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Non-invasive Transdermal Delivery Route Using Electrostatically Interactive Biocompatible Nanocapsules

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The outermost part of the skin, the stratum corneum, impedes the flux of toxins into the body and minimizes water loss, thus providing an excellent barrier function to our body.[1–3] Its barrier properties are due to its unique hierarchical structure mainly consisting of corneocytes and a multilamellar intercellular lipid-based matrix.[4,5] It is known that the diffusivity of low molecular weight active ingredients in the stratum corneum are around 10^{-9} to $10^{-13}\,cm^2 \cdot s^{-1}$, which is lower by orders of magnitude in comparison to the same system in water,[6] thereby precluding any inward flux. Therefore, it is difficult to effectively deliver active ingredients, such as drugs, vitamins, and antioxidants. Moreover, if their molecular weight is more than $\sim 10^3\,g \cdot mol^{-1}$, it is impossible to deliver them to deep skin layers by directly using their diffusion energy. To overcome this and deliver active ingredients into the skin, recent research has focused on using mechanical energy that can change the physical properties of the stratum corneum, ultimately increasing the diffusivity of actives;[7] in final applications, it has been inevitable to use complicated apparatus.

To date, many techniques, including iontophoresis,[8] electoporation,[9] sonophoresis,[10] microneedling,[11] and chemical enhancing,[12,13] have been developed to create improved transdermal delivery systems. However, it is still challenging to develop new techniques that allow us to non-invasively deliver actives with a sufficient concentration through the epidermal layers. Here we show how biologically active molecules can be delivered through the stratum corneum without the aid of any sophisticated delivery devices. The technique that is essential in our approach is that exploiting the electrostatic interaction between skin-compatible nanocapsules and the stratum corneum layer enables disturbance of its well-organized lipid lamellae, thus significantly improving molecular partitioning of the encapsulated actives. As a result, we needed to design and fabricate the utmost suitable capsules.[14,15] In this study, we rationalize our approach by showing how active molecules are slowly released from the capsules and penetrate the epidermis layers, as schematically illustrated in Figure 1a. Finally, we analytically characterize the actual concentration of active molecules and directly image their exact location in the skin.

In a typical procedure, we fabricated skin-compatible nanocapsules by using the in situ precipitation and encapsulation procedure (experimental details are provided in the Supporting Information (SI)). Basically, they consist of poly(D,L-lactide-*co*-glycolide) (PLGA, Resomer RG 504H, molecular weight: $Mw = \sim 5 \times 10^4\,g \cdot mol^{-1}$) and an active ingredient, Single EX Genistein 90. To provide PLGA with both interaction and colloidal stabilization sites, we incorporated a partially quaternizable amine group to one of PLGA chain ends with controllable concentrations, as shown in Figure 1b (see also Fig. S1, SI). We observed that incorporation of the amine group into PLGA chains does not change its biocompatibility (Fig. S2, SI). An important feature of using this amine-functionalized PLGA is that we are able to fabricate capsules with nanometer-length

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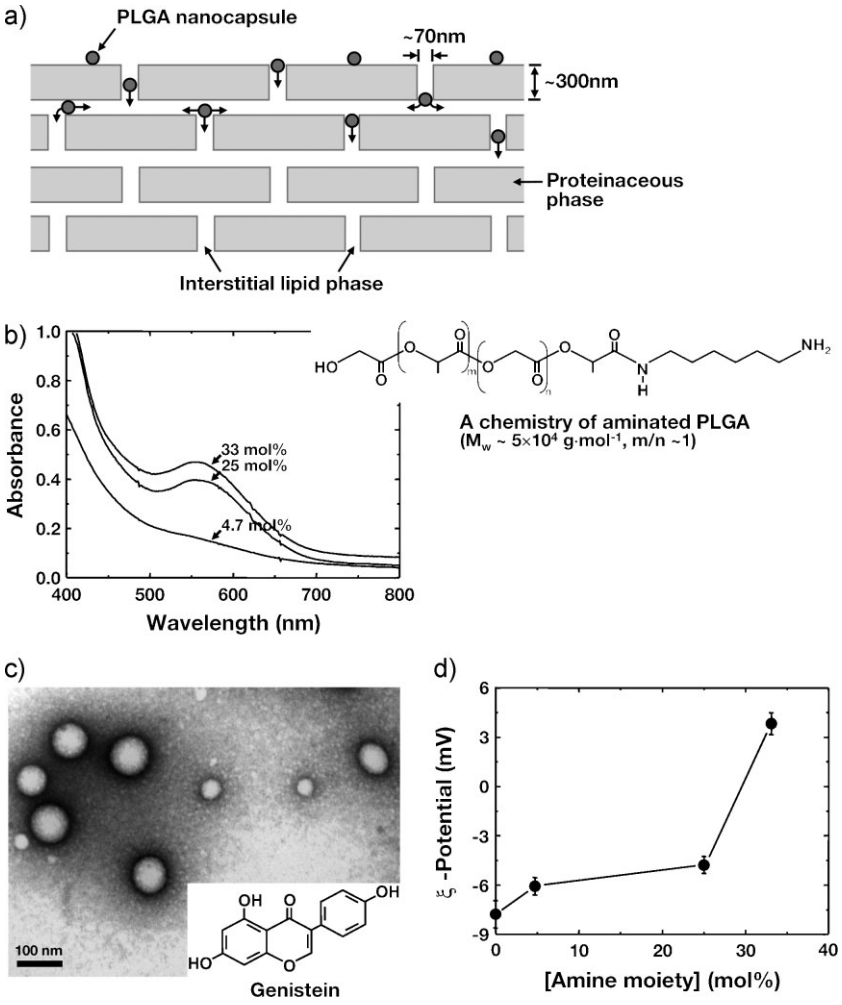

Figure 1. a) A schematic for drug diffusion through the interstitial lipid phase, based on the model of bricks-and-mortars. b) A quantitative analysis of the amine groups incorporated in poly(D,L-lactide-*co*-glycolide) (PLGA). c) A transmission electron microscopy image for the PLGA nanocapsules containing ~8.8 wt% genistein. This PLGA contained 33 mol% amine groups. d) Surface charges of genistein-loaded PLGA nanocapsules in a pH 5 buffer solution.

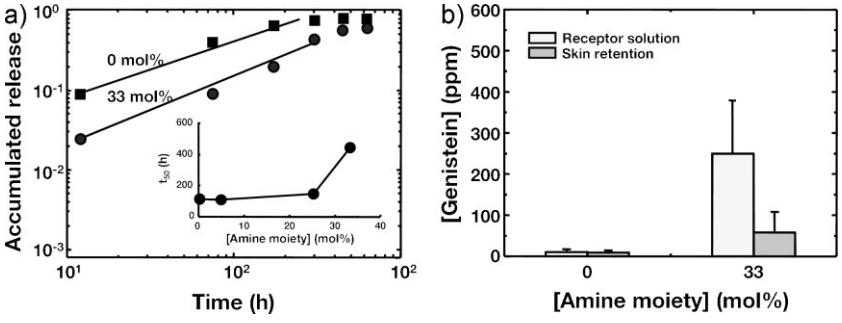

Figure 2. a) Accumulated release of genistein from PLGA nanocapsules. The inset is the shelf releasing time of genistein, t_{50}, with the amine moiety in PLGA. We defined t_{50} as the time for the genistein to release to 50% of the initial concentration. b) A quantified analysis of the penetration of genistein encapsulated in PLGA nanocapsules through the skin by Franz-type diffusion cells.

scales as well as different surface charges; on dropping the acetone solution containing genistein and PLGA into the aqueous solution with a surfactant, Tween 60, partially ionized hydroxyl groups of genistein interact with quaternized amine groups of PLGA,[16] separating the conjugating molecular species to generate particles with nanometer-length scales (Fig. 1c). We should point out that in this process our PLGA polymers form the matrix while encapsulating genistein therein; the nanocapsules are eventually covered by the PLGA phase, which was determined by analyzing their surface charges; from amine concentration of ~30 mol%, the zeta-potential value becomes positive, as shown in Figure 1d. We obtained a stable dispersion of nanocapsules even after complete removal of Tween 60, due to the charge–charge repulsion between the positive charges covering the capsules. We also confirmed that this fabrication method is widely applicable to other active ingredients and matrix materials with similar chemistry (Fig. S3, SI).

Our nanocapsules have the ability to easily load active ingredients in extremely small length scales. We determined that the capsules can trap the encapsulates well and controllably release them. To experimentally demonstrate this, we directly measured the release amount of genistein out of the nanocapsules as a function of storage time (Fig. 2a). We observed that genistein molecules, tightly trapped in the complex PLGA matrix, are released in an exponential manner, which is typical of diffusion-controlled behavior.[17,18] It is noticeable that the concetration of amine groups in PLGA determines the speeed of genistein release; this indicates that more molecular interactions result in less release, which is due to the reduced dissociation of genistein from the PLGA matrix. It is indeed advantageous to structure nanocapules that have nanometer sizes and positive charges for generating the electrostatic interaction with the stratum corneum layer, whose overall net charge is negative.[19,20] We confirmed this by measuring in vitro skin penetration, as shown in Figure 2b; it was a surprise to observe that our nanocapules dramatically increase skin penetration of genistein molecules, thus allowing us to overcome the barrier function of the stratum corneum.

To further characterize how our nanocapsules deliver active molecules through the skin, we imaged the exact locations of both probe molecules and capsule matrix materials. For this, we fabricated a model nanocapsule probe consisting of a molecular probe and a

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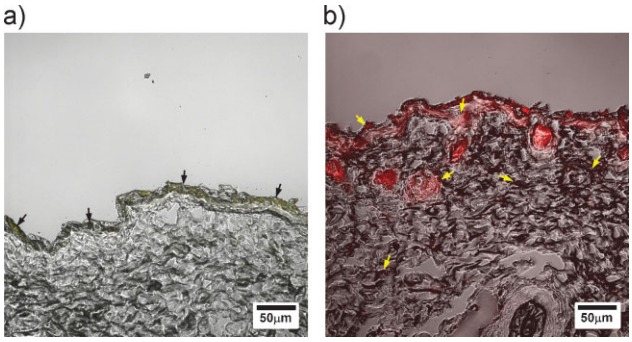

Figure 3. Distribution of probe molecules in the skin. For this, we fabricated model nanocapsules that encapsulate nile red molecules with the quaternized acryl copolymers tagged with lucifer yellow (lucifer yellow ethylenediamine). The concentration of nile red was set to 0.05 wt%. a) Detection of lucifer yellow in the skin. b) Detection of nile red in the skin. Arrows indicate the location of probe molecules.

conjugatable polymer; nile red was encapsulated with a quaternized acryl polymer, whose molecular weight is $\sim 10^5\,g\cdot mol^{-1}$. This polymer was also covalently tagged with lucifer yellow (an ethylenediamine derivative) by using an esterification reation.[21] In this case, we used the quaternized polymer instead of the aminated PLGA, which does not have high enough tagging sites. The diameter and zeta-ptential of these nanocapsules were ~100 nm and ~10 mV, respectively, which means that their surface characteristics were almost identical to those of our PLGA nanocpasules. Then, we treated the dorsal area of an Albino Hartley guinea pig with the nanocapsules and imaged their fluorescence with a confocal laser scanning microscope (Fig. 3). Surprisingly, we found that the capsule matrix stays in the stratum corneum layer and only nile red deeply permeates into the skin. To quantitatively chararcterize the diffusion amount of genistein into the skin, we selectively stripped out the stratum cormeum of the test skin by using the tape method (see SI).[22,23] By using the nanocapsules with positive charges, we detected more than $10\,\mu g\cdot cm^{-2}$ of actives in the stratum-corneum-stripped epidermis layer. In contrast, the nanocapsules with negative charges did not even deliver a small amount of actives to either the stratum corneum or the stripped epidermis. The importance of these observations implies that the generation of such strong interactions between the nanoparticles and stratum corneum, due to the charge effect, enables disturbance of the assembled lipid lamellar layers, thus favoring the diffusion of active molecules dissociating from the complex PLGA chains.

To evaluate the practical efficacy of the capsulated active ingredients in the skin, we monitored their preventive effects on UV-induced skin damage[24–26] in hairless mice. It is known that genistein, a sort of isoflavone found in soybean, possesses both tyrosine kinase inhibitory and antioxidant activities.[27] Previously, it has been reported that topical application of genistein prevents UV-induced skin aging in human skin in vivo;[24] however, since genistein is an unstable compound, it is easily oxidized losing its biological activity. Analyzing H&E (hematoxylin and eosin) stained sections, as shown in Figure 4, we found that UV irradiation not only remarkably increases skin thickness by $190 \pm 20\%$, comparable with vehicle-treated skin, but also induces the inflammatory cell infiltration of vehicle-treated skin from 1.7 ± 0.1 to 3.4 ± 0.3 within 48 h. This UV-induced skin thickening and inflammatory cell infiltration can be prevented by topically applying both fresh genistein and capsulated genistein; however, after a 2-week aging of genistein and its nanocapsules, only capsulated genistein successfully prevented both UV-induced skin thickening and inflammatory cell infiltration. In the experiments using TUNEL staining, we also found that only capsulated genistein prevented UV-induced apoptosis in hairless mice for a long time (see Fig. S4 and S5, SI). These in vivo test results clearly demonstrate that our carrier system enables maintenance of the efficacy of encapsulated active ingredients, while expressing their unique biological functions when topically applied to the skin.

In summary, this paper describes a robust means of fabricating skin-penetrating and compatible nanocarriers that have the ability to load biologically active ingredients and selectively release them through the epidermis lipid layer. The key to our fabrication approach is to use the molecular interaction between the active ingredients and polymer matrix, thus creating capsules with nanometer length scales as well as positive surface charges. Our nanocapsules, whose periphery is covered by positive surface charges, show an ability to disturb the tight lamellar layer of stratum corneum, which eventually enables better diffusion of encapsulated active molecules through the skin. The major advantages of using these types of nanocapsules are that we can not only manipulate the capsule properties, such as surface charges and its release of active ingredients, but also topically deliver them through the skin epidermis without any deterioration of the skin tissues. These characteristics highlight the robustness and versatility of our physicochemical approach, which could be used to develop novel and practical drug-carriers for applications in transdermal deliveries. Considering these advantages, we are now trying to develop a more extended technique that allows us to deliver hydrophilic macromolecules, which could be a significant scientific development in the field of transdermal delivery.

Experimental

Synthesis of Aminated PLGA and Fabrication of Genistein-Loaded Nanocapsules: The carboxylic acid terminal group of PLGA was replaced with a primary amine group, thus introducing an amine moiety to one of chain ends (Fig. S1, SI). In this study, we were able to incorporate amine group to more than 50 mol%. We loaded genistein in the PLGA nanocapsules by using the precipitation method. For this, we first dissolved 0.5 g of PLGA and 0.05 g of genistein in 30 mL of acetone. Then, this solution was dropped through a syringe into 60 mL of distilled deionized water that contains 0.22 wt% of Tween 60, which results in a fine dispersion of nanoparticles. We used Tween 60 to provide sufficient dispersion stability to the nanocapsules, while they separate to form nanoparticles in the continuous phase. After mixing the nanoparticle dispersion for 30 min, we repeatedly washed the nanocapsules with distilled deionized water using a centrifuge, which allowed us to completely remove the surfactant and impurities.

Ninhydrin Assay: To quantitatively characterize the content of amine groups in the aminated PLGA, we carried out the ninhydrin assay. First, we dissolved the ninhydrin reagent consisting of ninhydrin (0.8 g) and hydrindantin (0.12 g) in a solution made of lithium acetate buffer (pH 5.5, 10 mL), and dimethyl sulfoxide (30 mL). Then, we added this ninhydrin reagent (1 mL) to the tetrahydrofuran solution (1 mL) containing the

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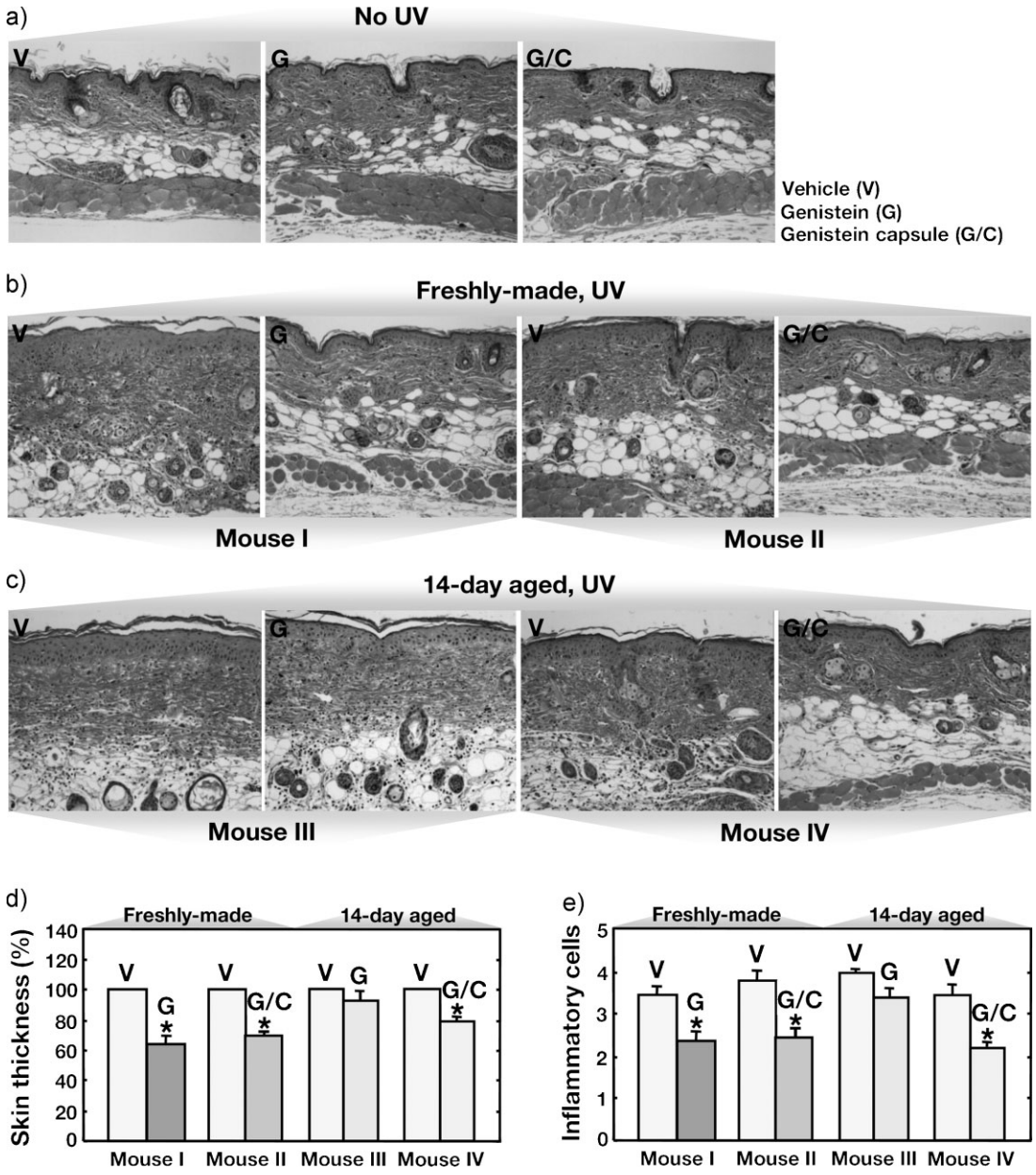

Figure 4. Photoprotective effects of genistein-loaded nanocapsules. a) The skins treated with a freshly made vehicle (V), a plain genistein formulation (G), and a genistein-loaded nanocapsule formulation (G/C). These samples were not irradiated with UV light. b) The skins treated with V, G, and G/C after UV irradiation. c) These skins were also treated with V, G, and G/C after UV irradiation, but in this case, we aged the samples for 14 days at room temperature before applying them to the skins. All the nanocapsules were made of PLGA with 33 mol% amine groups. d) The average skin thickness, which is from outer surface of epidermis to the lower end of subcutaneous fat layer. e) Inflammatory cell infiltration assessed at least three times as follows: 0 = no infiltration, 1 = sporadic, 2 = minimal, 3 = moderate, 4 = severe, and 5 = very severe infiltration. A **p*-value of < 0.05 was considered to be statistically significant.

aminated PLGA sample (0.1 g). To complete the reaction, we heated the mixture to 100 °C for 30 min. Finally we characterized the reaction of the ninhydrin reagent with the amine groups by detecting the absorbance spectra centered at 570 nm with an UV–vis spectrophotometer.

Visualization of Nanocapsules in the Skin: To visualize the nanocapsules in the skin, in this study, we used model polymer nanocapsules, in which quaternized acryl polymers were tagged with fluorescence probes; we synthesized methylmethacrylate/2-(methacryloyloxy) ethyl trimethyl ammonium chloride (METAC) copolymers (copolymerization ratio of methylmethacrylate to METAC was 95:5 by volume) and hydrolyzed the ester groups of the polymethylmethacrylate (PMMA) in the solution of NaOH and methanol (1/1, v/v), thus producing acrylic acid groups. Then, the acrylic acid groups were labelled with lucifer yellow ethylenediamine by using the amidation reaction. The molecular weight of these polymers was controlled to approximately 10^5 g · mol^{-1}, which is almost comparable to that of the aminated PLGA. Using these copolymers, we encapsulated ~0.05 wt% of nile red molecules. Then, the dorsal area of an Albino Hartley guinea pig treated with the dispersion of the nanocapsules for 20 h was biopsied in cold potassium buffered saline (PBS) and embedded in a cutting compound. Finally, the cross-sectional skin, whose thickness was ~10 μm, was imaged with a confocal laser scanning microscope.

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In vitro Skin Penetration Test: To exactly estimate how the nanocapsules help the active molecules penetrate the skin, we measured the concentration of active molecules that penetrated through an Albino Hartley guinea pig skin, in which we used in-vitro Franz-type diffusion cells (Lab Fine Instrument, Korea). For this, we prepared the donor solution filled with either active ingredient (either genistein) only or active ingredient-loaded nanocapsules. The concentration of active ingredients in the donor solution was adjusted to 559 ppm in all cases. Then, at a given time, we collected the receptor solution and measured the concentration of active ingredients therein by using the HPLC.

In vivo Photoprotection Tests: Photoprotective effects were observed by measuring the changes of UV-induced skin thickening and inflammatory cell infiltration. For UV radiation, F75/85W/UV21 fluorescent sun lamps, having an emission spectrum between 275 and 380 nm (peak at 310–315 nm), served as the UV source. A Kodacel filter (TA401/407; Kodak, Rochester, NY) was mounted 2 cm in front of the UV tube to remove wavelengths of less than 290 nm (UVC). Irradiation intensity at the mouse skin surface was measured using a UV meter (model 585100; Waldmann Co.). The irradiation intensity 30 cm from the light source was $1.0\,mW \cdot cm^{-2}$. For this experiment, we first prepared a vehicle (V) formulation consisting of 70 vol% ethanol and 30 vol% propylene glycol, and then formulated genistein (0.1 wt%, G) and genistein nanocapsules (net 0.1 wt% genistein, PLGA with 33 mol% amine moieties, C/G) in the vehicle solutions. Then, these three formulations were pretreated on either the right or left half of the dorsal back skin of hairless mice (four mice per group) for 48 h before UV irradiation ($200\,mJ \cdot cm^{-2}$) and post-treated for another 48 h after UV irradiation. After this treatment process, we stained the skin samples with H&E. Apoptotic cells were detected by the TUNEL method, in which we used the ApopTag in situ apoptotic detection kit (Chemicon). Then we determine the number of apoptotic TUNEL positive cells per field.

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