

## Time and Force Dependence of the Rupture of Glycoprotein IIb-IIIa-Fibrinogen Bonds between Latex Spheres

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**ABSTRACT** We studied the shear-induced breakup of doublets of aldehyde/sulfate (A/S) latex spheres covalently linked with purified platelet GPIIb-IIIa receptor, and cross-linked by fibrinogen. Flow cytometry with fluorescein isothiocyanate-fibrinogen showed that an average of 22,500 molecules of active GPIIb-IIIa were captured per sphere, with a mean  $K_d = 56$  nM for fibrinogen binding. The spheres, suspended in buffered 19% Ficoll 400 containing 120 or 240 pM fibrinogen, were subjected to Couette flow in a counter-rotating cone-plate rheoscope. Doublets, formed by two-body collisions at low shear rate ( $G = 8 \text{ s}^{-1}$ ) for  $\leq 15$  min, were subjected to shear stress from 0.6 to  $2.9 \text{ Nm}^{-2}$ , their rotations recorded until they broke up or were lost to view. Although breakup was time dependent, occurring mostly in the first 2 rotations after the onset of shear, the percentage of doublets broken up after 10 rotations were almost independent of normal hydrodynamic force,  $F_n$ : at 240 pN, 15.6, 16.0, and 17.0% broke up in the force range 70–150 pN, 150–230 pN, and 230–310 pN. Unexpectedly, at both [fibrinogen], the initial rate of breakup was highest in the lowest force range, and computer simulation using a stochastic model of breakup was unable to simulate the time course of breakup. When pre-sheared at low  $G$  for  $>15$  min, no doublets broke up within 10 rotations at  $70 < F_n < 310$  pN; it required  $>3$  min shear ( $>1110$  rotations) at  $F_n = 210$  pN for significant breakup to occur. Other published work has shown that binding of fibrinogen to GPIIb-IIIa immobilized on plane surfaces exhibits an initial fast reversible process with relative low affinity succeeded by transformation of GPIIb-IIIa to a stable high-affinity complex. We postulate that most doublet breakups observed within 10 rotations were from a population of young doublets having low numbers of bonds, by dissociation of the initial receptor complex relatively unresponsive to force. The remaining, older doublets with GPIIb-IIIa in the high-affinity complex were not broken up in the time or range of forces studied.

### INTRODUCTION

Human platelet glycoprotein IIb-IIIa is a member of the integrin family of cell adhesion receptors, a  $\text{Ca}^{2+}$ -dependent heterodimer complex (Jennings and Phillips, 1982) that binds several adhesive proteins such as fibrinogen (Marguerie et al., 1980; Bennett et al., 1982), von Willebrand factor (vWF; Fujimoto et al., 1982), and fibronectin (Plow and Ginsberg, 1981). Binding of soluble fibrinogen to GPIIb-IIIa requires activation of the platelet, through exposure to an agonist such as adenosine diphosphate (ADP) or thrombin, and leads to conformational changes in the receptor, enabling the complex to bind soluble ligand (Sims et al., 1991). GPIIb-IIIa binds to the dodecapeptide sequence located at the carboxyl terminus of the  $\gamma$ -chain of the fibrinogen molecule (Peerschke et al., 1986; Farrell et al., 1992; Hawiger, 1995), of which the last 4 amino acid residues (AGVD) are essential to this function (Farrell et al., 1992; Rooney et al., 1996).

Aggregation of ADP-activated platelets in flow in the range of physiological shear rates ( $25$  to  $2000 \text{ s}^{-1}$ ) is initiated by cross-bridging of divalent fibrinogen molecules

between colliding cells. It has been shown that fibrinogen occupancy on platelet GPIIb-IIIa is the first important determinant of the aggregation process (Xia and Frojmovic, 1994), and that subsequent cross-linking to GPIIb-IIIa on a second platelet also occurs via the  $\gamma$  chain carboxyl terminus, requiring the presence of the AGDV residues (Liu et al., 1997; Liu and Frojmovic, 1998).

The kinetics of binding of soluble fibrinogen to activated GPIIb-IIIa on platelets have been studied over the last 20 years. Equilibrium dissociation constants,  $K_d$ , varying from 30 nM (DiMinno et al., 1983) to  $5.6 \mu\text{M}$  (Kornecki et al., 1981) have been reported, although values between 100 and 200 nM are generally accepted. Peerschke (1992) and others have shown that fibrinogen binding to GPIIb-IIIa on platelets is a multiphase process, with an initial reaction that is reversible (by addition of divalent ions or excess unlabeled fibrinogen), but which with time becomes increasingly less reversible and, after 2 h, virtually irreversible. Even over much shorter times, on the order of minutes, using the techniques of surface plasmon resonance (Huber et al., 1995) and total internal reflection microscopy (Müller et al., 1993), it has been shown that binding of fibrinogen to GPIIb-IIIa immobilized on plane solid surfaces is a two-step process. Although adhesion of fibrinogen to the immobilized receptor in this technique occurs in flowing buffer, the hydrodynamic forces acting on the molecules are extremely small, in contrast to the significant normal and tangential hydrodynamic forces acting on flowing platelet aggregates and platelets adhering to the vessel wall in the circulation.

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In recent years, we have described a microrheological technique for studying the time and force dependence of the rupture of receptor-ligand bonds between latex spheres. We reported on the breakup of doublets of carboxyl-modified latex spheres bearing covalently linked synthetic blood group antigen and cross-linked by pentavalent IgM antibody (Tees and Goldsmith, 1996), and the breakup of aldehyde/sulfate latex spheres bearing covalently linked IgG antibody, cross-linked by divalent Protein G (Kwong et al., 1996). Doublets of latex spheres were observed and videotaped under a microscope while they were sheared in Couette flow in the annulus between a counter-rotating cone and plate rheoscope (Tees et al., 1993) until breakup or until they were lost to view. The normal force,  $F_n$ , acting along, and the tangential force,  $F_s$ , acting normal to the major axis of a doublet of rigid spheres at breakup were computed using fluid mechanical theory (Tha and Goldsmith, 1986):

$$F_n = \alpha_n(h) \eta G b^2 \sin^2 \theta_1 \sin 2\phi_1, \quad (1)$$

$$F_s = \alpha_s(h) \eta G b^2 \sin^2 \theta_1 \cdot \left\{ \frac{(2 \sin^2 \theta_1 \cos^2 \phi_1 - 1)^2 \sin^2 \phi_1 + \cos^2 \theta_1 \cos^2 \phi_1}{1 - \sin^2 \theta_1 \cos^2 \phi_1} \right\}^{1/2} \quad (2)$$

Here,  $\eta$  is the suspending medium viscosity,  $G$  the shear rate,  $b$  the sphere radius, and  $\theta_1$  and  $\phi_1$  the respective polar and azimuthal angles describing the orientation of the doublet axis with respect to the vorticity axis  $X_1$  of the shear field (Tha and Goldsmith, 1986).  $\alpha_n$  and  $\alpha_s$  are force coefficients that are weakly dependent on the minimum distance of approach,  $h$ , between sphere surfaces. Using a stochastic model of bond rupture (Bell, 1978), and a Poisson distribution for the number of bonds, breakup in Couette flow was simulated to relate the experimental data to the force dependence of the reverse, off-rate reaction coefficient.

The present paper reports results of applying the technique described above to determine the time and force dependence of rupture of fibrinogen-GPIIb-IIIa bonds, using doublets of aldehyde/sulfate latex spheres to which purified human GPIIb-IIIa receptor was covalently linked, and which were cross-linked by soluble fibrinogen.

## MATERIALS AND METHODS

### Latex spheres

These were surfactant-free hydrophobic aldehyde/sulfate latex spheres (Interfacial Dynamics Corporation Inc., Portland, OR) containing aldehyde groups grafted onto the surface of the hydrophobic sulfate charge-stabilized microspheres. The particles exhibit high sphericity and have approximately  $10^{10}$  aldehyde groups per sphere. Measurements of the size distribution of a population of suspended particles showed the average diameter to be  $4.93 \pm 0.46 \mu\text{m}$ , with a bimodal size distribution, 87% of the particles having a diameter of  $4.75 \pm 0.26 \mu\text{m}$  (SD), and 13% a diameter of  $6.10 \pm 0.23 \mu\text{m}$ .

The suspension of spheres contained a small number,  $<0.5\%$ , of doublets. These nonspecifically bound aggregates could not be broken up by either sonication or the application of high shear stress.

### Receptor and ligand

Glycoprotein IIb-IIIa was isolated from 18 units of outdated platelet-rich plasma (Canadian Red Cross) following a procedure outlined by Ramsamooj et al. (1990). The platelets were centrifuged at  $\sim 6000 \times g$  for 20 min, and the pellet washed in 100 ml of Tris-buffered saline (TBS), pH 7.4, and centrifuged at  $\sim 7000 \times g$  for 20 min. The pellet, estimated to contain about  $1.8 \times 10^{12}$  platelets, was suspended in 60 ml of CAT buffer (TBS containing 0.5 mM  $\text{Ca}^{2+}$  and the protease inhibitors leupeptin, 0.5 mM, and proteinin, 0.02 U/ml). The platelet suspension was then sonicated on ice and the cytosol removed by centrifugation at  $12,000 \times g$  at  $4^\circ\text{C}$  for 20 min. The supernatant containing the membrane fraction was centrifuged at  $35,000 \times g$  at  $4^\circ\text{C}$  for 90 min and the pellet suspended and solubilized in 20 ml CAT buffer containing 1% Triton-X100 reduced (Aldrich Chemical Co., Montreal, QC) overnight at  $4^\circ\text{C}$ . It was then again subjected to sonication on ice and centrifuged at  $100,000 \times g$  for 90 min. The supernatant was loaded onto a lentil-lectin column (Pharmacia Biotech, Montreal, QC) pre-equilibrated with CAT buffer containing 2 mM  $\text{Ca}^{2+}$  and 1% Triton-X100 reduced (RTT Buffer) and unbound protein eluted with the buffer at a flow rate of 15 ml/h. The GPIIb-IIIa was then eluted using the above buffer containing 2%  $\alpha$ -methylmannopyranoside. The eluate was concentrated about 10-fold (Centriprep 30, Amicon) and applied to an S300 Sephacryl gel filtration column (Pharmacia Biotech;  $0.95 \times 95 \text{ cm}$ ; void volume 60 ml) pre-equilibrated with a HEPES-saline buffer containing 8.8 g/L of  $n$ -octyl  $\beta$ -D-glucopyranoside, 2 mM  $\text{Ca}^{2+}$  with the above concentrations of the protease inhibitors. Samples were collected at a flow rate of 15 ml/h and the protein collected at 42 to 68 ml. Polyacrylamide gel electrophoresis showed that there was no detectable contamination of the GPIIb-IIIa by fibrinogen or thrombospondin.

Pooled samples of the eluants of known [GPIIb-IIIa] were divided into aliquots, stored at  $-70^\circ\text{C}$ , and freshly thawed when coupled to the spheres.

Human fibrinogen (KABI Diagnostica, Sweden) was purified by applying a solution to a DEAE cellulose column and subsequently concentrating the eluate, as previously described (Goldsmith et al., 1994; Lawrie et al., 1979).

Conjugation of fibrinogen with fluorescein isothiocyanate (FITC; Isomer I on celite 10%, Behring Diagnostics, La Jolla, CA) was carried out as previously described for labeled monoclonal antibodies (Frojmovic et al., 1991; Shattil et al., 1987). Aliquots of stocks of fibrinogen and FITC-fibrinogen were stored at  $-70^\circ\text{C}$  and freshly thawed on the day of use.

### Covalent coupling of protein to aldehyde/sulfate latex

Glycoprotein IIb-IIIa was covalently coupled to the spheres in a one-step reaction (Illum and Jones, 1985; Rembaum et al., 1978). The aldehyde groups of the latex form stable bonds with primary amino groups of the protein (e.g., lysine). A suspension of  $\sim 10^5$  spheres  $\mu\text{l}^{-1}$  was washed three times with 0.1 M HEPES buffer, pH 6.5. Twenty-five  $\mu\text{g ml}^{-1}$  GPIIb-IIIa was linked to the spheres in the buffer containing 1 mM hexapeptide, GRGDSP (Calbiochem Corp., La Jolla, CA), added to promote correct conformation of the receptor for linking of fibrinogen (Du et al., 1993; Xia and Frojmovic, 1994), and rotated at room temperature for 2 h. The spheres were washed 3 times in phosphate buffered saline (PBS), pH 6.5, containing 0.16% HEPES and 2.5% bovine serum albumin (BSA), and then rotated overnight at  $4^\circ\text{C}$  in order to block any nonspecific binding sites that remained on the sphere surface. The spheres were stored in PBS, pH 7.4, containing 0.06% HEPES, 0.1% BSA, and 0.1%  $\text{NaN}_3$ , at  $4^\circ\text{C}$  at final concentrations of  $\sim 10^5$  spheres  $\mu\text{l}^{-1}$ . After covalent linking, examination of the spheres in a hemocytometer revealed  $<0.5\%$  doublets in

the suspension. The spheres were used within 1 month of linking with GPIIb-IIIa.

## Suspending medium

Because Dextran was known to precipitate fibrinogen in blood (Rampling, 1974), it could not be used as a viscous cosolvent, as had been done for A/S latex spheres covalently bearing monoclonal IgG and cross-linked by Protein G (Kwong et al., 1996). Instead, Ficoll 400 (Pharmacia Biotech) at a concentration of 19% w/v was found to be a suitable viscous cosolvent, with a suspending medium viscosity of 20 mPa/s at 23°C. Observations of solutions of fibrinogen in 19% Ficoll under the microscope revealed a complete absence of the precipitated fibrinogen strands that were present in Dextran solutions. The density of the spheres,  $1.055 \text{ g cm}^{-3}$ , was slightly less than that of the 19% Ficoll ( $1.071 \text{ g cm}^{-3}$ ), and the particles rose with a velocity of  $\sim 0.6 \mu\text{m min}^{-1}$ . Because it took  $\sim 45$  min for all particles to settle onto the top cone wall, sedimentation did not significantly affect experiments on the breakup of individual doublets.

As documented below, when the receptor-linked spheres at concentrations between  $5$  and  $8 \times 10^3 \mu\text{l}^{-1}$  were suspended in buffered Ficoll in the presence of [fibrinogen]  $\leq 240$  pM and subjected to Couette flow at low shear rate ( $\sim 8 \text{ s}^{-1}$ ), they formed mostly doublets and a few triplets, even over a period of 30 min. Thus, fibrinogen concentrations of 120 and 240 pM were chosen for the experiments described below.

## Characterization of receptor attachment to A/S spheres

The presence of bound and activated GPIIb-IIIa on the latex spheres, functionally competent for ligand binding, was verified by binding its natural ligand, FITC-labeled purified fibrinogen. Twenty-microliter aliquots of a suspension containing 3000 spheres  $\mu\text{l}^{-1}$  in Tyrodes-albumin buffer, pH 7.4, were mixed with labeled fibrinogen at concentrations from 10 to 500 nM, the latter being the saturating concentration of ligand. After incubation for 30 min with ligand, the mixture was diluted with 10 volumes of Tyrodes-albumin to quench the reaction, and 3000 particles counted in a FACSCAN flow cytometer (Becton Dickinson Canada, Mississauga, ON) within 30 to 60 s to minimize post-dilution time-dependent changes (Frojmovic et al., 1991). Mean fluorescence values associated with FITC-fibrinogen binding to the GPIIb-IIIa-linked spheres were corrected for background binding by subtracting the fluorescence values in the presence of 1 mM GRGDSP, which completely inhibited fibrinogen binding. Active receptor surface density at 500 nM fibrinogen ranged from 16,750 to 34,120 sites per latex sphere (mean value  $22,470 \pm 6880$ ), and Scatchard analysis (linear plots; cf. Xia et al., 1996) showed that the corresponding dissociation constants  $K_d$  varied from 30 to 112 nM (mean value  $56 \pm 31$  nM).

Unfortunately, the mean fluorescence values at [fibrinogen] = 120 and 240 pM were not significantly above background, so the number of receptor-ligand bonds formed could not be computed. However, these could be estimated from the mean  $K_d$  value and the molar concentration of fibrinogen in solution. Assuming a linear relation between [fibrinogen] and the number of bonds, 97 and 48.5 fibrinogen-GPIIb-IIIa bonds per sphere are predicted at 240 and 120 pM ligand, respectively.

## Maximum number of bonds

The surface area for contact available for cross-bridging of fibrinogen is restricted by the geometry of the spheres and the surface receptor density. The closest distance of approach depends on the size of the GPIIb-IIIa molecule on the surface, which is known from electron micrographic data (an  $8 \text{ nm} \times 12 \text{ nm}$  globular head with two 18-nm flexible tails; Weisel et al., 1992). Neglecting the tails, that distance would be  $\sim 10$  nm. The

maximum separation between sphere surfaces, allowing for the length of the fibrinogen molecule = 47.5 nm (Doolittle, 1994), is then  $47.5 + 20 = 67.5$  nm. The maximum area for cross-linking is given by the surface area of the spherical cap,  $2\pi bj$ , of thickness  $j = (67.5 - 10)/2 = 28.8$  nm, is then  $0.43 \mu\text{m}^2$  (see Fig. 1), predicting an average of only 1.2 and 0.6 cross-bridges at 240 and 120 pM fibrinogen, respectively. Taking the tails into account, with a minimum distance of approach of 28 nm, yields a surface area of  $0.56 \mu\text{m}^2$ , predicting an average of 0.75 and 1.5 cross-bridges, respectively. The number of bonds expected is thus very low, whereas the corresponding number of free fibrinogen molecules available are computed to be 92 and 46, respectively, over the binding area.

## Procedures

As previously described (Tees et al., 1993; Tees and Goldsmith, 1996) breakup of doublets of latex spheres were carried out in a transparent, counter-rotating cone and plate rheoscope (model MR-1, Myrenne Instruments, Fremont, CA) with a nominal cone angle of  $2^\circ$ , mounted on a Leitz Diavert inverted microscope (Ernst Leitz Ltd., Midland, ON). The rheoscope cone and plate angular velocities, and hence the shear rate, were adjusted using a 10-turn variable resistor. Particle motions were observed close to the layer of zero translational velocity located in the midplane of the gap, at a distance between 0.7 and 1.0 mm from the center of rotation, and recorded on videotape using a CCD camera (model AVC-D7; Sony Canada Ltd., Montreal, QC) for subsequent analysis off-line.

Receptor-linked A/S latex spheres ( $5000\text{--}8000 \mu\text{l}^{-1}$ ) were suspended in PBS, pH 7.4, containing 20% Ficoll 400 and 0.1% albumin, and fibrinogen, previously diluted in 20% Ficoll, added at a final concentration of 240 or 120 pM. The suspension was mixed in a rotator for 1 h at room temperature before use. Thirty microliters of fresh suspension was then pipetted into the rheoscope, when at the most, 2 or 3 doublets could be seen upon rotating the cone and plate through  $360^\circ$ . Doublets were then allowed to form *de novo* as a result of two-body collisions at the lowest  $G = 8 \text{ s}^{-1}$  [corresponding to  $F_{n,\text{max}} \approx 17$  pN;  $\sin^2\theta_1 \sin 2\phi_1 = 1$  in Eq. 1 and  $\eta = 20$  mPa s]. After a doublet was found close to the midplane (usually within 10 min of the onset of shear), its rotational orbit was analyzed at high magnification, and the variable resistor speed setting required to produce the desired shear stress and force was determined (Tees et al., 1993). The doublet was then observed and videotaped at the preset shear rate at a lower magnification until breakup or until it disappeared from view. The rheoscope was subsequently cleaned out, fresh suspension pipetted in, and another doublet found and subjected to a predetermined hydrodynamic

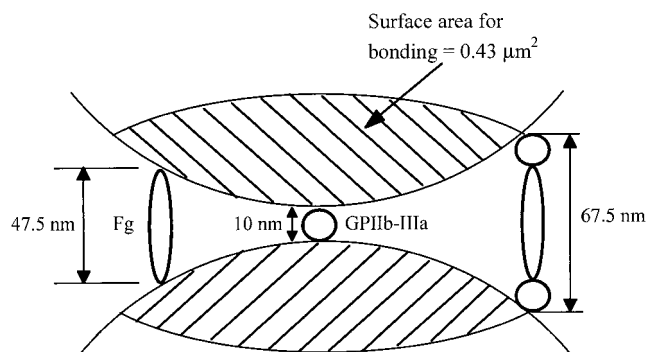


FIGURE 1 Schematic diagram (not drawn to scale) illustrating cross-bridging of divalent fibrinogen between GPIIb-IIIa receptor molecules covalently linked to latex microspheres,  $4.75 \mu\text{m}$  in diameter. The hatched section represents the area available for cross-bridges to form, based on the diameter of the receptor molecule (assumed = 10 nm globular head) and the length of the fibrinogen molecule, as shown.

**TABLE 1** Nonspecific vs. receptor-ligand-induced formation of doublets after 30 min shear at  $G = 8 \text{ s}^{-1}$ 

Ligand	Concentration of doublets				Concentration of triplets			
	$t = 0 \text{ s}$		$t = 30 \text{ min}$		$t = 0 \text{ s}$		$t = 30 \text{ min}$	
	$\mu\text{l}^{-1}$	A, %*	$\mu\text{l}^{-1}$	A, %	$\mu\text{l}^{-1}$	A, %	$\mu\text{l}^{-1}$	A, %
None	$14 \pm 7.5^\dagger$	$0.45 \pm 0.25$	$48 \pm 15$	$1.60 \pm 0.50$	$0^\dagger$	0	0	0
Fibrinogen 240 pM	$26 \pm 13^\dagger$	$0.67 \pm 0.43$	$168 \pm 64$	$5.60 \pm 2.13$	$0^\dagger$	0	$30 \pm 25$	$1.50 \pm 1.25$
Fibrinogen 240 pM 1 mM GRGDSP	$22 \pm 12^\ddagger$	$0.73 \pm 0.41$	$63 \pm 37$	$2.10 \pm 1.25$	$0^\ddagger$	0	0	0
Fibrinogen 240 pM	$39 \pm 27^\ddagger$	$1.29 \pm 0.88$	$178 \pm 23$	$5.93 \pm 0.77$	$0^\ddagger$	0	$35 \pm 31$	$1.74 \pm 1.55$

\*Percentage of A/S latex spheres in doublets or triplets.

$^\dagger \pm$  one standard deviation,  $n = 8$ .

$^\ddagger \pm$  one standard deviation,  $n = 5$ .

force. Only doublets consisting of 4.75- $\mu\text{m}$  diameter spheres of equal size were chosen for breakup studies.

## Data analysis

As in previous work with doublets of red cells and latex spheres, breakup was assumed to occur when  $F_n$  was a maximum, i.e., when the angle factor  $\sin^2\theta_1 \sin 2\phi_1$  in Eq. 1 was a maximum. The value of  $F_{n,\text{max}}$  was obtained from analysis of the experimentally recorded rotational orbit at high magnification and low shear rate (Tees et al., 1993). The shear rate at breakup or just before the doublet was lost to view was obtained from videotape analysis of the period of rotation  $T$ , given by Goldsmith and Mason (1967):

$$T = \frac{2\pi}{G} \left( r_e + \frac{1}{r_e} \right) \quad (3)$$

where  $r_e$  is the axis ratio of the ellipsoid having the same  $T$  as a doublet of touching rigid spheres. Measurements of the period of rotation of doublets of fibrinogen cross-linked latex spheres showed that the particles rotated with a measured  $TG$  close to the value, 15.61, predicted for doublets of rigidly linked spheres (Wakiya, 1971).

Where possible, statistical analyses were carried out using paired  $t$ -tests and standard deviations of the mean reported.

## RESULTS

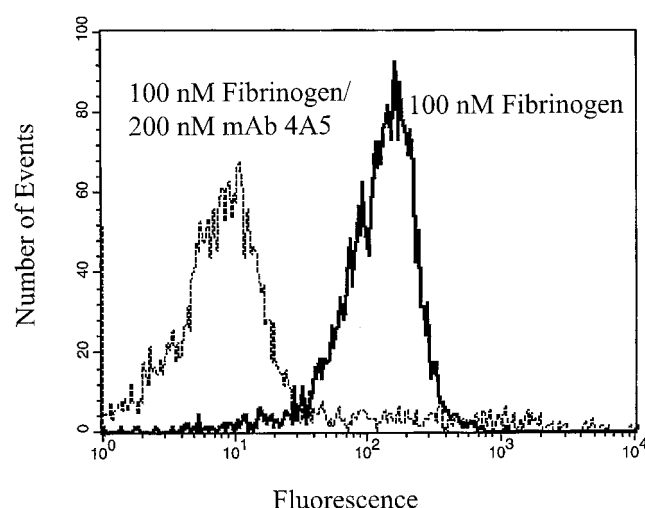
### Specific and nonspecific aggregation

At low shear rate, doublet formation of GPIIb-IIIa-linked spheres in Ficoll-buffer suspensions containing 240 pM fibrinogen was compared with that of native A/S spheres in the presence of fibrinogen, and with that of GPIIb-IIIa-linked spheres in the absence of fibrinogen. The number of doublets and higher order aggregates observed in the field of view were counted by slowly rotating the cone and plate through  $360^\circ$  in which  $0.16 \mu\text{l}$  of suspension was sampled. In the presence of 240 pM fibrinogen, either before the onset of or after 30 min shear, doublets of the native spheres were rarely seen. In the case of the GPIIb-IIIa-linked spheres in the absence of fibrinogen, as shown in Table 1, the mean increase of 34 doublets/ $\mu\text{l}$  (1.15% of spheres in doublets) in 30 min corresponds to a rate of formation of 1.1 doublet/ $\mu\text{l}/\text{min}$ , or 0.18 doublets per min in the suspension sampled. By comparison, in the presence of ligand the mean

increase was 142 doublets/ $\mu\text{l}$  (4.73% spheres in doublets) plus 30 triplets/ $\mu\text{l}$ , corresponding to a rate of formation of 4.7 doublets/ $\mu\text{l}$  and 1 triplet/ $\mu\text{l}$  per minute ( $\sim 0.7$  doublets and 0.16 triplets per minute in the suspension sampled). Thus, although nonspecific binding between GPIIb-IIIa-linked spheres was not negligible, it was small by comparison with that induced in the presence of fibrinogen.

### Inhibition of aggregation

The specific nature of the fibrinogen-GPIIb-IIIa binding and cross-linking in the suspending medium was demonstrated by testing the inhibition of doublet formation in suspensions containing GRGDSP. To this end, the fibrinogen-sphere suspension was incubated for one hour in the presence of 1 mM GRGDSP before being sheared. Table 1 indicates that the formation of doublets at  $G = 8 \text{ s}^{-1}$  at 240 pM fibrinogen was inhibited to a level comparable to that of the nonspecific aggregation documented above. Thus, after 30 min shear the rate of formation of doublets and triplets in the



**FIGURE 2** Fluorescence intensity histogram for FITC-fibrinogen binding to A/S latex spheres covalently linked with GPIIb-IIIa (right) and the inhibition of the binding by 200 nM monoclonal antibody 4A5 (left).



presence of GRGDSP was 1.4 and  $0 \mu\text{l}^{-1} \text{min}^{-1}$ , compared to 4.63 and  $1.16 \mu\text{l}^{-1} \text{min}^{-1}$  in suspensions of GPIIb-IIIa spheres having no added peptide.

Binding, as well as cross-linking of fibrinogen to activated GPIIb-IIIa on platelets occurs via the C-terminal dodecapeptide of the  $\gamma$ -chain of fibrinogen (Peerschke et al., 1986; Farrell et al., 1992; Hawiger, 1995; Liu et al., 1997; Liu and Frojmovic, 1998), and both can be totally inhibited by the monoclonal antibody 4A5 directed against the AGVD residues of the  $\gamma$ -chain (Matsueda and Bernatowicz, 1988). We therefore tested the inhibition of fibrinogen binding to GPIIb-IIIa on the latex spheres by mAb 4A5. GPIIb-IIIa spheres suspended in PBS buffer ( $1 \text{ mM Ca}^{2+}$ , pH 7.4) were incubated with 200 nM mAb 4A5 for 30 min, and then in the presence of 100 nM FITC-fibrinogen for 30 min. The suspensions were then quench-diluted with 10 volumes of PBS and 3000 particles counted in the FACSCAN flow cytometer. The mean fluorescence was compared with that in suspensions of spheres incubated with 100 nM FITC-fibrinogen in the absence of the antibody, and with 100 nM FITC-fibrinogen in the presence of 1 mM GRGDSP. As illustrated in Fig. 2, mAb 4A5 effectively blocked fibrinogen binding to GPIIb-IIIa, as was the case with GRGDSP (not shown).

### Temporal distribution of doublet breakup

As shown by Eq. 1, doublets in the rheoscope were subjected to a periodically varying applied force, with two periods of tensile and two periods of compressive stress in each orbit. Accordingly, the results of the temporal distribution of breakups were normalized by plotting time as the dimensionless number of rotations from the onset of shear,  $t/T$ . As previously found, the number of rotations for which a doublet was visible was roughly constant, between 10 and 15 rotations, at all  $G$ . Hence, the breakup statistics were compiled from the population of particles which broke up or were lost to view within 10 rotations of the onset of shear, corresponding to periods of 5.2 down to 1.1 s over the range of shear rate and applied normal force given below.

Figs. 3 *a* and 4 *a* show the fraction of the total number of doublets that survived in a given rotation, i.e., the surviving fraction of the total number observed in that rotation, plotted against the number of rotations at 240 and 120 pM fibrinogen, respectively. Shear rates in Couette flow varied from 30 to  $145 \text{ s}^{-1}$  (shear stress from 0.6 to  $2.9 \text{ Nm}^{-2}$ ) over a range of  $F_{n,\text{max}}$  from 70 to 310 pN. The data were binned into three force ranges:  $70 < F_n < 150 \text{ pN}$ ,  $150 < F_n < 230 \text{ pN}$ , and  $230 < F_n < 310 \text{ pN}$ . Table 2 gives the aggregate statistics for the fraction of all doublets observed that broke up within 10 rotations in each of the 3 force ranges.

The plots show that, at both fibrinogen concentrations, most of the breakups, from 65% to 80%, occurred in the first 2 rotations; however, as shown in Table 2, there is no obvious increase in the total fraction of breakups with

increasing applied force. Thus, at 240 pM fibrinogen, the aggregate fractions broken up in the first 10 rotations were 15.6, 16.0, and 17.0% in the low, medium and high force ranges, respectively. At 120 pM fibrinogen, where the extent of breakup was markedly higher, there was actually a decrease in the fraction broken up in the highest force range, values being 31.7, 30.9, and 20.7%, respectively. At 240 pM fibrinogen, 16.1% of all doublets (37 of 230) broke up within 10 rotations; at 120 pM fibrinogen, the fraction of breakups increased to 28.8% (36 of 125).

Most interesting in Figs. 3 *a* and 4 *a* is the observation, contrary to expectations, that the initial rate of doublet breakup is actually highest in the lowest force range. Thus, in going from the lowest to the highest force range, the fraction broken up in the first 2 of 10 rotations decreased

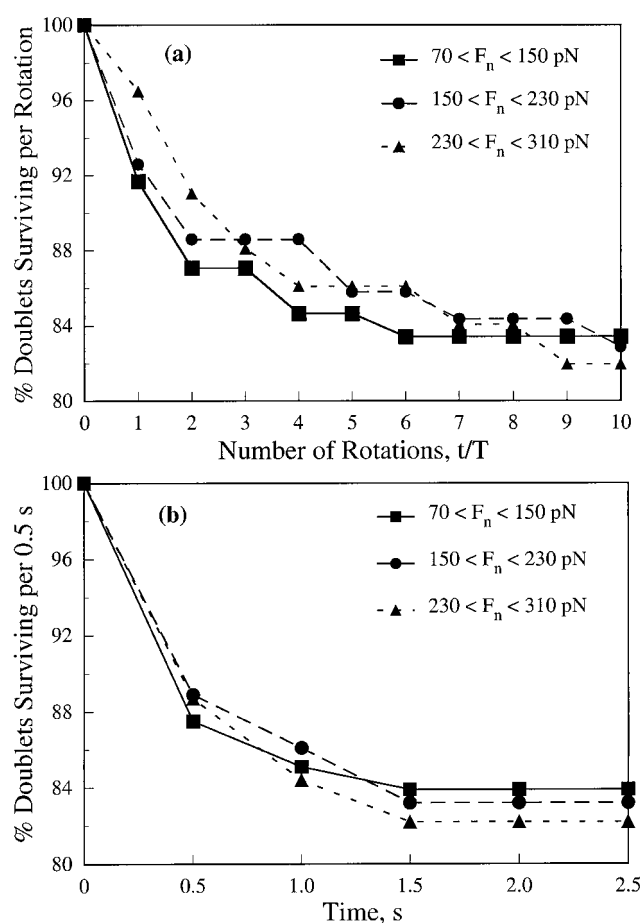


FIGURE 3 Time course over 10 rotations of the breakup of doublets of GPIIb-IIIa-linked A/S latex spheres, cross-linked by 240 pM fibrinogen in Couette flow. (a) Plot of the percentage of doublets surviving per rotation (i.e., of the total number observed in that rotation) against the number of rotations. (b) Plot of the percentage of doublets surviving in intervals of 0.5 s (percentage of doublets observed over an interval of 0.5 s which have survived) against time. The data were binned into three force ranges. Although there is very little difference in the extent of breakup after 10 rotations, it is evident that the initial rate of breakup is greatest in the lowest force range.

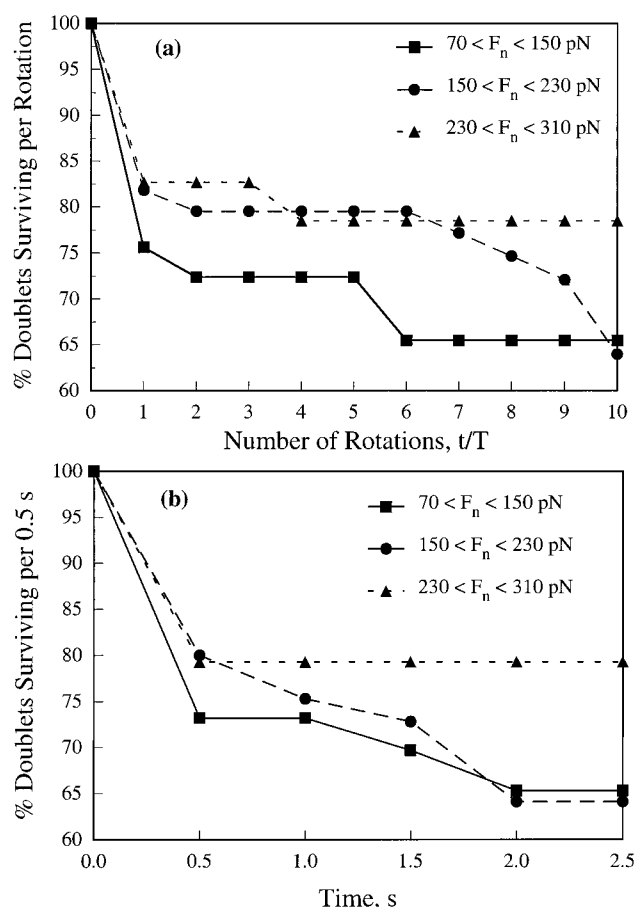


FIGURE 4 Plots, as in Fig. 3, of the time course of the breakup of doublets cross-linked by 120 pM fibrinogen. Here, the decrease in the initial rate of doublet breakup with increasing force is even more striking.

from 12.5 to 8.8% and from 26.8 to 17.2% at 240 and 120 pM fibrinogen, respectively. These observations are reflected in the values of the average number of rotations required to break up doublets in the first 10 rotations,  $\langle N_r \rangle$ , shown in Table 3. The values of  $\langle N_r \rangle$  increase with increasing force for the fibrinogen-GPIIb-IIIa doublets (although

not statistically significant;  $P > 0.07$ ) between the lowest and the higher force ranges.

These results are in striking contrast to those previously obtained with A/S latex spheres covalently coupled to monoclonal IgG antibody (Kwong et al., 1996), and cross-linked by 0.9 nM divalent Protein G (also a protein-protein bond) shown rebinned into the first two force ranges in Fig. 5a and Table 2. Here, the aggregate fraction broken up in the first 10 rotations increased from 33.3% in the low to 68.8% in the medium force range, and there is clearly a marked decrease in the fraction of doublets surviving after two rotations with increasing applied force: from 74.3% to 13.5% going from the low to the medium force range. Too few doublets were studied at  $F_n > 230$  pN to make a valid comparison. Also, as shown in Table 3, the values of  $\langle N_r \rangle$  significantly decreased for the IgG-Protein G doublets ( $P < 0.015$ ).

Similar force-dependent results can be seen in Fig. 5b for rebinned data previously obtained with doublets of 4.62  $\mu$ m diameter carboxyl modified latex spheres, covalently coupled to synthetic blood group B antigen trisaccharide, and cross-linked by monoclonal IgM anti-B antibody (carbohydrate-protein bond; Tees and Goldsmith, 1996).

### Effect of time of shear on breakup

When studying the breakup of individual doublets, the particle chosen for analysis was the first observed close to the midplane of the annulus between cone and plate, usually within 10 min of shear at low G. We wished to study the effect of time of shear at low G on the time and force dependence of subsequent doublet breakups. The suspension was therefore sheared for 15 or 30 min with 240 pM fibrinogen, before choosing a doublet for breakup study. Twenty doublets in each time range were analyzed. It was found that, after 15 min, breakups were observed: 7 out of the 20 broke up within 10 rotations in the force range  $F_n = 94$  to 272 pN, 6 of these within the first rotation. By contrast, after 30 min shear over a force range  $F_n = 117$  to 286 pN, none of the doublets broke up.

TABLE 2 Fraction of doublets breaking up within 10 rotations

Applied force (pN)	Fraction of break-ups					
	GPIIb-IIIa-240 pM fibrinogen		GPIIb-IIIa-120 pM fibrinogen		IgG-0.9 nM protein G*	
	Experiment	Simulation <sup>†</sup>	Experiment	Simulation <sup>‡</sup>	Experiment	Simulation <sup>§</sup>
$70 \leq F_n \leq 150$	0.156 ( $n = 96$ )	$0.174 \pm 0.019$ ( $n = 500$ )	0.310 ( $n = 41$ )	$0.322 \pm 0.012$ ( $n = 500$ )	0.333 ( $n = 54$ )	$0.387 \pm 0.024$ ( $n = 500$ )
$150 \leq F_n \leq 230$	0.160 ( $n = 81$ )	$0.162 \pm 0.012$ ( $n = 500$ )	0.309 ( $n = 55$ )	$0.339 \pm 0.032$ ( $n = 500$ )	0.688 ( $n = 32$ )	$0.686 \pm 0.005$ ( $n = 500$ )
$230 \leq F_n \leq 310$	0.170 ( $n = 53$ )	$0.186 \pm 0.022$ ( $n = 500$ )	0.207 ( $n = 29$ )	$0.398 \pm 0.018$ ( $n = 500$ )	—	—

$n$ , number of doublets.

\*Data from Kwong et al. (1996) were rebinned into the above force ranges.

<sup>†</sup> $c = 1.9 \times 10^{10} \text{ N}^{-1}$ ,  $t_o = 5 \text{ s}$ ;  $\langle N_b \rangle = 6$ ,  $t_f = 2 \text{ s}$ .

<sup>‡</sup> $\langle N_b \rangle = 4$ .

<sup>§</sup> $c = 9.5 \times 10^{10} \text{ N}^{-1}$ ,  $t_o = 175 \text{ s}$ ,  $\langle N_b \rangle = 3$ ,  $t_f = 100 \text{ s}$ .

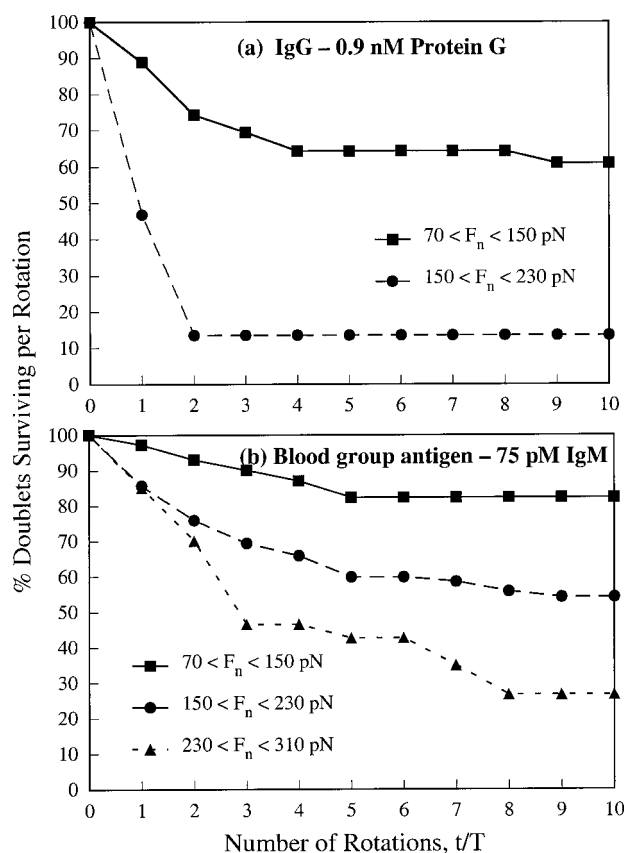


FIGURE 5 The kinetics of doublet breakup in Couette flow over 10 rotations previously documented in (a) doublets of IgG-coupled A/S latex spheres cross-linked by divalent Protein G, and in (b) doublets of carboxyl-modified spheres coupled with synthetic blood group B antigen trisaccharide and cross-linked by pentavalent IgM monoclonal anti-B antibody. Plot, as in Figs. 3 a and 4 a, show the time course of percentage of doublets surviving against number of rotations. Data rebinned from the results of Kwong et al. (1996) and Tees and Goldsmith (1996) show that both the initial rates of breakup and the percentage broken up after 10 rotations increase markedly with increasing applied force.

To ascertain whether these doublets would break up if exposed to longer times at high shear rate, population studies were carried out. In these, suspensions of spheres in 240 pM fibrinogen were sheared for 30 min at low  $G$ , and duplicate counts of the number of doublets and triplets made in the field of view over one rotation of the cone and plate. The suspensions were then subjected to a shear rate of 97

$s^{-1}$  (shear stress =  $1.94 \text{ N m}^{-2}$ ) corresponding to a maximum applied normal force  $F_{n, \text{max}} = 210 \text{ pN}$ , for 3 and 6 min ( $\sim 1100$  and  $2200$  rotations of a doublet), and again, duplicate counts of the number of aggregates made. The results, given in Table 4, show that there were reductions of 8.7% and 40.2% in the number of spheres in doublets and triplets after 3 and 6 min shear, respectively. Analysis of the data in Table 4 showed that after 3 min shear, the reduction in the number of spheres in aggregates is not statistically significant, whereas it is after 6 min of shear.

## Two-body collision efficiency

From the data obtained in population studies of the number of doublets formed after 30 min shear at  $G = 8 \text{ s}^{-1}$  (Table 4), the time average two-body collision capture efficiency was computed. The capture efficiency is defined as the fraction of the total number of two-body collisions that result in the formation of non separating doublets,  $\alpha = J_c/J$ , where  $J_c$  is the mean measured collision capture frequency per unit volume ( $0.103 \pm 0.044 \text{ s}^{-1} \mu\text{L}^{-1}$ ,  $n = 19$ ), and  $J$  the predicted total two-body collision frequency in a suspension of  $N$  equal-sized rigid spheres per unit volume (Smoluchowski, 1917):

$$J = \frac{16}{3} G b^3 N^2 = 36.8 \text{ s}^{-1} \mu\text{L}^{-1} \quad (4)$$

The collision efficiency =  $0.0028 \pm 0.0012$  ( $n = 19$ ),  $N = 8.0 \times 10^5 \mu\text{L}^{-1}$ , a value significantly lower than that, =  $0.012 \pm 0.0029$  ( $n = 117$ ), computed in suspensions of IgG-coupled A/S spheres containing 0.9 nM Protein G at the same shear rate and comparable sphere concentration and mean surface density (17,000 sites/sphere; Kwong et al., 1996; Long et al., 1999). It is also considerably lower than that found for platelets activated by  $0.2 \mu\text{M}$  ADP at low shear rates in platelet-rich plasma, computed using the initial measured rates of aggregation:  $\alpha \sim 0.2$  in the tube flow of cells at  $G = 42 \text{ s}^{-1}$  (Bell et al., 1989), and  $\sim 0.3$  in Couette flow at  $G = 100 \text{ s}^{-1}$  (Xia and Frojmovic, 1994). The most obvious reason for the above lower collision frequency is the low [fibrinogen] = 240 pM, only 0.0027% of that normally present in plasma ( $0.88 \mu\text{M}$ ), since it is

TABLE 3 Average number of rotations to break up within 10 rotations

Applied force pN	$\langle N_r \rangle \pm \text{SD}, s$		
	GPIIb-IIIa-240 pM Fg	GPIIb-IIIa-120 pM Fg	IgG-0.9 nM protein G*
$70 \leq F_n \leq 150$	$1.58 \pm 1.38$ ( $n = 15$ )	$1.17 \pm 1.78$ ( $n = 13$ )	$1.88 \pm 1.94$ ( $n = 18$ )
$150 \leq F_n \leq 230$	$2.62 \pm 2.83$ ( $n = 13$ )	$2.04 \pm 4.79$ ( $n = 17$ )	$0.69 \pm 0.38$ ( $n = 22$ )
$230 \leq F_n \leq 310$	$2.95 \pm 2.56$ ( $n = 9$ )	—	—

$n$ , number of doublets; —, too few particles studied to compare with the other values.

\*Data from Kwong et al. (1996) were rebinned into the above force ranges.

**TABLE 4** Population study of breakup over 3 and 6 min shear;  $F_{n,max} = 210$  pN

Duration of shear (min)	No. of aggregates/ $\mu$ l				No. of spheres in aggregates/ $\mu$ l	
	Doublets		Triplets		initial	final
	initial	final	initial	final		
3 ( $n = 12$ )	196 $\pm$ 71	192 $\pm$ 96	23 $\pm$ 23	14 $\pm$ 17	463 $\pm$ 197	423 $\pm$ 226*
6 ( $n = 7$ )	169 $\pm$ 86	98 $\pm$ 39	12 $\pm$ 14	7 $\pm$ 11	374 $\pm$ 200	224 $\pm$ 81 <sup>†</sup>

\*Not significantly different from initial value.

<sup>†</sup>Significantly different from initial value,  $P < 0.02$ .

known that the extent of aggregation, as well as  $\alpha$  decrease markedly as [fibrinogen] falls below 100 nM (Goldsmith et al., 1994; Xia et al., 1994).

## DISCUSSION

The time and force dependence of breakup observed with the fibrinogen cross-linked doublets was found to be strikingly different from that previously documented with systems in which protein-protein and protein-carbohydrate bonds were ruptured. Thus, not only did the aggregate fraction broken up after 10 rotations not significantly increase with increasing applied force (Table 2), but the initial rate of doublet breakup was actually greatest at the lowest  $F_n$  (Figs. 3 *a* and 4 *a*).

### Monte Carlo simulation of breakup kinetics

In our previous publications describing the kinetics of the failure of receptor-ligand bonds, the Bell (1978) exponential model of the force dependence of the lifetime of a bond was used in Monte Carlo simulations of doublet breakup in shear flow (Tees et al. 1993; Tees and Goldsmith, 1996). Accordingly, we attempted to carry out a computer simulation of GPIIb-IIIa-fibrinogen doublet breakup under shear in the rheoscope.

Bell used a model of Zhurkov (1965) for the force dependence of bond rupture based on empirical studies of the fracture of macroscopic solids, an extension of transition state theory for reactions in gases (Evans and Ritchie, 1997) in which it was proposed that intermolecular forces in a gas could be treated as a one-dimensional random walk in a potential energy well. The probability of escape depends on the depth,  $E_0$ , of the well and the natural frequency of the bond in vacuum,  $\tau_0$  ( $\sim 10^{-13}$  s for atoms in a solid,  $\sim 10^{-9}$  for atoms in a liquid). For a parabolic energy barrier it was shown that the escape or breakup time in the absence of an external force is given by:

$$t_0 = \tau_0 \exp(E_0/k_B T_K) \quad (5)$$

where the exponential represents the probability that thermal fluctuations provide enough energy for the barrier to be surmounted (for the transition state to be reached);  $k_B$  and  $T_K$  being the respective Boltzmann constant and the abso-

lute temperature. It was postulated that the external force per bond,  $f$ , acts directly along the reaction coordinate  $x$  to reach the value  $x_\beta$  at the transition state, and that the force reduces the energy barrier in a linear fashion; thus

$$t_b = \tau_0 \exp[(E_0 - fx_\beta)/k_B T_K] \quad (6a)$$

$$= t_0 \exp(-c \cdot f) \quad (6b)$$

where  $c = x_\beta/k_B T_K$ . Therefore, when  $f$  reaches the value  $f_b = E_0/x_\beta$ ,  $t_b = \tau_0$ , and bond failure is almost instantaneous. By identifying  $t_b$  with  $1/k_{off}$ , the reverse, or off-rate constant, the connection to bond reaction rates is made:

$$k_{off} = k_{off}^0 \exp(c \cdot f) \quad (7)$$

where  $k_{off}^0 = 1/t_0$ . The probability of bond breakage  $P_b$  in a short time interval,  $\Delta t$ , is then given by (Hammer and Apte, 1992)

$$P_b = 1 - \exp(-k_{off}\Delta t) = 1 - \exp\left[\frac{\exp(cf)}{t_0} \Delta t\right] \quad (8)$$

In the computer simulation,  $t_0$  (Eq. 5) and  $c$ , as well as the average time  $t_f$  to form a bond, and the average number of bonds  $\langle N_b \rangle$  were varied to fit the data collected in the experiments. We postulate that the force is equally divided among the bonds linking the spheres. Simulations of the first 10 rotations were carried out with sets of 500 to 1000 doublets with normal forces corresponding to the mean values in each of the force ranges: 110, 190, and 270 pN. Each rotation was divided into 1000 equal time steps, and for each step  $P_b$  was computed from Eq. 8 and the force per bond calculated from Eq. 1 for the current instantaneous values of  $\theta_1$ ,  $\phi_1$  and  $N_b$ . A random number between 0 and 1 was chosen from a uniform distribution for each bond remaining. If the number drawn was less than  $P_b$ , the number of bonds was reduced by one, and the force per bond on the remaining bonds recalculated. Thus, bonds are postulated to break sequentially. We know, from the population studies described above, that, after 60 min incubation with fibrinogen before studying breakups in the rheoscope, the GPIIb-IIIa-fibrinogen bonds established would be virtually impossible to rupture. Therefore, only the bond established upon collision with other spheres resulting in cross-linking of fibrinogen is the one likely to rupture. Also,



bearing in mind that the results at each [fibrinogen] had shown a very low dependence of breakup on applied force, simulations were run with  $c < 10^{11} \text{ N}^{-1}$  (it was known that at very low  $c$ , the fraction of breakups per rotation actually decreases with increasing force, likely due to the decrease in duration of shear in each orbit as shear rate increases; Tees et al., 1993), the parameter space searched was limited to  $t_0$  from 2 to 100 s,  $t_f$  from 0.01 to 50 s, and  $N_b$  from 1 to 6 bonds.

As shown in Table 2, the computations revealed that it was possible to achieve a tolerable best fit of the aggregate statistics for the fraction of breakup within 10 rotations, with the parameters  $x_\beta = 0.08 \text{ nm}$ ,  $t_0 = 5 \text{ s}$  ( $k_{\text{off}}^0 = 0.2 \text{ s}^{-1}$ ),  $t_f = 2 \text{ s}$ , and  $N_b = 4$  and 6 bonds for 120 and 240 pM fibrinogen, respectively. Given the estimated very low density of the number of fibrinogen cross-bridges between spheres, these values of  $N_b$  are implausible. Moreover, as illustrated in Fig. 6, *a* and *b*, the above bond parameters could not reproduce the characteristic kinetics in which most breakups occurred in the first 2 rotations, and the initial rate of doublet breakup was highest at the lowest force range. The higher initial rate of breakup could be reproduced using higher values of  $x_\beta$ , but then the simulation resulted in a significant force dependence of breakup.

Also tested in a Monte Carlo simulation was the possibility that the fibrinogen-GPIIb-IIIa linkage is a catch bond (Dembo et al., 1988), one in which the energy barrier to rupture increases with increasing applied force. Here, the simulation was carried out using negative values of  $c$  in the range  $-10^{10}$  to  $-10^{11} \text{ N}^{-1}$ . However, over a wide range of  $\langle N_b \rangle$ ,  $t_0$  and  $t_f$ , it was impossible to find a reasonable fit of the experimental results (not shown).

### Time-dependent changes in the kinetics of fibrinogen-GPIIb-IIIa bond formation

Unlike the doublets of IgG-coupled A/S spheres, the GPIIb-IIIa-fibrinogen doublets exhibited a marked increase in resistance to bond rupture at times  $>15 \text{ min}$  after the onset of shear at  $G = 8 \text{ s}^{-1}$ . We therefore turned to an alternate explanation of the results, one concerned with the time dependence of the resistance of the doublets to breakup. Peerschke (1992) and others have shown that fibrinogen binding to GPIIb-IIIa on platelets is a multiphase process, initially reversible but with time increasingly less reversible. Peerschke (1994) has also demonstrated that the loss of reversibility, thought to be associated with hydrophobic interactions between fibrinogen and the platelet membrane phospholipid bilayer (Marguerie et al., 1980), can occur even when the glycoprotein is immobilized directly on microtiter wells or immunocaptured by immobilized anti GPIIb or GPIIIa antibodies. Over much shorter times, Huber et al. (1995), using purified GPIIb-IIIa immobilized on a sensor surface and surface plasmon resonance technology, measured on line the time-dependent change in surface

coverage that occurred immediately upon contact with a solution of fibrinogen. Their data indicated an initial fast and reversible reaction (up to 400 s) to form a complex with a  $K_d = 165 \text{ nM}$  ( $k_{\text{on}}^0 = 3.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{\text{off}}^0 = 4.0 \times 10^{-2} \text{ s}^{-1}$ ). Over the subsequent 5 - 10 min, this complex was transformed into a more stable higher affinity complex with a  $K_d = 21 \text{ nM}$  ( $k_{\text{on}}^0 = 2.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{\text{off}}^0 = 4.2 \times 10^{-4} \text{ s}^{-1}$ ). It should be noted, however, that the plasmon resonance data were obtained in flow at  $Q \sim 1 \mu\text{l s}^{-1}$  through a two-dimensional chamber having a height,  $h$ , of only 20 to 30  $\mu\text{m}$  and width,  $w$ , of the order of 10 mm (Huber et al., 1992) which likely resulted in wall shear rates ( $G_w = 6Q/h^2w$ ), of the order of  $600 \text{ s}^{-1}$ . Thus, binding and detachment of the bond would have occurred under a wall shear stress,  $\tau_w$  of the order of  $0.7 \text{ Nm}^{-2}$  although the hydrodynamic force on the fibrinogen molecule (equivalent sphere radius  $\sim 20 \text{ nm}$ ) given by  $F_h = 1.7 \times 6\pi Gb^2\eta$  (Goldman et al., 1967) would be negligible, of the order of 0.009 pN.

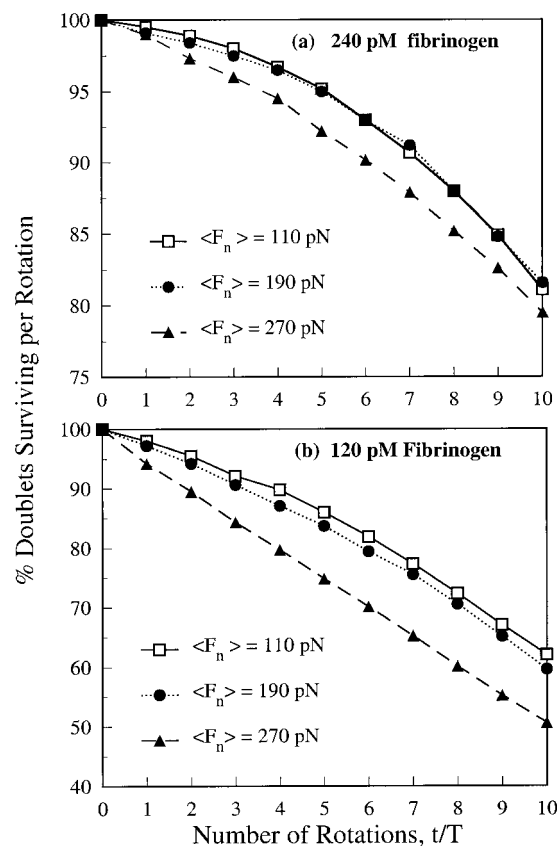


FIGURE 6 Plot of the simulated percentage of doublets surviving per rotation against number of rotations at (a) 240 and (b) 120 pM fibrinogen, computed using the mean normal force in each of the force ranges, and the best fit parameters used to simulate the percentage of doublets broken up after 10 rotations, as given in Table 2. It is evident that the simulated time course of doublet breakup differs markedly from that observed experimentally shown in Figs. 3 *a* and 4 *a*, respectively, as the simulated initial rates of breakup increase significantly with increasing force.

Over a much longer time scale (up to 2 h), Müller et al. (1993), using purified GPIIb-IIIa reconstituted in immobilized planar lipid bilayers, monitored the binding of fluorochrome-labeled fibrinogen by total internal reflection fluorescence microscopy at 24°C. Analysis of the kinetics revealed a fast reversible formation of a precomplex with a  $K_d = 50$  nM ( $k_{on}^0 = 4.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$  and  $k_{off}^0 = 2.2 \times 10^{-3} \text{ s}^{-1}$ ; compared to  $K_d = 20$  nM at 37°C using an immobilized receptor assay), values which strongly suggest that the precomplex corresponds to the second already stabilized complex found by Huber et al. (1995). Over time, the precomplex was converted into a stable complex ( $k_{on}^0 = 1.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ) which could not be dissociated after 2 h by the addition of excess of the peptide GRGDS.

### Application to results of doublet breakup

On the basis of the above work, the mean measured  $K_d = 56$  nM for GPIIb-IIIa on the A/S latex spheres would imply that, in the flow cytometer runs, after 30 min incubation with FITC-fibrinogen, we were dealing mostly with the second stabilized complex. In fact, we know that after 30 min incubation, FITC-fibrinogen cannot be displaced with 10× excess of unlabeled fibrinogen (Frojmovic, M. M., unpublished results). In the rheoscope, a small fraction (0.28%) of collisions between latex spheres result in the formation of doublets, due to cross-linking of fibrinogen between GPIIb-IIIa receptors involving the formation of a second fibrinogen-GPIIb-IIIa bond. Here, the results obtained may be explained if we postulate that the breakups observed were predominantly from the dissociation of the first reversible low affinity complex, which appears to be unresponsive to force in the range of  $70 < F_n < 310$  pN studied here. Most of the breakups occurred in the first 2 rotations (from 0.2 to 1 s after the onset of shear), presumably involving young doublets linked by only one or two bonds. Because most doublet breakups were studied from 5 to 10 min after the onset of shear, the suspension will also have contained older doublets in which the cross-linking bond had undergone the transition to the higher affinity complex, which we postulate could not be broken up within the time (from 1 to 5 s after 10 rotations) and force ranges studied.

That the initial rate of doublet breakage was greatest in the lowest force range could be explained by the fact that in a given rotation, the time the particle is subject to normal hydrodynamic forces is inversely proportional to the shear rate (Tees et al., 1993). If indeed, in the range of force investigated, the magnitude of the force has little effect on the rate of bond rupture ( $x_\beta$  in Eq. 6 *a* is very small), it is the time over which the force acts that will mainly determine the number of bonds ruptured. Because the duration,  $T$ , of each rotational orbit (Eq. 3) increases with decreasing  $G$ , the number of bonds ruptured per rotation may initially be greatest at the lowest shear rate. That after 10 rotations the total fraction of doublets broken up is virtually the same in

each force range can be explained by the fact that although the duration  $T$  of each rotational orbit decreases with increasing  $G$ , this is compensated by the increase in the number of rotations in a given time. Thus, the duration of exposure of the doublets to force in a given time of flow is independent of shear rate, and if we assume a low value for  $x_\beta$ , also of the magnitude of the force. After 10 rotations (1–5 s), the total fraction broken up would then be expected to be independent of the magnitude of the applied force. Indeed, as shown in Figs. 3 *b* and 4 *b*, a plot of the percentage of doublets surviving in intervals of 0.5 s plotted against time shows that the difference in the initial rate of breakup is now markedly smaller than when plotted against  $t/T$ .

The above explanation assumes an initial force resistant fibrinogen-GPIIb-IIIa bond over a range of shear stress from 0.6 to 2.9  $\text{Nm}^{-2}$ , whereas there is evidence that, for native platelets, the bond is not force-resistant. Thus, in Couette flow, Ikeda et al. (1991, 1993), have demonstrated that, in the absence of extrinsic activators, platelets in plasma will aggregate at shear stress  $>8 \text{ Nm}^{-2}$ , in a reaction mediated by vWF, but not by fibrinogen. Also, in Couette flow, we have shown that the initial collision efficiency in the fibrinogen-driven aggregation of platelets in citrated plasma activated with 5  $\mu\text{M}$  ADP, decreased from 0.18 to 0.05 as  $G$  increased from 300 to 1000  $\text{s}^{-1}$  (shear stress from 0.36 to 1.20  $\text{Nm}^{-2}$ ; Xia and Frojmovic, 1994). In the case of washed platelets containing no exogenously added fibrinogen but with vWF secreted on the cell surface, activated by 5  $\mu\text{M}$  ADP, the efficiency decreased from 0.09 to 0.07 over the same increase in  $G$  (Frojmovic et al., 1997). Thus,  $\alpha$  for surface secreted vWF-mediated aggregation at  $G = 300 \text{ s}^{-1}$  is already 50% of the maximal value for fibrinogen-driven aggregation, and at  $G = 1000 \text{ s}^{-1}$  is even 40% more efficient. These results suggest, but do not prove, that the GPIIb-IIIa bond is shear stress-sensitive in the range of applied normal force used in the experiments with A/S spheres.

An extension of the Monte Carlo simulation of bond rupture to encompass a two-stage model of doublet breakup has been considered: initial breakup of doublets having weak bonds and high  $K_d$ , succeeded by the break up of doublets with strong bonds and low  $K_d$ , complicated by the presence of a continuous transition from weak to strong bonds, as occurs with native activated platelets (Peerschke, 1992, 1994). We intend to explore such a model after present work on the time course of bond strengthening is complete and when experiments on breakup at significantly higher shear stress are underway, in order to study the breakup of the stronger bonds.

### Physiological relevance of the results

In flowing suspensions, normal platelets must be activated by agonists such as ADP and thrombin, which induce conformational changes in glycoprotein IIb-IIIa, allowing it to

bind soluble fibrinogen (Sims et al., 1991). In the case of the A/S spheres, the covalently linked receptor is in a conformation able to bind soluble fibrinogen, and this via the  $\gamma$ -chain of the receptor, as is the case with platelets. However, it is well known that the binding of fibrinogen induces additional conformational changes in the activated GPIIb-IIIa receptor that lead to the exposure of neo-epitopes called ligand-induced binding sites (LIBS; Frelinger et al., 1988) and, conversely, receptor-induced binding sites (RIBS) in the fibrinogen molecule (Zamarron et al., 1991; Ugarova et al., 1993). Some of these post-binding changes in the receptor-ligand complex are thought to be due to interactions with the lipid bilayer or other components of the platelet membrane (Marguerie et al., 1980). Yet, as shown by experiments with purified and immobilized GPIIb-IIIa on rigid surfaces, post-binding changes as reflected by a progressive irreversibility of the complex, also occur under quite unphysiological circumstances (Peerschke, 1992; Müller et al., 1993; Huber et al., 1995). Fibrinogen binding to the covalently bound receptor on the latex spheres, as judged by the ability to rupture the bond under force, was also shown to become more irreversible with time.

There is also the question of the effect of the viscous cosolvent, Ficoll 400, on the strength or the number of bonds formed, in view of the work of Yedgar and coworkers (Almagor et al., 1992). The increased viscosity might have an effect on the kinetic coefficients of receptor-ligand interactions and subsequent biochemical processes. Such questions were raised in our previous work on the breakup of the antigen-bearing CML latex spheres by monoclonal IgM antibody (Tees and Goldsmith, 1996). However, experiments showed that the likely effect of the increased viscosity was on the changes in ligand and receptor diffusivity, and therefore on the bond formation rate (fewer cross-bridges form), rather than on changes in the nature of the bond detachment mechanism and the off-rate constant.

The breakup results with doublets of A/S latex spheres point to the existence of a two-stage binding of fibrinogen to activated GPIIb-IIIa. We have suggested that bonds recently formed through cross-linking by fibrinogen, thereby producing young doublets (selected for study of breakup at high shear rate within minutes of being formed), were capable of dissociation under force, whereas the bonds in existence for longer times between the spheres of older doublets could not be broken.

Our results have little bearing on the flow behavior of, and bond strength within, platelet aggregates initially formed in response to damaged vessel wall (hemostatic response) because here, activation of platelets and aggregation must occur on a time scale of milliseconds. However, a different scenario exists for platelet aggregates found in regions of disturbed flow, where recirculation zones may form in which blood cells and small aggregates may have appreciable residence times, and shear rates are lower than in the mainstream (Karino and Goldsmith, 1979). Here, the

cells would more likely be irreversibly bound to each other. Thus, in the circulation, the time evolution of the fibrinogen-GPIIb-IIIa bond is likely to be as important a factor as the force dependence of the dissociation rate (tensile strength) of the bond.

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