Collagen I-A (3.0 mg/mL, Cellmatrix, Nitta Gelatin, Osaka, Japan) was suspended in PBS at 4 °C to give a final concentration of 30 μg/mL. A glass plate (diameter 24 mm, thickness 0.5 mm) was 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 r.t for 2 hr.

Measurement of the interaction of platelets with the collagen surface in the presence of H12-polyAlb using thrombocytopenic-imitation blood.

Blood withdrawn from healthy volunteers was treated with the thrombin inhibitor PPACK (final concentration of 40 μM) and was filtered through a leukocyte removal filter (NEO1J, Nihon Poll, Tokyo), which could remove platelets as well as leukocytes. The residual platelet concentration of the filtered blood was determined to be $5.0 \pm 3.0 \times 10^3$ /μL, and the final platelet concentration was adjusted to 20×10^3 /μL by addition of PRP, which was prepared by centrifugation (100 *g*, 15 min, 22 °C) of sodium citrate-treated blood. The platelet concentration was determined using an automated hematology analyzer (K-4500). This blood preparation was termed as thrombocytopenic-imitation blood.

The thrombocytopenic-imitation blood and H12-polyAlb (10 mg/mL, 70 μL)

mixtures were placed in a recirculating chamber mounted on an epifluorescent microscope (ECLIPS TE300, Nikon, Tokyo, Japan) equipped with a CCD camera, and the interaction of platelets with the collagen immobilized on the surface was observed. Single-frame images of adhesion and aggregation of platelets in the presence of H12- or cH12-polyAlb were obtained with an image processor, Argus-50 (Hamamatsu Photonics, Hamamatsu, Japan), and the surface coverage of the adhered platelets on the plate was calculated with an image processor, Argus-20 (Hamamatsu Photonics, Hamamatsu, Japan). All perfusion studies were performed at 37 °C.

Measurement of the tail bleeding time of the thrombocytopenic rats

All animal studies were approved by the Animal Subject Committee of Keio University, School of Medicine and performed according to NIH guidelines for the care and use of laboratory animals (NIH publicatgion 85-23 Rev. 1985). Experiments were carried out using male Wistar rats (230-250 g, CLEA Japan, Tokyo, Japan). A busulphan solution was prepared at a final concentration of 5 mg/mL in polyethylene glycol (average molecular weight 400) [32, 33]. Rats were anesthetized with diethyl ether and injected on Day 0 and Day 3 with 10, 15, or 20 mg/kg on each dosing day, to produce a total dosage of 20, 30, or 40 mg/kg

of busulphan, respectively. Blood samples for cell counting was obtained from ether-anesthetized rats by inserting a 25-gauge needle into a tail vein, and the cell concentration was determined using an automated hematology analyzer (K-4500).

On Day 10, thrombocytopenic rats were anesthetized with sevofrane, and the sample suspension was infused into the tail vein. The samples were H12-polyAlb, cH12-polyAlb or polyAlb at a dose of 4 mL/kg; saline was used to obtain the control value. Five minutes after administration, a 2.5 mm length x 1.0 mm depth template-guided incision (Quikheel™, Becton-Dickinson, San Jose, CA) was made 1 cm from the tip of the tail. A tail was immersed in a 50 mL cylinder of saline and the time taken for bleeding to stop was measured. In addition, cell concentrations were determined using an automated hematology analyzer (K-4500) before (-5 min) and after (30 min) samples injection.

Statistical Analysis

Statistical significance of Day 10 group vs. normal group as shown in Fig. 4, and H12-polyAlb group vs. saline group, polyAlb group, and cH12-polyAlb group as shown in Fig. 5 was tested with Tukey-Kramer tests. A P value of less than 0.05 was considered to be statistically significant. Statistical analytic software was used Stat View (SAS Institute Inc.).

RESULTS

Platelet aggregation study

Using an aggregometer we confirmed that H12 showed a concentration-dependent suppression of platelet aggregation [31]. In particular, when H12 was added at a concentration of 1 mM, platelet aggregation was significantly suppressed in comparison with the control experiment (Fig. 1). In contrast, cH12 at the same concentration did not inhibit platelet aggregation.

Characterization of H12-polyAlb and cH12-polyAlb

In the conjugation reaction, the mercapto group of the terminal cysteine of H12 or rH12 reacted with the PD group of the polyAlb particles which have diameters of 260 $\pm$ 60 nm, and 2-thiopyridone (2TP) was liberated after the formation of disulfide linkage due to the thiol-disulfide exchange reaction. Using the absorption of the liberated 2TP at 343 nm, the number of the H12 and cH12 molecules conjugated to one polyAlb was estimated to be approximately 9.6×10^3 and 9.1×10^3, respectively. The endotoxin concentration in the H12- or cH12-polyAlb suspension was below 0.2 EU/mL.

Measurement of the interaction of platelets with the collagen surface in the presence of H12-polyAlb using thrombocytopenic-imitation blood under flow conditions.

We prepared the thrombocytopenic-imitation blood and adjusted the number of platelets to 20 x 10^3 /μL. The platelets were labeled with a fluorescent marker, $DiOC_6$, in order to observe the adherence of platelets to the collagen surface at a shear rate of 150 s^{-1}. In the presence of the control polyAlb, the total surface coverage of platelets was 2.1 ± 0.4 % after 210 seconds of flow (Fig. 2). When platelets were allowed to flow over the collagen-immobilized surface in the absence of polyAlb, the same value was obtained. When the H12-polyAlb particles were added to the blood instead of the polyAlb, there was a significant increase in the surface coverage to 3.9 ± 1.1 %. By contrast, when cH12-polyAlb was used, the surface coverage did not change from that of the control polyAlb (2.4 ± 0.4 %).

Preparation of the thrombocytopenic rats

Rats that received busulphan at total doses of 40 mg/kg or 30 mg/kg developed profound thrombocytopenia with a decline in the level of platelets to half-maximal value on Day 7 or 8, respectively, which reached the lowest value between Day 10 and 14 (Fig. 3). Unfortunately, the rats became anorexic, lost weight, became anemic, and were all dead by

Day 12 and 16, respectively (data not shown). However, at a total dose of 20 mg/kg, thrombocytopenia was just as profound as at the higher doses of busulphan but all rats survived with no major problems. As shown in Fig. 3, the rats treated with the 20 mg/kg dose had a decline in platelet count to half-maximal value ($410 \pm 30 \times 10^3$ /μL) on Day 9. On Day 10, their platelet concentration was $200 \pm 30 \times 10^3$ /μL, which was equivalent to 1/4 to 1/5 of the platelet count of normal rats. There was a slight decrease in the leukocytes concentration of the busulphan-treated rats compared with that of uninjected control rats; however, the weight of busulphan-injected rats increased and their erythrocyte counts were unchanged (Table 1). Predictably, there was a gradual decrease in the platelet count of the busulphan-treated rats to Day 11 ($97 \pm 25 \times 10^3$ /μL); however, erythrocyte count also began to decline compared with that of uninjected control rats (data not shown). After the platelet count reached the lowest point on Day 14, the low count of $3 \pm 2 \times 10^3$ /μL persisted for at least 6 days before starting to rise gradually.

Measurement of the tail bleeding time of the thrombocytopenic rats in the presence of H12-polyAlb

The tail bleeding times of the normal rats ([platelet] = $810 \pm 90 \times 10^3$ /μL) and

thrombocytopenic rats on Day 10 after the busulphan injection ([platelet] = 200 ± 30 x 10^3 /μL) were 187 ± 51 and 609 ± 153 s, respectively (Fig. 4). On the other hand, platelet concentrations in the course of preparation of thrombocytopenic rats on Day 8.5 and 9 were 570 ± 40 and 410 ± 30 x 10^3 /μL, respectively, and their bleeding time was 288 ± 56 and 366 ± 153 s, respectively. In addition, after Day 11, thrombocytopenia became progressive rapidly but the measurement of bleeding time was hard to be standardized (data not shown). In fact, the bleeding time of the busulphan (20 mg/kg)-injected rats on Day 11 (platelet; 97 ± 25 x 10^3 /μL)was found to be immeasurable, since bleeding did not stop for more than 30 min. Therefore, for in vivo experiment we used animals on Day 10 after the busulphan treatment.

The intravenous administration of H12-polyAlb at a dose of 1 mg/kg slightly reduced the bleeding time to 581 ± 110 s in comparison with those of saline group (609 ± 153 s) or control polyAlb at a dose of 1 mg/kg (679 ± 102 s) (Fig. 5). At doses of 4 and 10 mg/kg, we confirmed a dose-dependent reduction of the bleeding time, and the time decreased significantly to 342 ± 73 and 288 ± 120 s, respectively. By comparison, control polyAlb groups at doses of 4 and 10 mg/kg were 553 ± 104 and 436 ± 102 s, respectively.

DISCUSSION

In the previous studies on platelet substitutes [2-5], despite their usefulness in reinforcing platelet aggregation and reducing bleeding time *in vivo*, their raw materials are dependent on human blood. In our previous studies, we have focused on H12, which is a synthetic peptide of human fibrinogen, and we conjugated H12 to the surface of FITC-labeled latex beads [31]. H12-latex beads in an erythrocyte suspension were allowed to flow over the activated platelets that were immobilized on a collagen surface at a shear rate of 150 s^{-1}. They adhered and accumulated to the surface in a time-dependent manner, whereas control latex beads did not adhere. The adhesion of H12-latex beads was suppressed in the presence of free H12 as an inhibitor of GPIIb/IIIa binding, showing that the adhesion was specific. Furthermore, the H12-latex beads showed minimal interaction with non-activated platelets based on the results of flow cytometric analyses of agglutination using the FITC-labeled latex beads. Therefore, H12 is a non-infective promising part as a recognition site of platelet substitutes. We prepared H12-particles with recombinant human serum albumin, which was not derived from human blood to obtain information about the possibility to construct biocompatible and biodegradable particles with hemostatic ability in thrombocytopenic conditions.

Firstly, we confirmed a specific binding of H12 in comparison with cH12 from a study

of the inhibition of fibrinogen-mediated platelet aggregation. Because H12 is a fibrinogen sequence, which is responsible for the recognition of GPIIb/IIIa on activated platelets [22-28], it significantly suppressed the platelet aggregation by competitive binding to the fibrinogen-binding site of the platelet, and the binding of H12 for GPIIb/IIIa was maintained even after the introduction of the N-terminal cysteine. On the other hand, cH12 showed a minimal inhibitory effect. These results were also supported by the other publications that the IC_{50} of inhibition of ^{125}I-fibrinogen binding to activated platelets by H12 and cH12 was 28 μM and 85 μM, respectively, indicating that binding avidity for GPIIb/IIIa of H12 was stronger than that of cH12 [23, 24].

We sufficiently conjugated either H12 or cH12 to the surface of polyAlb, and estimated the conjugation density on the polyAlb surface to approximately 46 x 10^3 or 43 x 10^3 molecules/μm^2, respectively. The conjugation densities were similar to that of H12-latex beads (diameter 1 μm) which enhanced platelet thrombus formation [31]. In thrombocytopenic-imitation blood flowing on the collagen surface, the surface coverage of platelets in the presence of control polyAlb was very low and the same in the absence of polyAlb. Furthermore, we confirmed that H12-polyAlb enhanced the thrombus formation of the remaining platelets, whereas, cH12-polyAlb did not. They indicate that the particles such

as the polyAlb and the cH12-polyAlb did not interfere the platelet adhesion and aggregation, and the enhancement effect was specific for H12 conjugated to the surface of polyAlb particles.

Next, we prepared the thrombocytopenic rats using busulphan in order to evaluate a hemostatic ability of H12-polyAlb *in vivo*. We succeeded in obtaining the extinction curve of platelets similar to that of the previous studies [32]. It was reported that a low hematocrit resulted in a significant prolongation in the bleeding time [34]. In fact, the bleeding time of the busulphan (20 mg/kg)-injected rats on Day 11 (platelet; $97 \pm 25 \times 10^3$ /μL)was found to be immeasurable, since bleeding did not stop for more than 30 min. From the data of hematologic indices, which the platelet counts were sufficiently decreased and the erythrocyte counts were maintained, as shown in Table 1, we determined that the appropriate dose of busulphan for the rats was 20 mg/kg and the incision of the tail was to be made on Day 10. The bleeding time of the thrombocytopenic rats on Day 10 was extended significantly in comparison with that of normal rats as shown in Fig. 4. The bleeding time approached to that of normal rats as increasing their platelet concentration. We confirmed that the bleeding time was correlated with their platelet concentration, and inferred that the tail bleeding time was an effective evaluation parameter for measuring the hemostatic capacity of the platelet

substitutes.

The endotoxin concentration in the H12- or cH12-polyAlb suspension was below 0.2 EU/mL, acceptable for the *in vivo* study. In preliminary experiments we studied the systemic clearance of H12-polyAlb. At a dose of 4 mg/kg (equivalent albumin concentration), the amount of FITC-labeled H12-polyAlb rapidly decreased to 88.8, 51.1, 27.2, and 9.0 % after 5, 10, 15, and 30 min, respectively (taken as 100 % just after injection) using a spectrofluorometer (FP-750, JASCO, Tokyo, Japan). The half-life of the H12-polyAlb was estimated to be approximately 10 min from the above data. Therefore, we measured the tail bleeding time 5 min after the injection. We are now studying the prolongation of the half-life of the H12-polyAlb using polyethylene glycol (PEG) modification.

Although not tested in animals with severe thrombocytopenia, we confirmed the hemostatic effect of H12-polyAlb in vivo using moderately thrombocytopenic rats, suggesting that the polyAlb may be a promising candidate for platelet substitutes (Fig. 5). The circulating H12-polyAlb at the dose of 4 mg/kg significantly reduced the bleeding time of the thrombocytopenic rats, whereas, at a lower concentration of 1 mg/kg, the hemostatic effect did not reach at a significant level. At a higher concentration of 10 mg/kg, however, the bleeding time of H12-polyAlb was significantly reduced in comparison with that of saline

group, but not the polyAlb group, because the bleeding time of the control polyAlb was also slightly reduced, indicating that the polyAlb itself possessed a hemostatic activity at its higher concentration. When we used PEG-modified polyAlb, the bleeding time became comparable to that obtained with saline group (manuscript in preparation), suggesting that negative charge of the control polyAlb at pH 7.4 may nonspecifically cause the shortening of the tail bleeding time.

We estimated that the hemostatic ability of the H12-polyAlb at a dose of 4 mg/kg (approximately 1.0×10^{11} particles) would correspond to 1/20th of the hemostatic ability of one platelet, which was based on the bleeding time of the thrombocytopenic rats and platelet concentration. Furthermore, we confirmed that hematologic indices were stable before and after the administration of H12-polyAlb.

In conclusion, H12-polyAlb was shown to preferentially interact with an activated platelet surface via GPIIb/IIIa receptors and to facilitate platelet accumulation at sites of hemostasis. Furthermore, H12-polyAlb had the hemostatic ability in the tail bleeding model of thrombocytopenic rats. Thus, H12-polyAlb may be a suitable candidate for an alternative to human platelet concentrates infused into thrombocytopenic patients.

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FIGURE CAPTIONS

Fig. 1 ADP-induced (20 μM) platelets aggregation (200 x 10^3 /μL) was monitored with an aggregometer in the presence of 1 mM H12 or cH12, or PBS (control).

Fig. 2 Comparison of surface coverage of platelets in the thrombocytopenic-imitation blood on the collagen surface after addition of H12-polyAlb, cH12-polyAlb, or polyAlb at a shear rate of 150 s^{-1} (N = 4). The amount of surface coverage was recorded after blood was circulated for 210 s. The concentration of platelets was 20 x 10^3 /μL. *P < 0.05 for H12-polyAlb group vs. saline group, polyAlb group, and cH12-polyAlb group.

Fig. 3 Dose-response effect of busulphan on rats. Rats were injected with busulphan at a total dose of 20 mg/kg (○), 30 mg/kg (△), or 40 mg/kg (□) as described in Materials and Methods. Tail vain blood was collected at intervals and platelet concentration measured. Arrows show injection day of busulphan (N = 5).

Fig. 4 Correlation of tail bleeding time (white bars) with platelet concentration (○) in the rat

(N = 5). *P < 0.05 for Day 10 group vs. normal group.

Fig. 5 Effects of the administration of H12-polyAlb on tail bleeding time (white bars). The administered amount of H12-polyAlb was 1, 4, 10 mg/kg equivalent of albumin. ○; platelet concentration in the rats (N = 6-10). *P < 0.05 or **not significant for H12-polyAlb vs. polyAlb group at the same dose.

Table 1 Weights and hematological parameters before and after (on day 10) busulphan injection.

	Initial weight (g)	Final weight (g)	RBC ($x10^3/\mu L$)	WBC ($x10^3/\mu L$)	PLT ($x10^3/\mu L$)
normal (n = 6)	251 ± 11	304 ± 15	7160 ± 300	12.7 ± 2.8	809 ± 86
busulphan 20 mg/kg (n = 6)	258 ± 10	288 ± 12	7000 ± 230	8.1 ± 1.2	198 ± 28