Chapter 6
Albumin Microsphere: A Novel Carrier for the Artificial Platelet

6-1 General introduction of albumin microsphere

In drug delivery systems (DDS), various kinds of carriers have been designed and prepared. Those carriers are (i)liposomes or vesicles, (ii) micelles, (iii) microspheres, etc., and some of them have already been clinically used. (i)Phospholipid vesicles are promising candidates with high blood compatibility. They can encapsulate water-soluble drugs or genes[1] in their inner aqueous phase and can bind receptor proteins such as immunoglobulin or lectin on their surface[2]. (ii) Polymeric micelles from amphiphilic block copolymers have been developed to carry hydrophobic drugs[3]. (iii) There are various kinds of microspheres such as microcapsules, hydrogel microparticles[4], latex micro-beads[5], etc. Dendrimers could also be new candidates as nano-particles with quite regulated sizes[6]. Of course, those with high biocompatibility and biodegradability have been extensively studied as carriers for receptor proteins. On the other hand, the surface modification of the vesicle with poly(ethylene glycol) (PEG) is considered to be effective for the prevention of aggregation, the prolongation of blood circulation time, and the suppression of the binding of plasma proteins[7]. However, we should consider the reduction of the recognition ability of the bound proteins by the modification and dissociation of the receptor protein-conjugating lipids from the surface of the vesicle because of their relatively high hydrophilicity. Polymer micro-particles physically adsorb proteins such as immunoglobulins[8] or glycoproteins[9]. They are used as reagents for diagnosis such as immunoassay because of the easiness of preparation. The surface conditions can be easily controlled, responding to the nature of the proteins such as their isoelectric points, hydrophobicity, and specific structural interaction. However, protein conjugation with covalent bonding to those microparticles is generally more difficult and more complicated. On the other hand, dendrimers are also effective materials for conjugation because it is possible to introduce various functional groups at their surfaces. However, synthetic processes are somewhat time-consuming and expensive simultaneously.

We selected albumin microspheres (AMS) as carriers for functional proteins because of their high blood compatibility, high biodegradability, and long experience in clinical use[10]. Recetly, recombinant human serum albumin (rHSA) has become available. It has many functional groups on its surface, and various protein-conjugation methods could be applied. We prepared AMS in aqueous solutions to retain the hydrophilicity on their surface for high dispersing ability and high capability for conjugation of water-soluble functional proteins. We used *N*-succinimidyl 3-(2-pyridyldithio)propionate(SPDP) as a smart cross-linker[11], which reacts with the amino groups of proteins to introduce a disulfide group. The coupling of the proteins to AMS was carried out with disulfide linkage. Furthermore, the advantage of this reaction is that the reaction ratio of SPDP can be quantified from the amount of 2-thiopyridone (2TP) released at the cleavage of the 2-pyridyldithio (PD) groups by the thiol-disulfide exchange reaction. The conjugation ratio of the functional proteins and AMS can be analyzed from the amount of 2TP.We used cytochrome *c* having a typical absorption in the visible region as a probe protein and water-soluble rGPIbα. rGPIbα is a recombinant fragment of amino acid residues, 1-302, of glycoprotein (GP) Ibα of the platelet membrane and has a role in primary hemostasis with recognition ability with a von Willebrand factor (vWf)[12]. The receptor protein had

already been conjugated on the surface of phospholipid vesicles and the specific interaction with vWf was confirmed. The purpose of this study is to prepare AMS with rGPIbα[13-16] which could aid in primary hemostasis as a prototype of platelet substitutes.

6-2 Results and Discussion

6-2-1 Preparation of the size-controlled albumin microsphere

Figure 6-1 is the relationship between the stirring period and the average diameter of the AMS. After 15, 30, 45, and 60min, the AMS grew to 65±1, 126±3, 194±7, and 240±8 nm, respectively. Finally, the AMS of 240 ±10 nm was obtained after 90 min as shown in Figure 6-2a.

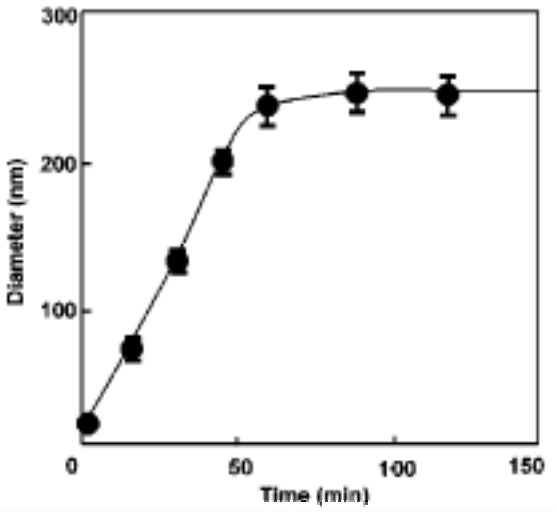

Figure 6-1 Change in AMS diameter during stirring at 37 ◦C, pH 6.02.AMS gradually grew at 37 ◦C, pH 6.02, to 240 ±10 nm after 60 min.

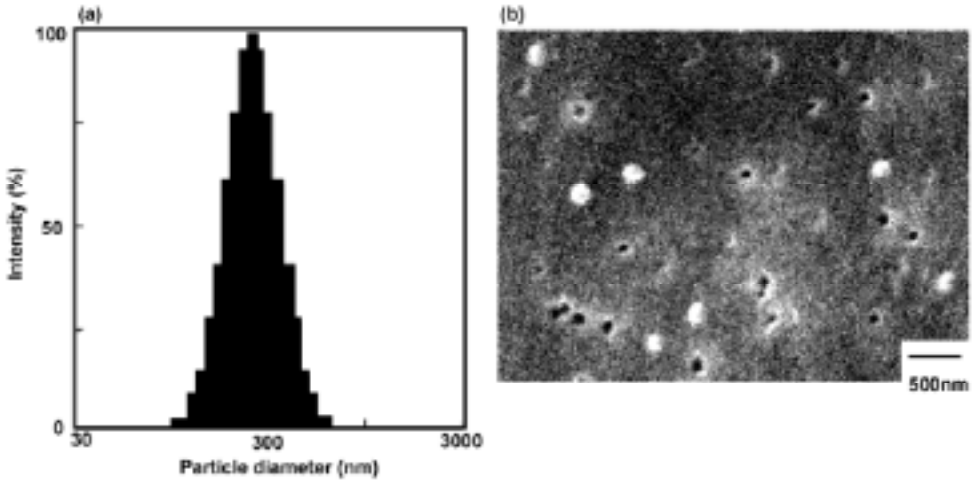

Figure 6-2 (a) Distribution of particle diameters of AMS. (b) Scanning electron micrograph of AMS.

The scanning electron microscopic observation identified the spherical AMS particles with a

predicted size as shown in Figure 6-2b. Human serum albumin (HSA) has 17 pairs of disulfide bonds and one thiol group at ^{34}Cys, and its molecular weight is 66.5 kDa[17]. Those disulfide and mercapto groups, which usually exist inside the HSA, should be exposed to the aqueous phase after conformational conversion from the N-form to the B-form under alkaline conditions. Thus, the thiol-disulfide exchange reaction occurs extensively at the intra- and intermolecular level[18]. By lowering the solution pH, the electrostatic repulsion decreases among the negatively charged B-form albumin, resulting in aggregation. Further stirring at 40 °C is considered to promote the intermolecular disulfide bonding and further aggregation. Because further stirring causes significant precipitation of the AMS aggregates, we determined an appropriate stirring period, according to the states of the albumin solution and the size distribution. Under our experimental conditions, at least 60 min is necessary to prepare AMS of 250 nm diameter. The remaining reactive thiol groups were deactivated by the addition of iodoacetamide to stop the further reaction and aggregation.

6-2-2 Activation of albumin microsphere by SPDP

We adopted an HPLC method to determine precisely the introduction ratio of the PD groups to the AMS and cytochrome *c*, not the spectrophotomeric method. The conventional spectrophotomeric method is for determining the introduction ratio of the PD group from the absorbance of 2TP at 343 nm without separating DTT. At present, this method is not considered to be accurate because the absorbance of 2TP at 343 nm was changed by reaction conditions such as pH, additives, and concentrations, etc. Furthermore, the base of the peak attributed to DTT at 280nm overlaps with 343 nm in PBS (pH 7.4)[19]. Therefore, Na and co-workers[20] treated PD-bound proteins with DTT and separated the resulting 2TP from DTT with a reverse phase HPLC. On the other hand, the quantitative analysis of the thiol group with an Ellman's reagent would sometimes overestimate the introduction ratio of the PD group due to the inter- and intramolecular reduction of disulfide bonds. From these arguments, we measured 2TP at 343 nm after separation from DTT with a size-exclusion HPLC using a calibration curve for 2TP. Figure 6-3 shows the relationship between the introduction ratio of the PD group to AMS and the SPDP feed. The linear relation indicates that the reaction was completed within 30min in this concentration range.

The yield of the reaction of SPDP with AMS was about 60% on average. Because 15% of the feed SPDP has already been deactivated before the reaction, the compensated yield would be 71%. The number of HSA molecules attached on the surface of AMS was calculated from the approximation of the packing of albumin molecules (8×8× 3 nm) in a sphere of 240 nm diameter. The number of the surface HSA molecules on one AMS particle was calculated to be one-fifteenth of the number of HSA molecules (ca. 90, 000) constructing one AMS. Therefore,4.8 PD groups were calculated to be introduced into the one HSA covering the surface of AMS. Because the number of amino groups is 59 for one HSA, the introduction of the PD group is controllable by the amount of added SPDP.

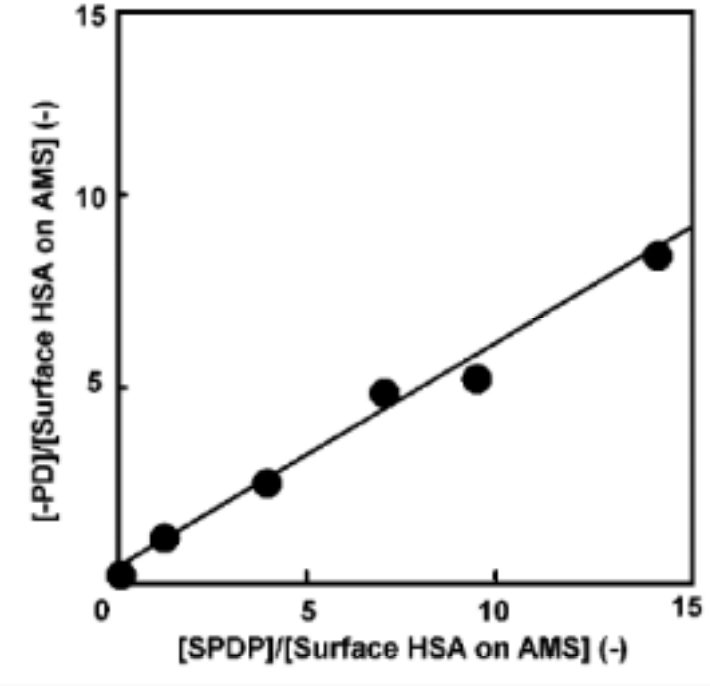

Figure 6-3 Control of the amount of the PD groups bound to cytochrome c. PD-AMS-cytochrome c was reduced by DTT, and the liberated 2TP was thenquantified by size-exclusion HPLC at 343 nm.

6-2-3 Conjugation of cytochrome c onto albumin microsphere

Figure 6-4 is the relationship between the introduction ratio of the PD group to cytochrome *c* and the SPDP feed. The relation was completely linear, also indicating the completion of the reaction within 30 min. The yield of SPDP binding to cytochrome *c* was 80%.

As mentioned above, the compensated yield would be approximately 100%. From the relation, we could introduce one PD group into one cytochrome *c*. The difference in the PD-conjugation yields between AMS and cytochrome *c* should be due to the different reaction rates caused by the different concentrations of those proteins. Namely, the concentrations of the surface HSA and SPDP were 9.3 μM and 82 μM, respectively, and those of cytochrome *c* and SPDP were 411 μM and 508 μM, respectively. Therefore, the AMS reacts with SPDP very slowly compared with the cytochrome *c* reaction. The hydrolysis of *N*-hydroxysuccinimide ester in the aqueous environment would be another reason. The above reaction aminolysis with SPDP should compete with the hydrolysis because the half-life of the hydrolysis in a pH7.5 solution was reported to be 14 min. If the aminolysis reaction would be relatively slow, the effective SPDP should be decreased due to the deactivation by hydrolysis.

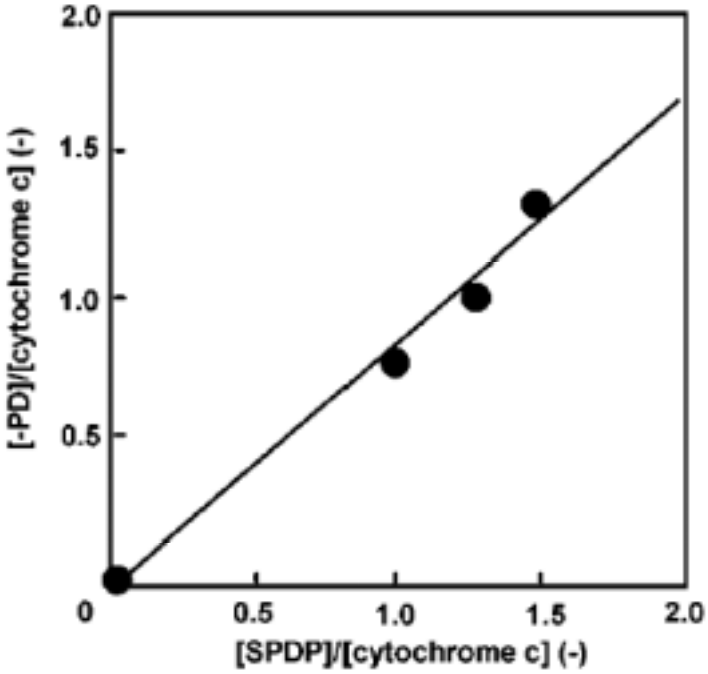

Figure 6-4 Control of the amount of the PD groups bound to AMS.PD-AMS was reduced by DTT, and the liberated 2TP was thenquantified by size-exclusion HPLC at 343 nm.

After the treatment of the PD-cytochrome *c* with DTT, the resulting SH-cytochrome *c* ([cytochrome *c*] =100 μM) reacted with the PD-AMS at 37 °C. The final concentrations of the PD group of AMS and the SH group of cytochrome*c* were 20 and 12 μM, respectively. Figure 6-5 shows the elution profiles of the samples after 30 min and 2, 7, and 11h. The conjugation yields of cytochrome *c* with AMS were 2, 8, 25, and 30%, respectively, as shown in Figure 6-6. Because the yield did not increase even after 11 h, and there was no release of the 2TP after the further addition of cytochrome *c*, we considered that the reaction had been saturated.

Such a slow reaction compared with SPDP reactions would reflect the diffusion-limited reaction between macromolecules and particles. The saturation seems to be due to the shielding of the reactive PD bound onto the AMS surface by cytochrome *c* which had already been conjugated. From the measurement of the amount of liberated 2TP, about 25, 000 cytochrome *c* molecules were calculated to be bound to one AMS particle. This means that 1.4 molecules of cytochrome *c* would be conjugated to one HSA molecule covering the surface of the AMS, and 3.4 PD groups of the HSA would remain. From the size of cytochrome *c*, the surface of the AMS was estimated to be completely covered with cytochrome *c*. Therefore, this number should be a reasonable limit. The UV-visible spectra of ferro- and ferricytochrome *c* conjugating on the surface of AMS were identified with those of unconjugated ones. The amount of cytochrome *c* calculated from the differential absorbance and the differential molar absorption coefficient at 550 nm between ferro- and ferricytochrome *c* was almost the same as that calculated from 2TP. This indicates that the redox function of cytochrome *c* was preserved after

conjugation to AMS.

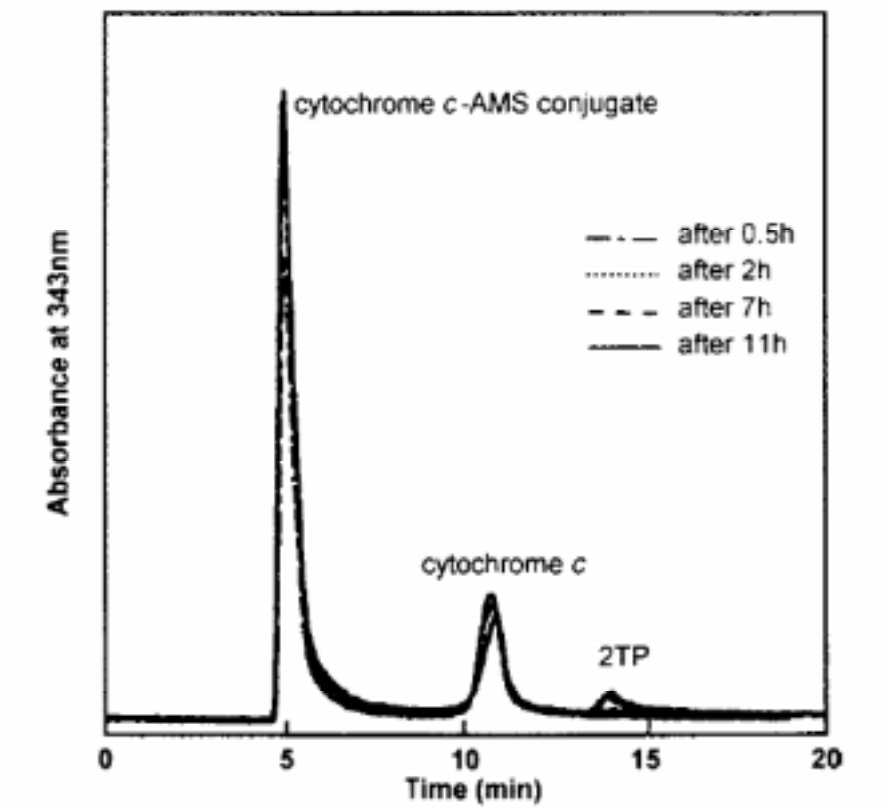

Figure 6-5 Size-exclusion HPLC chromatograms of AMS-cytochrome c conjugate. Cytochrome c-AMS conjugate, cytochrome c, and 2TPwere detected at 5, 11, and 14 min.

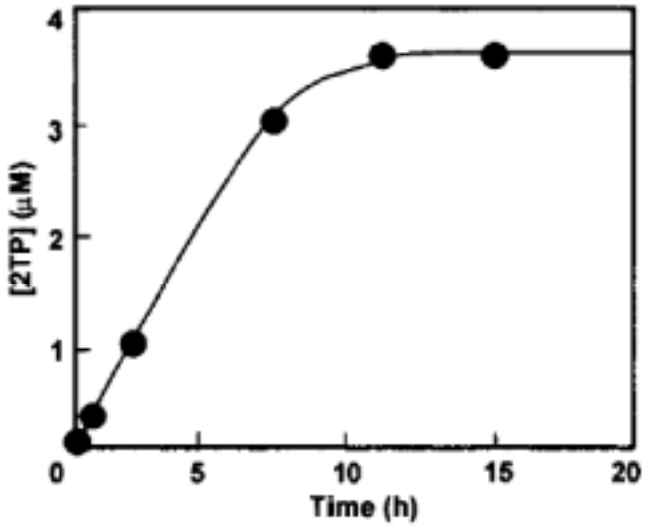

Figure 6-6 Conjugation of cytochrome c to AMS. The conjugation reaction was saturated during stirring for 11 h.

6-2-4 Conjugation of GPIbα onto albumin microsphere

In the same way as the case of cytochrome c, rGPIbα was conjugated to AMS. The number of the conjugated rGPIbα to one AMS was calculated to be 2500 from the amount of 2TP. The difference in the conjugation numbers between cytochrome *c* and rGPIbα can be explained in terms of the different molecular weights and shapes. Cytochrome *c* is a globular protein with a molecular weight of 12.5kDa, whereas rGPIbα is theoretically45kDa. Moreover, from the native PAGE analysis, the rGPIbα existed as oligomers such as dimer, trimer, tetramer,etc., by intermolecular cross-linking through one or three mercapto groups of the rGPIbα. Therefore, the oligomerization of the rGPIbα would result in a one-order smaller number of conjugations in comparison with cytochrome *c*.

6-2-5 Formation of co-aggregation of GPIbα -bound albumin microsphere with vWf

We evaluated the function of the rGPIbα conjugated to the AMS with an aggregometer, which is used for the study of platelet aggregation. Small, medium, and large aggregates indicate particle diameters of >9, >30-40, and >35-60 μm, respectively. rGPIbα-AMS supplemented with vWf was aggregated by the addition of ristocetin, which could be confirmed from the increase in the transmittance of middle aggregates (Figure 6-7a). This aggregation was inhibited when the anti-GPIbα antibody, GUR20-5, which recognizes a conformational-dependent epitope within the residues 1-293 of GPIbα, was added (Figure 6-7b).

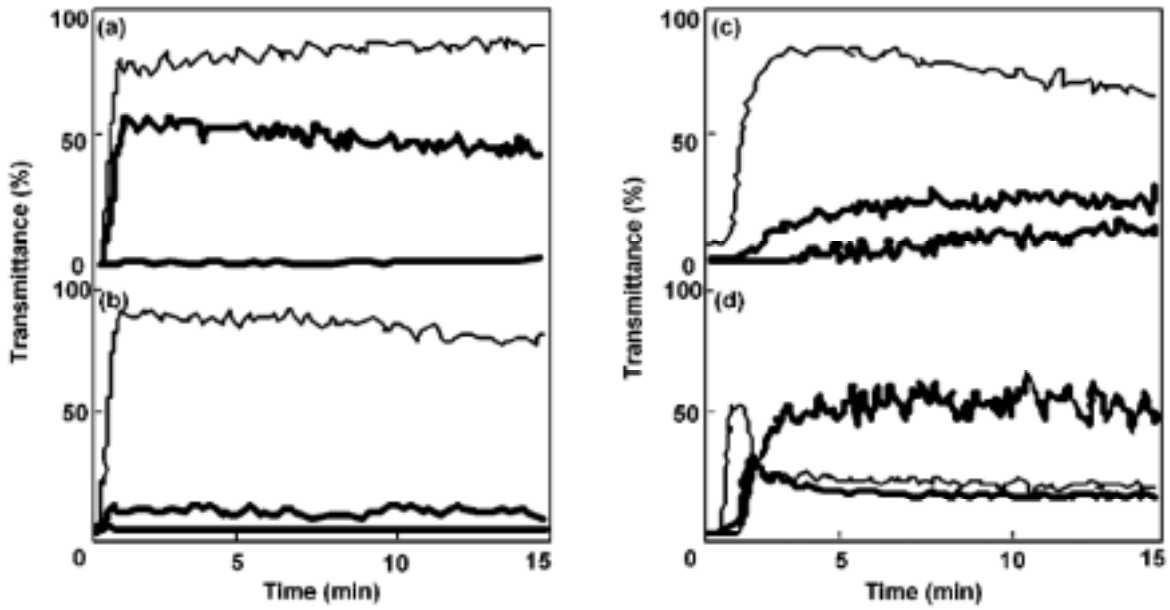

Figure 6-7 Aggregation of rGPIbα-AMS in the presence of vWf by the addition of ristocetin detected by PA-100. rGPIbα-AMS ([rGPIbα]) 4.0 μ/mL, [HSA]) 1.0 mg/mL] was first mixed with 50 μM/mL vWf and 1.0 mg/mL ristocetin was added thereafter (a). Aggregation of rGPIbα-AMS under the above conditions was inhibited by 50 μM/mL GUR20-5 (b). Platelet aggregation by rGPIbα-AMS with ristocetin was monitored byPA-100. PRP at low platelet counts (4.0 107/mL) was mixed with rGPIbα-AMS ([rGPIbα]) 0.27 μM/mL, [HSA]) 150 μM/mL] (c), or control AMS (d), and 1.5 mg/mL ristocetin was then added to the mixture.

This indicates that the aggregation was caused by a specific rGPIbα-vWf binding. Platelet aggregation is usually impaired when platelet concentration is low. We examined the effect of rGPIbα–AMS on platelet aggregation at low platelet concentration using PA-100. Ristocetin was added to the mixture of rGPIbR-AMS and 10-fold diluted platelets. rGPIbα-AMS shows the quick formation of small and middle aggregates and the conversion of those aggregates to large aggregates(Figure 6-7c). This indicates that nearly a hundred platelets would be involved in the aggregates with rGPIbα-AMS. Figure 6-7d is the result of a control experiment using rGPIbα–freeAMS. The small aggregates were detected because ristocetin should interact directly with AMS to cause weak aggregation. On the basis of these results, the function of rGPIbα was preserved after conjugation with AMS, and the resulting rGPIbα-AMS could participate in the formation of platelet thrombi at the site of vascular injury as a potential candidate of platelet substitutes such as rGPIbα-liposomes.

6-3 Materials and methods

Synthesis of albumin microspheres (AMS)

A25 g/dL sample of recombinant human serum albumin (rHSA) was kindly donated by Yoshitomi Pharmaceutical Industries, Ltd.(Osaka, Japan), and dialyzed against pure water for 24 h to remove the stabilizers such as *N*-acetyl D,L-tryptophan and sodium caprate. After dilution with saline, 1 g/dL rHSA was prepared as a sample. To the rHSA solution (25 mL) was gradually added a 0.1 N NaOH solution of 800 μL until the pH became 10.65. After being heated at 80 °C for a set period, the solution was cooled to room temperature in an ice bath for about 5 min. The pH of the solution was adjusted to pH 6.04 (21 °C) by stepwise addition (200 μL, about 900 μL total) of 0.1 N HCl solution. During stirring of the solution for 90 min at 37 °C, the solution gradually became turbid. The solution was dialyzed against phosphate-buffered saline (PBS)for 15 h at 5 °C, mixed with excess iodoacetamide (25 mg), and stirred for 60 min at room temperature. After the iodoacetamide was removed by dialysis followed by concentration, an AMS dispersion ([HSA]) 1.0 g/dL, pH 7.4) was prepared. Yield: 90%.To 1 mL of the AMS dispersion 88 μL of a 1.0 mM*N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Wako Pure Chemical Co. Japan) ethanol solution was added and stirred for 30min at 21 °C. After the separation of unreacted SPDP and byproducts by gel permeation chromatography (GPC, SephadexG-25, 10 mm o.d. 70 mm h), pyridyl disulfide-bound AMS (PDAMS,[HSA]) 0.47 wt %, 1.4 mL) was obtained. Ten microliters of 1 M dithiothreitol (DTT, Wako Pure Chemical Co.) was added to the PD-AMS dispersion (500 μL). 2-Thiopyridone (2TP) liberatedfrom the PD-AMS was quantified by an HPLC method, and the amount of the introduced PD group was thus determined. To 1 mL of a 0.5 g/dL cytochrome *c* (Wako Pure Chemical Co.)PBS solution was added 53 μL of a 10 mM SPDP ethanol solution, and the mixture was stirred for 30 min. After the separation of the unreacted SPDP and byproducts by GPC (Sephadex G-25, 10 mmo.d. 70 mm h), 1.4 mL of a PD-cytochrome *c* solution([cytochrome *c*]) 210 μL) was obtained. The quantitative analysis of the introduced PD group was performed as described above. DTT treatment was carried out for the PD-cytochrome *c* solution(500 μL) to reduce the PD group to a mercapto (SH) group. To700 μL of the PD-AMS solution

([HSA]) 0.47 g/dL) was added 100 μL of a SH-cytochrome *c* solution for the conjugation of cytochrome *c* to AMS. The rGPIbα was also kindly donated by Yoshitomi Pharmaceutical Industries, Ltd. As described above, the PD group was bound to the protein, converted into the mercapto group, and reacted with PD-AMS.

Measurements

The concentration of the PD-introduced proteins was measured by a bicinchoninic acid method (BCA protein assay kit, Pierce Chemical, IL) for AMS and UV absorbance at 215 nm for cytochrome *c*. After treatment of the PD-introduced proteins with DTT, 20 μL was applied to the size-exclusion HPLC (TSKGELG3000SWXL column, 7.8 mm o.d. 300 mm h, 1 mL/min, PBS). The elution curve of the proteins was drawn from the absorbance at 215 nm, and that of liberated 2TP was done at 343nm. It is noted that, though the base of the DTT peak and the peak of 2TP were overlapped, this could be ignored under our conditions because the influence was within an acceptable error range. An aggregometer, PA-100 (Kowa, Nagoya, Japan), was used for the assessment of aggregation of rGPIbα-AMS[21]. rGPIbα-AMS ([GPIbα]) 4.0 μM/mL, [HSA]) 1.0 mg/mL) was first mixed with 50 μM/mL vWf, and 1.0 mg/mL ristocetin was added thereafter. To confirm that the aggregation was a specific phenomenon caused by vWf-GPIbα binding, an inhibition study was carried out using50 μM/mL murine anti-GPIbα monoclonal antibody, GUR20-5. The intensities of scattering light from small, middle, and large aggregates were monitored and recorded. Platelet-rich plasma at low platelet counts (4.0 107/mL) was mixed with rGPIbα-AMS ([GPIbα]) 0.27 μM/mL, [HSA]) 150μM/mL) or control AMS, and 1.5 mg/mL ristocetin was then added to the mixture. The intensities of scattering light from small, middle, and large aggregates were monitored and recorded with the PA-100.

6-4 Conclusions

We were able to synthesize AMS with regulated sizes from rHSA in aqueous solutions by pH change and heat treatment. This method is feasible for large-scale production with a quite high yield. The resulting AMS has functional groups on the surface for the conjugation of various functional proteins. We used SPDP and the thiol-disulfide exchange reaction for the quantitative conjugation of cytochrome *c* or rGPIbα to the AMS. The advantage of this method is the ease of obtaining the amount of the introduced PD moieties and the amount of the conjugated proteins by size-exclusion HPLC from the liberated 2TP. Therefore, we can control the degree of those reactions. We confirmed that the function of those proteins was well preserved. The AMS having rGPIbα is applicable as a prototype for platelet substitutes.

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