MMA/MPEOMA/VSA copolymer as a novel blood-compatible material: *ex vivo* platelet adhesion study

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MMA/MPEOMA/VSA copolymers with both pendant polyethylene oxide (PEO) side chains and negatively chargeable side groups were synthesized by random copolymerization of methyl methacrylate (MMA), methoxy PEO monomethacrylate (MPEOMA; PEO mol. wt, 1000), and vinyl sulfonic acid sodium salt (VSA) monomers with different monomer composition to evaluate their blood compatibility. MMA/MPEOMA copolymer (with PEO side chains) and MMA/VSA copolymer (with negatively chargeable side groups) were also synthesized for the comparison purpose. The synthesized copolymers were coated onto polyurethane (PU) tubes (inner diameter, 4.6 mm) by a spin coating. The platelet adhesion of the MMA/MPEOMA/VSA copolymer-coated tube surfaces was compared with that of tube surface coated with MMA/MPEOMA or MMA/VSA copolymer with similar MPEOMA or VSA composition, using an *ex vivo* canine arterio–artery shunt method. The platelet adhesion was evaluated by radioactivity counting of technetium (^{99m}Tc)-labeled platelets adhered on the surfaces after 30 and 120 min of blood circulation. The MMA/MPEOMA/VSA copolymer (monomer molar ratio 9/0.5/0.5 or 8/1/1) was better in preventing platelet adhesion on the surface than the MMA/MPEOMA or MMA/VSA copolymer with similar MPEOMA or VSA composition, probably owing to the combined effects of highly mobile, hydrophilic PEO side chains and negatively charged VSA side groups.

Introduction

Surface-induced thrombosis remains as one of the main problems in the development of blood-contacting devices. When a foreign surface comes in contact with blood, the initial blood response is adsorption of blood proteins, followed by platelet adhesion and activation of the coagulation pathways, leading to thrombus formation. A particularly effective polymer for the prevention of protein adsorption and platelet adhesion appears to be polyethylene oxide (PEO; or polyethylene glycol (PEG) when the molecular weight is less than about 10 000) [1], probably owing to its minimum interfacial free energy with water, hydrophilicity, high surface mobility and steric stabilization effects, and unique solution properties and molecular conformation in water [2–4]. PEO surfaces have been prepared by many different methods, including physical adsorption of PEO-containing amphiphilic block or graft copolymers onto hydrophobic substrates, blending small amounts of PEO or PEO-containing block or graft copolymers with matrix materials, and covalent coupling or graft copolymerization of PEO derivatives to substrates, as reviewed elsewhere [2]. Many research groups have also reported that sulfonate [5–9] or sulfonated PEO [10–14] incorporation to substrates reduces protein adsorption or platelet adhesion owing to its negative charge character in aqueous solution: large portions of blood protein and platelet surfaces are negatively charged and thus they may be repulsed electrostatically on negatively charged surfaces [15, 16].

In our previous study [17], novel copolymers (MMA/MPEOMA/VSA copolymers) with both pendant PEO side chains and negatively chargeable side groups (not PEO chain with negative charge end group, but independent PEO chain and negative charge group) were synthesized by random copolymerization of methyl methacrylate (MMA), methoxy PEO monomethacrylate (MPEOMA), and vinyl sulfonic acid sodium salt (VSA)

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monomers with different monomer composition. The synthesized copolymers were coated onto polyurethane (PU) films and their interaction behaviors with blood proteins (albumin, γ-globulin, fibrinogen, and plasma proteins) and platelets were compared *in vitro*.

In this study, MMA/MPEOMA/VSA copolymers were coated onto the inner surfaces of PU tube (inner diameter, 4.6 mm) by a spin coating method to evaluate the effect of both PEO side chains and negatively charged side groups on blood compatibility using an *ex vivo* canine arterio–artery (A–A) shunt method. The platelet adhesion on the MMA/MPEOMA/VSA copolymer-coated PU tube surfaces was compared with that of tube surface coated with MMA/MPEOMA copolymer (with PEO side chains) or MMA/VSA copolymer (with negatively chargeable side groups) with similar MPEOMA or VSA composition.

Experimental

Synthesis of copolymers

Fig. 1 shows the synthetic scheme of MMA/MPEOMA, MMA/VSA, and MMA/MPEOMA/VSA copolymers. The copolymers were synthesized by radical polymerization of monomers (15.0 wt % in dimethyl sulfoxide (DMSO) at 60 °C for 48 h with monomer feed ratios listed in Table I. 2,2′-azobis-isobutyronitrile was used as an initiator. The average molecular weights of the synthesized copolymers were estimated by gel permeation chromatography (GPC) and the monomer composition in the copolymers was determined by ^{1}H-nuclear magnetic resonance spectroscopy (NMR), as shown in Table I. The detailed synthetic method and characterization of the copolymers were described elsewhere [17].

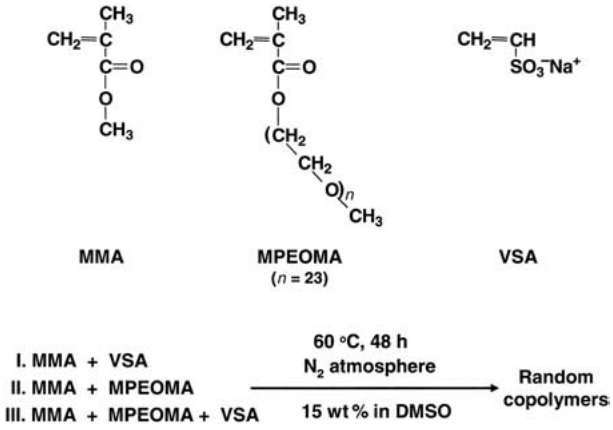

Figure 1 Synthetic scheme of MMA/MPEOMA, MMA/VSA, and MMA/MPEOMA/VSA copolymers.

PU tube fabrication and coating with copolymers

A spinning tube-fabricating device was designed by our laboratory for both uniform PU tube fabrication and the coating of tube inner surface with the copolymers (Fig. 2(A)). To fabricate PU tube, polyurethane (PU; Pellethane 2363-80AE, Dow Chemical, USA) was dissolved in tetrahydrofuran into 5 wt % and the PU solution was injected into a cylindrical brass mold (7 cm length, 5 mm inner diameter) rotating at 3500 rpm by a motor-driven spinner (Fig. 2(B)). After drying for 2 h while keeping the rotation of the mold, the PU tube was peeled out from the mold in water. The tube thickness could be controlled by adjusting the amount of injected PU solution. When 8 mL solution was injected into the mold a PU tube with 0.2 ± 0.01 mn thickness was obtained.

To prepare a copolymer-coated PU tube, PU solution (5 wt %) was injected into a brass mold rotating at 3500 rpm, then dried for 2 h as above. Then 1 mL of 0.5 wt % copolymer (MMA/MPEOMA, MMA/VSA, or

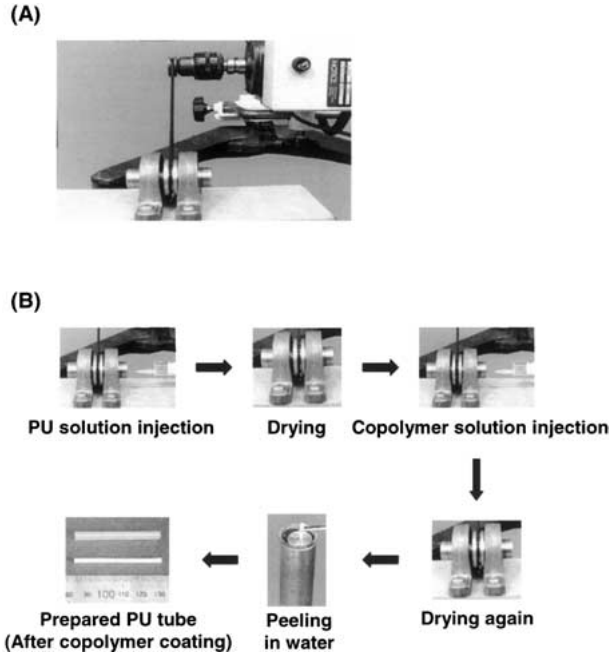

Figure 2 (A) A spinning tube-fabricating device. (B) Procedures of PU tube fabrication and copolymer coating onto the inner surface of PU tube using a spinning tube-fabricating device.

TABLE I Composition and molecular weights of copolymers synthesized[a]

Copolymer name	Composition (mol %)		M_w[c]	M_w/M_n[c]
	Monomer feed	Copolymer[b]		
MMA/MPEOMA	9/1	8.9/1.1	435 000	2.4
MMA/VSA	9/1	9.0/1.0	312 000	1.7
MMA/MPEOMA/VSA	9/0.5/0.5	9.3/0.4/0.3	437 000	2.4
	8/1/1	8.1/0.9/1.0	433 000	2.5

[a]All copolymers, water-insoluble.
[b]By ^{1}H-NMR measurement.
[c]By GPC measurement.

MMA/MPEOMA/VSA copolymer) dissolved in methylene chloride was directly injected into the PU tube while keeping the same rotation. After drying for another 30 min, the copolymer-coated PU tube was peeled out from the mold in water (Fig. 2(B)). The prepared copolymer-coated PU tubes were vacuum dried for three days to remove water and any remaining residual solvent. The uniformity of the copolymer coating on the inner surface of PU tube was examined by the observation of SEM (Model 2250N, Hitachi, Japan) and a measurement of water contact angles using a contact angle goniometer (Model 100-0, Rame-Hart, USA) on the different positions along the tube length.

Ex vivo platelet adhesion on copolymer-coated tube surfaces

Shunt preparation. Two control and four different copolymer-coated PU tube segments (each, 2 cm in length, 4.6 mm inner diameter and 5.0 mm outer diameter) were connected in series to a U-shaped shunt for *ex vivo* platelet adhesion test (Fig. 3). The copolymers used for the coating of PU tube inner surface are listed in Table I. The prepared shunts, each with randomly arranged six tube segments, were equilibrated in phosphated buffered saline (PBS: pH 7.4) for 24 h before the blood contacting experiment.

Surgical procedure. Ten healthy adult mongrel dogs weighing 25–30 kg were selected and cared for according to the Korean Regulations for the Care and Use of Laboratory Animals. The carotid artery of each dog was used for the *ex vivo* test by an A–A shunt method. Each dog was injected with autologous technetium (^{99m}Tc)-labeled platelet solution (see below) through a venous cannula inserted in the forefoot vein. Following anesthetic induction with ketamine, an endotracheal tube was inserted and anesthesia was maintained with intravenous ketamine. Following a midline cervical incision, the carotid artery was bilaterally exposed. The shunt was interposed into the carotid artery after clamping the proximal and distal positions. With careful deairing procedure, the clamps were released and blood flow resumed through the shunt. Two groups of animals, each group with five animals, were sacrificed after 30 min and 120 min of blood circulation, respectively, and then the shunts were removed for the data analysis.

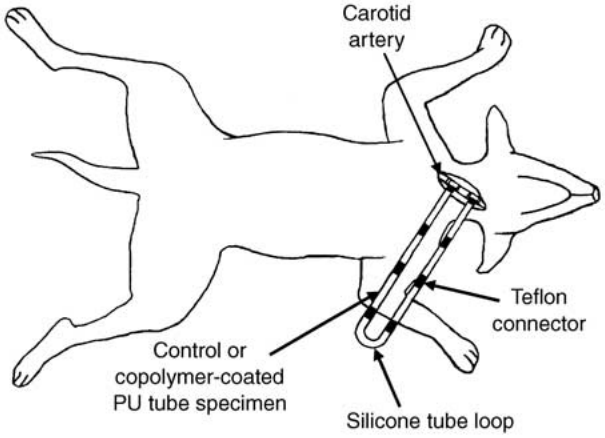

Figure 3 Schematic diagram showing *ex vivo* A–A shunt (two control and four different copolymer-coated PU tube segments were randomly connected in each shunt).

Labeling of platelets with ^{99m}Tc. For each animal, 60 mL of venous blood was drawn into a sterile plastic syringe containing 12 mL acid–citrate–dextrose (ACD) as an anticoagulant. The blood sample was centrifuged at 300 × *g* for 20 min to obtain platelet-rich-plasma (PRP). Then, the PRP was further centrifuged at 2000 × *g* for 20 min to separate the platelets from the platelet-poor-plasma (PPP). The platelet pellet was resuspended with 1 mL PPP and ^{99m}Tc 40 mC was added. This solution was incubated in a water bath for 30 min at 37 °C. After incubation, 10 mL of PPP was added into the solution and centrifuged at 2000 × *g* for 20 min. The resultant pellet was resuspended with 10 mL PPP and the labeling effect (LE) of the solution was calculated. After LE was calculated, the ^{99m}Tc-labeled platelet solution was reinjected into the vein of the experimental animal immediately.

Evaluation of platelet adhesion. The shunt removed from each animal after 30 or 120 min of blood contact was gently flushed with PBS. Then, the control and different copolymer-coated PU tube segments of the shunt were cut into the same length (1 cm) from the middle sections. Radioactivity from the ^{99m}Tc-labeled platelets adhered on the inner surface of each tube segment was counted immediately in a gamma counter and the value was converted into the number of platelets adhered. The data obtained were averaged from five separate animal experiments and expressed as mean ± standard deviation. Student's *t*-test was used to determine the significance of differences in platelet adhesion on the tube segment surfaces using the SAS system for windows V8 (SAS Institute, Inc., USA). A *p*-value less than 0.05 was considered statistically significant.

Results and discussion

Characterization of copolymer-coated PU tubes

PU tubes prepared by a spinning method were smooth (both inner and outer surfaces) and had uniform diameter and thickness along the tube length. The tube thickness could be controlled by the amount of PU solution injected into the cylindrical mold of the spinning tube fabricating device. The PU tubes with 4.6 mm inner diameter and 0.2 mm thickness were prepared for the copolymer coating and the following *ex vivo* platelet adhesion test. The MMA/MPEOMA, MMA/VSA, and MMA/MPEOMA/VSA copolymers were coated on the inner surfaces of the PU tube by the same spinning method used for the PU tube fabrication. Copolymer solutions with different concentration (0.1–1.0 wt %) were applied for the tube coating and the uniformity of the coating was examined by the observation of SEM and a measurement of water contact angles along the tube length. From the SEM observation (not shown), it was found that the copolymer solutions with the concentrations of 0.5–1.0 wt % are desirable for the uniform and smooth coating. From the result of water contact angle measurements (Fig. 4), it also was observed that the

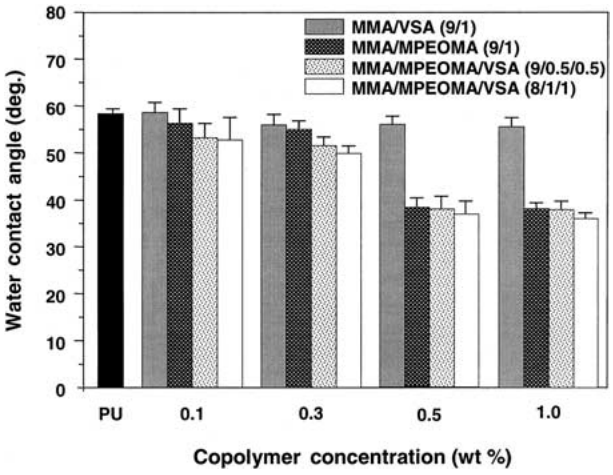

Figure 4 Water contact angles of the inner surface of copolymer-coated PU tubes as a function of applied copolymer concentration ($n = 5$).

water contact angles of MPEOMA-containing copolymer-coated PU tube surfaces decreased with the increasing copolymer concentration up to 0.5 wt % and then did not show further decrease. The decrease in the contact angles (and thus the increase in wettability or hydrophilicity) on the tube surfaces is owing to the hydrophilic property of MPEOMA chains exposed on the surfaces. The VSA seems not to be so hydrophilic and thus the MMA/VSA copolymer-coated PU surface did not show significant decrease in the contact angles compared to the control PU surface. The contact angles of the copolymer-coated tube surfaces did not show position-dependant changes along the tube length, indicating the uniform surface coating. The stability of the copolymers coated on the inner surface of the PU tubes was examined for more than one month in aqueous solution. All the copolymers used in this study are water-insoluble and thus were firmly attached on the tube surfaces without weight loss and peeling.

For an *ex vivo* platelet adhesion test, the PU tubes coated with 0.5 wt % copolymer (MMA/VSA, MMA/MPEOMA, or MMA/MPEOMA/VSA copolymer) solution were used. VSA and MPEOMA on the MMA/VSA and MMA/MPEOMA copolymer-coated surfaces, respectively will have similar surface densities since their mole ratios in the copolymers are the same (9/1). The MMA/MPEOMA/VSA copolymer-coated surfaces also will have surface densities, for VSA and MPEOMA together in MMA/MPEOMA/VSA copolymer (9/0.5/0.5) or separately in MMA/MPEOMA/VSA copolymer (8/1/1), similar to the MMA/VSA or MMA/MPEOMA copolymer-coated ones.

Evaluation of *ex vivo* platelet adhesion on copolymer-coated tube surfaces

Short-term evaluation of the blood compatibility of copolymer-coated PU tube surfaces was conducted using an *ex vivo* canine A–A shunt method. After 30 and 120 min of blood circulation, the platelet adhesion on the inner surface of PU tube segments of each shunt was examined by radioactivity counting of ^{99m}Tc-labeled platelets adhered on the surface and then the conversion of the value into the number of platelets (Fig. 5). The

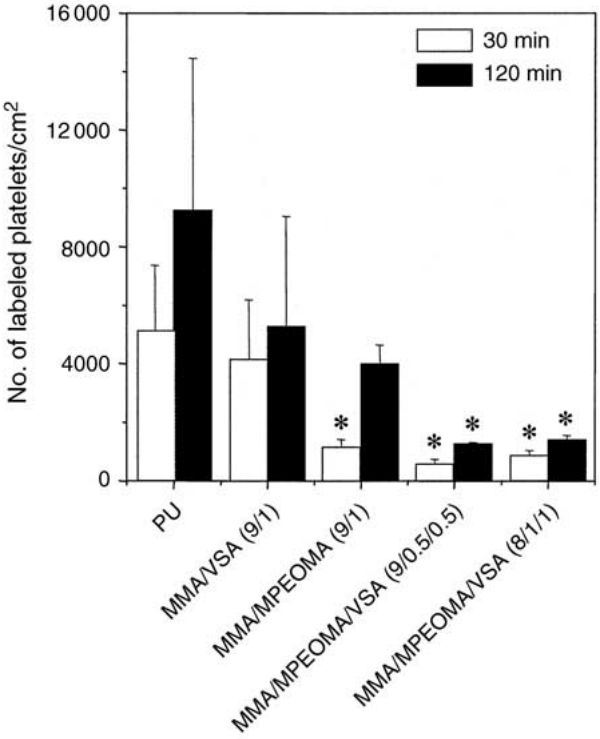

Figure 5 Number of labeled platelets adhered on control and copolymer-coated PU tube surfaces after 30 and 120 min blood circulation ($n = 5$). *$p < 0.05$.

MMA/VSA copolymer-coated tube surface showed less amount of platelet adhesion on the surface than the control PU tube surface ($p = 0.69$ and 0.71 for 30 and 120 min adhesion, respectively), but it showed a larger amount of platelet adhesion than MMA/MPEOMA copolymer-coated surface ($p < 0.05$ and 0.18 for 30 and 120 min adhesion, respectively), even though the negative charge (VSA) density on the MMA/VSA copolymer-coated surface is similar to the PEO density on the MMA/MPEOMA copolymer-coated one. This result indicates that PEO is more effective than negative charge in preventing the platelet adhesion on the copolymer-coated surfaces used in this study even though the statistical analysis shows only marginally significant except for the MMA/MPEOMA copolymer-coated surface with 30 min adhesion, perhaps due to the small number of samples. Similar results also were observed from the studies of the adsorption of plasma proteins and the adhesion of platelets *in vitro* using the same copolymer-coated PU film surfaces [17]. One possible explanation of poor effect of MMA/VSA copolymer-coated surface on the prevention of platelet adhesion is that some portion of negative charge group (VSA) in MMA/VSA copolymer-coated surface may be buried due to its short side chain. If a vinyl monomer with spaced negative charge group can be used instead of VSA (if available) for the synthesis of copolymer, it may be possible for less platelet adhesion. MMA/MPEOMA/VSA copolymer-coated surfaces were better in preventing platelet adhesion than the MMA/MPEOMA copolymer-coated surface ($p < 0.05$). Little platelet adhesion was observed even on the MMA/MPEOMA/VSA (9/0.5/0.5) copolymer-coated surface with half surface density of each VSA or MPEOMA compared to that of MMA/VSA (9/1) or MMA/MPEOMA (9/1)

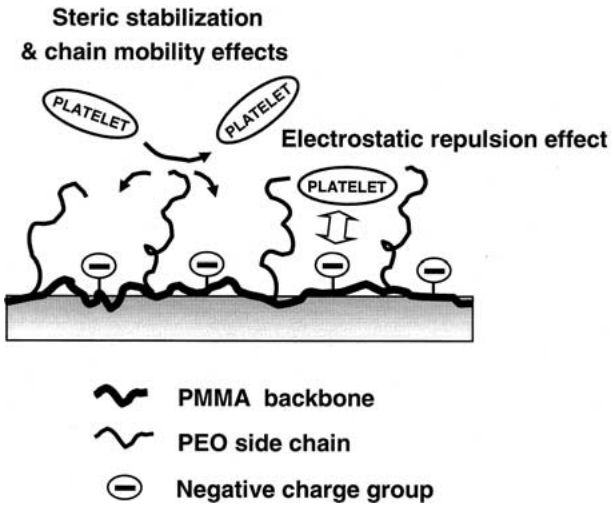

Figure 6 Possible mechanisms for the prevention of platelet adhesion on MMA/MPEOMA/VSA copolymer surface.

copolymer-coated surface, respectively. This result was somewhat different from that obtained *in vitro:* the *in vitro* result of the MMA/MPEOMA/VSA (9/0.5/0.5) copolymer-coated surface showed much higher platelet adhesion than *ex vivo* result of this study [17]. We are not clear yet why this difference occurred, but this may be derived from the different circumstances of platelet adhesion in both tests. For *ex vivo* test, platelets in whole blood were adsorbed on the copolymer-coated surface, while for *in vitro* test, platelets in platelet-rich-plasma were adsorbed. The effective prevention of platelet adhesion on the MMA/MPEOMA/VSA copolymer surfaces, particularly the MMA/MPEOMA/VSA (8/1/1) copolymer surface with sufficient PEO and VSA density for both *in vitro* and *ex vivo* tests may be caused from the combined effects of highly mobile, hydrophilic PEO side chains and negatively charged side groups in aqueous solution, as suggested in Fig. 6. Many proteins or platelets approaching the surface may be repulsed by highly mobile PEO chains exposed in aqueous solution. PEO in water has rapid motions and a large excluded volume compared with other water-soluble polymers. As we consider the structures of water-soluble polymers such as PEO, polyacrylic acid, polyacryl amide, polyvinyl alcohol, and polyvinyl pyrrolidone, we can expect that PEO will be the most flexible in water among those polymers because only PEO has flexible ether linkages on the backbone chain and does not have bulky side groups attached to the backbone. The dynamic behavior of PEO in water compared to other water-soluble polymers was also observed from the measurement of nuclear magnetic relaxation times [18–20]. PEO has a large excluded volume in water and thus a high steric stabilization effect, as evidenced by the high value of second virial coefficient or low value of polymer–solvent interaction parameter [2]. PEO surface in water with rapidly moving hydrated PEO chains and a large excluded volume tend to repel protein or platelet molecules which approach the surface. Although some portion of proteins or platelets may penetrate the PEO chains at the surface, they will be faced with the negatively charged surface and thus not be easily attached onto the surface; large portions of protein or platelet surface have native charges and thus will be repeled electrostatically from the surface. If MMA/MPEOMA or MMA/VSA copolymers with a higher MPEOMA or VSA composition (more than 9/1) can be used, it may be possible for more effective prevention of platelet adhesion than MMA/MPEOMA/VSA copolymers used in this study (9/0.5/0.5 or 8/1/1). However, the copolymers with those compositions unfortunately were water-soluble and thus could not be used as a stable coating material [21].

From this short-term *ex vivo* platelet adhesion study as well as a previous *in vitro* plasma protein adsorption and platelet adhesion studies [17], we conclude that MMA/MPEOMA/VSA copolymer with both pendant relatively long PEO chains (PEO mol. wt. 1000) and negatively chargeable sulfonate groups may be a very good candidate as a blood-compatible coating material. We still need further long-term systematic studies to understand the blood interactions with the copolymer surfaces.

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