two-tailed Student's *t*-test and taken as $p < 0.05$.

3. Results

3.1. Rolling behavior of anti-ICAM/carriers

We measured the rolling velocity of 1- μ m carriers functionalized with anti-ICAM at three surface densities using real-time fluorescence microscopy. The microscope was focused on the cell surface and only carriers that appeared as round spheres and were observed to move over the cells for a minimal threshold distance $\geq 100 \mu$ m were considered to be rolling. Rolling behavior of control IgG/carriers was excluded from the study, given that they rolled distances on the cell surface below the minimal threshold limit before they rapidly disappeared from the focal plane and entered the free stream flow. Anti-ICAM/carriers under flow conditions were observed to display several distinct patterns: (i) moving in the flow at high speed without visible interactions with EC (similarly to IgG/carriers and bare carriers); (ii) rolling continuously over EC; (iii) rolling first and then binding firmly, and (iv) rolling first and detaching thereafter as they traveled along the cell surface.

Figure 2 shows a time-sequence example of rolling patterns of 4100 Ab/ μ m² anti-ICAM/carriers after perfusion at a shear stress of 0.1 Pa for 30 min. At this initial time, several carriers are already bound to the cells while others are rolling (spherical carriers marked by closed arrows) or moving freely at high velocities in the stream (appearing as streaks marked by open

arrows). Carrier 1 is a particle that originally rolled over the EC and then bound firmly (circle) to their surface after rolling a distance of 5 μm in 0.7 seconds. Alternatively to rolling followed by firm arrest, some carriers were observed to roll over EC for some distance and later detach, e.g., carriers 2 and 3 in frame t_2 . Carrier 2 enters the field in frame t_2 and rolls for all the remaining frames while carrier 3 rolls from frame t_2 to frame t_3 but in frame t_4 is already seen as a streak object, suggesting its detachment from the cells, and is no longer visible in frame t_5 .

The rolling motion of carriers appeared to be affected by the contour or shape of the cells, as not all the carriers traveled in the same direction of flow. For example, carrier 1 in Fig. 2 did not move parallel to the direction of flow, but instead it appeared to roll upwards as it rolled over the cells. This behavior was observed to diminish as the shear stress increased, e.g., at 0.5 Pa most of the carriers at either antibody density traveled in the direction of flow (not shown), a phenomenon similar to that observed in leukocytes by other researchers [50,51]. The motion of carriers in any direction not parallel to the principal direction of flow may be due to Brownian motion instead of due to interactions with the cell surface. In order to investigate this behavior further we used bare carriers at 0.1 Pa. These carriers followed the direction of the flow with very little non-parallel motion. We interpret this to indicate that there was very little direct interaction of bare carrier with the cells, and the very small deviations in carrier motion detected were the result of small variations in the flow field due to the contours of the cells. Bare carriers did not reside on the cell surface, and any Brownian motion present was of such small amplitude as to be non-observable. For antibody or IgG coated carriers, most carriers moved over the cells in a continuous motion, and very few carriers were observed to move in a biphasic behavior, as described by others [6,27,48]. The few carriers that demonstrated a biphasic pattern were those coated at 4100 Ab/ μm^2 .

Table 2 shows the rolling velocity quantified for carriers presenting three different anti-ICAM surface-densities. Only very few 370 Ab/ μm^2 carriers were observed to roll over the cells at 0.5 Pa ($n = 15$ carriers), while there were no notable differences in the number of carriers rolling between 1100 vs. 4100 Ab/ μm^2 anti-ICAM/carrier, at either shear stress ($n > 50$ carriers). Thus, reducing antibody surface density below ~ 1000 Ab/ μm^2 greatly diminishes the probability of a carrier to engage into productive adhesive interactions with the cells. In general, the rolling velocity decreased and increased, respectively, by increasing anti-ICAM surface density and shear stress in activated cells.

In resting cells very few carriers appear to roll over the cell surface even at 0.1 Pa. We did not observe any examples of either carrier biphasic motion or carriers that rolled and subsequently became firmly bound to the cells. We were able to calculate rolling speeds for 4100 Ab/ μm^2 carriers rolling over resting cells (38.4 ± 3.6 $\mu\text{m/s}$), which was faster than the speed of the carriers of the same antibody density rolling over activated cells. Even carriers having high antibody density, e.g., 4100 Ab/ μm^2 , exerted minimal binding to resting cells, similar to that of non-specific IgG/carriers (1.99 ± 0.9 vs. 0.9 ± 0.2 carriers/cell at 180 min).

3.2. Firm binding of anti-ICAM/carriers to endothelial cells

To investigate how antibody surface-density on carriers influences endothelial anchoring, we assessed binding kinetics at 0.1 and 0.5 Pa. These values of shear stress are within the range of shear stresses occurring in capillaries, venules and small arteries where we expect most attachment of therapeutic carriers to occur *in vivo* [18,33,38]. Experiments were also done at 1 Pa with 4100 Ab/ μm^2 carriers using activated cells. Binding in that case was low and comparable to levels of non-specific IgG binding (2.11 ± 1.3 vs. 0.9 ± 0.2 carriers/cell at 180 min). Because binding at this higher shear stress was minimal, we concentrated our work on experiments performed using shear stresses of 0.1 and 0.5 Pa. The number of anti-ICAM/carriers bound per cell increased as time progressed, Fig 3 and Fig 4, which was specific as no

significant binding of IgG/carriers to cells was observed within the same time period. As an example, Fig. 3 shows cell binding of IgG/carriers vs. anti-ICAM/carriers at 0.1 Pa. The microscope plane was focused on the endothelial plasmalemma. Under these visualization conditions, even those few images of IgG/carriers that could appear bound to the cells were less bright than the images of the cell–gelatin coated surface bound anti-ICAM/carriers, due to their shorter residence in the cell surface (see Section 2). This is the case, given that these images show the average light intensity per pixel of a total of 20 or 30 frames, hence, less intensity of light indicates that a carrier was not bound to the cell surface for all the averaged frames. This reveals that some IgG/carriers may not be firmly bound to EC, but transiently associated with cells at a given time. Bare carriers did not bind to EC, but they did bind to the spaces in between the cells or in open spaces on the gelatin-coated surface.

Figure 4 shows average light-intensity images and their quantification, regarding binding kinetics of anti-ICAM/carriers at 0.1 or 0.5 Pa. Despite the basal expression of ICAM-1 in resting cells ($\sim 10^4$ molecules/cell [47]), carrier binding was low (1.99 ± 0.9 carriers/cell, at 180 min) under this condition, indicating a high selectivity toward pathologically altered EC. Hence, we thereafter focused on activated cells.

As expected, both increasing the antibody surface-density of anti-ICAM/carriers and decreasing shear stress resulted in an increase binding of the carriers to EC. Carriers with 4100 and 1100 Ab/ μm^2 had significant binding to EC ($p < 0.05$) compared to IgG carriers for time points after 5 min (Fig. 4(B, C)). At both shear stresses, 370 Ab/ μm^2 anti-ICAM/carriers exhibited no specific binding over the basal level exerted by IgG/carriers ($p > 0.05$) (Fig. 4(B, C)). At 0.1 Pa, 4100 Ab/ μm^2 carriers bound to EC significantly ($p < 0.05$) better than 1100 Ab/ μm^2 carriers at all time points (Fig. 4(B)). The differences in the number of carriers bound per cell between 1100 and 4100 Ab/ μm^2 carriers became greater after 30 minutes, reaching a maximum difference of 59.2% at 0.1 Pa. Regarding experiments at 0.5 Pa, at early time points (0–15 min) both 1100 and 4100 Ab/ μm^2 carriers bound similarly to cells ($p > 0.05$), but after 15 min the differences in the binding of 1100 and 4100 Ab/ μm^2 anti-ICAM/carrier increased considerably (43.2% increase from 1100 to 4100 Ab/ μm^2 at 180 min, $p < 0.05$) (Fig. 4(C)).

Binding of both 1100 and 4100 Ab/ μm^2 anti-ICAM/carriers increased linearly with time, e.g., at a binding rate (K_b) of 0.145 and 0.217 carriers/cell/min, respectively, at 0.1 Pa, or 0.075 and 0.110 beads/cell/min, respectively, in the case of 0.5 Pa. Hence, K_b decreased with increased shear stress, specifically, K_b decreased by half. When compared for each independent time point, 1100 Ab/ μm^2 carriers bound similarly at 0.1–0.5 Pa between 2 and 45 min, but after this time the binding at 0.1 Pa clearly surpassed that at 0.5 Pa by 1.7 fold. In the case of 4100 Ab/ μm^2 carrier, they bound more efficiently at 0.1 Pa than at 0.5 Pa for all the time points tested (also, 1.7 fold greater). As a control, binding was close to zero for 370 Ab/ μm^2 carriers at both shear stresses, suggesting a non-specific interaction similar to that of IgG/carriers.

3.3. Carrier detachment

Figure 5 shows the detachment of carriers presenting different antibody surface-densities from activated EC as shear stress is increased. Anti-ICAM/carriers with 370 Ab/ μm^2 started to detach at low shear stresses and had over 50% detachment at 1.5 Pa. Nevertheless, even at 13 Pa, 20% of the carriers were still attached to the cells. Detachment of the carriers was expected to occur suddenly after values of shear stress enough to break the bonds formed by carriers and the cells. This steep detachment occurs between 0.6–1.0 Pa for 370 Ab/ μm^2 carriers. On the contrary, detachment of 1100 and 4100 Ab/ μm^2 carriers was minimal after the initial increase of shear, when only 20% and 10% of carriers detached, respectively. However, to our surprise, 1100 and 4100 Ab/ μm^2 anti-ICAM/carriers did not further detach from the cell surface even at extremely high shear stresses (above physiological relevant levels), suggesting that the hydrodynamic forces experienced by these carriers were not enough to break the bonds engaged

by the anti-ICAM/carriers and cellular ICAM-1. To better understand the relationship between antibody surface density on the carriers and their detachment, we used the following theoretical model.

3.4. Theoretical analysis of carrier detachment

Using Eq. (7) we calculated the minimum number of bonds needed to prevent anti-ICAM/carrier detachment from EC at increasing shear stresses, shown in Fig. 6 for carriers of three different sizes. Detachment depends on the hydrodynamic surface area of the carrier exposed to the flow. Because of this, larger carriers will need more engaged bonds to prevent detachment, e.g., 1, 5 and 15 anti-ICAM-ICAM-1 bonds are needed to prevent detachment of 0.1, 1 and 2- μm carriers, respectively, at 1 Pa (Fig. 6(A)).

The density of targeting antibody molecules on the carrier surface is a crucial factor in determining the number of antibodies, which can be available to form a bond with ICAM-1 on the EC surface. From (8) we observed that the higher the antibody density the greater the probability (P_a) that a carrier will engage receptors on the cell and by increasing S_b , P_a reduces which indicates that it decreases exponentially with F_{hf} . Once bound, carriers with higher numbers of engaged bonds can withstand higher shear stresses. Using Eq. (2) we determined the maximum number of bonds that 1- μm anti-ICAM/carriers can engage, depending on its antibody surface density and other parameters from Table 1. For instance, a maximum of 10, 31 and 116 bonds can be engaged by carriers having 370, 1100 and 4100 $\text{Ab}/\mu\text{m}^2$, respectively.

Due to the method of coating carriers with antibodies (i.e., hydrophobic attachment of anti-ICAM to polystyrene beads), the antibody orientation on the carriers is random, for which some antibodies may not be favorably oriented and accessible to form bonds with ICAM-1 in the cell surface. Using Eq. (2) a 1- μm diameter carrier with 370 $\text{Ab}/\mu\text{m}^2$ can establish at maximum 16 bonds with a cell. This is sufficient to prevent detachment at a shear stress of up to 3.2 Pa (see Fig. 6). However, experimental data shown in Fig. 5 demonstrates that most 370 $\text{Ab}/\mu\text{m}^2$ carriers detach from cells between 0.6–1.5 Pa. Instead, assuming that half of the anti-ICAM molecules are correctly oriented on the carrier surface ($C_f = 2$), 370 $\text{Ab}/\mu\text{m}^2$ carriers could likely withstand 1.5 Pa. Yet, it is reasonable to assume that some carriers will detach at a lower shear stress because not all of the bonds possible will actually be engaged. Some carriers may also be sequestered within ridges or valleys of the contoured cell surface and thereby be exposed to a smaller hydrodynamic force, enabling them to remain attached despite a higher bulk shear stress.

Figure 6(B) shows the percentage of antibody molecules that need to establish engagement with ICAM-1 from the total number of possible bonds, to prevent carrier detachment at different shear stresses ($100 \times B_d/B_{\text{bound}}$ vs. τ). One hundred percent of the antibodies available to establish bonds need to be engaged to prevent detachment of 370 $\text{Ab}/\mu\text{m}^2$ carriers at 1.5 Pa. However, at 4.5 Pa, 90% and 25% of the possible bonds need to be established for 1100 and 4100 $\text{Ab}/\mu\text{m}^2$ carriers, respectively. This will explain why 1- μm carriers with higher antibody densities ($>1100 \text{ Ab}/\mu\text{m}^2$) do not detach even at very high levels of shear and can withstand very high hydrodynamic forces once bound at the EC surface.

4. Discussion

In this work, we have explored flow dynamic parameters of prototype carriers targeted to ICAM-1, a convenient and representative model for which an extensive number of fragmentary targeting studies are available [5,10,13,48]. In particular, we determined the rolling velocities, binding kinetics and detachment of 1- μm anti-ICAM/carriers presenting three different antibody surface-densities, at two different levels of shear with resting and activated EC. Analysis of this study, focused on optimization of drug targeting, may benefit from analogies

with leukocyte adhesive interactions with endothelium under flow. Endothelial adhesion molecules including selectins and ICAM-1 are being explored as both the targets for drug delivery and as adhesive counterparts for rolling and firm adhesion of leukocytes [4,5,13,16, 29]. By analogy to these processes governed by multivalent molecular interactions, functionalized carriers for drug delivery have the ability to bind to EC, yet they are also subjected to detaching forces of blood flow [23,48,58]. Like leukocytes, these carriers can potentially bind either temporary or firmly to the endothelial wall depending on the number of effective bonds engaged in such interaction, and likely a threshold number of such bonds are required to prevent the carrier from moving or detaching from the endothelial surface. Digital analysis of real-time fluorescence imaging of our experimental model indicates that anti-ICAM/carriers initially rolled over EC and eventually became firmly bound to the cell surface (see Fig. 2). These results are similar to those of other groups, which used antibody-coated particles to simulate parameters of white blood cells interaction with the endothelium [15, 17], which validates our model.

A strategy of using fixed cells in a parallel-plate flow chamber was used to investigate endothelial-targeting parameters at a physiological temperature, without the influence of other parameters involving rapid mobilization of ICAM-1 from the plasma membrane [37,42]. For instance, anti-ICAM/carriers are known to be internalized by EC within minutes after binding to the cell surface [42], and this process rapidly retrieves surface ICAM-1 into intracellular vesicles, which then becomes inaccessible for carrier binding [37]. All these concurrent events would have influenced and mislead our interpretation of the targeting parameters studied in this work.

We observed that some $4100 \text{ Ab}/\mu\text{m}^2$ carriers demonstrated biphasic motion over activated EC, but the majority of the carriers were observed to roll smoothly over EC. Sakhalkar et al. observed that $1\text{--}2.5 \mu\text{m}$ diameter particles coated with anti-ICAM exhibited a biphasic motion while interacting with the cells [48]. An explanation for the different dynamic interactions observed between anti-ICAM/carriers and EC in that work, regarding biphasic motion or smooth rolling, may be due to the distance of the particle to the surface of the cell and/or to the size of the particles. Both the distance between the cell and the particle and the particle size in [48] are higher than that of our work. This may cause a higher lift and increased the hydrodynamic forces acting to detach the particles, thus raising the likelihood of detachment occurring before firm adhesion results. Another possibility is that the biphasic motion was present but the residence time was short and thus was not captured by our camera even in the live preview due to a time resolution limit. If the residence time was short ($<60 \text{ ms}$) then the biphasic motion could be present without our having detected it.

The number of bonds needed for a carrier to bind firmly to EC depends on the level of shear that the carriers experience and the individual bond force for the antibody–target (“ligand–receptor”) pair [1]. Increasing the number of antibodies in the surface of anti-ICAM/carriers and/or the ICAM-1 receptors in the EC surface should increase the probability of adhesion, or P_a (see Eq. (8)), thus slowing carriers in motion, as shown in this work and by others [15,17]. The results presented here show that, indeed, having more antibodies on the surface of the carriers decreases their rolling velocity and increases their binding efficiency. As expected, there is a minimal limiting number of antibody molecules needed to establish effective specific binding under flow (e.g., binding of carriers with $370 \text{ anti-ICAM Ab}/\mu\text{m}^2$ showed binding levels similar to the non-specific binding of IgG/carriers to EC at the shear stresses studied). However, an antibody density well below saturation on the carrier surface ($1100 \text{ Ab}/\mu\text{m}^2$) still provides effective and specific binding to EC (Fig. 4). Table 3 shows the percentage of the area occupied by antibodies in the surface of the carriers employed in this work. Carriers with $1100 \text{ Ab}/\mu\text{m}^2$ have only 15.6% of their total surface occupied by anti-ICAM. This indicates that it is possible to co-couple drugs at the carrier surface together with targeting antibodies, and still

achieve significant targeting to EC, as it has been shown in previous works using animal models [18].

In this study binding of carriers to resting EC was minimal and markedly increased (~21 fold) in cells activated with TNF- α . These results show high carrier selectivity toward activated EC under flow conditions, which imitate rates of shear stress typical to a variety of blood vessels in arterial and venous vasculature. ICAM-1 is expressed at a basal level in resting conditions and this expression is increased 10–100 fold in activated EC cultures [33,47]. While others have seen a more modest 10-fold increase [47], in our cell models, ICAM-1 expression increases from resting to activated EC by a factor of 60 (in cells in suspension measured by FACS) or 100 (in adherent cells measured by radioisotope tracing) [33], up to a maximum of 1.6×10^6 ICAM-1 molecules per cell [36].

Although binding of carriers to EC in resting conditions is categorized here as minimal compared to that of activated EC, it shall be noted that approximately 2 carriers bound per cell corresponds to approximately 3200 carriers/mm² (EC area was measured at $612.29 \pm 110.67 \mu\text{m}^2$). These values are over 150 fold higher than those previously reported for the binding of similar anti-ICAM/carriers to resting EC [48]. In that study the increase of carrier binding from resting to activated EC was a modest 2 fold. This observed difference may be due to the different incubation time with the inflammatory agent (12 h vs. 4 h in [48]), which may dramatically influence the level of expression of ICAM-1.

The possibility of coupling a variety of other functional agents on the surface of carrier particles, along with targeting antibodies, is particularly interesting in the case of carriers with a considerable surface area, such as those carriers in the micron-size range used in this work. We have observed that the characteristic threshold length for spherical drug delivery carriers may be 1 μm . Spherical carriers larger than this characteristic length show non-specific binding *in-vivo* probably due to mechanical lodging within vessels as opposed to targeted adhesion to EC [38]. Nevertheless, different types of particles (degradable polymers, latex, microbubbles, etc.) above 1 μm in diameter have been used to simulate leukocyte rolling and binding, and to study endothelial targeting of contrast imaging with ultrasound [23,38,48,58]. In these model systems, increasing shear stress was found to decrease the endothelial binding of the functionalized particles [21,48,58], which was a pattern we also observed here with 1- μm carriers.

The size and shape of the carriers are also important considerations under flow conditions and can be tunable parameters for targeted delivery optimization [12,49]. In future work, we plan to study the influence of the carrier size and shape on their endothelial targeting under flow conditions. Since these parameters will also determine the hydrodynamic forces that the carriers experience and carriers with a given geometry could be designed depending on the desired vasculature to be targeted and the therapeutic application. For instance, smaller carriers that are less susceptible to hydrodynamic forces (see Fig. 6) may be preferable for drug delivery to large vessels, while larger carriers may be preferred for targeting capillaries or tumor vasculature with imaging probes, where size of the probe is important [54].

In addition to binding, we also tested whether anti-ICAM-1/carriers bound to the EC surface detached from the cells, e.g., depending on their antibody surface-density. The magnitude of the hydrodynamic force a carrier can withstand while remaining attached depends mainly on (1) the strength of, and the degree of stretch an anti-ICAM–ICAM-1 bond can withstand, and (2) the number of effective bonds that these carriers can establish in comparison to the critical hydrodynamic forces [3] that they experience. Regarding the shear stress that 1- μm anti-ICAM/carriers can withstand without detaching from the EC surface (Fig. 6), this is relatively high in the case of 1100 and 4100 Ab/ μm^2 carriers which did not detach from EC even at high shear

stress (5.0 Pa). However, 370 Ab/ μm^2 carriers detached at relative low shear stresses (~ 0.6 Pa), which from Eq. (3) and Fig. 6(B) we can observe to correspond to over 55% of B_{bound} engaged.

Interestingly, although 370 Ab/ μm^2 anti-ICAM/carriers started to detach at 0.6 Pa, 20% of these carriers were still attached at very high levels of shear. This heterogeneous detachment of carriers could be reflective of fluctuations in the chemical reaction between the bonds [8], making some bonds stronger than others. This phenomenon has been previously observed and discussed [8]. Another reason for this behavior could relate to the adhesion of the antibodies to the surface of the carriers. Based on the simple model of antibody absorption, about one third of the molecules can be oriented unfavorably for antigen binding (e.g., by Fc-fragment outwards). This orientation heterogeneity is accompanied by a random distribution of the total number of antibody molecules per carrier, which can reasonably vary by 5–10% within the reproducibility of our detection method using radiolabeled IgG. Together these factors may account for the binding behavior of carriers not being entirely uniform. This suggests that even those carriers presenting low antibody surface-density (which, hence, have a low probability of binding to EC) may engage enough bonds to prevent their detachment once they are bound to the cell surface, but in relatively low quantities (e.g. 20% as shown in Fig. 5).

Equation (8) gives a probability of adhesion that depends on several factors. We have concentrated our focus on the importance of the antibody surface density, the size of the carrier, and S_b . Increasing F_{hf} increases the force that each bond experiences. Previous works [3,11] demonstrate that the detachment process is not linear since the detachment of bonds also has non-linear kinetics. Even so, by increasing S_b , P_a in fact decreases because F_{hf} increases. The smaller the value of P_a , the easier it is to detach carriers from cells. There exists a critical force to detach carriers from the cells [3,8]. This critical force for the overall detachment can be extrapolated by estimating the bond strength of each anti-ICAM–ICAM bond, which is taken from literature as 150 pN [1]. Using this bond strength we have modeled the maximal shear stress carriers can withstand with a fixed initial number of engaged bonds.

There are at least three possible breaking points in order for a carrier to detach from the EC: (1) ICAM-1 can be detached from the cell membrane with the carrier, (2) the anti-ICAM–ICAM bond can break, and (3) the bond between anti-ICAM and carrier can break. It is difficult to assess the specific break point within these experiments, but cell fixation makes it unlikely that ICAM-1 can detached from the cell membrane without proteolytic cleavage. It is also unlikely that the weak spot is the bond between anti-ICAM and the carrier, because even sonication of anti-ICAM/carriers does not cause detachment of radiolabeled antibody (our unpublished results). We therefore believe that antibody receptor bonds are the most likely yield spot. Current research in our lab focuses on assessing these different interactions by atomic force microscopy.

It has been shown that these bonds behave like a spring and have a range of force values depending on stochastic nature of the bond formation and stretch [3]. Nevertheless, the analysis used here to understand the maximum bond force takes into account the dimensions of the particle and antibody and has been used by others [11] previously. It gives an adequate estimate of the forces needed to prevent detachment and the values for the bond strength have been calculated from [1] for R6.5 anti-ICAM antibody.

5. Conclusion

The work presented here shows that hydrodynamic forces resulting from the shear stress, as well as the surface-density of the targeting antibody on the carrier, both influence carrier binding to EC. We were able to quantify and qualitatively describe the dynamic adhesion and

binding kinetics of anti-ICAM/carriers to EC under flow conditions. These carriers were shown to withstand high levels of shear at the two higher antibody surface-densities. These results will help design a more complete computational model for carrier binding and detachment. This quantitative characterization of the dynamics of carrier binding to EC under shear flow conditions demonstrates that carrier targeting is a function of experimentally tunable parameters that may be optimized to enhance therapeutic capabilities of endothelium-targeted carriers.

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Fig. 1. Schematic representation of the binding of an antibody coated carrier to endothelial cell receptors under flow conditions. The value of δ_b is an estimated length of the antibody–receptor bond, which varies depending on the compression and stretching of the bond in dynamic conditions. F and T are the drag force and torque that the carriers and engaged bonds experience under uniform shear stress, respectively.

Fig. 2.

Rolling patterns of anti-ICAM/carriers over HUVEC under flow conditions. Fluorescent 1- μm diameter anti-ICAM carriers ($4100 \text{ Ab}/\mu\text{m}^2$; $6.825 \times 10^8 \text{ carriers/ml}$) were injected into a parallel-plate flow chamber containing a TNF- α activated HUVEC monolayer, perfused at 0.1 Pa (the top-left large arrow indicates the flow direction). Representative frames taken every 0.2 s show carriers which are initially firmly adhered to the cell monolayer (oval mark), non-bound carriers freely flowing in the media (3, open arrows), and carriers rolling on the endothelial surface (1, 2; arrowheads), one of which finally firmly adheres to the cells (1, circle). Carrier 3 originally appears as rolling in frames t_2 and t_3 , but in frame t_4 appears as a streak and no longer present in t_5 . The length bar is 5 μm .

Fig. 3. Binding of IgG vs. anti-ICAM carriers to HUVEC under flow. Time-lapse fluorescence microscopy images of anti-ICAM/carriers or control IgG/carriers ($4100 \text{ Ab}/\mu\text{m}^2$) binding to TNF- α activated HUVEC at 0.1 Pa. Scale bar is 5 μm . (The colors are visible in the online version of the article.)

Fig. 4.

Effect of antibody surface density of anti-ICAM/carriers on their binding kinetics under flow. (A) Time-lapse phase-contrast and fluorescence microscopy merged images of anti-ICAM/carriers binding to TNF- α activated HUVEC at 0.1 Pa. Quantitative analysis of binding kinetics is demonstrated for (B) 0.1 Pa and (C) 0.5 Pa. Data represent mean $\pm$ standard errors ($n = 30$ carriers). In some cases small error bars are hidden by the symbols. (The colors are visible in the online version of the article.)

Fig. 5. Shear stress-induced detachment of anti-ICAM carriers from HUVEC. Data represent mean $\pm$ standard errors ($n = 30$ carriers).

Fig. 6.

Estimated number of anti-ICAM–ICAM-1 bonds required to prevent carrier detachment under flow. (A) The drag force and torque were calculated using the schematic in Fig. 1 and Eq. (7). The minimum number of engaged bonds needed to prevent detachment (B_d) was calculated using an estimated value of bond strength for anti-ICAM-1 R6.5–ICAM-1 (150 pN as per [46]). (B) The number of bonds needed to prevent detachment, B_d , was divided by the total number of engaged bonds, B_{bound} , for the different antibody carrier surface densities, and plotted against shear stress. The shear stress at 100% detachment is the maximum shear stress that 1- μm carriers can withstand at a specific surface antibody density. Continuous line = 370 Ab/ μm^2 ; dotted line = 1100 Ab/ μm^2 ; dashed line = 4100 Ab/ μm^2 .

Table 1
Parameters of anti-ICAM/carrier-ICAM-1 model

Carrier chemistry	Polystyrene latex
Size	1 μm (diameter)
Surface antibody density	370, 1100, 4100 Ab/ μm^2
Carrier concentration	6.825×10^8 carriers per ml
Shear stress	0.1 vs. 0.5 Pa
Viscosity of media	1.6×10^{-3} Pa s
Bond length	18 ± 1 nm
Bond force	150 pN

Table 2
Rolling speeds of anti-ICAM/carriers over HUVEC ($\mu\text{m/s}$)

Shear stress (Pa)	Antibody density on carriers ($\text{Ab}/\mu\text{m}^2$)		
	370	1100	4100
0.1	42.0 ± 3.0	28.3 ± 2.42	19.4 ± 1.4
0.5	156.0 ± 20.6	117.8 ± 10.2	82.5 ± 6.4

Note: Student's *t*-test: $p < 0.05$ in all cases; mean $\pm$ std. error ($n \geq 15$).

Table 3

Antibody coating of carriers 100% carrier surface saturation = 7000 Ab/ μm^2 as per [36]

Ab/ μm^2	mAb/carrier	% Saturation	B_{bound}
4100	12,881	58.0	116
1100	3456	15.6	31
370	1182	5.2	10