bearing receptor proteins of the platelet surface recognize the collagen surface under shear rates. The rolling and adhesion properties will be discussed depending on the stiffness or membrane fluidity of the particles.

Present Status of the Development of Red-Blood-Cell Substitutes [1,2]

Hb-vesicles that encapsulate concentrated hemoglobin with a phospholipids bilayer membrane have a similar structure to red blood cells, and are expected to be used soon in clinical tests because the degree of safety and efficacy are considered to be high. Although effective use of the hemoglobin from donated and expired blood should be promoted at the present stage, recombinant human hemoglobin will be used in the future. During hemoglobin purification from red blood cells, stroma including the glycoproteins which determine a blood type, proteins other than hemoglobin, and the viruses are removed by heating or filter processing. By encapsulating hemoglobin with a stabilized phospholipids membrane with POE-lipid, liquid-state preservation for 2 years is guaranteed at room temperature under nitrogen atmosphere [3], and with dry powder, further prolonged preservation is possible. These points are advantages for an artificial oxygen carrier.

The design of the red-blood-cell substitutes (POE-modified hemoglobin vesicles) are summarized in Fig. 1. Hb-vesicles are dispersed into a saline solution and enclosed with the bottle in a state of deoxidization. The hemoglobin concentration is 10 g/dl and is close to that of human blood. Moreover, because hemoglobin molecules are encapsulated, the colloid osmotic pressure of the solution is zero. Therefore, when regulation of colloid osmotic pressure

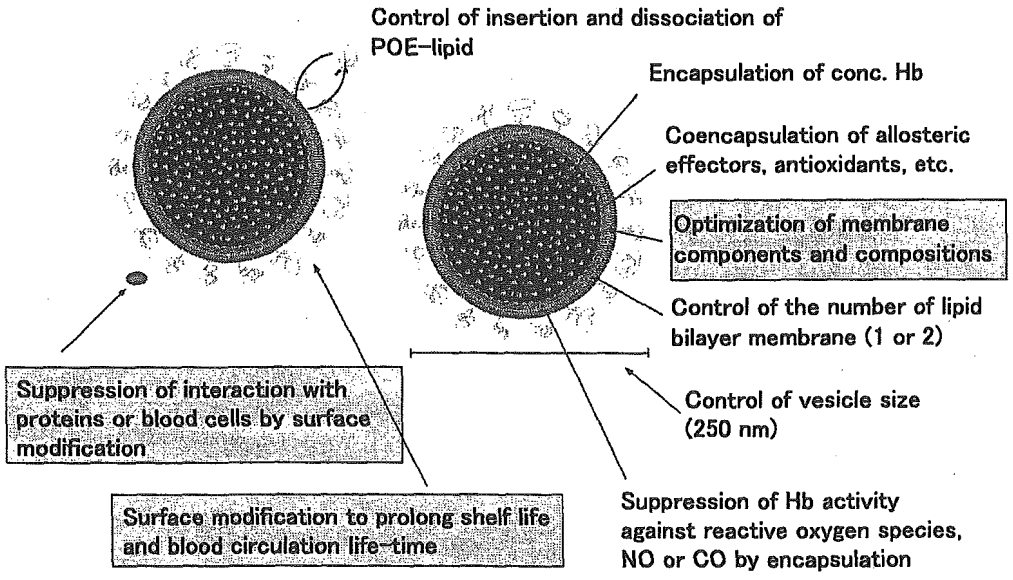

FIG. 1. Design of POE-modified hemoglobin vesicles (Hb-vesicles) as red-blood-cell substitutes

is needed, a solution of colloids such as albumin and polysaccharide will be used with the Hb-vesicle dispersion. The particle diameter is strictly adjusted to 250 nm. The degree of oxygen affinity is adjusted to a suitable value by coencapsulating an allosteric effector such as pyridoxal 5′-phosphate. The optimization of the composition of the lipid components resulted in high encapsulation efficiency of hemoglobin in the Hb-vesicle, a stability of 2 years in a liquid state, the prevention of hemolysis, an appropriate lifetime in blood circulation, and avoidance of platelet and complement activation. Furthermore, large-scale manufacturing has been improved by the introduction of freeze-thawing and freeze-drying operations which can control a molecular assembling state before encapsulating hemoglobin molecules.

Present Results of Safety and Efficacy Tests

Although in vivo testing was carried out using rats or hamsters, we confirmed the fundamental safety and oxygen transporting ability. Safety tests using primates is in progress. When 90% of the volume of rat blood was exchanged by the albumin dispersion of the Hb-vesicles, the oxygen partial pressure of the renal cortex was maintained as was blood pressure [4]. On the other hand, when the blood was exchanged by an albumin solution in the same concentration, the fall of blood pressure and oxygen partial pressure of the renal cortex became noticeable at 70% exchange, and all rats died just after 90% exchange.

In the hamster 80% exchange transfusion examination with the albumin dispersion of the Hb-vesicles, the noninvasively measured oxygen partial pressure of the subcutaneous tissue microcirculatory system was maintained at 5 times or more than that of the control albumin group although it fell to 60%–70% before exchange [5]. The contraction of a resistance blood vessel and the rise of blood pressure was not confirmed at all, but it was confirmed with modified hemoglobin products. Because the Hb-vesicle has a size that does not penetrate a blood vessel, there is no influence on the activity of nitrogen oxide as an endothelium-derived relaxation factor [6]. Furthermore, the Hb-vesicles cannot penetrate the sinusoidal vessels of liver (several holes 10–200 nm in size are open in the blood vessels) like old red blood cells, but are metabolized by Kupfer cells of liver and macrophages in reticuloendothelial systems. On the other hand, acellular hemoglobin molecules in the liver influenced liver microcirculation by eliminating carbon monoxide as a gaseous vasodilator, caused overgeneration of bilirubin, and suppressed bile secretion [7,8]. The half-life of Hb-vesicles in human blood circulation was estimated to be about three days on the basis of the results in rats, rabbits, and monkey. Moreover, from details of the blood biochemistry examination and pathology examination in the single and repetitive administration, we confirmed the transitional rise of lipase in connection with lipid decomposition, the transitional rise of a cho-

lesterol value, iron content, and the transitional hypertrophy of a reticuloendothelial system [9]. It was concluded that Hb-vesicles are expected to function adequately in cases of extensive bleeding.

Development of Platelet Substitutes

The history of platelet substitutes is short compared with that of red blood cells, with few examples of research. A platelet has complicated functions, such as adhesion specific to the bleeding site, expansion, aggregation, secretion, and the activation of a blood coagulation system. Needless to say, we cannot make platelet substitutes that have these all. However, a bleeding tendency is strongly apparent in such bleeding diseases as Bernard-Soulier syndrome and thrombasthenia, in which adhesion and aggregation ability are lacking. In these conditions, a hemostatic effect can be expected by the infusion of particles having functions such as adhesion and the aggregation of platelets due to the assistance of the function of the remaining platelets. Although clinical tests were carried out with human red blood cells [10] or albumin microcapsules [11] conjugating fibrinogen, and with the dried powder of human platelets [12], all clinical tests were suspended due to problems of safety and efficacy. Moreover, since the blood components of human origin were used, the risk of infection cannot be avoided completely. The platelet substitutes created by our group use liposomes and recombinant human albumin as biocompatible particles. They also use recombinant proteins of the part of platelet membrane or synthetic oligopeptides by conjugating to those particles for the purpose of accumulation to the bleeding site involving native platelets, expecting to achieve hemostasis.

The mechanisms of platelet adhesion differ between the blood flow of high shear rate and that of low shear rate. As shown in Fig. 2, the hemostasis of the platelets to bleeding in a high shear rate begins from a platelet recognition of the von Willebrand factor (vWf) bound to the collagen in the subcutaneous tissue of a blood vessel exposed to the bleeding site, followed by platelet adhesion and rolling. This recognition ability comes from the GPIb/V/IX complex containing GPIbα on the surface of a platelet [13]. Next, the platelets will progress and a granule will be secreted if GPIaIIa ($\alpha_2\beta_1$ integrin) or GPVI on the surface of the platelet directly interacts with the collagen and is activated. Fibrinogen recognizes the activated GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin), constructs a crosslink between blood platelets, forms an aggregate, and serves as primary hemostasis. Hemostasis is completed by formation (secondary hemostasis) of the fibrin clot by induction of the coagulation system.

The target platelet substitutes bear the water-soluble part of receptor proteins such as GPIbα which recognizes collagen through vWf under the blood

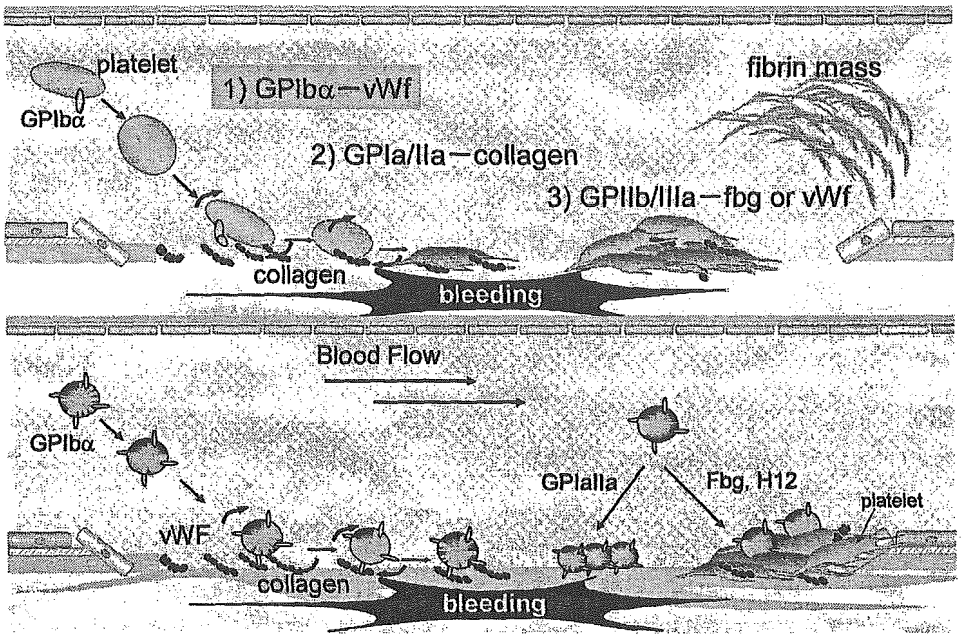

FIG. 2. Design of platelet substitutes studied from functions of natural platelets

flow of high shear rates and GPIaIIa which recognizes collagen directly at low shear rates. Instead of GPIIb/IIIa on the surface of an activated platelet, they bear the fibrinogen or its oligopeptide to assist the platelet aggregation as ligands of the GPIIb/IIIa.

If an anti-GPIIb/IIIa antibody is added to a platelet dispersion to inhibit GPIIb/IIIa activity, the platelets roll on the vWf-immobilized plate along the flow direction by the interaction of rGPIbα on the platelet and the vWf. Interestingly, phospholipid liposomes conjugating rGPIbα roll on the vWf-immobilized plate as well [14]. The number of the rolling liposomes increased with the shear rate, indicating the characteristic of rGPIbα. Moreover, the rolling speed was correlated with the membrane fluidity of the liposomes. That is, the rolling speed of "soft" liposomes was low, whereas that of the "hard" liposomes was high [15]. However, the rGPIbα-liposomes did not continue rolling but departed from the plate after rolling some length. This was remarkable as the "soft" liposomes. When the amount of rGPIbα on the surface of the liposome after the experiment was measured, it was suggested that rGPIbα-lipid should dissociate from the bilayer membrane during the rolling on the vWf-plate. Now rGPIbα-lipid which cannot dissociate serves as a point of a molecular design.

On the other hand, the rGPIaIIa-liposomes directly recognize collagen under the blood flow of low shear rates and adhered to (stopped at) the collagen-immobilized plate [16]. In this case, the number of the adhering liposomes decreased as the shear rate rose. However, liposomes conjugating both rGPIbα and GPIaIIa adhered on the collagen plate under the blood flow from low to high shear rates [17]. If the liposomes having platelet activation factors

or coagulation factors in the internal aqueous phase accumulate at the bleeding site, they will be able to contribute effectively to hemostasis by releasing their contents. We also focused on using polymerized albumin particles as effective platelet substitutes and obtained some unique in vitro and in vivo results [18,19].

Conclusions

For red-blood-cell substitutes the present target is the supportive treatment of transfusion therapy in emergency, and nonclinical and clinical studies will be scheduled within 2 years.

On the other hand, the research of platelet substitutes has just started. There is a conflict between the carrier design for the extension of circulation lifetime and the carrier design to show the hemostatic activity by recognizing the bleeding site; therefore, we need to resolve this conflict and to design platelet substitutes for prophylactic or chronic treatments. And we also need a method to confirm that the candidate does not create a thrombus in blood circulation.

Although profitability is important in the development of blood substitutes, one that is based on a long-term view with consideration for human beings is expected first. At present, as many discoveries about the dynamic function of platelets have accumulated in a short period of time with the progress of biotechnology and opto-electronics, and the manufacturing technology of recombinant proteins or carriers is progressing, a usable product is sure to be invented in the near future.

Acknowledgments. The author thanks Drs. E. Tsuchida, H. Sakai, K. Sou, Y. Teramura, and Y. Okamura at Waseda University, and Drs. K. Kobayashi, Y. Ikeda, M. Suematsu, H. Horinouchi, M. Handa, and M. Murata at Keio University for useful discussions and suggestions. This work was supported in part by Health and Labor Sciences Research Grants (Research on Pharmaceutical and Medical Safety); the Ministry of Health, Labor, and Welfare, Japan; and Grants-in-Aid from the JSPS, Japan, and 21COE "Practical Nano-Chemistry" from MEXT, Japan.

References

1. Tsuchida E (ed) (1995) Artificial red cells: materials, performances and clinical study as blood substitutes. Wiley, Chichester
2. Tsuchida E (ed) (1998) Blood substitutes: present and future perspectives. Elsevier, Amsterdam

3. Sakai H, Tomiyama K, Sou K, et al (2000) Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. Bioconjug Chem 11:425–432
4. Sakai H, Takeoka S, Park SI, et al (1997) Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. Bioconjug Chem 8:23–30
5. Sakai H, Takeoka S, Wettstein R, et al (2002) Systemic and microvascular responses to hemorrhagic shock and resuscitation with Hb vesicles. Am J Physiol 283:H1191–H1199
6. Sakai H, Hara H, Yuasa M, et al (2000) Molecular dimensions of Hb-based O-2 carriers determine constriction of resistance arteries and hypertension. Am J Physiol 279: H908–H915
7. Wakabayashi Y, Takamiya R, Mizuki A, et al (1999) Carbon monoxide overproduced by heme oxygenase-1 causes a reduction of vascular resistance in perfused rat liver. Am J Physiol 277:G1088–G1096
8. Kyokane T, Norimizu S, Taniai H, et al (2001) Carbon monoxide from heme catabolism protects against hepatobiliary dysfunction in endotoxin-treated rat liver. Gastroenterology 120:1227–1240
9. Sakai H, Horinouchi H, Tomiyama K, et al (2000) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. Am J Pathol 159:1079–1088
10. Agam G, Livne AA (1992) Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia. Eur J Clin Invest 22:105–112
11. Levi M, Friderich P, Ten CW, et al (1999) Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits. Nat Med 5:107–111
12. Chao F, Reddick RL, Bode AR, et al (1996) Infusible platelet membrane microvesicles: a potential transfusion substitute for platelet. Transfusion 36:536–542
13. Soslau G, Class R, Morgan DA, et al (2001) Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. J Biol Chem 276:21173–21183
14. Nishiya T, Murata M, Handa M, et al (2000) Targeting of liposomes carrying recombinant fragments of platelet membrane glycoprotein Ibα to immobilized von Willebrand factor under flow conditions. Biochem Biophys Res Commun 270:755–760
15. Takeoka S, Teramura Y, Okamura Y, et al (2002) Rolling properties of rGPIbα-conjugated phospholipid vesicles with different membrane flexibilities on vWf surface under flow conditions. Biochem Biophys Res Commun 296:765–770
16. Kainoh M, Tanaka T (2002) Production of soluble integrin α2β1 heterodimer complex functionally in vitro and in vivo. Biochem Biophys Res Commun 290:305–310
17. Nishiya T, Kainoh M, Murata M, et al (2002) Reconstitution of adhesive properties of human platelets in liposomes carrying both recombinant glycoproteins Ia/IIa and Ibα under flow conditions: specific synergy of receptor-ligand interactions. Blood 100: 136–142
18. Takeoka S, Teramura Y, Ohkawa H, et al (2000) Conjugation of von Willebrand factor-binding domain of platelet glycoprotein Ib alpha to size-controlled albumin microspheres. Biomacromolecules 1:290–295
19. Teramura Y, Okamura Y, Takeoka S, et al (2003) Hemostatic effects of polymerized albumin particles bearing rGPIa/IIa in thrombocytopenic mice. Biochem Biophys Res Commun 306:256–260

人工血液(赤血球，血小板)の最近の進歩

武 岡 真 司*

1．はじめに

日進月歩の科学技術の進歩によってウイルスの検出感度や不活化度が向上しても，新たなウイルス出現やプリオン問題，そして少子高齢化問題は，献血システムの大きな不安要因になっており，安全な血液代替物に対する期待となっている．血液には血漿成分と血球成分があり，血漿成分の代替は進んでいるものの，血球成分(赤血球，白血球，血小板)の代替には機能と安全性を兼ね備えた微粒子系(分子集合体)の開発が必要であるため時間がかかっている．わが国の人工血液の本格的な研究は，平成9年度から厚生科学研究高度先端医療研究事業に『人工血液開発分野』が設置され，人工赤血球，人工血小板，人工抗体の三部門にて活発な研究展開が進められ現在に至っている．人工血液は，現行の血液製剤を補完して安全な製剤の安定供給に寄与することから，人工血液の製品化に向けた研究開発の促進が国の基本方針(平成14年7月24日決議)となっている．また，製品化が実現すれば，21世紀医療の進歩に大きな影響を与えうるものと期待される．

筆者の所属する共同研究グループでは，人工赤血球と人工血小板の研究を行っている．前者は酸素分圧に応じて酸素を吸収・脱着する分子(ヘモグロビンなど)を担持し，血中に長く留まって安全かつ安定に酸素運搬機能を発現し続ける担体である．後者は血管損傷部位や活性化血小板のみを認識する分子を担持し，血中に長く留まって血管損傷部位に特異的に粘着して止血機能を発現する担体である．従って，担体には適当な血液適合性や血中滞留性が必要であり，分解性や代謝物の低毒性は当然保証されなければならない．このような観点から，リン脂質分子の集合体(リポソーム)や遺伝子組み換えヒトタンパク質の重合体や架橋体を選択した．

2．人工赤血球開発の現状とヘモグロビン小胞体の製造

人工赤血球としてパーフルオロカーボン乳剤や修飾ヘモグロビンなどが検討されその一部は臨床使用されてきたが，機能や安全性の観点から満足できるものではなかった．現在われわれが開発を進めている，高濃度ヘモグロビンをリン脂質の二分子膜にて包み込んだ，赤血球と類似構造のヘモグロビン小胞体(図1)は，最も安全度と機能が高い酸素運搬体であろう[1,2]．現段階では期限切れの献血血液から精製したヘモグロビンの有効利用が進められているが，将来的には遺伝子組換えヒトヘモグロビンが使用されるであろう．われわれの方法では，赤血球からヘモグロビンを精製する際に，血液型を決める型物質やヘモグロビン以外の蛋白質，ウイルス(もし含まれたとしても)が加熱やフィルター処理にて除去されている．この際，一酸化炭素化処理を施してヘモグロビンを安定化していることが特徴である．他方，膜成分である混合リン脂質には分散安定化剤としてポリエチ

* 早稲田大学理工学部応用化学科

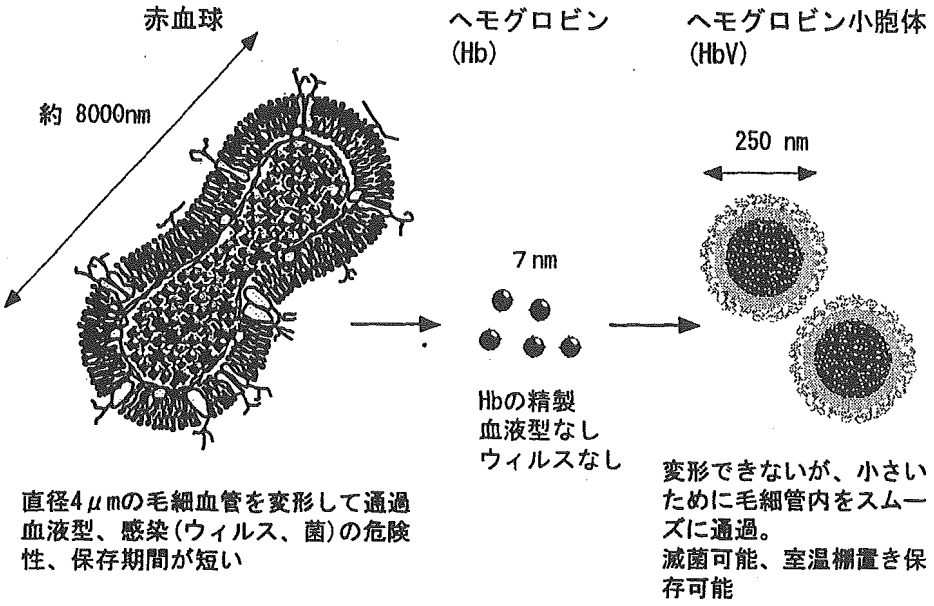

図1　ヘモグロビン小胞体の概念図

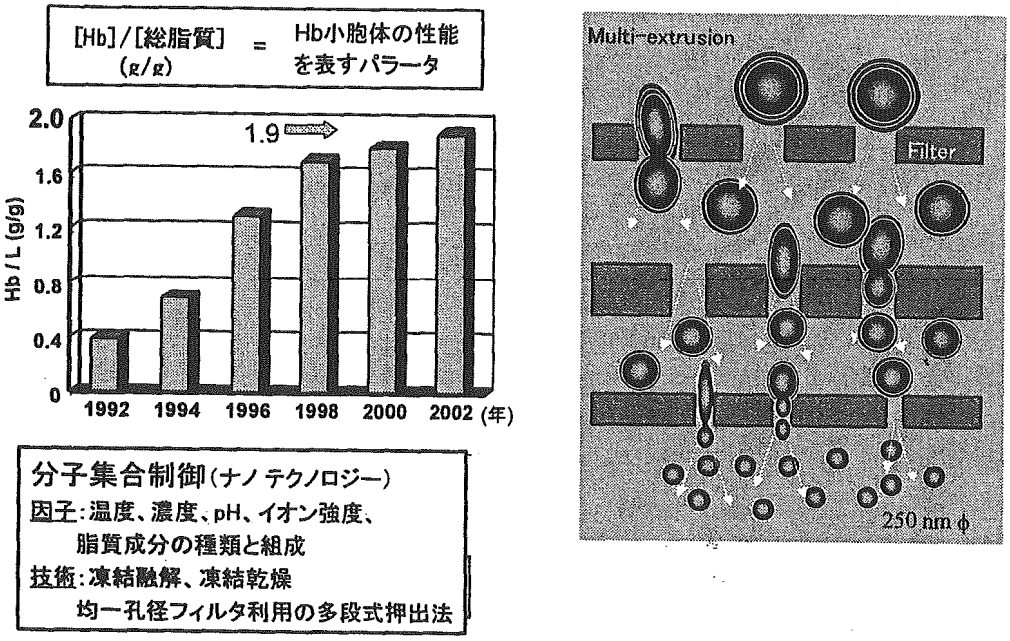

図2　ヘモグロビン小胞体の厳密な粒径制御と高性能化

レングリコール(PEG)結合脂質が含まれており，予め分子集合制御技術によって目的サイズの約2倍の空小胞体とし，これを精製した濃厚ヘモグロビン溶液(40g/dL)に分散，均一な貫通孔を持つメンブランフィルターに高圧透過(エクストルージョン法)させて，目的サイズの小胞体に高濃度ヘモグロビンを内包させたヘモグロビン小胞体が調製される．その際，溶液のpH，イオン強度，温度，圧力，そして孔サイズの組合せが重要なパラメーターであり，その厳密な設定によって内包効率が決まる．この十年，筆者らはパラメーターの調節に専念し，約5倍の内包効率の向上に成功した(図2)．そして，ヘモグロビン小胞体は，未内包ヘモグロビンの限外ろ過処理による除去操作，脱一酸化炭素化処理，脱酸素化処理を経て容器に充填され，室温で2年間の液状保存(赤血球製剤では採血後3週間の冷蔵保存)が保証される．乾燥粉末では更に長期間の保存が可能であるため，これが人工物の大きな長所とされている．現在，製造プラントの設計が最先端の化学工学技術を駆

表1　ヘモグロビン小胞体の物理化学的特徴

	ヘモグロビン小胞体 (20%アルブミン製剤と混合後)	ヒト血液 (赤血球)
粒径 (nm)	220 - 280	(8000)
P_{50} (Torr)	27 - 34[a]	26 - 28
[Hb] (g/dL)	10 ± 0.5 (8.6 ± 0.4)	12 - 17
[総脂質] (g/dL)	5.3 - 5.9 (4.6 - 5.4)	1.8 - 2.5[b]
[Hb]/[総脂質] (g/g)	1.6 - 2.1	6.7[c]
[PEG-脂質] (mol%)	0.3	—
metHb (%)	< 3	<0.5
粘度 (cP at 230 s^{-1})	2 - 3 (3 - 4)	3 - 4
晶質浸透圧 (mOsm)	300	Ca.300
膠質浸透圧 (Torr)	0 (20)	20 - 25
pH (37℃)	7.4	7.2 - 7.4
エンドトキシン (EU/mL)	<0.1	—
パイロジェン	検出なし	—

[a]調節可，[b]赤血球膜の全成分，[c]Hb と膜成分の重量比

使して行われている．

3．ヘモグロビン小胞体の物性と安全性評価

製造されたヘモグロビン小胞体製剤の性状を表1にまとめた．ヘモグロビン小胞体は生理食塩水に分散され，脱酸素状態で容器に封入されている．製剤のヘモグロビン濃度は10g/dLであり，ヒト血液のそれに近い．また，ヘモグロビン分子がカプセルに封入されているため製剤の膠質(コロイド)浸透圧はほとんどゼロである．従って，膠質浸透圧の調節が必要となる場合にはアルブミンや多糖類などのコロイド製剤と併用となる．溶液粘度は，アルブミンに分散させて膠質浸透圧をヒト血液に揃えるとヒト血液と同等になる．

粒径は250nmに調節されており赤血球の約30分の1程度であるので，梗塞部位の透過など赤血球にはない機能が期待できる．酸素親和度はアロステリック因子，ピリドキサール5'-リン酸の共封入によりヒト血液と同等の値に調節されている．脂質類の成分組成や含量には，ヘモグロビンのカプセル化効率，常温で2年間液状保存できる安定性[3]，血流中での溶血の回避と適度な血中滞留時間，血小板や補体の活性化の回避など，に対する工夫が施されており，従来の小胞体における課題が解決されている．

4．動物試験による機能と安全性の評価

ヘモグロビン小胞体に関する評価試験が，筆者の所属する早稲田大学理工学総合研究センター土田英俊名誉教授の研究グループと慶應義塾大学医学部小林紘一教授，末松 誠教授のグループとの共同研究にて行われ，その良好な成績から臨床応用の可能性が見えてきた．主にラットやハムスターを用いた試験であるが，基本的な安全性と酸素輸送効果は充分確認できており，現在，企業側の参画も得て霊長類や犬を用いた安全性や効力確認試験が進められている．以下に90%交換試験の結果を紹介する．ラット全血液量の90%をアルブミン単独で交換した場合には70%交換あたりから血圧と腎皮質酸素分圧の低下が顕著となって全例死亡したが，ヘモグロビン小胞体をアルブミン溶

液に分散させた系にて90%交換した場合には血圧，腎皮質酸素分圧ともに維持された[4]．ヘモグロビン小胞体のアルブミン分散液によるハムスター80%交換輸血試験では，非侵襲に測定した皮下微小循環系の組織酸素分圧は交換前の60～70%に低下するものの，対照アルブミン投与群よりも5倍以上の値が維持されていた[5]．安全面では，ヘモグロビン小胞体では修飾ヘモグロビンに認められる抵抗血管の収縮と血圧亢進は全く認めなかった．これは，ヘモグロビン小胞体は血管を透過しない大きさであり，血管内皮由来弛緩因子である一酸化窒素への影響はほとんどないと考えられている[6]．更には，ヘモグロビン小胞体は赤血球と同様に肝臓の類洞血管(数10～200nm程度の孔が血管に開いている)を透過できず，老廃赤血球と同様肝臓のクッパー細胞や脾臓などの細網内皮系にて代謝される．他方，カプセル化されていないヘモグロビンは，これを容易に透過して内因性COを消去し，ビリルビンの過剰生成と胆汁分泌機能の低下を招来した[7,8]．ラット，ラビット，カニクイザルによる血中半減期からヒトでの類推では，半減期は3日程度と見積もられる．また，単回，反復負荷投与による血液生化学試験や病理試験での詳細から，ヘモグロビン小胞体成分である，脂質分解に関わるリパーゼの亢進，コレステロール値の上昇，細網内皮系の肥大が一過性に認められた以外の変動は認められておらず，出血時の緊急対応としての輸血の代替にて十分なる機能を発現するものと期待されている[9,10]．

5．人工血小板の開発の現状とストラテジー

人工血小板(血小板代替物)の開発は，赤血球代替物と比較して歴史が浅く研究例も少ない[11]．血小板は出血部位に対し特異的粘着，伸展，凝集，放出，血液凝固系の活性化等の複雑な機能を持ち，これらの全てを兼備した血小板代替物の構築は事実上不可能といえよう．しかし，粘着，凝集能が欠如した出血性疾患Bernard-Soulier症候群や血小板無力症において出血傾向が強く認められることから，血小板の粘着と凝集機能に着目して，これらの機能を付与させた担体の投与によっても少数残存する血小板の機能を補助できるものと考えられている．これまでにフィブリノーゲンのヒト赤血球担持体[12]やアルブミンマイクロカプセル担持体[13]，ヒト血小板の乾燥粉末[14]が臨床試験に供されたものの，作用機序が不明瞭であることも問題点となり，全て中断されている．また，ヒト由来の血液成分に依存している面も解決しなければならない課題であろう．

筆者らが慶應義塾大学医学部池田康夫教授のグループと共同研究している人工血小板では，生体投与可能なリポソームや遺伝子組み換えヒトアルブミン重合体を担体としてその特徴を利用し，血小板膜蛋白質の一部の遺伝子組換え蛋白質や合成オリゴペプチドを担持させて血小板を巻き込んだ出血部位への集積による止血能の発現を期待している．

血小板による止血は，高ずり速度の血流と低ずり速度の血流では機構が異なる．図3に示したように，高ずり速度の出血に対する血小板の止血は，出血部位に露出する血管内皮下組織であるコラーゲンに結合したフォンビルブランド因子(vWf)に対して，血小板が認識して接着して転がることから始まる．*in vitro*観測にて抗GPⅡb/Ⅲa抗体を添加してGPⅡb/Ⅲaの機能を阻害した血小板では，vWf固定化基板上を流動方向に沿って転がる現象が見られ，この認識能は血小板表面のGPIb/V/Ⅸ複合体のGPIbα部が担っている[15]．次に，血小板表面のGPIaⅡa($\alpha_2\beta_1$インテグリン)やGPⅥが直接コラーゲンと相互作用して血小板は粘着し，そこで活性化されると血小板は伸展して顆粒を放出するが，最も重要な過程はGPⅡb/Ⅲa($\alpha_{IIb}\beta_3$インテグリン)が活性体となる現象である．フィブリノーゲンは，この活性化GPⅡb/Ⅲaを認識して，血小板間を架橋し凝集体を形成して一次止血となる．引き続く凝固系の誘導によるフィブリン塊の形成(二次止血)によって止血が完成する．われわれは，患者に残存する血小板や凝固系の有効利用を前提にして，当面目標とする人工血小板としては，高ずり速度の血流下でコラーゲンをvWfを介して認識するGPIbα，

1次止血(血小板血栓)

1) 接着(tethering→rolling) GPIbα − vWf
2) 粘着(adhesion) GPIa/IIa − collagen
3) 凝集(aggregation) GPIIb/IIIa − fbg or vWf

2次止血(フィブリン血栓)

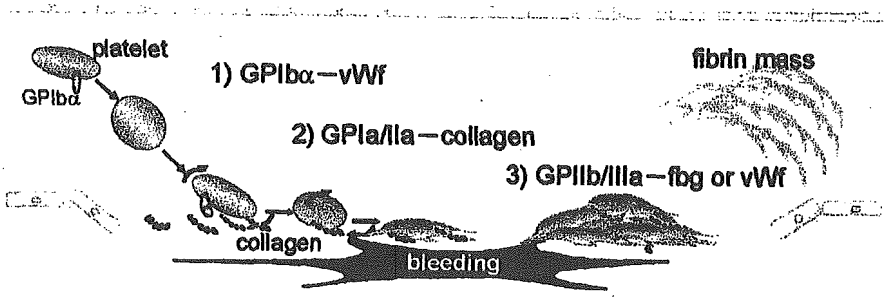

図3　血小板の止血機構

低ずり速度にてコラーゲンを直接認識するGPIaIIa，活性化血小板上のGPIIb/IIIaを認識して血小板凝集体の形成をサポートするフィブリノーゲンやその認識部位を担持させた担体を開発のターゲットとした．

6．リポソーム(小胞体)利用の血小板代替物

小胞体表面に血小板膜蛋白質の遺伝子組換え体(rGPIbα[16])を結合させると，血小板止血過程の初期が再現できる．この小胞体の調製方法を図4と図6に示した．エタノールアミン型リン脂質(DPPE)にSPDPを用いてピリジルジスルフィド基を導入し，これをコリン型リン脂質とコレステロールの混合脂質と混合して薄膜を形成，水和・分散体からエクストルージョン法によって小胞体を形成，rGPIbαに導入したメルカプト基とチオール・ジスルフィド交換反応によってrGPIbαを小胞体表面に結合させた．

得られたrGPIbα担持小胞体は，血小板と同様にvWf基板上を転がる性質を持ち[17]，転がる小胞体の数は高ずり速度の方が多くなる，rGPIbαの特徴が確認できた．興味深いことに，その転がり速度は小胞体を構成するリン脂質二分子膜の柔軟性(membrane flexibility)と相関しているようである[18]．すなわち，“柔らかい”小胞体では転がり速度は低くなり，“硬い”小胞体では転がり速度は高くなった．これは血流中ではずり応力により“柔らかい”小胞体は変形しやすくvWf基板と小胞体間の接触面積が増大したために，変形しにくい“硬い”小胞体よりも転がり速度が低下したためと考えられた．このように，rGPIbαの担持により小胞体に血小板機能の一部を付与でき，しかもその機能を制御する方法も見出すことができた．

他方，低ずり速度の血流下でコラーゲンに直接結合する血小板膜蛋白質の遺伝子組換え体(rGPIaIIa)を同様に結合させた小胞体では，コラーゲン基板を特異的に認識して粘着(停止)する挙動が西谷らによって確認された[19,20]．この系ではずり速度が高くなるにつれ粘着数は減少するが，rGPIbαとrGPIaIIa共に担持させた小胞体では低ずり速度から高ずり速度までコラーゲン基板を粘着する系が構築されている[20]．更に，小胞体の内水相に血小板の活性化や凝固系を誘導する因子を内包させておいて，出血部位に集積した小胞体がこれらを放出できれば，有効に止血に貢献できるであろう．

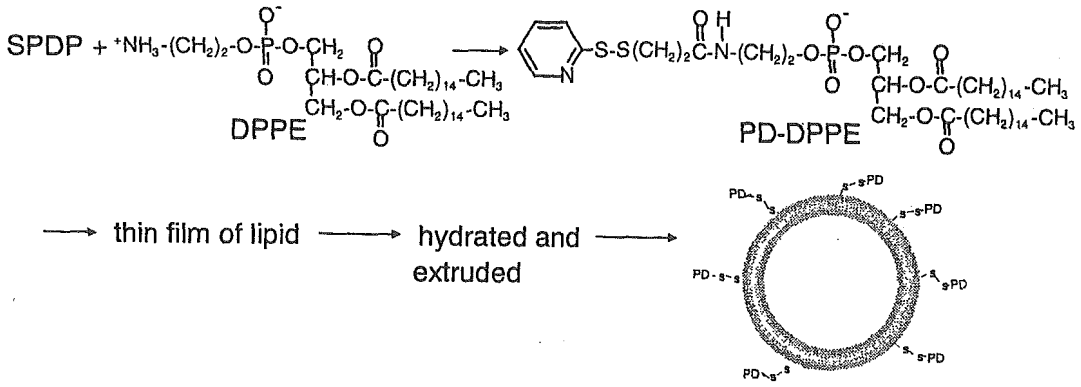

図4　リン脂質小胞体の調製法

7．アルブミン重合体を用いた血小板代替物

ヒト血清アルブミンは血漿蛋白質の中で最も多量(5 g/dL)に存在する蛋白質であり，コロイド浸透圧の調節や栄養物や代謝物などの運搬，などの機能を担っている．これを利用した微粒子は生体適合性・生分解性を有するため，すでに1950年代から静注用製剤としてアルブミン大凝集体が血流動態観測用プローブや血管造影剤などに，噴霧乾燥法によって調製したアルブミンマイクロカプセルは超音波診断用増感剤として，アルブミンマイクロスフェアは徐放性の薬物担体として利用されてきた．しかし，アルブミン粒子は，高温や有機溶媒による不可逆的な変性や界面活性剤や架橋剤を用いるため，粒径制御や除去操作が煩雑であった．われわれは図5に示すような，①遺伝子組換えヒト血清アルブミン(rHSA)を単量体として，rHSAをジスルフィド結合にて重合する方法を用いて，②重合度の制御によりナノスケールからマイクロスケールの粒径制御が可能，③水溶液中でのpHと温度の制御にて重合するためクリーンであり，得られた粒子の表面は親水性，④アルブミン変性がほとんどない重合体を得る方法を確立した[21]．そして，得られたアルブミン重合体表面にSPDPを用いてピリジルジスフィド基を導入し，メルカプト基を導入したレセプタータンパク質と結合させた(図6)．

アルブミン重合体は内部が充填された無定形な綿雪のような形態をとっており，出血部位での充填効果が期待できる．表面にrGPIbαを結合させたところ，小胞体のようなvWf基板を転がる挙動は全く認められず，高ずり速度下でも粘着する挙動が認められた．ラテックスビーズにrGPIbαを結合させた系でも粘着することから，担体が重合体である場合と膜構造を持つ場合ではrGPIbα機能の発現の仕方が異なることが示唆された．他方，rGPIaIIaを担持させたアルブミン重合体は低ずり速度下で粘着し，X線照射にて血小板数を正常値の1/5程度に減少させたマウスに投与したところ，コントロール群の出血時間(730±198秒)と比較して，投与量依存的に出血時間の短縮が認められた(例えば，2.4×10^{11} particles/kgでは出血時間は337±46秒)[22]．

さらに粘着して活性化した血小板同士を架橋するフィブリノーゲンを結合させた微粒子は減少し

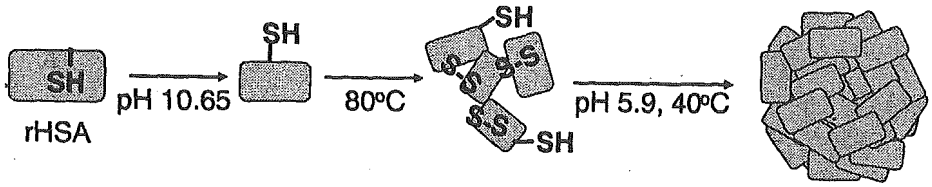

図5　アルブミン重合体の調製法

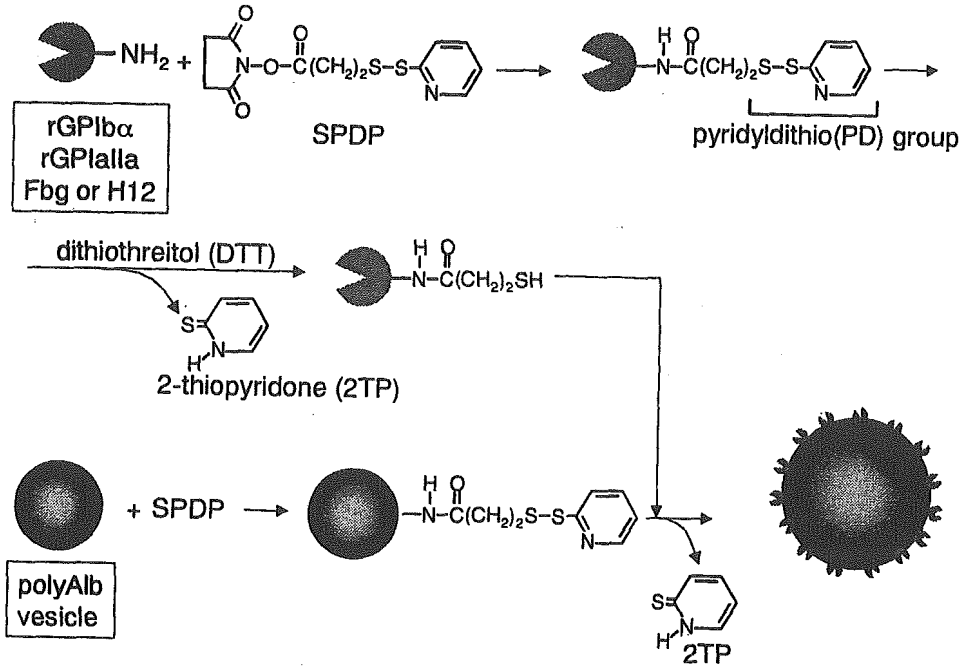

図 6　担体への認識部位の担持方法

た残存血小板の凝集を補助として期待できる[23]．活性化血小板の固定化基板を作成し，フィブリノーゲン結合アルブミン重合体を流動させたところ基板上に一様に粘着し，抗 GPⅡb/Ⅲa 抗体を添加した系やアルブミン重合体のみの系では粘着は認められなかった．血小板数が正常値の 1/5 程度に調節された血小板減少モデル血液にフィブリノーゲン結合アルブミン重合体を添加すると，その濃度増大と共に流動血小板の粘着数が増大したことから，フィブリノーゲン結合アルブミン重合体は血小板粘着増強効果を有する微粒子であることが示唆された．しかし，フィブリノーゲンは不安定なタンパク質であり，水溶液中では速やかに失活してしまうことが課題となった．われわれはフィブリノーゲンのγ鎖 C 末端アミノ酸序列(HHLGGAKQAGDV)を担体に結合させた微粒子はアルブミン重合体でも小胞体でも，フィルブリノーゲン機能が安定に発現されることを確かめた[24]．

抗がん剤であるブスルファンをラットに 20mg/kg を 2 回に分けて投与すると，投与後 10 日後に，血小板数のみが 1/5 まで再現性よく低下する．5 分前あるいは 3 時間前に予め H12 結合アルブミン重合体を投与してから尾静脈からの出血時間を測定すると出血時間が投与濃度依存的に有意に短縮された[25]．また，アルブミン重合体の表面をポリエチレングリコール鎖にて修飾し，この末端の一部に H12 を結合した系では，3 時間後でもその効果が持続していることが確認された．したがって，フィブリノーゲンの代替をするオリゴペプチドの担持した微粒子でも有意な止血効果を示すことが確認された．

8．ま と め

人工赤血球は救急救命における輸血療法の補完を当面の目標としており，役目が終われば比較的速やかに代謝臓器で代謝され，生合成される自身の赤血球と置き換わるものである．今後，臨床試験に進むために製剤の物性規格や製造方法の確定，非臨床試験，臨床試験のためのガイドラインやプロトコール作成へと進むことになるであろう．他方，人工血小板の開発は国内外共に浅く，まだ緒に就いたばかりの段階である．今後，①血中滞留時間の延長のための担体設計と認識機能発現を高めるための担体設計は一般に相反するので，その工夫が必要となること，②出血部位を認識して止血能を発揮する担体は，血流中で血栓形成が起こらないことを評価する系の確立，③急性時に局

所的な効果を発現する製剤，あるいは慢性的な全身からの出血を予防する製剤設計のための要件の確認など，*in vivo* 評価のフィードバックの段階を踏むことになろう．

このような人工赤血球，人工血液の実現は，わが国の医療貢献はもとより，安全な血液が不足している多くの国に対しても大きな国際貢献と成り得る．民間で行うには採算性も重要であるが，まずは長期的そして全人類的な視野に立った開発を期待したい．現在ではバイオテクノロジーやオプトエレクトロニクスの進歩により血小板の動的な機能に関する多くの情報が短期間に蓄積され，化学工学の進歩によって遺伝子組換え蛋白質の大量製造や担体の製剤化技術が飛躍的に進歩しているため，必ずや実用可能な系が創製されるものと信じている．

引用文献

1) 土田英俊，酒井宏水，武岡真司他：酸素輸液(人工赤血球)，医学のあゆみ，2003；**205**：558-566
2) 土田英俊，宗慶太郎，酒井宏水他：酸素輸液(人工赤血球)の安全度と体組織への酸素供給，麻酔，2003；**52**：S55-S66
3) Sakai H, Tomiyama K, Sou K, *et al.*: Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state, *Bioconjugate Chem.*, 2000；**11**：425-432
4) Sakai H, Takeoka S, Park SI, *et al.*: Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjugate Chem.*, 1997；**8**：23-30
5) Sakai H, Takeoka S, Wettstein R, *et al.*: Systemic and microvascular responses to hemorrhagic shock and resuscitation with Hb vesicles. *Am. J. Physiol.*, 2002；**283**：H1191-H1199
6) Sakai H, Hara H, Yuasa M, *et al.*.: Molecular dimensions of Hb-based O-2 carriers determine constriction of resistance arteries and hypertension. *Am. J. Physol.*, 2000；**279**：H908-H915
7) Wakabayaski Y, Takamiya R, Mizuki A, *et al.*: Carbon monoxide overproduced by heme oxygenase-1 causes a reduction of vascular resistance in perfused rat liver. *Am. J. Physol.*, 1999；**277**：G1088-G1096
8) Kyokane T, Norimizu S, Taniai H, *et al.*: Carbon monoxide from heme catabolism protects against hepatobiliary dysfunction in endotoxin-treated rat liver. *Gastroenterology*, 2001；**120**：1227-1240
9) Sakai H, Horinouchi H, Tomiyama K, *et al.*: Hemoglobin-vesicles as oxygen carriers-Influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am. J. Phatol.*, 2000；**159**：1079-1088
10) 研究代表者 土田英俊，厚生労働科学研究費補助金　医薬安全総合研究事業「臨床応用可能な人工赤血球の創製に関する研究」(H12-医薬-009)平成12年度～14年度，総括研究報告書
11) 村田　満：人工血小板(血小板代替物)．血液・免疫・腫瘍，**6**：35-39，2001
12) Agam G, Livne A.A: Erythrocytes with covalently-bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia. *Eur. J. Clin. Invest*, **22**：105-112, 1992
13) Levi M, Friderich P, Ten C.W *et al.*: Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits. *Nat. Med.*, **5**：107-111, 1999
14) Chao F, Reddick R.L, Bode A.R *et al.*: Infusible platelet membrane microvesicles : a potential transfusion substitute for platelet. *Transfusion*, **36**：536-542, 1996
15) Soslau G, Class R, Morgan D.A, Foster C, Lord S.T, Marchese P, Ruggeri Z.M: Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. *J. Biol. Chem.*, **276**：21173-21183, 2001
16) Murata M, Ware J, Ruggeri Z.M: Site-directed mutagenesis of a soluble recombinant fragment of platelet glycoprotein Ibα demonstrating negatively charged residues involved in von Willebrand factor binding. *J. Biol. Chem.*, **266**：15474-15480, 1991
17) Nishiya T, Murata M, Handa M, Ikeda Y: Targeting of liposomes carrying recombinant fragments of platelet membrane glycoprotein Ibα to immobilized von Willebrand factor under flow conditions. *Biochem. Biophys. Res. Commun.*, **270**：755-760, 2000
18) Takeoka S, Teramura Y, Okamura Y, Tsuchida E, Handa M, Ikeda Y: Rolling properties of rGPIbα-conjugated phospholipid vesicles with different membrane flexibilities on vWf surface under flow conditions. *Biochem. Biophys. Res. Commun.*, **296**：765-770, 2002
19) Kainoh M, Tanaka T: Production of soluble integrin $\alpha_2\beta_1$ heterodimer complex functionally *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.*, **290**：305-310, 2002
20) Nishiya T, Kainoh M, Murata M, Handa M, Ikeda Y: Reconstitution of adhesive properties of human platelets

in liposomes carrying both recombinant glycoproteins Ia/IIa and Ibα under flow conditions : specific synergy of receptor-ligand interactions. *Blood*, **100** : 136-142, 2002

21) Takeoka S, Teramura Y, Ohkawa H, Ikeda Y, Tsuchida E : Conjugation of von Willebrand factor-binding domain of platelet glycoprotein Ib alpha to size-controlled albumin microspheres, *Biomacromolecules*, **1**, 290-5, 2000

22) Teramura Y, Okamura Y, Takeoka S, Tsuchiyama H, Narumi H, Kainoh M, Handa M, Ikeda Y, Tsuchida E : Hemostatic effects of polymerized albumin particles bearing rGPIa/IIa in thrombocytopenic mice. *Biochem. Biophys. Res. Commun.*, **306** : 256-260, 2003

23) Takeoka S, Teramura Y, Okamura Y, Handa M, Ikeda Y, Tsuchida E : Fibrinogen-conjugated albumin polymers and their interaction with platelets under flow conditions. *Biomacromolecules*, **2** : 1192-1197, 2001

24) Takeoka S, Okamura Y, Teramura Y, Watanabe N, Suzuki H, Tsuchida E, Handa M, Ikeda Y : Fibrinogen γ-chain dodecapeptide-conjugated latex beads under flow. *Biochem. Biophys. Res. Commun.*, **312** : 773-9, 2003

25) Okamura Y, Takeoka S, Teramura Y, Maruyama H, Tsuchida E, Handa M, Ikeda Y : Hemostatic effects of fibrinogen-gamma chain dodecapeptide-conjugated polymerized albumin particles *in vitro* and *in vivo*, *Transfusion*, 2004. in press

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 312 (2003) 773–779
BBRC
www.elsevier.com/locate/ybbrc

Function of fibrinogen γ-chain dodecapeptide-conjugated latex beads under flow

Shinji Takeoka,[a] Yosuke Okamura,[a] Yuji Teramura,[a] Naohide Watanabe,[b] Hidenori Suzuki,[c] Eishun Tsuchida,[a,*] Makoto Handa,[b] and Yasuo Ikeda[b]

[a] *Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan*
[b] *Blood Center and Department of Internal Medicine, School of Medicine, Keio University, Tokyo 160-8582, Japan*
[c] *Medical Research and Development Center, The Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan*

Received 17 October 2003

Abstract

In order to perform a fundamental study of platelet substitutes, novel particles that bound to activated platelets were prepared using two oligopeptides conjugated to latex beads. The oligopeptides were CHHLGGAKQAGDV (H12), which is a fibrinogen γ-chain carboxy-terminal sequence (γ 400–411), and CGGRGDF (RGD), which contains a fibrinogen α-chain sequence (α 95–98 RGDF). Both peptides contained an additional amino-terminal cysteine to enable conjugation. Human serum albumin was adsorbed onto the surface of latex beads (average diameter 1 μm) and pyridyldisulfide groups were chemically introduced into the adsorbed protein. H12 or RGD peptides were then chemically linked to the modified surface protein via disulfide linkages. H12- or RGD-conjugated latex beads prepared in this way enhanced the in vitro thrombus formation of activated platelets on collagen-immobilized plates under flowing thrombocytopenic-imitation blood. Based on the result of flow cytometric analyses of agglutination, PAC-1 binding, antiP-selectin antibody binding, and annexin V binding, the H12-conjugated latex beads showed minimal interaction with non-activated platelets. These results indicate the excellent potential of H12-conjugated particles as a candidate for a platelet substitute.

Keywords: Platelet substitute; H12; RGD; Thrombocytopenic-imitation blood; Flow condition; Latex beads; Flow cytometry; PAC-1; P-selectin; Annexin V

Platelet transfusion plays an important role in the supportive therapy of patients with thrombocytopenia caused by cancer or hematologic malignancies, or during surgical procedures. However, the shortage of platelets has always been a serious issue because of the short storage life of platelet concentrates (three days in Japan). Furthermore, there is the issue of the risk of viral and bacterial infections by transfusion. Platelet substitutes such as infusible platelet membranes (IPMs) [1], solubilized platelet membrane protein-conjugated liposomes [2], fibrinogen-conjugated red blood cells [3], fibrinogen-coated albumin microcapsules [4], and arginine–glycine–asparaginic acid (RGD) peptide-bound red blood cells [5] have been developed to solve these problems. However, despite their usefulness in enhancing platelet aggregation and reducing bleeding time in vivo, because these platelet substitutes are all derived from human blood, the risk of infection cannot be avoided.

We have focused on recombinant platelet membrane proteins such as glycoprotein GPIbα [6,7] and GPIa/IIa complex [8–10] for identifying the site of interaction of platelets with von Willebrand factor (vWf) binding to the collagen and have conjugated these proteins to phospholipid vesicles (liposomes) [11–14] or polymerized albumin particles (polyAlb) [15,16] as carriers. We found that rGPIbα-conjugated polymerized albumin particles (polyAlb) accumulated on a surface on which vWf was immobilized, (vWf-immobilized) [16] and that fibrinogen-conjugated polyAlb facilitated the recruitment of flowing platelets in polyAlb aggregates after their attachment to the activated platelet-immobilized surface in vitro [15]. These findings confirmed that such

* Corresponding author. Fax : +813-3205-4740.
E-mail address: eishun@waseda.jp (E. Tsuchida).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.10.184

conjugates could replicate the function of platelets in primary hemostasis. However, fibrinogen from human blood is not stable and its activity in solution is extremely low.

The sites of fibrinogen that are proposed to bind to the GPIIb/IIIa complex on the activated platelet surface are a dodecapeptide (HHLGGAKQAGDV, H12) corresponding to a γ-chain carboxy-terminal segment (γ400–411) and a tetrapeptide RGD containing sequences such as RGDF and RGDS corresponding to α-chain residues α95–98 and α572–575, respectively [17]. H12 is a sequence existing only in a fibrinogen domain [18,19], whereas RGD is also a ubiquitous cell recognition sequence shared with other adhesive proteins [20]. These peptides inhibit platelet aggregation and fibrinogen-binding to activated platelets [18,19,21]. Recently, Hallahan et al. [22] reported that H12 and RGD were bound to the activated GPIIIa on the endothelium of tumors after exposure to ionizing radiation.

In this paper, for the purpose of constructing practical platelet substitutes targeting activated platelets for thrombus formation, we selected H12 or RGD containing peptides, with an added amino-terminal cysteine, as a recognition site. We prepared and evaluated the binding ability of the H12- or RGD-conjugated latex beads to the collagen-surface in thrombocytopenic-imitation blood under flowing conditions. Latex beads are very useful carriers for in vitro studies because of their regulated size and surface properties.

Materials and methods

Reagents. A fibrinogen γ-chain dodecapeptide (C-HHLGGAKQ AGDV, H12) and an RGD-containing peptide (C-GGRGDF, RGD) were synthesized using a solid-phase synthesizer by BEX (Tokyo, Japan). Latex beads (Fluoresbrite plain, 200 nm or 1 μm diameter) and *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) were purchased from Polysciences (Warrington, PA) and Pierce Chemical (Rockford, IL), respectively. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$), which is a platelet fluorescent dye, was purchased from Molecular Probes (Eugene, OR). An anticoagulant D-phe-pro-arg-chloromethylketone (PPACK) was obtained from Calbiochem (San Diego, CA). FITC-PAC-1, FITC-antiP-selectin (CD62P) antibody, and FITC-annexin V were purchased from Becton–Dickinson (San Jose, CA), BD-Pharmingen (San Diego, CA), and Sigma–Aldrich (Saint Louis, MO), respectively. Recombinant human serum albumin (rHSA) was kindly donated by Mitsubishi Pharma (Osaka, Japan).

Preparation of H12- or RGD-conjugated latex beads. Latex beads were mixed with an rHSA solution (50 mg/mL) and incubated at 20 °C for 2 h to coat the surface of the latex beads with rHSA. After the separation of the free rHSA by centrifugation (13,000*g*, 5 min, 4 °C, 3 times), the rHSA-coated latex beads (rHSA-latex beads) were dispersed into phosphate-buffered saline (PBS, pH 7.4, 500 μL). A solution of SPDP in ethanol (5 mM, 5 μL) was added to the rHSA-latex bead dispersion (4.0×10^6/μL, 500 μL) and incubated for 30 min at 20 °C. The unreacted SPDP and the by-products were separated by centrifugation (13,000*g*, 5 min, 4 °C, 3 times), and the pyridyldisulfide bonded rHSA-latex beads (PD-latex beads) were obtained. A dispersion of PD-latex beads (4.0×10^6/μL, 500 μL) was mixed with either a solution of H12 (10 mM, 8 μL) or an RGD solution (10 mM, 8 μL) and reacted at 20 °C for 12 h. The unreacted reagents were removed by centrifugation (13,000*g*, 5 min, 4 °C) to obtain the purified H12- or RGD-conjugated latex beads (H12-, RGD-latex beads, 2.0×10^6/μL, 1 mL). The concentration of H12 or RGD conjugated on the latex beads was determined by quantification of the 2-thiopyridone (2TP) that was liberated by a thiol-disulfide exchange reaction using high pressure liquid chromatography (HPLC) on a TSK-GEL G3000SW_{XL} column (7.8 mm o.d. × 300 mmH in PBS at 1 mL/min), by measuring the absorbance of the column flow at 343 nm.

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 2.0×10^5/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).

A 20 μM ADP solution was added to the PRP containing H12 or RGD solutions adjusted to set concentrations 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, Japan) 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 h, carefully rinsed with PBS, and then immersed in bovine serum albumin (20 mg/mL) at 20 °C for 2 h.

Preparation of platelet-immobilized surface. The PRP (see Materials and method) was mixed with a 3:20 volume of acid-citrate-dextrose composed of 2.2% (w/v) sodium citrate, 0.8% (w/v) citric acid, and 2.2% (w/v) glucose (ACD) containing 1 μM prostaglandin E_1 (PGE_1, Sigma, St. Louis). The suspension was centrifuged (2200*g*, 10 min, 22 °C) and the plasma was replaced with a Ringer's-citrate-dextrose solution (RCD solution, composition: 0.76% (w/v) citric acid, 0.090% (w/v) glucose, 0.043% (w/v) $MgCl_2$, 0.038% (w/v) KCl, and 0.60% (w/v) NaCl, pH 6.5) containing 1 μM PGE_1. The platelets were resuspended in the RCD solution and centrifuged (2200*g*, 10 min, 22 °C), and the concentrated platelets were resuspended at 2.0×10^5/μL in a Hepes–Tyrode buffer (H–T buffer, pH 7.4). A collagen-coated glass plate was immersed into this platelet suspension for 1 h at 37 °C and rinsed carefully with PBS.

Measurement of interaction of H12- or RGD-latex beads with platelets immobilized on glass surface using reconstituted blood. Blood containing 10% volume of 3.8% (w/v) sodium citrate was centrifuged (100*g*, 15 min, 22 °C) to remove PRP and the PRP was replaced with an equal volume of a 0.9% (w/v) NaCl solution containing 10% (v/v) ACD (10% ACD-saline). The red blood cell suspension was centrifuged (2200*g*, 10 min, 22 °C) and washed with 10% ACD-saline three times to remove the buffy coat and plasma completely. After the final centrifugation, the red blood cells were resuspended in H–T buffer to produce a 50% hematocrit (Hct). The residual platelet concentration was determined to be $(5.0 \pm 2.0) \times 10^3$/μL.

The reconstituted blood and FITC-labeled H12- or RGD-latex bead (1.0×10^5/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 the latex beads with the platelets immobilized on the surface was observed. All perfusion studies were performed at 37 °C. Single-frame images of H12- or RGD-latex beads on the plates were obtained with an image processor, Argus-20 (Hamamatsu Photonics, Hamamatsu, Japan).

Measurement of the interaction between platelets and H12- or RGD-latex beads with the collagen surface using thrombocytopenic-imitation blood. Thrombocytopenic-imitation blood was prepared as follows. Blood, which was treated with the thrombin inhibitor PPACK (40 μM) were filtered with 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 $(6.0 \pm 2.0) \times 10^3/\mu L$, and the final platelet concentration was adjusted to $2.0 \times 10^4/\mu L$ by PRP addition. This blood preparation was termed thrombocytopenic-imitation blood.

In the perfusion study, either platelets or latex beads were labeled with a fluorescent marker (platelets: $DiOC_6$, LB: FITC). H12- or RGD-latex beads (adjusted to $1.0 \times 10^5/\mu L$), was mixed with the thrombocytopenic-imitation blood, and the interaction between platelets and H12- or RGD-latex beads with the collagen-coated surface was observed as described above. Calculation of the surface coverage of platelets or H12- or RGD-latex beads on the plates was carried out with an Argus-20 image processor.

Observation of the glass plates after perfusion study by scanning electron microscopy. Platelets and latex beads that adhered on the glass plates after the perfusion study were washed with H–T buffer, 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 with a freeze dryer (Hitachi ES-2020, Hitachi, Tokyo) using *t*-butyl alcohol. After coating with osmium tetroxide (approximately 5 nm thick) using an osmium plasma coater (NL-OPC80, Nippon Laser and Electronics Lab., Nagoya, Japan), the samples were examined with a Hitachi S-4500 field emission scanning electron microscope at an accelerating voltage of 10 kV.

Agglutination assay with H12- or RGD-latex beads by flow cytometry. A mixture of PRP (final concentration $2.0 \times 10^4/\mu L$) and H12- or RGD-latex beads, each labeled with fluorescein isothiocyanate (FITC), or FITC-labeled latex beads (200-nm diameter, final concentration $1.0 \times 10^5/\mu L$) was rotated at 200 rpm using a Bioshaker (BR-13UM, Tietech, Nagoya) at 37 °C for 30 min. Before and after rotation, the samples were fixed with formaldehyde (final concentration 1.5% (v/v)). Platelets were gated to their characteristic forward versus side scatter, and 20,000 platelets were analyzed using a FACSCalibur flow cytometer (Nihon Beckton–Dickinson, Tokyo). Percentage agglutination was quantified as the fraction of fluorescent-positive platelets that were rendered fluorescent due to the binding of FITC-labeled latex beads. Each experiment was performed at least three times.

PAC-1, antiP-selectin antibody, and annexin V binding to platelets induced by H12-latex beads. A mixture of PRP (final concentration $1.0 \times 10^5/\mu L$) and non-labeled H12-latex beads (200-nm diameter, final concentration $1.0 \times 10^5/\mu L$) was stirred with a cuvette using an aggregometer at 37 °C for 30 min. Before and after stirring, FITC-antiP-selectin antibody or FITC-annexin V was added at ca. 0.5 μg to the mixture (ca. 10^6 platelets), incubated at 37 °C for 10 min, and then fixed with formaldehyde (final concentration 1.5% (v/v)). On the other hand, to the mixture (ca. 10^6 platelets) FITC-PAC-1 (ca. 0.5 μg) was added and stirred at 37 °C for 10 min, and then fixed with formaldehyde (final concentration 1.5%). As a positive control, ADP- (final concentration 20 or 100 μM) or thrombin-stimulated platelets (final concentration 0.5 U/mL) were prepared using PRP or washed platelets, respectively. The platelets were gated to their characteristic forward versus side scatter and 20,000 platelets were analyzed using a FACSCalibur flow cytometer. PAC-1, anti P-selectin antibody, and Annexin V binding were quantified as a fraction of the fluorescent positive platelets. Each experiment was performed at least three times.

Results and discussion

The degree of recognition of the free H12 or RGD for the activated platelets was judged by their inhibition of the fibrinogen-mediated platelet aggregation. This is because those peptides have fibrinogen sequences, which are considered to be responsible for the recognition of GPIIb/IIIa on the activated platelet [17], and should suppress the platelet aggregation due to competitive binding to the fibrinogen-binding site of the platelet with fibrinogen. We used an aggregometer to measure the platelet aggregation induced by addition of 20 μM ADP to the PRP. Though both H12 and RGD showed concentration-dependent suppression of the platelet aggregation, H12 completely inhibited the platelet aggregation at a concentration of 4 mM, whereas RGD did so at 1 mM (Fig. 1). This indicated that the RGD had a strong inhibitory effect compared with H12.

Kloczewiak et al. and Hawiger et al. [17,19] reported that the IC_{50} of inhibition of ^{125}I-fibrinogen binding to activated platelets by H12, RGDS, and RGDF was 28, 10, and 2 μM, respectively, indicating that the order of binding avidity for GPIIb/IIIa was H12 < RGDS < RGDF. Our results also show that RGDF-containing peptide, CGGRGDF in our case, has a higher affinity for GPIIb/IIIa of the platelet than that of H12. Notably, the binding affinity of the two oligopeptides for GPIIb/IIIa was maintained even after the introduction of the N-terminal cysteine.

The mercapto group of the terminal cysteine of the H12 or RGD reacted with the PD group of the rHSA that was physically adsorbed on the surface of latex beads, and 2-thiopyridone (2TP) was liberated after the formation of the disulfide linkage due to the thiol-disulfide exchange reaction. We could therefore estimate the number of oligopeptides conjugated on the latex beads from the measurement of the liberated 2TP using HPLC and the absorption at 343 nm. The number of H12 or RGD molecules conjugated to one latex bead was estimated to be 2.0×10^5 and 1.8×10^5, respectively, showing that we successfully conjugated a sufficient amount of oligopeptides to the latex beads. Using a Coulter N4 Plus submicron particle sizer (Beckman–Coulter, Miami), we ascertained that the size and the

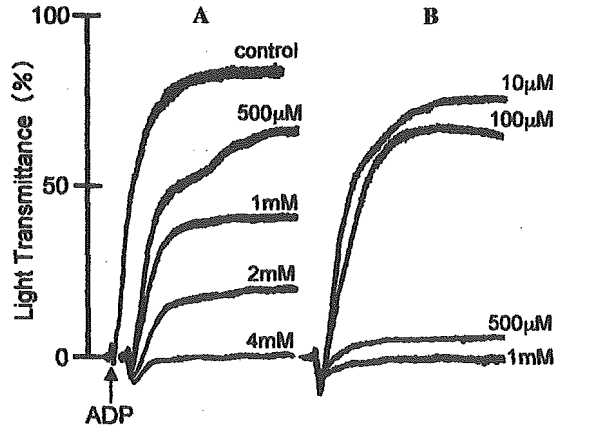

Fig. 1. ADP-induced (20 μM) aggregation of PRP ($2.0 \times 10^5/\mu L$) in the presence of H12 (A) or RGD (B).

stability of the dispersion state of the latex beads were not significantly changed after the adsorption of albumin and the conjugation of two kinds of the oligopeptide.

After the FITC-labeled H12-latex beads or RGD-latex beads were mixed with the reconstituted blood as described in Materials and methods, the mixture was allowed to flow over the activated platelets that were immobilized on a surface at a shear rate of $150\,s^{-1}$. They adhered and accumulated on the surface in a time-dependent manner, whereas the control albumin-adsorbed latex beads did not adhere on the surface (Fig. 2). The adhesion of the H12-latex beads and RGD-latex beads was suppressed in the presence of free H12 and RGDF, respectively, as inhibitors of GPIIb/IIIa binding. We confirmed that this adhesion was specifically caused by the interaction between the H12 or RGD and GPIIb/IIIa present on the surface of the activated platelets. The number of adherent latex beads decreased with increasing shear rate (data not shown). This interaction resembled the phenomenon when activated platelets interacted with fibrinogen under flow [23] and was different from that of GPIbα-conjugated latex beads where the number of adherent latex beads increased with increasing shear rate [7].

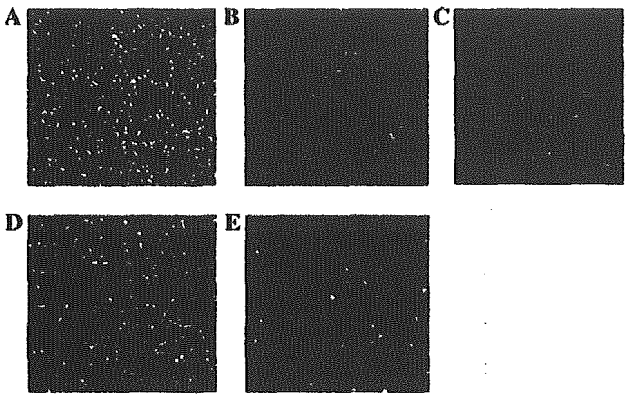

Fig. 2. Images of platelet-immobilized surface after reconstituted blood (Hct. 50%, platelet concentration $= 5.0 \pm 2.0 \times 10^3/\mu L$) were passed over the surface mixed with sample latex beads of varying composition as follows (A) H12-latex beads, (B) H12-latex beads after addition of free H12 (4 mM), (C) latex beads, (D) RGD-latex beads, and (E) RGD-latex beads after addition of free RGD (1 mM), at a shear rate of $150\,s^{-1}$. (A–E) images were obtained after blood was circulated for 180 s.

The aim of this study was to provide information that can be used for creation of artificial platelets capable of reinforcing thrombus formation under thrombocytopenic conditions. We prepared the thrombocytopenic-imitation blood and adjusted the number of platelets and latex beads to $2.0 \times 10^4/\mu L$ and $1.0 \times 10^5/\mu L$, respectively. The platelets were labeled with a fluorescent marker, $DiOC_6$, in order to observe the platelets instead of latex beads. In the presence of the control albumin absorbed latex beads, the surface coverage of platelets gradually increased to $3.1 \pm 0.4\%$ after 180 s of flow. This was the same value ($3.0 \pm 0.6\%$) when platelets were allowed to flow in the absence of the latex beads. This indicated that the control latex beads did not interfere with platelet adhesion. When the H12-latex beads or RGD-latex beads were used instead of the control latex beads, the surface coverage increased significantly to $5.1 \pm 0.3\%$ and $5.6 \pm 0.4\%$, respectively (Fig. 3A).

Next, FITC-labeled H12- or RGD-latex beads and non-labeled platelets were used in the same experiments. The initial binding rates of the labeled latex beads were

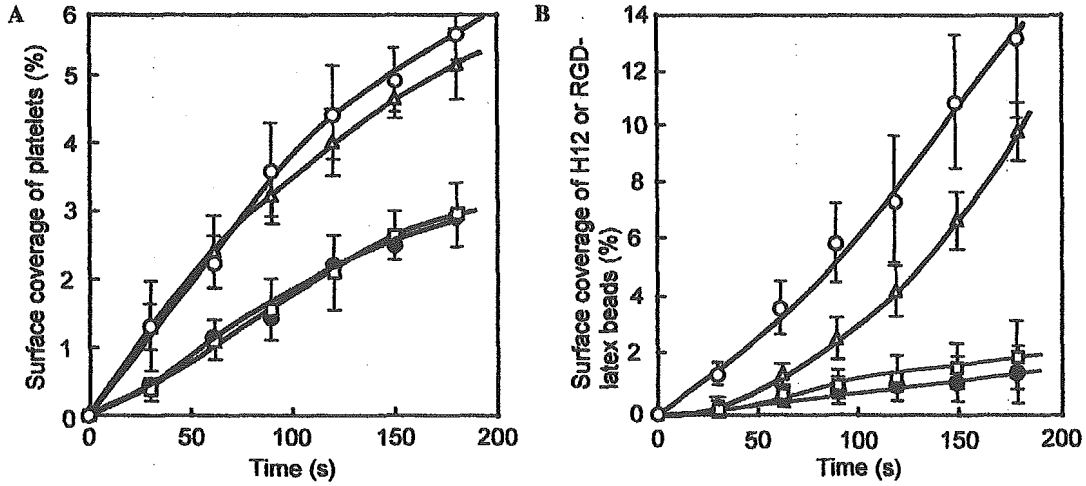

Fig. 3. (A) Time course studies of the surface coverage of platelets in the thrombocytopenic-imitation blood after addition of H12-latex beads (△), RGD-latex beads (○), latex beads (□), and PBS (●), at a shear rate of $150\,s^{-1}$. The concentrations of platelets and latex beads were $2.0 \times 10^4/\mu L$ and $1.0 \times 10^5/\mu L$, respectively. (B) Time course studies of the surface coverage of latex beads in the thrombocytopenic-imitation blood after addition of H12-latex beads (△), RGD-latex beads (○), and latex beads (□), with a platelet concentration of $2.0 \times 10^4/\mu L$ at a shear rate of $150\,s^{-1}$. (●) Shows the surface coverage of H12-latex beads when the concentration of platelets was $(6.0 \pm 2.0) \times 10^3/\mu L$.

slow and then increased dramatically with time. The percentages of the surface coverage for H12-latex beads and RGD-latex beads were $10.1 \pm 1.2\%$ and $13.1 \pm 2.6\%$, respectively. In these experiments we sometimes observed overlapped dots of FITC-labeled latex beads during flow which we considered to be due to the adhesion of several latex beads to one activated platelet. The adhesion of latex beads was inhibited when the platelet concentration was $6.0 \pm 2.0 \times 10^3/\mu L$ (Fig. 3B). This suggested that the adhesion sites of the latex beads decreased due to the reduced attachment frequencies of the circulating platelets on the collagen-immobilized plate. No adhesion of latex beads was observed when the control latex beads were used.

The above results suggest that the adhesion of H12-latex beads and RGD-latex beads is initiated by the activated platelets, which had already adhered on the surface of the collagen-immobilized plate. The H12- or RGD-latex beads adhering to the platelets would bring about subsequent adherence of flowing platelets. The thrombus formation would then grow because of the latex beads. We also observed the surface of the plates used in the experiments with a scanning electron microscope. There were many small aggregates where platelets first adhered on the collagen-immobilized surface; following this, H12- or RGD-latex beads became involved with the adherent and activated platelets. Furthermore, we could observe some latex beads sandwiched between the activated platelets as shown in Figs. 4A and B. Conversely, the interaction of the control latex beads with platelets was not observed in the platelet aggregates (Fig. 4C). This suggested that H12- or RGD-latex beads accelerated the thrombus formation of the remaining and flowing platelets on the collagen-immobilized surface in thrombocytopenic-imitation blood.

Therefore, our interest focused on whether the H12- or RGD-latex beads would cause the agglutination of non-activated platelets. Beer et al. [24] reported that the CGGRGDF-bound polyacrylonitrile beads showed agglutination with non-activated platelets, and D'Souza et al. [25] reported that non-activated platelets were agglutinated with the free 16-amino acid sequence containing H12 when it was added to the washed platelets. We mixed the bare latex beads, H12-latex beads, or RGD-latex beads ($1.0 \times 10^5/\mu L$) with non-activated platelets ($2.0 \times 10^4/\mu L$) and rotated the mixture for 30 min at 37 °C. The percentage agglutination measured by flow cytometry did not increase for the bare latex beads and the H12-latex beads but did increase for the RGD-latex beads to $2.9 \pm 1.3\%$ (fivefold from baseline) as shown in Fig. 5. We confirmed that the H12-latex beads showed minimal agglutination with non-activated platelets. This observation suggests that H12 could not enter into and interact with non-activated platelets because H12 was conjugated to large carriers, such as latex beads, depriving it of the necessary degree of freedom.

Moreover, we considered whether the H12-latex beads would cause the activation of platelets under severer conditions than in the above experiments, so we performed an experiment where the mixture of platelets and the H12-latex beads was stirred. The binding ratio

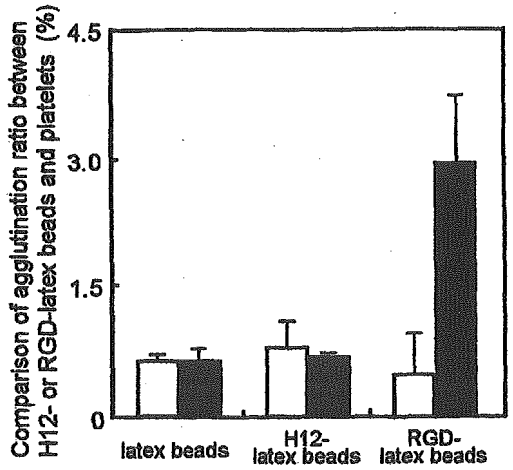

Fig. 5. Comparison of agglutination ratio of H12- or RGD-latex beads and platelets by flow cytometry. Zero minute (open bar) and 30 min (filled bar), after mixing with H12- or RGD-latex beads and platelets.

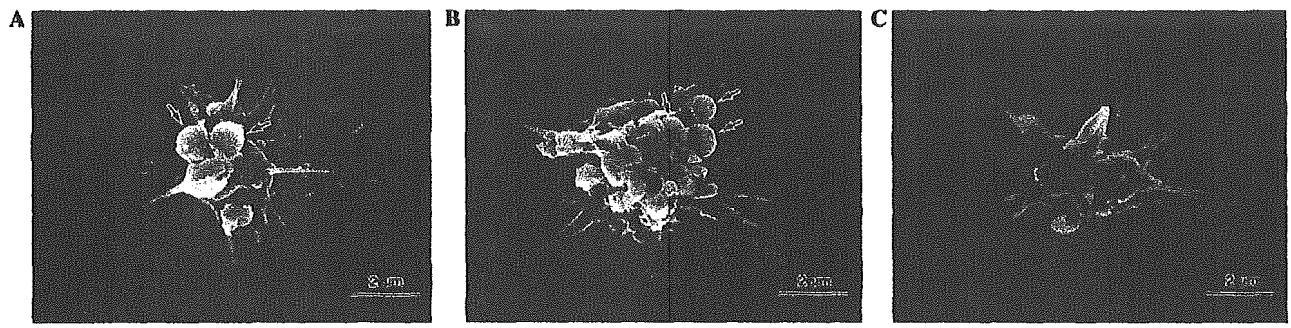

Fig. 4. SEM images obtained after platelets mixed with various bead formulations were passed over collagen immobilized on a glass surface. (A) Platelets and H12-latex beads, (B) platelets and RGD-latex beads, and (C) platelets and latex beads. Arrows in the SEM images are the latex beads.