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Correlation Between Composition of the Outer Layer and Phase Asymmetry for Vesicles Ruptured by Phospholipase D

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Abstract Spherical phospholipid bilayers, vesicles, were prepared by using the layer-by-layer double emulsion technique, which allows individual layers to be formed asymmetrically. Phases of the layers were adjusted by selecting the lipid tail group. The head group composition of the vesicle outer layer varied 0–100 % of phosphatidylcholine (PC) by 10 % under the condition that the diameter of the vesicle was kept constant. On the outer layer of the vesicle, the phospholipase D (PLD) reacted to convert PC to phosphatidic acid. The reaction induced a curvature change of the vesicles, which eventually led them to rupture. Response time from the PLD injection to the rupture was measured against the different compositions of the outer layer at each phase (solid and liquid) using the fluorescence intensity change of pH-sensitive dye encapsulated in the vesicles. From this measurement, the rupture caused by the PLD reaction was analyzed with respect to the phase asymmetry of the layers and the composition of the outer layer. These results were interpreted with the lipid density and stability of the layers. It was observed that the solid phase of the outer layer had a variance in response time according to the phase of the inner layer, whereas the liquid phase did not. Additionally, the response of the solid phase of the outer layer at the liquid phase of the inner layer was faster than at the solid phase of the inner layer as a result of its stability.

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Abbreviation

PLD	Phospholipase D
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
PA	Phosphatidic acid
DOPE	Dioleoylphosphatidylethanolamine
DPPE	Dipalmitoylphosphatidylethanolamine
OHPE	Oleoylhydroxyphosphatidylethanolamine
PHPE	Palmitoylhydroxyphosphatidylethanolamine
DOPC	Dioleoylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DOPA	Dioleoylphosphatidic acid
DPPA	Dipalmitoylphosphatidic acid

Introduction

Phospholipase D (PLD) is a membrane-active enzyme that is related to a variety of cellular processes, such as membrane/vesicle trafficking, actin cytoskeleton rearrangements, glucose transport, superoxide production, secretion, cellular proliferation, and apoptosis (McDermott et al. 2004; Exton 2002). As a result, PLD is implicated in a range of diseases including cancer, inflammation, and myocardial disease (Huang and Frohman 2007; Tappia et al. 2006; Scott et al. 2009; Brown et al. 2007). By PLD action upon phosphatidylcholine (PC), PC is cleaved into alcohol and phosphatidic acid (PA, a potent mitogen) that may be essential for the formation of certain types of transport vesicles or that may be constitutive of vesicular transport to signal transduction pathways. Because this hydrolysis leads to changes in the lipid content of membranes, PLD may have a physiological

function through the further metabolism of PA to diacylglycerol (DAG) and lysophosphatidic acid (McDermott et al. 2004; Brown et al. 2007).

Lipid layers are known as widely used models for cell surfaces and for investigating molecular events in membranes because the method of preparation of the layers has been well established, and highly sensitive analytical techniques can be applied to investigate the events (New 1990; McConnell et al. 1986; Sackmann 1996; Brian and McConnell 1984). The lipid layers have been applied to many areas of biomedical research, such as cell recognition, membrane-mediated catalysis, effects of anesthetics, and antimicrobial peptide activity (Giesen et al. 1991; Mou et al. 1994; Miszta et al. 2008; Fang et al. 2000; Cheow and Hadinoto 2011). Furthermore, the phospholipases have been investigated at the layers, i.e., the effect of the enzymes on the wetting properties and the configuration of the layers, and the activity of the enzymes (Jurak and Chibowski 2010; Chen et al. 2009; Chemburu et al. 2008).

The hydrolysis triggered by PLD is a critical step for the fusion that is essential for cellular processes. The hydrolysis induces the change in the composition of the membranes and eventually ruptures the vesicles because the hydrolysis results in the occurrence of smaller head groups at the outer layer of the vesicles. Therefore, the hydrolysis leads to a decrease in the curvature, and rupture is finally attained. Recently, it has been found that the hydrolysis response depends on the phase asymmetry of the single-component outer layer and the symmetric-phase outer layer, respectively (Park 2011, 2012a, b). However, little is known about how phase asymmetry correlates with the composition of the outer layer for the PLD-induced vesicle rupture. An investigation of this correlation may contribute to an understanding of the physical behavior of these enzymes in terms of quantitative analysis. We thus aimed to investigate the correlation between the phase asymmetry and the composition for the PLD-induced vesicle rupture. For the solid phase of the outer layer, the response was changed up on the phase of the inner layer; this was not the case for the liquid. In addition, for the solid phase of the outer layer, the response of the liquid phase of the inner layer was faster than the solid phase of the inner layer.

Experiments

Micelle Solution Preparation

Dioleoylphosphatidylethanolamine (DOPE), dipalmitoylphosphatidylethanolamine (DPPE), oleoylhydroxyphosphatidylethanolamine (OHPE), palmitoylhydroxyphosphatidylethanolamine (PHPE), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidic acid (DOPA), and dipalmitoylphosphatidic acid (DPPA) were purchased from Avanti Polar Lipids (Alabaster, AL), and used without further purification. DOPA or DPPA was chosen for the desired phase of the inner layer. One of PAs was dissolved in 10 ml of *tert*-butyl methyl ether at 10 mg/ml, followed by adding 100 μl DI water of 5 mM pyranine, 50 mM NaCl, and 1 mM $CaCl_2$ at pH 9.0. The micelles with PAs were prepared by extrusion through the 50 nm pores of 78 mm diameter PTFE membranes above the transition temperature of the chosen PA. The micelle diameter was 75 ± 10 nm from measurement with ELS-8000 (Otsuka Electronics Co. Ltd., Osaka, Japan). For the measurement, the viscosity and the refractive index of the *tert*-butyl methyl ether were 0.23 cP and 1.3686, respectively (Lide 2005).

Vesicle Solution Preparation

Several drops (<10 μl) of the micelle solution were added through a 22 gauge needle inserted into the 10 ml aqueous solutions of 50 mM NaCl and 1 mM $CaCl_2$ at pH 5.0, followed by dropping the 10 mg/ml *tert*-butyl methyl ether solution of a desired ratio of DOPC, DOPE, and OHPE (or DPPC, DPPE, and PHPE) continuously. As components for the outer layer, PC and PE were selected because only PC was reacted by PLD. However, if DPPC was replaced with DPPE only, the radius of the vesicles would be increased as a result of the difference in the geometries between DPPC and DPPE. Therefore, PHPE (lyso-PE) was utilized for the vesicle preparation with DPPC and DPPE, so the radius could be kept constant. Because the PLD reaction was based on the change in curvature of the vesicles, it was essential that the radius remained constant. The final lipid concentration of the aqueous solution was 1 mg/ml. During the addition, the aqueous solution was magnetically stirred and exposed only to the nitrogen stream. After the solution was centrifuged ($3{,}700\times g$) to remove the phospholipids that did not form the vesicles, the liposome solution was obtained from the supernatant of the solution. These procedures are well known as a methodology to prepare vesicles (New 1990). The diameter of the vesicles was also measured into 80 ± 10 nm. The change in the diameter from the micelle to the vesicle was consistent with the expectation that it was from the lipid layer that formed on the micelle surface.

Encapsulation Confirmation

In addition to the change in diameter, a lack of leakage of the pyranine molecules indicated that each layer was not disturbed. The encapsulation was confirmed by a fluorometer (Perkin-Elmer, Boston, MA) with 460 nm excitation and 520 nm emission wavelengths. After the addition of pH 3 DI water drops to the vesicle solution, the

fluorescence intensity was not varied. Otherwise, the fluorescence intensity at 510 nm would be changed tremendously when several drops of aqueous solutions of 50 mM NaCl and 1 mM $CaCl_2$ at pH 3.0 were added to the vesicle solution.

Rupture Response Measurement

A total of 1 nM of PLD, purchased from Sigma Aldrich (St. Louis, MO), was selected as a PLD concentration because a concentration of >1 nM of PLD had little effect on the response time for the rupture (Park 2011). Because it is known that the reaction causes a rupture of the vesicles, and because the pyranine (pH-sensitive fluorescence dye) was encapsulated inside the vesicles, the fluorescence intensity was monitored in real time with a Wallac Victor3 multiwell fluorimeter (Perkin-Elmer, Waltham, MA). Because the pyranine molecules have different fluorescence intensities when they are exposed to different pH solutions within the rupture, the tremendous change in the intensity difference between the vesicle solution with the PLD injection and with only the buffer solution injection means that the vesicles rupture. Therefore, the intensity was observed so we could investigate the correlation between the phase asymmetry and the composition for the PLD-induced vesicle rupture. For each ratio of the phospholipids at each phase of the inner layer, we measured when the rupture occurred after the injection of the PLD molecules.

Layer Composition Measurement

The HPLC analyzer consisted of a Gel Silica 60 column (particle size 5 µl; ID 47 mm; length 15 cm; Tosoh Co., Tokyo, Japan) and an HPLC system (Waters Associates, Milford, MA) containing a Type 600 solvent delivery system, a Type U6K injector, a Type 490 variable wave length detector, and a Type 740 data module. Elution was performed with a solution of acetonitrile–methanol–85 % phosphoric acid (130:5:1.7, v/v/v) at a flow rate of 1 ml/min at room temperature. A desired solution was dissolved in 100 µl of methylene chloride, and 10 µl of the solution was injected into the HPLC system. The peak area of each component was compared for the solution before centrifugation and for the supernatant after centrifugation.

Permeability Measurement

Permeability reflects the proton flux across the lipid bilayer of the vesicles. The permeability depends on the phase and the radius of the layer. Several drops of aqueous solutions of 50 mM NaCl and 1 mM $CaCl_2$ at pH 3.0 were added to the pyranine-encapsulated vesicle solution of 50 mM NaCl and 1 mM $CaCl_2$ at pH 5.0. The fluorescence intensity was monitored with respect to time with a fluorometer with 460 nm excitation and 520 nm emission wavelengths.

Results and Discussion

Toward the investigation of the PLD reaction on the lipid layer, the pyranine molecules, dissolved in aqueous solution, were encapsulated inside the vesicles. After the addition of pH 3 DI water drops, the fluorescence intensity was not varied before treatment with Tween 20. With this treatment, the intensity immediately changed (Fig. 1). Therefore, the encapsulation was successfully achieved.

The inner layer was prepared at the desired phase with the phospholipids whose transition temperature was considered, before the outer layer of the vesicle was formed (Park and Ahn 2008). At room temperature, when the PLD reaction was performed, dioleoyl lipids were used for the liquid phase because their transition temperature was much lower than room temperature, whereas dipalmitoyl lipids were used for solids. The phase of each layer was adjusted, and an asymmetric phase of the layers was made. Therefore, there are four types of vesicles prepared for this study (Fig. 2). The ratio of the phospholipids at the outer layer was confirmed with the HPLC system. In Fig. 3, the stock solution was provided before centrifugation, and the liposome solution was from the supernatant. Although the areas for every component after the centrifugation were reduced except PA, the degree of the decrease was proportional. The proportional decrease means that the ratios of the phospholipids at the outer layer were still almost identical with those of the DOPC, DOPE, and OHPE

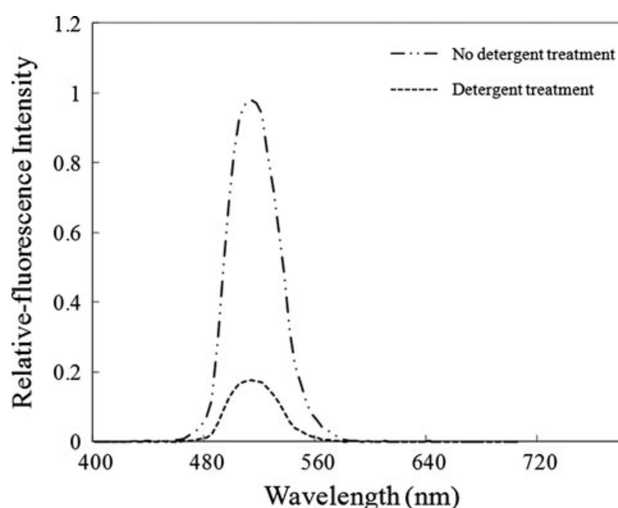

Fig. 1 Fluorescence intensity change after the addition of pH 3 DI water drops

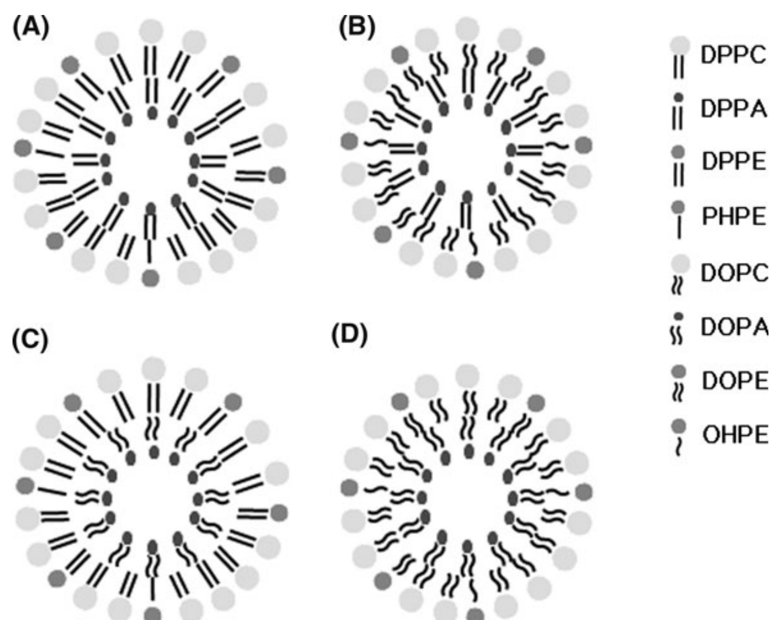

Fig. 2 Schematic diagram for four types of the vesicles made with different components at phase asymmetry lipid layer. **a** DPPA inner layer and DPPC, DPPE, PHPE outer layer. **b** DPPA inner layer and DOPC, DOPE, OHPE outer layer. **c** DOPA inner layer and DPPC, DPPE, PHPE outer layer. **d** DOPA inner layer and DOPC, DOPE, OHPE outer layer

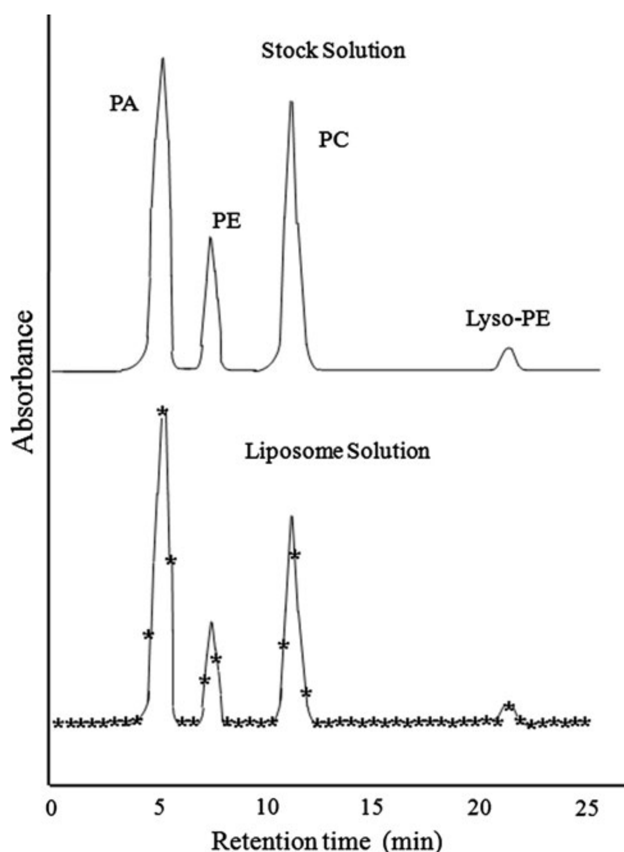

Fig. 3 HPLC peaks for each component. Lipid stock solution before centrifugation (*top*) and supernatant of the solution after centrifugation (*bottom*)

solution added for vesicle preparation. As we expected, the more negative component corresponded to shorter retention as a result of surface interaction with silica spheres whose surfaces had inherently negative dipoles. The retention time for OHPE was longest as a result of the molecular weight. The results for each component were consistent with that published previously (Kurumi et al. 1991; Singleton and Stikeleather 1995; Park 2012a, b). PA peak after the centrifugation varied little after centrifugation. The invariance means that the inner layer made with PAs was not disturbed during the centrifugation. This result is consistent with the confirmation of encapsulation.

Apart from the composition, other factors also influenced the reaction, such as the number of vesicles, PLD concentration, ionic concentration of vesicle solution, vesicle radius, and vesicle stability. Therefore, these factors were determined before conducting experiments for the correlation between the layer phase and the composition of the outer layer. The concentration of the phospholipids and the radius of the vesicles were constant through this experiment, and it is known that 10 mg/ml of the phospholipids correspond to 10^7–10^8 vesicles (Park 2007). Other factors have previously been investigated (Park 2011). At a 1 mg/ml solution of phospholipids, 1–10 nM PLD concentration led to little change in the reactivity. The 50 mM NaCl and 1 mM $CaCl_2$, one of biomimetic conditions, was selected for the ionic concentration because the reactivity was dependent on concentration only at lower concentrations (Brown et al. 2007; Webb et al. 2010; Cheow et al. 2010; Furt and Moreau 2009). The stability was adjusted independently of the composition. Stability is described in the next paragraph because it depended on the composition of the vesicles.

In order to investigate the effect of the outer layer composition on the response of the PLD reaction, the ratio of saturated PE to DPPC was varied. The PLD reaction was

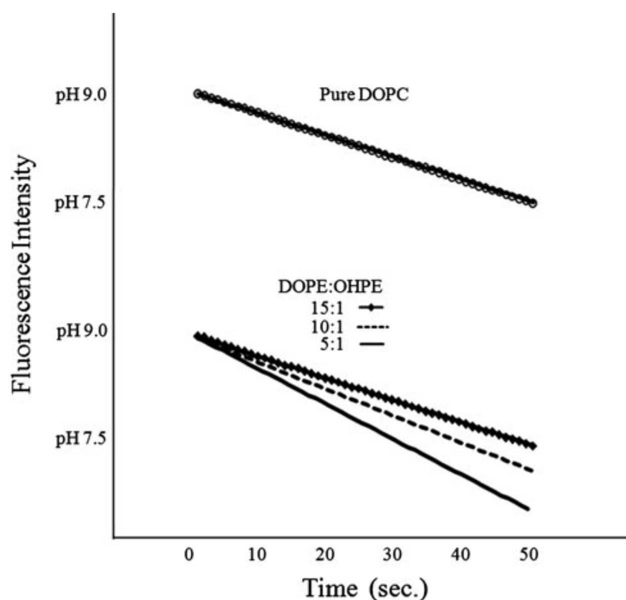

Fig. 4 Fluorescence intensity change with respect to time for the vesicles of the outer layer made with pure DOPC (*top*) and made with mixture of DOPC, DOPE, OHPE at different ratios of DOPE to OHPE (*bottom*)

conducted at 11 conditions (pure saturated PE to pure DPPC by 10 %). However, at each ratio, it was also necessary to determine the ratio of DPPE to PHPE where the radius of the vesicles was kept identical to that of pure DPPC vesicles. Therefore, the composition of the mixed outer layer was adjusted. In order to find out the composition of the identical radius, permeability was observed at 5:1, 10:1, and 15:1 ratios of DPPE to PHPE for each saturated PE condition by measuring the fluorescence intensity change with respect to time (Fig. 4). The ratio of saturated PE to DPPC at a specific ratio of DPPE to PHPE had little effect on the stability. It was found that the stability of the vesicles at a 15:1 ratio of DPPE to PHPE was almost identical with that of the vesicles made with pure DPPC. This result was consistent with the expectation from the geometries of the lipids that the ratios of lipid volume to head group area and lipid length are 0.5–1, 1, and <0.33333 for DPPC, DPPE, and PHPE, respectively (Cevc and Marsh 1987). Similarly, the stability of pure DOPC was identical with that of a 15:1 ratio of DOPE to OHPE. These results are also consistent previous research (Park 2012a, b).

Table 1 Response time (s) from PLD injection (DOPE:OHPE) to vesicle rupture

Phase	Pure, DOPC	90 % DOPC, 10 % PE	80 % DOPC, 20 % PE	70 % DOPC, 30 % PE	60 % DOPC, 40 % PE	50 % DOPC, 50 % PE	40 % DOPC, 60 % PE	30 % DOPC, 70 % PE	20 % DOPC, 80 % PE	10 % DOPC, 90 % PE	Pure, PE
Solid phase of inner layer	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.9	1.3	2.7	50
Liquid phase of inner layer	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.9	1.3	2.7	50

PE consisted of DOPE and OHPE in a 15:1 ratio*PLD* phospholipase D, *DOPE* dioleoylphosphatidylethanolamine, *OHPE* oleoylhydroxyphosphatidylethanolamine, *PE* phosphatidylethanolamine

Table 2 Response time (s) from PLD injection (DPPE:PHPE) to vesicle rupture

Phase	Pure, DPPC	90 % DPPC, 10 % PE	80 % DPPC, 20 % PE	70 % DPPC, 30 % PE	60 % DPPC, 40 % PE	50 % DPPC, 50 % PE	40 % DPPC, 60 % PE	30 % DPPC, 70 % PE	20 % DPPC, 80 % PE	10 % DPPC, 90 % PE	Pure, PE
Solid phase of inner layer	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.8	1.6	More than 2 days
Liquid phase of inner layer	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.5	1.3	~30

PE consisted of DPPE and PHPE at a 15:1 ratio

PLD phospholipase D, *DPPE* dipalmitoylphosphatidylethanolamine, *PHPE* palmitoylhydroxyphosphatidylethanolamine, *PE* phosphatidylethanolamine

Reactivity was monitored at the various composition conditions for each phase of the inner layer using the fluorescence response time. The time of pure DOPC vesicles was consistent with the previous result (Park 2011). Thirty percent PC-to-non-PC condition with a liquid phase of the outer layer led to a gradual increase in the response time (Tables 1 and 2), which means that reactivity was decreased gradually from 30 % PC to a non-PC condition. This result had also been found previously (Park 2012a, b). The reason for the decrease from 30 % may be explained by the PC density required for collision with PLD. According to this hypothesis, the decrease from 20 % for the saturated lipids may also be explained. Interestingly, for the liquid phase of the outer layer, the reactivity was found to be independent of the phase of the inner layer. This result seems to mean that the unsaturated outer layer has invariance in the density upon the phase of the inner layer. At a specific composition with a certain inner layer phase, it was found that the response time was slower for the unsaturated outer layer. This finding appears to be caused by less PC density and higher diffusivity of the liquid phase.

However, for the solid phase of the outer layer, the response times were varied according to the phase of the inner layer. This observation suggests that the density of the saturated outer layer would be changed on the basis of the phase of the inner layer. Besides lipid density, mechanical stability also appears to affect the response time. When we only consider density, the response time of the solid phase of the outer layer would be faster for the solid phase of the inner layer than the liquid phase of the inner layer because both solid layers have a higher density of lipids. However, the response times were slower for the solid phase of the inner layer. These results may be interpreted as related to the stability of the vesicles, which is less for the liquid phase of the inner layer as a result of the mismatches between the hydrophobic tails of layer phase asymmetry.

Conclusion

In this study, the correlation between phase asymmetry and the outer layer composition for the PLD-induced vesicle rupture was investigated by fluorescence intensity change. Before this investigation, the composition of lyso-PE to total PE was found as a condition that the vesicles made with the mixed lipids were as stable as those made with pure PC. The rupture caused by the PLD reaction was analyzed with respect to the phase asymmetry of the layers and the composition of the outer layer. These results were interpreted in terms of the lipid density and layer stability. We observed that the solid phase of the outer layer had a variance in the response time that depended on the phase of the inner layer, whereas the liquid phase did not. Additionally, the response for the solid phase of the outer layer was faster with the liquid phase of the inner layer as a result of its stability.

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