Preparation of Therapeutic Phospholipids Through Porcine Pancreatic Phospholipase A_2-Mediated Esterification and Lipozyme-Mediated Acidolysis

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ABSTRACT: Highly unsaturated fatty acid-containing phospholipids (HUFA-PL), which serve to increase the deformability of human red blood cells, were prepared through porcine phospholipase A_2-mediated esterification of the lysophosphatidylcholine, which was derived from soy phosphatidylcholine (PC), and by Lipozyme-mediated acidolysis. Through these processes, phospholipase A_2, with formamide as a water mimic, enhanced the incorporation of HUFA into position *sn*-2 of PC and suppressed hydrolysis of the synthesized PL. On the other hand, Lipozyme-mediated acidolysis between position *sn*-1 of soy PC and HUFA was enhanced by a combination of water and propylene glycol. Simultaneously, the recovered PL products showed decreased hydrolysis of newly synthesized health-beneficial HUFA-PL.

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KEY WORDS: Acidolysis, deformability, docosahexaenoic acid, eicosapentaenoic acid, esterification, nonaqueous solvents, phospholipids, red blood cell, unsaturated fatty acids.

Highly unsaturated fatty acid-containing phospholipids (HUFA-PL) are currently receiving attention because of their novel physiological functions. Yazawa and co-workers (1) reported that the decrease in the weight of adipose tissue among the major organs (perirenal adipose tissue, paraepididymal adipose tissue) after the administration of the eicosapentaenoic acid-containing PL (EPA-PL) suggests a specific effect of this novel chemical form of EPA. Suzuki and co-workers (2) reported that docosahexaenoic acid-containing phosphatidylcholine (DHA-PC), isolated from rainbow trout embryos, induces differentiation of murine undifferentiated tumor cells. Kohno *et al.* (3) observed that the rate of retinoic acid-induced differentiation of HL-60 human leukemia was accelerated by HUFA-PC. 5-Lipoxygenase is known to catalyze the first step in leukotriene production. Matsumoto *et al.* (4) showed that DHA-PC can inhibit this enzyme. Their study indicated that *sn*-1 18:1/*sn*-2 DHA-PC is the most potent inhibitor of 5-lipoxygenase. The inhibition of 5-lipoxygenase by *sn*-1 18:1/*sn*-2 DHA-PC was noncompetitive, and *sn*-1 18:1/*sn*-2 DHA-PC showed no effect on 12- or 15-lipoxygenase or cycloxygenase. These results suggest that the endogenously existing DHA-PC may affect 5-lipoxygenase activity, and thus control leukotriene biosynthesis *in vivo*. Enhancement of discriminatory shock-avoidance learning in rats was observed by Izaki *et al.* (5) when the rats were injected intraperitoneally with *sn*-1 18:1/*sn*-2 DHA-PC. To assess the effect of DHA on the permeability of living T27A tumor cells, the tumor cells were modified by fusion with lipid vesicles composed of *sn*-1 18:0/*sn*-2 DHA-PC, with vesicles composed of *sn*-1 18:0/*sn*-2 18:1-PC, and with the nonfusogenic control 16:0/16:0-PC. The DHA-containing cells were about 1.9 times more permeable to ^{51}Cr than the 16:0/16:0-PC, and 1.5 times more permeable than the 18:1-altered cells (6).

The present work shows that nonaqueous solvents that generate multiple hydrogen bonds (water mimics) can be partially substituted for water as lipase and phospholipase activators to produce phospholipids that are beneficial to health.

EXPERIMENTAL PROCEDURES

Chemicals. Lipase (EC 3.1.1.3) from *Mucor miehei* Lipozyme IM-60 (48.0 BIU/g) was a generous gift from Novo Nordisk Bioindustries Inc. (Bagsvaerd, Denmark). Porcine pancreatic phospholipase A_2 (protein content 38.8%) and lysophosphatidylcholine (LPC) were generous gifts from Kyowa Hakko Kogyo Ltd. (Tokyo, Japan). Phospholipase D was donated by Asahi Chemical Industry Co., Ltd. (Fuji, Japan). Free EPA (purity 90%) and free DHA (purity 88%) were kindly supplied by Nippon Chemical Feed Ltd. (Hakodate, Japan). Soy PL (purity, 95%) was obtained from Avanti Polar-Lipids Inc. (Alabaster, AL). Ethylene glycol (>95.0%, moisture <0.85%), formamide (>98.5%, moisture <0.5%), propylene glycol (>95.0%, moisture <0.22%), *N,N*-dimethylacetamide (>98.0%, moisture <0.11%), and *N,N*-dimethylformamide (>99.5%, moisture <0.2%) were employed as water mimics and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and solvents were reagent-grade.

Esterification of LPC. Reactions were initiated by adding 23 mg of porcine pancreatic phospholipase A_2 to a closed reaction vial that contained varying amounts of water mimics (or water as reference), 110 mg LPC, 180 mg EPA or DHA,

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and 5500 mg glycerol (moisture 0.15%). Reaction mixtures were subjected to magnetic stirring and incubated at 25°C for 48 h. Reactions were terminated by adding 288 mL of chloroform/methanol/water (10:5:3, vol/vol/vol). The chloroform layer was then recovered, concentrated, and subjected to a silica Sep-Pak cartridge (Waters Associate Co., Ltd., Milford, MA). Free fatty acids were recovered with chloroform/methanol (10:1, vol/vol) as the eluent; PC were isolated with methanol as the eluent. Aliquot amounts of the recovered PC were applied to Chromarod S III (Iatron Laboratories, Inc., Tokyo, Japan) and were developed in a mixture of chloroform/methanol/water (65:25:4, vol/vol/vol). Relative amounts of the lipid classes were analyzed through thin-layer chromatography (TLC)–flame-ionization detection (FID) (Iatroscan TH-10; Iatron Laboratories).

Standard curves between weight and area (FID response) were constructed by subjecting known quantities of each lipid class to the same system. PC yield was calculated as follows:

$$\text{PC yield (\%)} = [\text{synthesized PC (mg)/substrate LPC (mg)}] \times 100 \quad [1]$$

Incorporation rates of EPA or DHA to LPC were defined as the EPA or DHA increase in PC fatty acid composition as determined by gas-chromatographic analysis (7).

Acidolysis reaction of soy PC. Twenty-three mg of Lipozyme IM-60 was dried with phosphoric anhydride at 25°C for 24 h and placed in a screw-capped test tube. Varying amounts of water mimics, 0.5/mL of dried hexane, containing 60 mg of EPA or 66 mg of DHA, and 10 mg of soy PC were added to the Lipozyme to initiate the reaction at 40°C by immersion in a reciprocal water bath shaker (2-cm stroke at 75 rpm). Reactions were terminated by adding 5 mL of acetone/ethanol (1:1, vol/vol), and also by passing the reactant through a 0.45-μm pore size filter to remove the Lipozyme with 30 mL of chloroform/methanol (1:1, vol/vol). The recovered lipids from the chloroform layer were subjected to a silica Sep-Pak cartridge in the same manner. Relative amounts of the lipid classes also were analyzed in the same manner. FID responses were constructed. Recoveries of PC were calculated as follows:

$$\text{PC recovery (\%)} = [\text{recovered PC (mg)/applied PC (mg) before acidolysis}] \times 100 \quad [2]$$

Incorporation rates (conversions) of EPA or DHA into PC were defined as the EPA or DHA increase in PC fatty acid composition as determined by gas-chromatographic analysis (8).

Transphosphatidylation of PC. Transphosphatidylation was carried out by the method of Juneja *et al.* (9) with slight modification as follows: Fifty mg soy PC or HUFA-PC was dissolved in 2.2 mL ethyl acetate. Then, 200 mM acetate buffer (pH 5.6) containing 87.8 mg ethanolamine salt, 11.9 mg bovine serum albumin, and 80 units phospholipase D from *Streptomyces* sp. were added to initiate the reaction. Stirring was conducted at 1,200 rpm and 30°C. Incubation was terminated by adding chloroform/methanol/water (10:5:3, vol/vol/vol) after 4 h of reaction, and the recovered phosphatidylethanolamine (PE) was purified by preparative TLC with chloroform/methanol/water (65:25:4, vol/vol/vol) as solvent. To prepare phosphatidylserine (PS) through transphosphatidylation, 1.7 mL of ethyl acetate, containing 50 mg of PC, 0.8 mL of 200 mM acetate buffer (pH 5.6) with 5 mg $CaCl_2$, and 250 mg of L-serine were used. Recovery and purification of PS were done in the same manner as with PE. Through these modifications, it was possible to prepare PC, PE, and PS with the same acyl combinations.

Hydrogenation of PL. Hydrogenation was achieved by bubbling hydrogen gas directly into absolute ethanol solution in the presence of 5% Rh-Carbon catalyst (Kawaken Fine Chemical Co., Ltd., Tokyo, Japan). The reaction was carried out for approximately two hours under ambient conditions. Then chloroform was added until the saturated lipids dissolved, the solution was filtered through filter paper, and finally through a 0.45-μm pore size filter to remove the catalyst.

Treatment of human red blood cells with various PL. Sixty μL of individual PL suspensions (an equal volume of sonicated mixture of presonicated 4 mM PL suspension and dimethylsulfoxide) were added to 6 mL of washed human red blood cells (hematocrit value was adjusted to 2%). They then were incubated for 3 h at 37°C by immersion in a reciprocal waterbath shaker (2-cm stroke at 80 rpm). After incubation, excess PL were removed through rinsing with 10 mM Na_2HPO_3, 125 mM NaCl (pH 7.4) phosphate buffer saline (PBS). They were suspended again in PBS to adjust the hematocrit value up to 10%. Human plasma (0.2 mL) that had been separated immediately after venipuncture was added to 0.5 mL of individual PL suspension, and then mixed. These red blood cell suspensions, containing plasma, were treated with Nucleopore filters (Corning Costar Co. Ltd., Cambridge, MA) and filtrates were used for the evaluation of red blood cell deformability.

Evaluation of the deformability of human red blood cells. The flow rate of human red blood cells on silicon substrate microchannels was measured to evaluate their deformability after they were treated with soy PL, hydrogenated PL, DHA- or EPA-PL.

The silicon substrate surface has a compartment, the so-called well, which is partitioned by banks (as illustrated in Fig. 1A) (10). Microgrooves, where red blood cells pass through, are formed in the single-crystal silicon as shown in the close-up view of the banks in Figure 1B. The cross-sectional area of each microgroove corresponds to the cross-sectional area of the narrowest human capillary vessel. Red blood cells pass through the hole on the bottom of the well, then flow through the microgrooves on the banks, as shown in Figure 1C. Reducing pressure of 7.4 mm Hg (10 cm H_2O) is applied, and the time required for 10 μL to flow through is measured to assess the deformability (11).

RESULTS AND DISCUSSION

Effect of water mimics on esterification of LPC. As a preliminary study, we examined *N,N*-dimethylacetamide, propylene glycol, ethylene glycol, dimethylformamide, and formamide as water mimics on the esterification of LPC prepared from

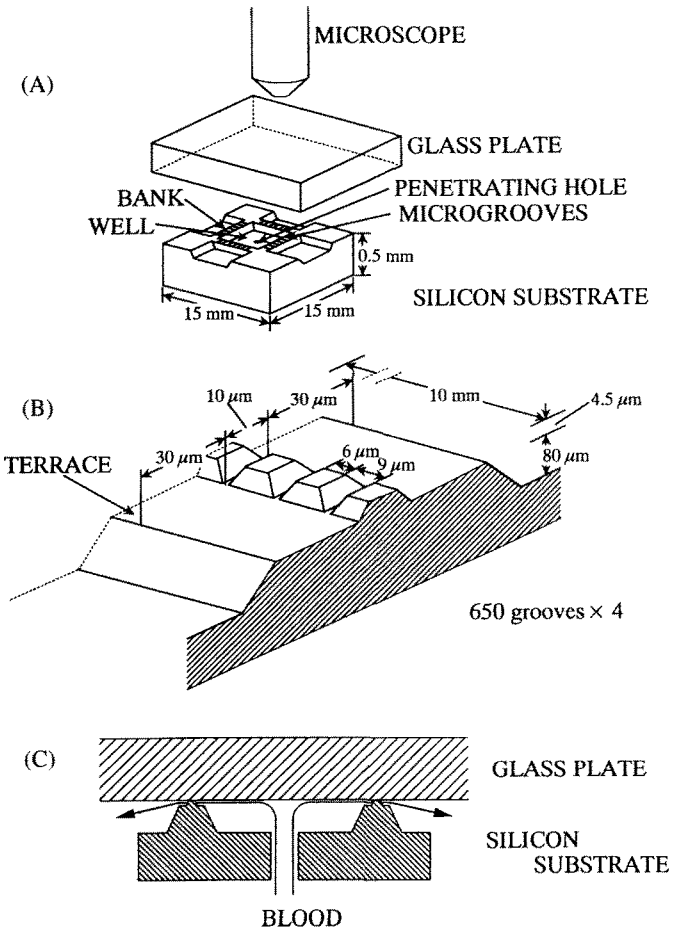


FIG. 1. Outline structure of the artificial capillary model (Refs. 10,11). (A), Outline structure. The silicon substrate surface has compartments (wells) partitioned by banks. (B), Close view of a bank. The level areas (terraces) are placed at two sides of the bank at the same level to the groove depth. (C), Blood passage. Contact of the upper surface of the bank to the glass plate can be made watertight with mechanical pressing alone because of the optical flatness of both surfaces.

soy PC. We found that a small amount of formamide resulted in a high yield and enhanced incorporation. As shown in Figure 2, the yield reached 60% with the use of formamide, while the water-added system remained at 30%. It was possible to incorporate DHA or EPA into position *sn*-2 up to the theoretical maximum (12). Addition of more water increased the initial rate of the reaction but impaired the ultimate yield.

Effect of water mimics on acidolysis of PC. We also examined the aforementioned water mimics on acidolysis of soy PC (8). With a water and propylene glycol combination, the incorporation of EPA reached 40% conversion after 48 h reaction (Fig. 3A). This corresponds to 80% of the theoretical maximum incorporation level (because Lipozyme is known to incorporate the desired fatty acid exclusively into position *sn*-1 of PC). The water and propylene glycol combination also enhanced higher recovery (80%, Fig. 3B).

Deformability of red blood cells treated with various PL. Deformability assessment was carried out for all the enzymatically synthesized PL, soy PL, and hydrogenated PL. The

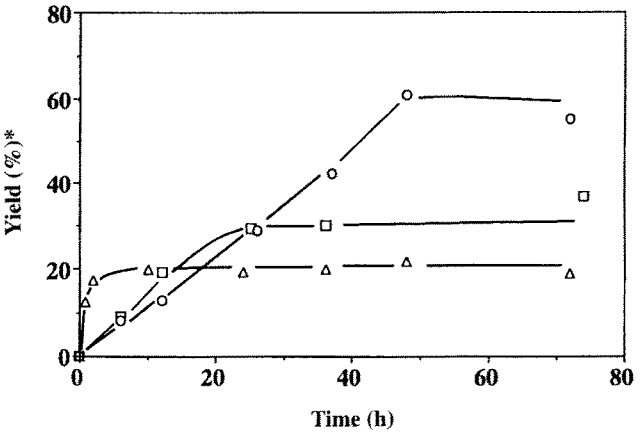


FIG. 2. Effect of formamide on phospholipase A_2-mediated esterification. Reaction conditions: phospholipase A_2, 23 mg; lysophosphatidylcholine, 110 mg; eicosapentaenoic acid, 180 mg; glycerol, 5500 mg; 3 μmol $CaCl_2$. ○, Formamide; □, tris–HCl buffer, 0.2 mL; △, tris–HCl buffer, 0.5 mL. The buffer used was 0.2 M tris–HCl, pH 8.0. See Equation 1 in the text.

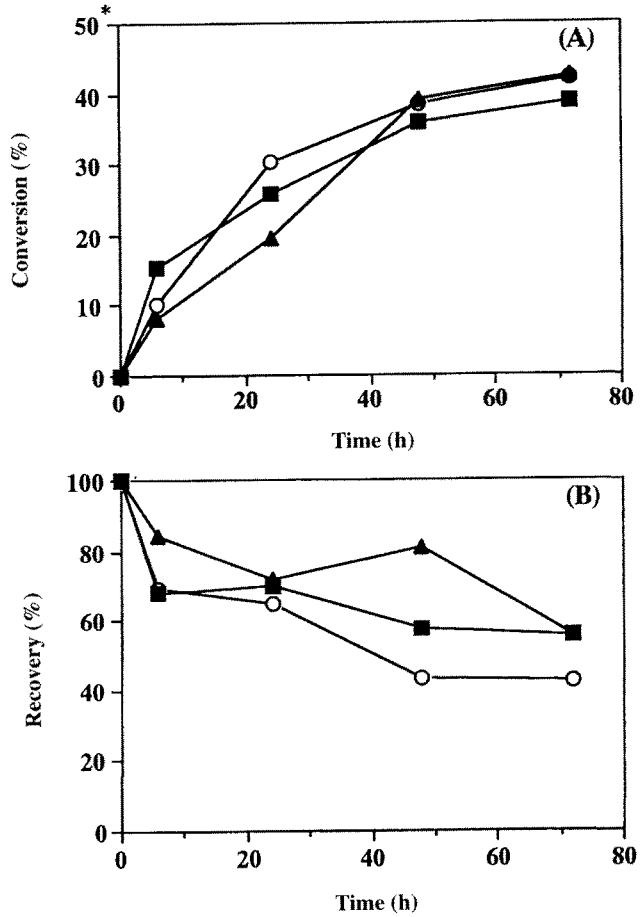
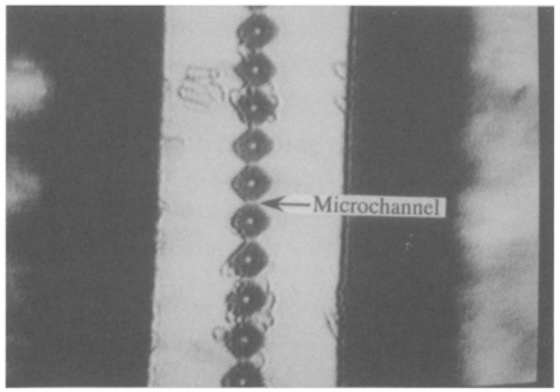
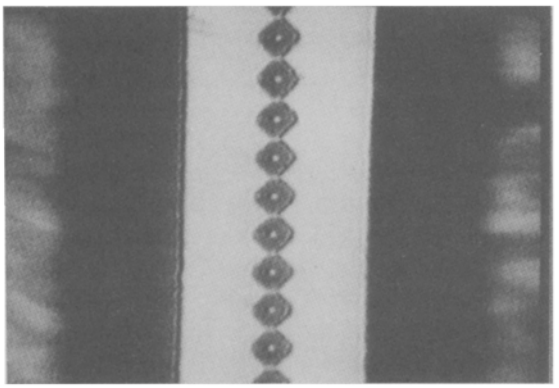


FIG. 3. Effect of combined usage of water and propylene glycol on Lipozyme IM60-mediated acidolysis to incorporate eicosapentaenoic acid (EPA) into soy phosphatidylcholine. (A), Percent incorporation of EPA; (B), percent recovery of EPA-incorporated product. Reaction conditions: hexane (0.5 mL) solution containing soy phosphatidylcholine (10 mg), EPA (60 mg), and Lipozyme IM60 (23 mg by initial weight). ○, Water (1.0 μL/system); ■, propylene glycol (1.0 μL/system), Water activity (aw) ≅ 0; ▲, propylene glycol (0.5 μL) with water (0.5 μL). *Theoretical maximum of EPA incorporation corresponds to 50% because Lipozyme exclusively incorporates EPA into position *sn*-1 of the phospholipid. Lipozyme from Novo Nordisk (Bagsvaerd, Denmark).



← flow direction

(A) Red blood cells treated with HPC



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(B) Red blood cells treated with *sn*-2 DHA-PE

FIG. 4. Microscopic observation of red blood cells treated with hydrogenated phosphatidylcholine (HPC) (A) and *sn*-2 docosahexaenoic acid-containing phosphatidylethanolamine (DHA-PE) (× 1,000) (B) in microgrooves of the artificial capillary model (Ref. 11) (× 1,000). Measured conditions: groove length of the microchannel, 10 μm; negative pressure applied into the reservoir, 7.4 mm Hg (10 cm H_2O); temperature, ambient (24°C).

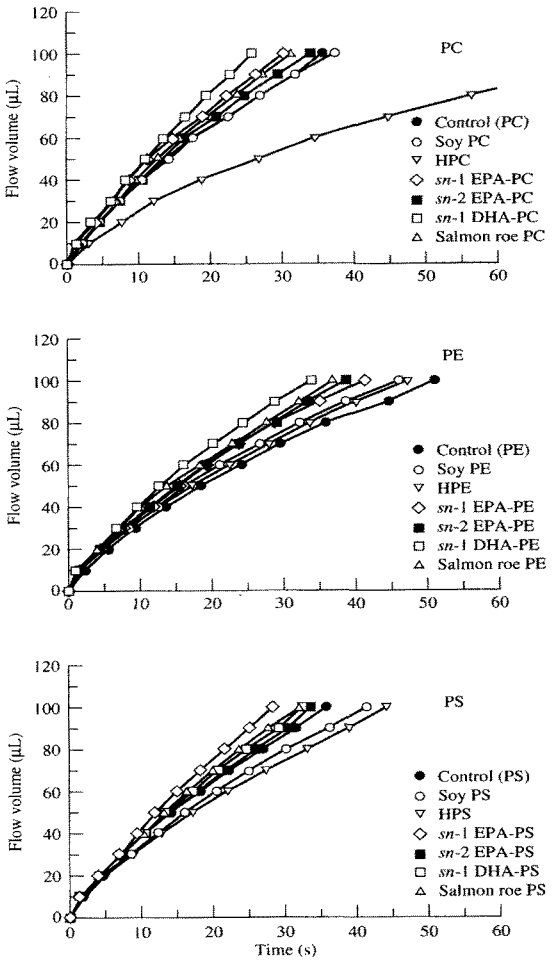

FIG. 5. Flow curves of red blood cells treated with various phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) for the evaluation of deformability through the artificial capillary model. Measured conditions are the same as in Figure 4 (Ref. 11). HPC, hydrogenated PC; *sn-1* EPA-PC, eicosapentaenoic acid (at *sn*-1)-containing PC; *sn*-2 EPA-PC, EPA (at *sn*-2)-containing PC; *sn*-2 DHA-PC, docosahexaenoic acid (at *sn*-2)-containing PC; salmon roe PC, naturally occuring PC prepared from chum salmon roe; soy PE, PE prepared through transphosphatidylation of Soy PC; HPE, hydrogenated PE; *sn*-1 EPA-PE, EPA (at *sn*-1)-containing PE; *sn*-2 EPA-PE, EPA (at *sn*-2)-containing PE; *sn*-2 DHA-PE, DHA (at *sn*-2)-containing PE, salmon roe PE, PE prepared through transphosphatidylation of salmon roe PC; soy PS, PS prepared through transphosphatidylation of soy PC; HPS, hydrogenated PS; *sn*-1 EPA-PS, EPA (at *sn*-1)-containing PS; *sn*-2 EPA-PS, EPA (at *sn*-2)-containing PS; *sn*-2 DHA-PS, DHA (at *sn*-2)-containing PS; Salmon roe PS, PS prepared through transphosphatidylation of salmon roe PC.

arrow in Figure 4 shows one of the microgrooves where blood cells pass through. Transit of saturated fatty acid PL-treated human red blood cells was slow, resulting in eventual blockage of the passages. The microgrooves have smaller diameters than the red blood cells, thus, a decrease in deformability of the red blood cells causes blockage of the passages (as seen in Fig. 4A). We could follow the flow of each red blood cell on videotape recorder (VTR), because the flow rate slowed down considerably. In contrast, transit of red blood cells which were treated with our synthesized HUFA-PL went very quickly (Fig. 4B), and it was impossible to follow the flow of each red blood cell on VTR.

We compared the flow rate of various enzymatically synthesized PL-treated red blood cells in the artificial capillary model with naturally occurring PL, soy PL, and hydrogenated PL. As shown in Figure 5, the flow rate of EPA-containing PS and DHA-containing PC or PE were fastest among the PL examined. Therefore, they were considered to be the most effective lipid molecular species to improve the deformability of human red blood cells, whereas hydrogenated PL tend to impair their deformability.

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