

Technical note

# Influences of ammonia plasma treatment on modifying depth and degradation of poly(L-lactide) scaffolds

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## Abstract

Hydrophobicity of poly(L-lactide) scaffolds is a main drawback in obtaining a sufficient mass of seeded cells for satisfying the requirements of tissue engineering. Plasma treatment is a useful technique to enhance the hydrophilicity of the scaffolds. However, the effect of this technique on the modifying depth and degradation of the scaffolds should be considered. In this paper, the influence of NH<sub>3</sub> plasma treatment on the modifying depth and degradation of scaffolds were investigated. The results showed that the modifying depth of the scaffolds increased with treating time and the plasma power ranging from 20 to 80 W influenced the depth slightly. However, the degradation of the scaffolds increased with increasing treatment time and plasma power. The results also showed that the plasma intruded the scaffolds gradually from top to bottom. For a 4 mm thick scaffold, the optimized treatment condition was 20 W of power in a 30 Pa ammonia atmosphere for 30 min of treating time. Under this condition, the integrity of scaffold could be relatively well kept. NH<sub>3</sub> plasma treatment enabled the penetration of cells into scaffolds and facilitated the proliferation of cells in them.

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## 1. Introduction

One of elementary functions of cell scaffolds of the tissue engineering is to obtain evenly distributed and sufficient seeded cells itself, which was found to be closely correlated with pore size, pore geometry, pore interconnection and hydrophilicity/hydrophobicity of the scaffolds. For example, although poly(lactic acid) (PLA) possesses good biocompatibility, biodegradability and mechanical property, a high hydrophobic polyester—PLA scaffold disables for penetrating of cell suspensions into insides of the scaffold. Furthermore, nutrient supplying, waste removal and ingrowths of cells into the scaffold are also influenced disadvantageously by the hydrophobicity of the scaffold. The bulk modification of the material is one of feasible methods for solving the problem. In our earlier work, a hydrophilic component poly(ethylene glycol) (PEG) had been introduced into PLLA to form poly(L-lactide)-PEG multi-block copolymers with high molecular weight [1,2].

The resulting fabricated three-dimensional scaffolds with appropriate PEG content were in favor of mass transportation and cell proliferation [3]. Another important route for solving the problem is to modify the surface property of the materials. Various methods such as surface coating [4], surface chemical modification [5], plasma treatment [6,7] and hybrid modification [8] and so on were well performed [9]. For example, the anhydrous ammonia plasma treatment can improve not only the surface hydrophilicity, but also the surface morphology of the scaffold. In result, the cell affinity [10,11] and cell adhesion force of the scaffold had been greatly improved by such plasma treatment [12]. However, it had also been found that the plasma treatment condition could influence not only the modifying depth, but also the degradation of the PLA scaffold [13]. So to control the parameters of the plasma treatment becomes important.

In the present work, poly(L-lactide)—a useful but hydrophobic biodegradable polymer was used as model polymer and a series of experiment variations were applied in order to understand the relationship between modifying depth and degradation of the PLLA scaffolds.

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The resulting parameters of the plasma modification could be designed by concerning molecular weight of the polymer and processing method.

## 2. Materials and methods

### 2.1. Materials

L-lactic acid (80%, Shanghai Yierbao, China) was used as purchased. Stannous octoate (Sigma Chemical Co.) as a polymerization catalyst was used as received. Hexadecanol (C.P. grade) was supplied by Beijing Chemical Factory and freeze-dried before use. NaCl granules were sieved in 200–250  $\mu\text{m}$  of size.

### 2.2. Synthesis of PLLA

L-lactide was synthesized from L-lactic acid according to the literature method [2,10]. The resulting lactide was recrystallized three times before polymerization. PLLA ( $M_n = 40,000$ ) was synthesized at 140  $^{\circ}\text{C}$  for 10 h under vacuum in a sealed tube using stannous octoate as catalyst and hexadecanol as molecular weight modulator.

### 2.3. Preparation of scaffolds

Scaffolds were manufactured by an improved solid–liquid separation method. Different amount of sieved NaCl granules was added into 5% (w/v) solution of PLLA in dioxane, then the slurries were maintained at  $-5^{\circ}\text{C}$  for over 2 h to induce the solid–liquid phase separation completely. After the solvent was removed by freeze-drying for 3 days, the matrix was put into distilled water to leach out the NaCl. The distilled water was renewed every 3 h until no chloric ion could be detected by adding drops of  $\text{AgNO}_3$  solution. The dried scaffolds were then kept in a desiccator for characterization and usage.

### 2.4. Measurement of modifying depth of the plasma treatment

According to the previous method [13], the PLLA scaffolds with a similar thickness (4.0 mm) were modified by ammonia plasma treatment under the condition described above. Then put the scaffolds into blue ink (Beijing ink factory, Beijing) immediately for 5 min. The dyed scaffolds were then taken out from the blue ink and the free ink in the scaffolds was removed by a filter paper. After the scaffolds were dried under vacuum, they were broken into two halves via frozen in liquid nitrogen. Finally, the ink dyeing depths were measured by using a micrometer and recorded by taking pictures of the surface and cross section of the scaffolds.

### 2.5. SEM observation of PLLA scaffolds

The plasma-treated PLLA scaffolds were sputter-coated with gold and examined under a scanning electron microscope (Hitachi S-530).

### 2.6. Degradation measurement of the plasma-treated PLLA scaffolds

The PLLA scaffolds with the same thickness (1.0 mm) were modified by ammonia plasma treatment under the condition described above. Then the scaffolds were dissolved in chloroform and determined by gel permeation chromatography (GPC) measurement. The GPC measurement was carried out on a Waters 510 apparatus equipped with Shodex GPC K-800 series columns. Chloroform was used as the eluent at a flow rate of 1.0 ml/min. Calibration of the molecular weight of the copolymer was based on polystyrene standards.

### 2.7. Cell proliferation

Mouse 3T3 fibroblasts were cultured in a 50 ml cell culture flask with Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 0.05 mg/ml ascorbic acid, 0.3 mg/ml L-glutamine (Gibco), 3.7 mg/ml  $\text{NaHCO}_3$  (AR grade, Beijing chemical factory) and 100 units/ml each of penicillin and streptomycin. Cell culture was maintained in a gas-jacket incubator equilibrated with 5%  $\text{CO}_2$  at 37  $^{\circ}\text{C}$ .

### 2.8. Cell proliferation on $\text{NH}_3$ modified scaffolds

The  $\text{NH}_3$  plasma modified scaffolds were pre-wetted by a 75% ethanol aqueous solution for 2 h, and then the ethanol was exchanged with an excess amount of phosphate-buffered saline (PBS). Then the scaffolds were cut in to pieces (1.5  $\times$  3.5 cm) and located in culture dishes (6 cm) and then the culture dishes were transferred to ultra-cleaning workstation for 2 h of ultraviolet lighting. A cell suspension with  $5 \times 10^6$  cell/ml of cell density was seeded evenly into the scaffolds with a micro-pipette. The cell-seeded scaffolds were maintained at 37  $^{\circ}\text{C}$  under 5%  $\text{CO}_2$  for 4 h and then 5 ml of culture medium was added to the culture dishes. The cell-seeded scaffolds were maintained at 37  $^{\circ}\text{C}$  under 5%  $\text{CO}_2$  for 3 h and then 10 ml of new culture medium was added into the culture dishes. After the cells culturing for 2 days, the viability and proliferation of the fibroblast cells was determinate by MTT assay.

In total, 150  $\mu\text{l}$  of fresh culture medium was added to each well after the original culture medium was removed with a macro-pipette. A total of 5  $\mu\text{l}$  of MTT solution (5 mg/ml) was freshly added to culture well incubated at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 4 h. The upper medium was removed carefully and the intracellular formazan was solubilized by adding 2 ml of 0.04 mol/l HCl/iso-propanol to each well. The absorbance of produced formazan was measured at 570 nm with microplate reader (Texcan, Aus). Three disks were applied for each pore size of scaffolds.

## 3. Results and discussion

### 3.1. Modifying depth of the plasma treatment

Dependences of modifying depth of the plasma treatment on treating time and plasma power of the treatment were listed in Table 1. Two features could be easily observed. The first feature was that the plasma modification could not be completed within a short time, and the modifying depths increased with increasing plasma treating time. Under the similar 20 W of plasma power, the modifying depths from less than 0.1 mm for 1 min of treating time increased to about 4 mm for 30 min of the time. The second feature was that the plasma power only has little effect on the modifying depth, and the modifying depth did not significantly increased with increasing the plasma power. Under the similar 10 min of treating time,

Table 1  
The effect of plasma treatment conditions on the depth of PLLA scaffold

Treating power (W)	Modifying depth of scaffolds <sup>a</sup> (mm)					
	1 min	5 min	10 min	20 min	25 min	30 min
20	<0.1	0.2 $\pm$ 0.1	0.8 $\pm$ 0.2	1.1 $\pm$ 0.2	2.0 $\pm$ 0.3	4.0
50	<0.1	0.2 $\pm$ 0.1	0.9 $\pm$ 0.1	1.2 $\pm$ 0.1	2.3 $\pm$ 0.3	4.0
80	<0.1	0.3 $\pm$ 0.1	0.6 $\pm$ 0.2	1.2 $\pm$ 0.2	2.2 $\pm$ 0.1	4.0

<sup>a</sup>Thickness of the scaffolds were 4.0 mm.

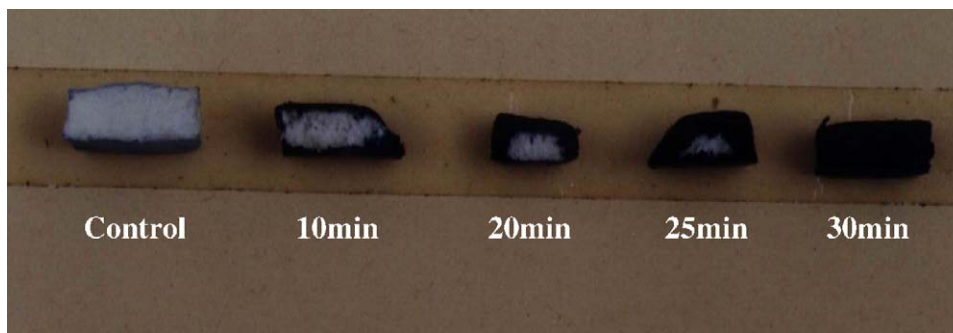


Fig. 1. Effect of plasma treating time on modifying depth of PLLA scaffolds (by 20 W of plasma power).

the modifying depth had contrarily decreased with further increasing the plasma power from 20 and 50 to 80 W.

The two features could be explained as follows. The generation of active species inside of the scaffold was controlled by the interconnection and tortuosity of channels or pores of scaffolds though plasma could be created outside of the scaffolds as soon as the radio frequency source began to work. The energetic species density had increased with the increase of plasma power, but simultaneously the energetic species density could be also decreased under some high power because the chance of annihilation of energetic species had enhanced compared to under a low plasma power. That is to say, the energetic species density had an optimal value under some power.

The inference of plasma treating time on modifying depth measured by using a colored aqueous solution—ink dyeing method as shown in Fig. 1. For the control, the ink could only dyes the most outside layer of the scaffold since the poor hydrophilicity of the internal scaffold. However, after 30 min of plasma treatment the scaffold was completely dyed. The dark color in center of the scaffold indicated that the plasma treatment was completely throughout the whole scaffold. It was clear that the modifying depth increased with plasma treating time.

### 3.2. The degradation measurement of the PLLA scaffolds after exposed to plasma treatment

Both the data in Table 1 and Fig. 1 indicated that the modifying depth increased with plasma treating time. However, it could be found that the degradation of the scaffolds had also happened accompanying with the plasma treatment process. Especially, under some severe plasma treatment conditions, the degradation was not tolerant any more. The SEM observation results of plasma-treated scaffolds were shown in Fig. 2.

It could be seen that the integrity of scaffold was maintained under 20 W of plasma power for 30 min. With the increase of plasma power, severe damage occurred on the surface and inside of the scaffolds. Many large cavities (indicated by arrows) had generated throughout the whole scaffold and the pores become not integrated. It was

considered that because energy of the energetic species increased with increasing plasma power, in result the chemical bond energy exceeded greatly. Furthermore, the heat generated by the high plasma power assisted the chemical chain scission. So the thin walls of pores of the scaffolds were inevitably broken under both the high power and long treating time, and the large cavities generated.

The weight loss of the plasma modified scaffolds (height 1 mm) was shown in Fig. 3. It could be seen that the weight loss percentage of the scaffolds also increased with increasing treating time and plasma power. Under 20 W of plasma power for 30 min, the weight loss of the scaffold was about 5.7%. However, under 80 W of plasma power for 30 min the weight loss of the scaffold increased to 45%. Furthermore, under 80 W the weight loss of the scaffold sharply increased from about 20–45% within 5 min. The data indicated that a high plasma power could not benefit for further enhancing the modifying depth. On the contrary, a high plasma power would lead to severe damage of the scaffold easily compared with low plasma power.

The molecular weight of the PLLA scaffold under various plasma treatment conditions were determined by GPC measurement as shown in Table 2. It was interested to find that the molecular weight of the scaffolds had not decreased with the treating time. The changes of the molecular weight were the Mw decreased firstly and then increased. For example, original Mw of a scaffold was 274,000. After plasma treating under 20W for 20 min the Mw reduced to 188,000, but when the treating time prolonged to 30 min the Mw increased to 206,000. It was considered that under the condition of 20 W for 20 min, the thin walls or small struts of the scaffolds first degraded into lower Mw chain but did not collapse, because of the remained polymer with lower Mw could still keep the morphology structure of the scaffold. But in the treating time was prolonged to 30 min, since the scaffold degraded further and the degradation product with low Mw was pumped away, only higher molecular weighted polymer of the scaffold has remained. In result, both the weight loss and Mw of the remained scaffold were higher than that of the 20 min treated scaffold.

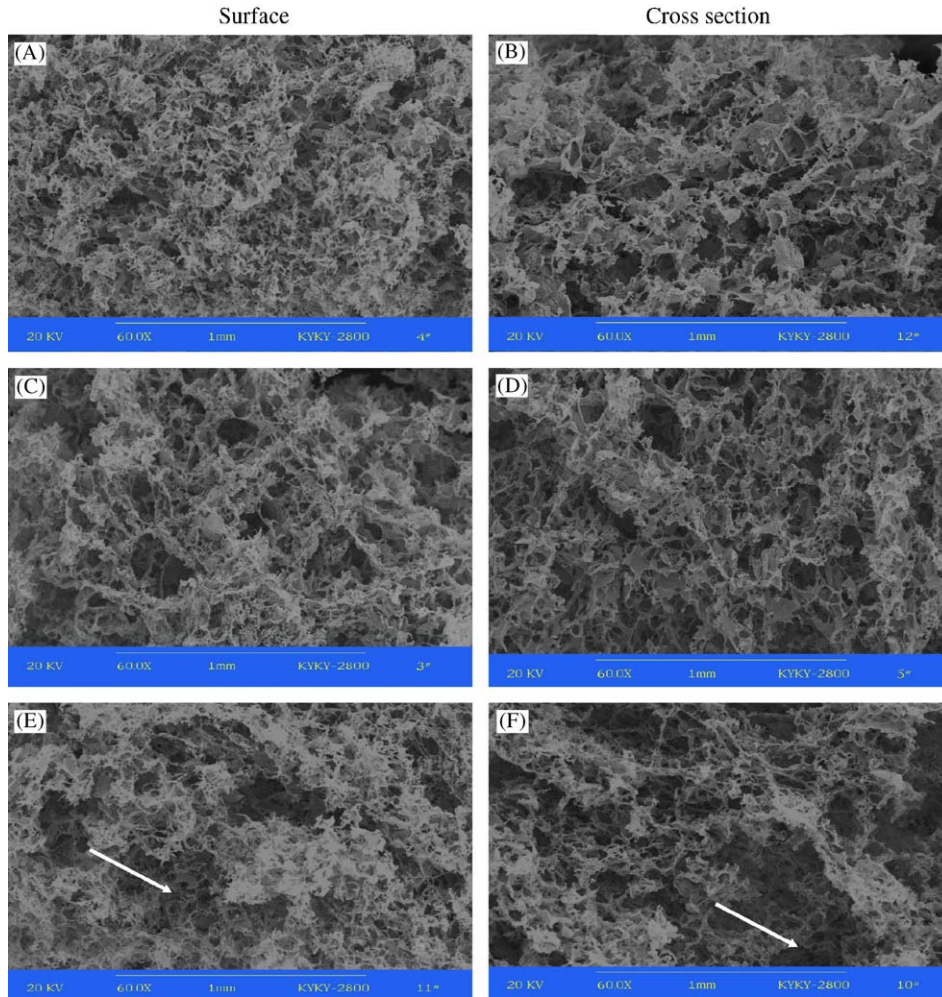


Fig. 2. SEM images of scaffolds after plasma treatment: (A,B) control; (C,D) under 20 W for 30 min; (E,F) under 50 W for 30 min.

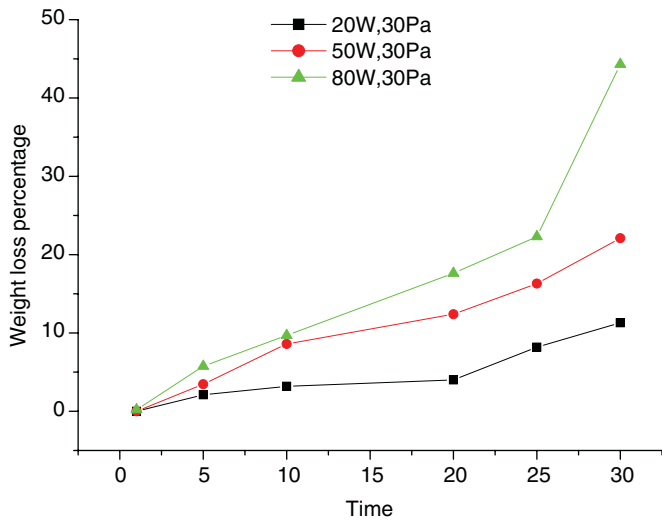


Fig. 3. Effect of NH<sub>3</sub> plasma treatment condition on weight loss of PLLA scaffold.

In order to examine the molecular weights of polymer inside of the scaffold treated by plasma, a scaffold composed of four layers scaffolds (each layer in 1 mm

Table 2  
Effect of NH<sub>3</sub> plasma treatment conditions on molecular weight of PLLA scaffold

Treating time (min)	20 W		50 W		80 W	
	Mw ( $\times 10^{-4}$ ) <sup>a</sup>	Mw/Mn	Mw ( $\times 10^{-4}$ ) <sup>a</sup>	Mw/Mn	Mw ( $\times 10^{-4}$ ) <sup>a</sup>	Mw/Mn
0	27.4	1.26	27.4	1.26	27.4	1.26
10	18.7	1.36	20.4	1.33	19.5	1.29
20	18.8	1.33	18.8	1.38	14.9	1.34
30	20.6	1.30	15.5	1.41	18.3	1.30
40	20.0	1.28	19.4	1.35	— <sup>b</sup>	— <sup>b</sup>
50	20.5	1.30	— <sup>b</sup>	— <sup>b</sup>	— <sup>b</sup>	— <sup>b</sup>

<sup>a</sup>Determined by gel permeation chromatography (GPC) measurement.  
<sup>b</sup>Not measured, because the sample degraded too severe to be collected.

thickness) in layer-by-layer arrangement was designed and restructured to simulate a 4 mm thickness of scaffold. After every plasma treatment, the molecular weights of various layers of the mimetic scaffold were determined and the results were listed in Table 3.

Table 3  
The molecular weight of sequential layer of the mimetic scaffold<sup>a</sup>

Layers	Distance to air surface (mm)	20 W		50 W	
		Mw ( $\times 10^{-4}$ ) <sup>b</sup>	Mw/Mn	Mw ( $\times 10^{-4}$ ) <sup>b</sup>	Mw/Mn
Control		27.4	1.26	27.4	1.26
Layer A	0–1.0	17.7	1.20	14.2	1.39
Layer B	1.0–2.0	25.9	1.38	25.3	1.44
Layer C	2.0–3.0	26.5	1.32	26.8	1.41
Layer D	3.0–4.0	27.3	1.28	27.1	1.33

<sup>a</sup>Treatment time: 30 min.

<sup>b</sup>Determined by gel permeation chromatography (GPC) measurement.

It could be seen that after plasma treatment the Mw of all four layers has reduced, and the order of Mw was  $A < B < C < D$ . But the Mw of the D layer was near same with that of control. On the other hand, it also could be found that when the plasma power enhanced from 20 to 50 W, although degradation of Layer A increased and Mw of the Layer A reduced from  $17.7 \times 10^4$  to  $14.2 \times 10^4$ , the Mw of other layers were nearly similar. It meant that degradation of the scaffold will be accompanied by the plasma modification, but the degradation degree reduced with increasing the distance to air surface, i.e. it reduced with increasing the depth of the scaffold.

### 3.3. Effect of plasma treating depth on cell proliferation of PLLA scaffold

The above mimetic scaffold composed of four layers of 1 mm thickness scaffolds in layer-by-layer arrangement was modified by  $\text{NH}_3$  plasma-treatment under 20 W for 30 min. Due to compare the cell proliferation on different layers of the scaffold, the cell culture test was carried out in the every layer. The PLLA scaffold without plasma treatment was set as control. The results of cell proliferation (Fig. 4) shown that the cells in the D layer grew slowly than in other layers. It was considered that because the layer D was far away from the  $\text{NH}_3$  plasma source and was also exposed to  $\text{NH}_3$  plasma for less time than other layers. In result the number of polar groups introduced onto the layer and hydrophilicity of the layer was lower than that of other layers, and cell affinity of the layer was also worse than that of other layers [14–17]. In another experiment carried out by our group, the data also showed that the number of polar groups introduced onto poly(D,L-lactide-co-glycolide) (PLGA) films and the cell affinity did increase with the ammonia plasma treating time (from 2 to 20 min) (data not shown here). It proved that both effects of modification and degradation of the plasma treatment on different parts of a scaffold are different. So due to obtain a homogeneous plasma-treated scaffold, which with higher modifying depth and lower degradation degree, to use a mild plasma power and a shorter treating time but to treat both sides of the scaffold was suggested.

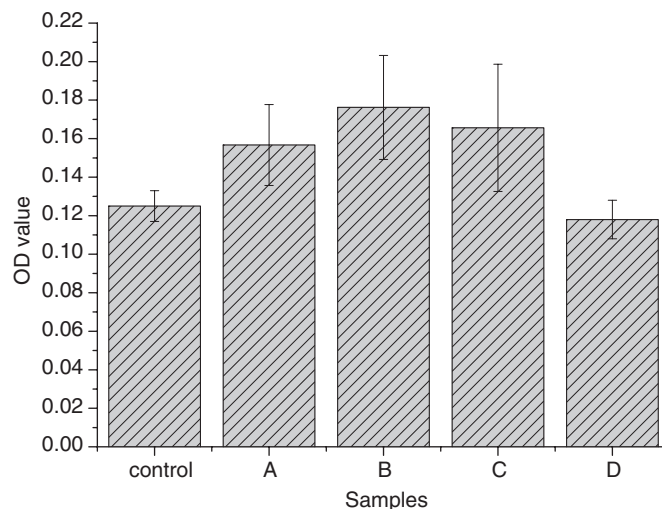


Fig. 4. Dependences of cell proliferation on  $\text{NH}_3$  plasma treating depth of PLLA scaffold (under 20 W for 30 min).

## 4. Conclusions

Plasma treatment can effectively modify surface of a scaffold, but degradation of the scaffold would be accompanied. The effect of  $\text{NH}_3$  plasma on modifying depth and degradation of PLLA scaffold was that, the plasma modifying depth increased with the treating time, but the plasma power ranged from 20 to 80 W influenced the depth slightly. However, the degradation of the scaffolds increased with the increase of the plasma power and treating time. The results also showed that the plasma gradually intruded into the scaffold from top to bottom instead of a rapid process. For 4 mm height scaffold, the optimized condition was 20 W of the plasma power treated for 30 min under 30 Pa of  $\text{NH}_3$  atmosphere. In this condition, the integrity of scaffold structure was relatively kept well. The  $\text{NH}_3$  plasma treatment actually enabled to penetrate into inside of the scaffold and facilitate cell proliferation in the whole scaffolds.

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