solution of SPDP in ethanol (5 mM, 5 μL) was added to the suspension of the latex beads (4.0 x $10^6$/μL) and incubated for 30 min at r.t. The unreacted SPDP and the by-products were separated by centrifugation, and the pyridyl disulfide (PD)-bonded rHSA-coated latex beads were collected. The suspension of the latex beads (4.0 x $10^6$/μL) was mixed with a solution of H12 (10 mM, 8 μL) and reacted at 25 °C for 12 hr by the thiol-disulfide exchange reaction. The unreacted H12 was removed by centrifugation to purify the latex beads bearing H12 (H12-latex beads, 2.0 x $10^6$/μL). The concentration of the H12 conjugated to the latex beads was determined by indirect quantification of the 2-thiopyridone (2TP) that was liberated during a thiol-disulfide exchange reaction using high pressure liquid chromatography (HPLC) on a TSK-GEL G3000$SW_{XL}$ column (7.8 mm o.d. x 300 mm h in PBS at 1 mL/min), by measuring the absorbance of the column flow at 343 nm.

***Latex beads bearing rGPIbα:*** A solution of SPDP in ethanol (5 mM, 5 μL) was added to an rGPIbα solution (4.3 mg/mL, 200 μL) and incubated for 20 min at r.t.. A dithiothreitol solution (final concentration; 20 mM) was added to the PD-rGPIbα solution to obtain SH-rGPIbα after separation with GPC (sephadex G25). The solution of the SH-rGPIbα (1.0 mg/mL) was mixed with a suspension of the PD-bonded rHSA-coated latex beads obtained as described above, and reacted at 25 °C for 12 hr. The unreacted reagents were removed by centrifugation to separate the latex beads bearing rGPIbα (rGPIbα-latex beads, 2.0 x $10^6$/μL).

The concentration of the rGPIbα conjugated on the latex bead was determined with a sandwich enzyme-linked immunosorbent assay (ELISA) with GUR 20-5 and horseradish peroxidase-conjugated GUR83-35.[19]

***Latex beads bearing both H12 and rGPIbα.*** A solution of SPDP in ethanol (20 mM, 10 μL) was added to a suspension of rHSA-coated latex beads (4.0 x $10^6$ /μL) and incubated for 30 min at r.t. The suspension of the PD-latex beads (4.0 x $10^6$ /μL) was mixed with an aqueous solution of H12 (4 mM, 8 μL) and reacted at 25 °C for 12 hr. The unreacted reagents were removed by centrifugation and the purified H12-latex beads (2.0 x $10^6$ /μL) were obtained. One or two mL of a SH-rGPIbα solution (1.0 mg/mL) was added to the dispersion of the H12-latex beads, and incubated for 12 hr at r.t. The latex beads bearing both H12 and rGPIbα (H12/rGPIbα-latex beads) were purified by centrifugation. The concentration of H12 or rGPIbα conjugated on the latex bead was determined by HPLC or a sandwich ELISA, respectively, as described above.

**Platelet aggregation study**

Blood withdrawn from healthy volunteers was mixed with 10% volume of 3.8% (w/v) sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (100*g*, 15 min, 22 °C), and the platelet concentration of PRP was adjusted to 2.0 x $10^5$ /μL by platelet-poor

plasma (PPP) prepared by centrifugation (2200*g*, 10 min, 22 °C). The platelet concentration was determined using an automated hematology analyzer (K-4500, Sysmex, Kobe, Japan). An ADP solution was added to the PRP containing H12-latex beads or H12/rGPIbα-latex beads (final concentrations, 2.0 x $10^3$ /μL) at a final concentration of ADP 3 μM, and the light transmittance was measured with an aggregometer (Hema Tracer T-638, Nico Bioscience, Tokyo).

**Preparation of collagen-immobilized surface**

Collagen I-A (3.0 mg/mL, Cellmatrix, Nitta Gelatin, Osaka) was suspended in PBS at 4 °C to give a final concentration of 30 μg/mL. Glass plates (diameter 24 mm, thickness 0.5 mm) were immersed into the collagen suspension at 4 °C for 8 hr, carefully rinsed with PBS, and then immersed in a bovine serum albumin solution (20 mg/mL) at 20 °C for 2 hr.

**Measurement of the interaction of platelets and the H12- or rGPIbα-latex beads with collagen surface**

Blood, which was treated with a thrombin inhibitor PPACK (final concentration, 40 μM), was filtered with a leukocyte removal filter (NEO1J, Nihon Poll, Tokyo) to remove platelets as well as leukocytes. The residual platelet concentration of the filtered blood was determined to be (6.0 ± 2.0) x $10^3$ /μL, and the final platelet concentration was adjusted to 2.0

x $10^4$ /μL by the addition of PRP. The platelet concentration was determined using an automated hematology analyzer (K-4500). Blood thus prepared was termed thrombocytopenia-imitation blood.

In the perfusion study, either platelets or the latex beads were labeled with a fluorescent marker (platelets: $DiOC_6$, LB: FITC). The thrombocytopenia-imitation blood in the presence of the H12- or rGPIbα-latex beads (1.0 x $10^5$/μL) was placed in a recirculating chamber mounted on an epifluorescence microscope (ECLIPS TE300, Nikon, Tokyo) equipped with a CCD camera, and the interaction of the platelets or latex beads with the collagen immobilized on the surface was observed at 37 °C. The surface coverage of platelets or H12- or rGPIbα-latex beads on the plates was calculated with an Argus-20 image processor (Hamamatsu Photonics, Hamamatsu). All measurements were performed in triplicate, and results showed average surface coverage and all experimental data into parentheses.

**Observation of the H12-latex beads or the rGPIbα-latex beads adhering on the platelet thrombus with a scanning electron microscope**

Aggregates of platelets and latex beads adhering on the glass plates after the perfusion study were washed with a Hepes buffer (pH 7.4), fixed with 1 % (v/v) glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M phosphate buffer (pH 7.4) for 30 min, and post-fixed with 1 % (w/v) osmium tetroxide in the same buffer for 30 min. The

samples were dehydrated with a graded ethanol series and then dried in a freeze-dryer (Hitachi ES-2020, Hitachi, Tokyo) using *t*-butyl alcohol. For immunostaining, the plates were incubated with 0.1 % (w/v) bovine serum albumin in PBS for blocking. They were incubated with mouse anti human GPIb/IX antibody (SZ2, Immunotech, 10 μg/mL, PBS) at 4 °C overnight. The plates were washed three times with PBS and incubated with goat-anti human IgG coupled to colloidal gold (Amersham bioscience Inc., 15 nm, 1:100 final dilution) at r.t. for 60 min. After coating with osmium tetroxide (ca. 5 nm thick) using an osmium plasma coater (NL-OPC80, Nippon Laser & Electronics Lab., Nagoya), the samples were examined with a Hitachi S-4500 field emission scanning electron microscope (SEM) at an accelerating voltage of 7 kV.[27] Colloidal gold-labeled latex beads on the platelet thrombus (the number of thrombus: 120) were counted as the rGPIbα-latex beads, and the non-labeled latex beads were counted as H12-latex beads. The glass plates were marked to distinguish the direction of blood flow, which was from left to right of the SEM image.

The distribution of each bead on the platelet thrombus as shown in Fig. 4 (a) was determined as follows. Firstly, we drew a line connecting the center of a thrombus and the center of a latex bead. Secondly, from the point where the line met the thrombus edge, a tangent line was drawn. Finally, a second line (1) was drawn parallel to the tangent line and passed through the center of latex beads.

Next, we determined the blood flow as flux and we drew the flux of reflection from

the point where the flux of incidence was intersected with the line (1). The flux of reflection was divided with X and Y components. We defined the distribution of the latex beads as "front" when the X component was negative, and as "side" when the X component was positive. Furthermore, when the latex beads were located to the downstream side of the thrombus, and the flux did not directly collide with the latex beads, we defined the distribution as "back".

## RESULTS

### Characterization of the H12-latex beads, the rGPIbα-latex beads, and the H12/rGPIbα-latex beads

The mercapto group attached to the N-terminal of H12 as cysteine was reacted with the PD group conjugated to the lysine residues of rHSA, which was physically absorbed on the surface of the latex bead. The number of H12 molecules conjugated to one latex bead was estimated to be approximately $2.0 \times 10^5$ (by HPLC). Similarly, the mercapto group of rGPIbα was conjugated to the latex beads via a disulfide linkage with the rHSA absorbed on the latex bead. The number of rGPIbα molecules on the surface of the latex bead was calculated to be approximately $1.0 \times 10^4$. In the preparation of the H12/rGPIbα-latex beads, SH-rGPIbα molecules were reacted with the PD group (approximately $1.0 \times 10^5$/particle) remaining on the surface of the H12-latex beads. However, the resulting number of the rGPIbα molecules

did not reach 1.0 x$10^4$ and was estimated to be 5.0 x $10^3$, in spite of the addition of excess SH-rGPIbα in comparison with that of rGPIbα-latex beads preparation. In this study, two preparations of the H12/rGPIbα-latex beads were used. One preparation was estimated to contain 1.7 x $10^3$ molecules of rGPIbα per bead and the other 5.0 x $10^3$ molecules per bead.

**Platelet aggregation study**

Using an aggregometer, we confirmed the function of H12 on the H12-latex beads and the H12/rGPIbα-latex beads as shown in Fig. 1. In the case of the control albumin-absorbed latex beads, the transmittance of ADP-induced platelet aggregation was 40% (Fig. 1a). When the H12-latex beads were added instead of the control, the transmittance increased significantly to approximately 70% (Fig. 1b). In the case of the H12/rGPIbα-latex beads, the increase in transmittance was considerably smaller and the transmittance approached the control value, especially for the H12/rGPIbα-latex beads conjugated with the large number of rGPIbα molecules (Fig. 1c and d). The rGPIbα-latex beads showed the same transmittance as the control (data not shown).

**The interaction of platelets, H12- and rGPIbα-latex beads with collagen surface**

We studied a mixture of H12-latex beads and rGPIbα-latex beads, both of which were labeled with FITC, and allowed to flow over the collagen surface at various shear rates. We

used the thrombocytopenia-imitation blood where the total number of platelets and latex beads were adjusted to 2.0 x $10^4$ /μL and 1.0 x $10^5$ /μL, respectively. At first, we confirmed that no adhesion was observed at various shear rates when the control latex beads were allowed to flow as shown in Fig. 2a (●). When the H12-latex beads were made to flow over the surface instead of the control at a shear rate of 150 $s^{-1}$, the average surface coverage after 3 min increased significantly to 5.1 % [3.9, 4.9, 6.6 %]. The average surface coverage decreased to 1.2 % [0.7, 1.2, 1.6 %] at 800 $s^{-1}$ and 1.0 % [0.3, 1.1, 1.7 %] at 1600 $s^{-1}$ with the increasing shear rate (Fig. 2a (◊)). In contrast, the rGPIbα-latex beads adhered immediately and accumulated on the collagen surface. The average surface coverage of the rGPIbα-latex beads increased with the increasing shear rate and was 2.8 % [2.1, 3.0, 3.4 %] at 150 $s^{-1}$, 4.3 % [3.2, 3.9, 5.8 %] at 800 $s^{-1}$, and 5.0 % [4.1, 5.0, 5.8 %] at 1600 $s^{-1}$ (Fig. 2a (▵)). For a mixture of H12- and rGPIbα-latex beads, the arithmetic mean surface coverage was calculated to be 4.0 % [3.5, 3.7, 4.8 %] at 150 $s^{-1}$, 2.8 % [2.0, 2.8, 3.5 %] at 800 $s^{-1}$, and 3.0 % [2.9, 3.0, 3.1 %] at 1600 $s^{-1}$ (Fig. 2a (□)). However, when a mixture of H12-latex beads (5.0 x $10^4$ /μL) and the rGPIbα-latex beads (5.0 x $10^4$ /μL) was used, the surface coverage was surprisingly greater than the theoretical arithmetic mean surface coverage with increasing shear rate. The average surface coverage was 3.5 % [2.2, 3.7, 4.5 %] at 150 $s^{-1}$, 3.7 % [2.9, 3.4, 4.9 %] at 800 $s^{-1}$, and 4.6 % [3.9, 4.1, 5.9 %] at 1600 $s^{-1}$ (Fig. 2a (○)).

Next, we evaluated the adhesion and aggregation of platelets labeled with $DiOC_6$ instead

of labeled latex beads under the same experimental conditions as above. In the presence of the control latex beads, the average surface coverage of platelets was 2.3 % [1.2, 2.2, 3.6 %] at a shear rate of 150 $s^{-1}$, and gradually decreased with increasing shear rate and was 1.0 % [0.3, 1.1, 1.7 %] at 800 $s^{-1}$ and 0.8 % [0.5, 0.6, 1.4 %] at 1600 $s^{-1}$ (Fig. 2b (●)). In the absence of the control, the coverage did not change at all under all observed shear rates (data not shown). When the H12-latex beads were made to flow over the collagen surface, the platelet surface coverage increased significantly to 4.8 % [3.3, 5.2, 6.0 %] at a low shear rate of 150 $s^{-1}$ and gradually decreased with increasing shear rate, 1.1 % [0.7, 1.2, 1.5 %] at 800 $s^{-1}$ and 1.0 % [0.4, 0.5, 2.2 %] at 1600 $s^{-1}$ (Fig. 2b (◊)). In contrast, in the presence of the rGPIbα-latex beads, the platelet surface coverage increased with increasing shear rate, 2.4 % [1.8, 1.9, 3.4 %] at 150 $s^{-1}$, 2.8 % [1.3, 3.2, 3.8 %] at 800 $s^{-1}$, and 3.7 % [2.7, 3.8, 4.5 %] at 1600 $s^{-1}$ (Fig. 2b (△)). On the other hand, the arithmetic mean platelet surface coverage for a mixture of H12-latex beads and rGPIbα-latex beads was 3.6 % [2.6, 3.5, 4.7 %] at 150 $s^{-1}$, 1.9 % [1.2, 1.9, 2.6 %] at 800 $s^{-1}$, and 2.3 % [1.5, 2.1, 3.4 %] at 1600 $s^{-1}$ (Fig. 2b (□)). Although the platelet surface coverage of the mixed system did not change from the arithmetic mean surface coverage especially at low shear rate such as 150 $s^{-1}$ (3.3 % [2.6, 2.8, 4.6 %]), the surface coverage tended to be higher than that of arithmetic mean as the shear rate increased, 2.2 % [1.6, 2.2, 2.9 %] at 800 $s^{-1}$ and 3.2 % [2.0, 2.2, 4.7 %] at1600 $s^{-1}$ (Fig. 2b (○).

H12-latex beads were labeled with a fluorescent marker; FITC, to monitor the

time-course of adhesion of the H12-latex beads in the mixed system at a shear rate of 1600 $s^{-1}$. The control latex beads did not adhere to the collagen on the plate as shown in Fig. 3 (□). The H12-latex beads did not initially adhere to the plate; however, the average surface coverage slightly increased to 1.8 % [1.5, 1.8, 2.2 %] after 4 min of flow (Fig. 3 (△)). In the case of the mixed system of the rGPIbα-latex beads and the H12-latex beads, the initial binding rate of the H12-latex beads was small and then dramatically increased with time reaching 4.2 % [3.2, 4.1, 5.2 %] after 4 min (Fig. 3 (○)).

**Evaluation of adherence of H12-latex beads or rGPIbα-latex beads on the platelet thrombus by immunogold scanning electron microscopy**

By immunogold scanning electron microscopy, we could immunocytochemically distinguish the rGPIbα-latex beads from the H12-latex beads, adhering on the platelet thrombus in the mixed system after flowing at a shear of 1600 $s^{-1}$. The average number of each latex bead adhering to one thrombus was determined. There were abundant platelet thrombi with adhering latex beads, and the total number of the latex beads adhering on one thrombus was estimated to 6.5 $\pm$ 2.6 particles (Fig. 4 (b)). The rGPIbα-latex beads adhered approximately 3 times more frequently than the H12-latex beads. Next, we determined the distribution of each latex bead as one of three kinds of adherent distribution on the platelet thrombus as described in the experimental section (Fig. 4 (a)). The average adhesion numbers

of the rGPIbα-latex beads located at the front, the side, and the back on one platelet thrombus were 1.7 ± 0.5, 2.3 ± 0.4, and 1.5 ± 0.6 particles, respectively, showing that they tend to distribute on the side (Fig. 4 (b)). Whereas, the numbers of the H12-latex beads in the front, the side, and the back were 0.9 ± 0.4, 0.6 ± 0.3, and 1.7 ± 0.6 particles, respectively, showing that they especially tend to distribute on the back.

## DISCUSSION

In the previous studies on platelet substitutes,[1-5] although the substitutes were shown to be useful in reinforcing platelet aggregation and reducing bleeding time *in vivo*, they were derived from human blood. We have focused on H12, which is a synthetic oligopeptide of human fibrinogen, and conjugated it to the surface of FITC-labeled latex bead.[19] The H12-latex beads in an erythrocyte suspension adhered to the surface and accumulated in a time-dependent manner when they were allowed to flow over the activated platelets immobilized on a collagen surface at a shear rate of 150 $s^{-1}$, whereas the control latex beads did not adhere at all. The adhesion of the H12-latex beads was suppressed in the presence of free H12 as an inhibitor of GPIIb/IIIa binding, indicating that the adhesion was a specific interaction between the H12 and the activated GPIIb/IIIa on the immobilized platelet surface. Furthermore, the H12-latex beads showed minimal interaction with non-activated platelets based on the results of the flow cytometric analyses of agglutination using the FITC-labeled

latex beads. Therefore, H12 is a promising candidate as a platelet substitute. However, this effect was limited to work at low shear rates (*e.g.* 150 $s^{-1}$).

Our previous studies have shown that the rGPIbα-polyAlb or the rGPIbα-vesicles accumulated or rolled on the VWF-immobilized surface especially at high shear rates (*e.g.* 1600 $s^{-1}$).[7, 10] We considered that it was possible to combine H12 and rGPIbα as recognition sites for the purpose of constructing practical platelet substitutes which work in a wide range of shear rates.

We prepared three kinds of latex beads; H12-latex beads, rGPIbα-latex beads, and H12/rGPIbα-latex beads, which had both H12 and rGPIbα on the same latex bead. Latex beads were preferentially used as a model carrier because of their homogenous size and ease of detection by electron microscopic observation.

With respect to the H12-latex beads, we conjugated H12 to the surface of latex beads at a density of ca. 6.4 x $10^4$ /$\mu m^2$. This density was sufficient for the H12-conjugates to enhance the size of platelet aggregates via crosslinking between the activated GPIIb/IIIa of platelets.[19] With respect to the rGPIbα-latex beads, the surface density of rGPIbα such as ca. 3.2 x $10^3$ /$\mu m^2$ was sufficient to interact with VWF-immobilized on the glass surface.[10] In order to prepare the H12/GPIbα-latex beads, we attempted to react SH-rGPIbα with a sufficient number of the PD groups remaining on the surface of the H12-latex bead. However, we did not obtain the GPIbα density of ca. 3.2 x $10^3$ /$\mu m^2$, but ca.1.6 x $10^3$ /$\mu m^2$, indicating

that the rGPIbα could not sufficiently bind due to the steric hindrance of H12 already bound to the latex beads. We prepared two kinds of H12/rGPIbα-latex beads where the density of rGPIbα was estimated to be 5.4 x $10^2$ and 1.6 x $10^3$ /μm$^2$, respectively.

Firstly, we confirmed the function of H12 on the H12-latex beads and the H12/rGPIbα-latex beads using an aggregometer. ADP, which is an agonist of platelet aggregation, caused concentration-dependent platelet aggregation, resulting in the elevation of the transmittance of the platelet suspension.[28] We added ADP at a final concentration of 3 μM, which caused platelet shape change and first aggregation of platelets, but the effect was weak and did not cause the second aggregation of platelets. In the case of the control albumin-adsorbed latex beads (final concentration 2.0 x $10^3$ /μL), the transmittance of ADP-induced platelet aggregation was 40 % (Fig. 1). This value was the same as that in the absence of the latex beads (data not shown), indicating that the latex beads did not interfere with the platelet aggregation. H12-latex beads significantly increased the transmittance, indicating that the H12 on the latex beads connected to the activated GPIIb/IIIa on the ADP-stimulated platelets and cross-linked platelets because many molecule of H12 were conjugated to the latex bead. However, the transmittance in the presence of H12/rGPIbα-latex beads was lower than the transmittance in the presence of H12-latex beads, and was decreased with increasing rGPIbα conjugated on the latex bead. This suggested that the function of the H12 (Mw 1.3 kDa) could be suppressed by steric hindrance of rGPIbα (Mw 50 kDa) bound to

the latex bead. Therefore, we concluded that the conjugation of both H12 and rGPIbα to a single latex bead is not effective and that the conjugation of H12 should employ a spacer such as polyethylene glycol. However, this would also reduce the conjugation of rGPIbα by steric hindrance of the H12-polyethylene glycol chain.

From the above conclusion, we considered that the mixture of H12-latex beads and rGPIbα-latex beads would likely respond to a wide range of shear rates. First, we confirmed the lack of adhesion of the control latex beads to the collagen at various shear rates as shown in Fig. 2a (●), indicating that the latex beads did not interact with the collagen or activated platelets, and the surface coverage of platelets gradually decreased with the increasing shear rate (Fig. 2b (●)). This trend is the normal behavior of the platelets flowing over the collagen surface in this condition and the value was the same in the absence of the control (data not shown). Control latex beads did not participate in platelet adhesion and aggregation on the collagen surface.

In the case of the H12-latex beads at a shear rate of 150 $s^{-1}$, the average surface coverage increased approximately five-fold compared with control (Fig. 2a (◊)). The adhesion decreased considerably with increasing shear rate. This particular behavior resembled the behavior of the platelets aggregated on the platelet-immobilized surface by fibrinogen crosslinking under flow.[29] We also confirmed that the fibrinogen-conjugates preferentially interacted with the platelets at low shear rates.[6]

In the same experiment, we observed the platelet adhesion and confirmed that the H12-latex beads enhanced the thrombus formation of the collagen surface at a shear rate of 150 $s^{-1}$, and the effect gradually disappeared with the increasing shear rate (Fig. 2b (◊)). As expected from the previous report, the H12-latex beads as a model of platelet substitutes interacts with the activated platelets to form larger thrombi at low shear rates.

On the other hand, the average surface coverage of the rGPIbα-latex beads increased with increasing shear rate (Fig. 2a (△)); this corresponds to the well-known shear dependence of the interaction between GPIbα and VWF. Furthermore, in the presence of rGPIbα-latex beads, the platelet surface coverage significantly increased in comparison with that of the control latex beads, especially at a shear rate of 1600 $s^{-1}$, indicating that the rGPIbα-latex beads also enhanced thrombus formation with the remaining platelets at high shear rates (Fig. 2b (▵)). Therefore, we confirmed that H12-latex beads alone or the rGPIbα-latex beads alone were not effective in enhancement of platelet aggregation or thrombus formation at a wide range of shear rates.

We proposed the use of a mixture of the H12- and rGPIbα-latex beads to confer function at a wide range of shear rates, because the surface coverage was expected to be theoretically the arithmetic mean of both H12-latex beads and rGPIbα-latex beads as shown in Fig. 2a and 2b (□). In fact, the slope of the surface coverage curve in the case of the mixed system was smaller than that of each single system, indicating that the beads in the mixed

system were able to adhere at wide range of shear rates (Fig. 2a and 2b (○)). However, surprisingly, the average surface coverage was significantly higher than the arithmetic mean with increasing shear rates. If H12-latex beads and rGPIbα-latex beads were independently worked, the surface coverage at a shear rate of 1600 $s^{-1}$ would have been the same as the arithmetic mean.

In order to determine the reason for the above observation, we used H12-latex beads labeled with a fluorescent marker, FITC, and valuated the time-course of adhesion of H12-latex beads in the mixed system at a shear rate of 1600 $s^{-1}$. We had determined that H12-latex beads alone could not adhere at high shear rate Fig. 2a (◊). Surprisingly, the initial binding rate of the H12-latex beads in the mixed system was slow and then increased dramatically with time (Fig. 3 (○)).

In order to consider the above phenomena, we immunocytochemically observed the platelet thrombus involving H12-latex beads or rGPIbα-latex beads after flowing at a shear of 1600 $s^{-1}$ using a scanning electron microscope. We visually reconfirmed that the number of rGPIbα-latex beads adhering to a thrombus was more than that of H12-latex beads, and that H12-latex beads adhered even at high shear rates. Furthermore, rGPIbα-latex beads tended to locate on the side of a platelet thrombus, whereas H12-latex beads tended to locate at the back (Fig. 4). It has been known that the stagnation and reattachment points are generated at the downstream (back) of stenosis in vessels.[30, 31] The point of thrombus formation is usually the

point of fluid stagnation, that is, low shear rates. In particular, because blood is a non-Newtonian fluid, the burble and reattachment points tend to occur in more stagnant regions. Furthermore, fluid velocity is zero at the point of collision (front) with the stenosis. We considered that this property could be exploited for adhesion of latex beads in the process of platelet thrombus formation. The reason that H12-latex beads in the mixed system adhered to the collagen even at the shear rate of 1600 $s^{-1}$ was considered to be as follows: firstly, rGPIbα-latex beads could adhere to collagen surface at high shear rates and in doing so enhance the platelet thrombus formation. Secondly, blood fluid would tend to become stagnant at the front or back of the thrombus, and H12-latex beads could adhere to the sites even at high shear rates. At the same time, adhesion of rGPIbα-latex beads also would be enhanced at the side of the thrombus. Finally, platelet thrombus formation would be enhanced by their cooperative effects.

## CONCLUSION

A mixture of H12-latex beads and rGPIbα-latex beads was found to enhance thrombus formation at a wide range of shear rates by their cooperative effects in comparison with each of the beads used singly. After further evaluating the function of the H12- or rGPIbα-latex beads *in vitro*, we are planning to test more biocompatible particles such as phospholipid vesicles or polymerized albumin particles for *in vivo* investigation. Thus, such biocompatible

particles may be suitable candidates for an alternative to human platelet concentrates transfused into thrombocytopenic patients.

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