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Vibrational characterization of pheomelanin and trichochrome F by Raman spectroscopy

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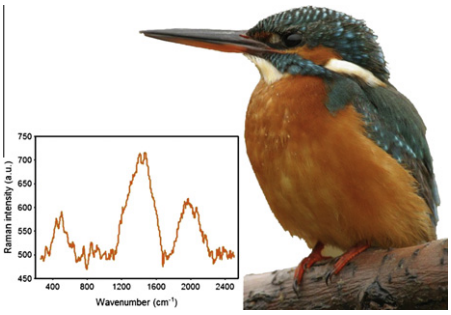
HIGHLIGHTS

- We characterize for the first time the Raman spectrum of pheomelanin.
- The Raman spectra of pheomelanin differ from those of eumelanin and trichochrome F.
- We found Raman signal in feathers and hairs without the need of isolating the pigment.
- Raman spectroscopy represents a non-invasive tool to detect and distinguish melanins.

GRAPHICAL ABSTRACT



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ABSTRACT

We characterize for the first time the vibrational state of natural pheomelanin using Raman spectroscopy and model pigment synthesized from 5-S-cysteinyldopa. The shape of the Raman spectrum was very different from that of eumelanin. Four Raman bands were visible in the 500–2000 cm^{-1} wavenumber region about 500, 1150, 1490 and 2000 cm^{-1}, which we assigned to the out-of-plane deformation and the stretching vibration of the phenyl rings, to the stretching vibration of C–N bonds or the stretching and wagging vibration of CH_2, and to overtone or combination bands. Interestingly, we also show that the Raman spectrum of synthetic trichochrome F, a pigment that may be produced along with pheomelanin during pheomelanogenesis, is different from that of pheomelanin and similar to the spectrum of eumelanin. We could detect Raman signal of both eumelanin and pheomelanin in feathers and hairs where both pigments simultaneously occur without the need of isolating the pigment. This indicates that Raman spectroscopy represents a non-invasive method to detect pheomelanin and distinguish it from other pigments. This may be especially relevant to detect pheomelanin in animal skin including humans, where it has been associated with animal appearance and classification, human phototypes, prevention of skin diseases and cancer risk.

Introduction

Vertebrate animals synthesize the pigment melanins in two chemical forms: eumelanin, a polymer of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxilic acid (DHICA) units, and pheomelanin, a polymer of benzothiazine derivatives [1]. Although most natural melanins are mixed polymers of both pigments, there is considerably more knowledge on the structure and physical properties of eumelanin than on those of pheomelanin, as the former has taken up most research on melanins in the last decades [2,3]. This is exemplified by the optical properties of eumelanin, which have been investigated with a greater diversity of spectroscopic and imaging techniques e.g., [4–6] than the optical

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properties of pheomelanin (see, however, [7,8]), maybe due to the potential applications of eumelanin as a bioelectronic material [5]. Some optical properties of melanins are necessary to determine their ultrastructure [3] and functionality [9], and others allow distinguishing them from other chromophores in natural pigmented tissues [4,10]. Given the phototoxicity and the capacity to increase cancer risk of pheomelanin [11,12], together with recently proposed adaptive functions of this pigment in removing excess cysteine [13], a deeper knowledge of the optical properties of pheomelanin appears to be of paramount importance.

Optical measurements of natural melanins are largely based on their absorption properties (e.g., [10,14,15]), but this has several limitations because melanins have no distinctive absorption peaks that allow distinguishing them from other pigments. Toral et al. [10] made discriminant analyses that allowed the separation of melanins and carotenoids in feathers based on the shape of their reflectance spectra, but also highlighted the difficulty in distinguishing between pheomelanin and other pigments with similar reflectance profiles such as iron oxide and porphyrins. Although absorption properties of eumelanin and pheomelanin pigments have similar spectra, absorption decreasing progressively with wavelength from 300 to 1100 nm [16], Ozeki et al. [17] pointed out that commonly used spectrophotometric methods for melanin determination measure the color intensity of melanin but not the absolute amount of melanin. The absorbance at 500 nm (A_{500}) of a solution of melanins, Soluene-350 and water can be used to qualify the total combined amount of eu- and pheomelanins, and the ratio A_{650}/A_{500} is also useful in estimating the eumelanin/total melanin ratio in hair from different species. Thus, the spectrophotometric method provides a convenient way to qualitatively (not quantitatively) characterize eu- and pheomelanins produced in follicular melanocytes, but the A_{650}/A_{500} ratios are not suitable for detecting subtle differences in the chemical composition of eumelanins in hair [17]. Furthermore, spectrophotometric methods require chemical treatments of samples (e.g., with Soluene-350, [17]), which implies costs and destroying at least part of the samples. This requires that alternatives to absorption measurements are used for detection of melanins. Raman spectroscopy, which measures the change in energy of an irradiating laser photon that is scattered from a sample molecule, may constitute a solution. This is because, when a molecule is excited from a ground state to a virtual energy state, the light is scattered inelastically and the wavelength shift is specific to particular bonds in the molecule. For a molecule to exhibit a Raman effect, it is necessary that a change in the polarization potential is produced.

The vibrational characterization of eumelanin through Raman spectroscopy has been made in different studies [4,18–20], showing that its most outstanding features are two bands at about 1380 and 1580 cm^{-1} similar to D and G bands, respectively, observed in the Raman spectrum of disordered graphite (the Raman spectrum of black fungal pigments may exhibit an additional band about 1250 cm^{-1} because it is synthesized from the polymerization of phenols instead of 5,6-dihydroxyindole as in animals; [20]). These bands have thus been attributed to the stretching vibration of the hexagonal carbon rings, the stretching vibration of the C–C bonds within the rings and the C–H vibrations in the methyl and methylene groups in the eumelanin polymers [4]. This represents a non-invasive method to characterize, and may also be used to quantify, eumelanin present in organic and inorganic materials. By contrast, the Raman spectrum of pheomelanin has never been characterized, maybe because of the difficulty to avoid damage to melanins under Raman excitation if the instrumentation requires high illumination power density and long data acquisition times [4,21]. If anything, previous papers tangential to this topic are those by Bilińska [22], who reported the infrared spectrum of melanins isolated from human hair containing eu- and pheomelanin, and by Minitha et al. [23], who obtained the Raman spectrum of a phenothiazine, a compound similar to the pheomelanin units but with two benzene rings instead of one as benzothiazines have. As eumelanin and pheomelanin are found mixed in most biological tissues [3], the application of Raman spectroscopy as a non-invasive method to detect melanins would be only completely useful if the Raman spectra of both pigments are known. This would be especially relevant for tissues such as skin, where the presence of pheomelanin is known to increase, together with other genetic factors, cancer risk [11,12,24,25]. Here we show for the first time that pheomelanin exhibits a specific Raman spectrum that can be obtained without the need of extracting the pigment from the biological tissues where it is embedded.

Material and methods

We first searched for the Raman signal of synthetic pheomelanin. For this, we analyzed pheomelanin derived from 5-S-cysteinyldopa (5SCD), the most abundant cysteinyldopa isomer that is formed when cysteine is added to dopaquinone, i.e. the first step of the pheomelanogenesis pathway [3]. During the process, 5SCD undergoes redox exchange with o-quinones leading to different benzothiazine species, which are oxidized to pheomelanins [3]. Pheomelanin was synthesized from DOPA (3,4-dihydroxyphenylalanine) and cysteine using tyrosinase as a natural oxidant following Wakamatsu et al. [26].

It is necessary to determine, however, if the Raman spectrum is actually caused by the molecular vibration of pheomelanin or by that of trichochromes, as small amounts of these pigments are formed by oxidation of cysteinyldopas during pheomelanogenesis [3,27,28]. Trichochromes are defined dimers with a benzothiazine moiety, while pheomelanin is an ill-defined polymer mostly formed by benzothiazine and benzothiazole moieties [28,29]. Therefore, we used Raman spectroscopy to determine the vibrational characterization of trichochrome F. We used trichochrome F synthesized according to Napolitano et al. [29].

A surface of around 0.5 cm^2 of pheomelanin or trichochrome F powder was analyzed in a Thermo Fisher DXR Raman microscope operating in the Museo Nacional de Ciencias Naturales (MNCN-CSIC, Madrid, Spain) with a point-and-shoot Raman capability of 1 μm spatial resolution and using an excitation laser source at 780 nm of 20 mW power. The single spectra were obtained using a 100× confocal objective, a slit aperture of 50 μm and a grating of 400 lines/mm. These conditions produced an average spectral resolution of 2.2–4.4 cm^{-1} in the wavenumber range of 100–2500 cm^{-1}. An integration time of 3 s × 48 accumulations allowed getting an acceptable S/N ratio and a photobleaching time of 30 s. The system was operated with OMNIC 8.1 software. Calibration and aligning of the spectrograph were checked using pure polystyrene.

Once characterized the Raman spectrum of synthetic pheomelanin, we determined if it can also be detected *in vivo* without isolating the pigment from the issues where it is embedded. With this aim, we analyzed feathers and hairs of different species with Raman spectroscopy. We analyzed orange breast feathers of domestic chicken *Gallus gallus domesticus* and orange flank feathers of zebra finches *Taeniopygia guttata*, as degradative analyses have shown that the color of these feathers is due to the presence of pheomelanin [28,30]. We also analyzed dorsal feathers of red-legged partridges *Alectoris rufa*, where degradative analyses have also shown the presence of pheomelanin (own unpublished data). Additionally, we analyzed hairs of wild boars *Sus scrofa*, where the presence of pheomelanin has chemically been shown [31]. Lastly, we analyzed red human hairs in which relatively high contents of pheomelanin have been reported from degradative

analyses [32]. As control, we analyzed black belly feathers of zebra finches and black hairs of humans and wild boars, which have relatively high contents of eumelanin [30–32], as well as dorsal feathers of red-legged partridges which contain both eu- and pheomelanin (own unpublished data). Samples from chicken, zebra finches and red-legged partridges were obtained from adult specimens kept in captivity at *Dehesa Galiana* experimental facility (Instituto de Investigación en Recursos Cinegéticos-CSIC-UCLM-JCCM, Ciudad Real, Spain). Wild boar hairs were taken from wild specimens shot in Doñana National Park, southwestern Spain, as part of a larger study on the epidemiology of tuberculosis in ungulates [31]. The red human hairs were collected from an American female (19 year old, [32]), and the black human hairs were collected from a Colombian female (19 year old). The Raman beam was focused at the barbs and barbules of feathers, to the basal (brownish, pheomelanin) and distal (black, eumelanin) portions of wild boar hairs, and to random portions of human hairs.

Results and discussion

We succeeded in obtaining Raman signal from the synthetic pheomelanin, with defined bands in the wavenumber range of 500–2000 cm^{-1}. The shape of the Raman spectrum of pheomelanin is very different to that of eumelanin [4,18,19], showing three 'humps' (Fig. 1). Four Raman bands were found at about 500 cm^{-1} (from four Raman beams at different points of the pheomelanin powder: 501, 501, 507 and 694 cm^{-1}), about 1150 cm^{-1} (1141, 1147, 1147 and 1159 cm^{-1}), about 1490 cm^{-1} (1482, 1482, 1488 and 1488 cm^{-1}) and about 2000 cm^{-1} (1940, 1952, 2011 and 2040 cm^{-1}) (Fig. 1). The band about 500 cm^{-1} is close to the band found at 521 cm^{-1} in a synthetic phenothiazine by Minitha et al. [23], and also to the band at 518 cm^{-1} found by Centeno and Shamir [19] in eumelanin extracted from cuttlefish *Sepia officinalis*. These authors assigned these bands to the out-of-plane deformation of the phenyl rings, which occurs at this wavenumber range. Pheomelanin, like benzothiazine and eumelanin, has phenyl rings in its structure [3], so the band found here about 500 cm^{-1} is probably due to the out-of-plane vibration of the aromatic rings too. We consider that the record of 694 cm^{-1} in one of our four measurements (Fig. 1) was due to noise caused by dust in the sample.

The band at about 1150 cm^{-1} may be due to the stretching vibration of the C–N bond or to the stretching and wagging vibration of CH_2 (methylen groups) as assigned by Minitha et al. [23] to the band at 1159 cm^{-1} of phenothiazine, which coincides with one of our measurements of pheomelanin. Alternatively, this band may be the same that was found by Centeno and Shamir [19] in eumelanin at 1135 cm^{-1} and assigned to the in-plane deformation of the N–H bond in the pyrrole ring or to ring breathing. However, pheomelanin has N–H bonds in its benzothiazine units but not as part of aromatic rings like eumelanin [3], so it is not likely that the band found here at about 1150 cm^{-1} is due to the vibration of the N–H bond.

The third Raman band of pheomelanin was found at about 1490 cm^{-1}, which may be due to the stretching vibration of the phenyl ring as this band should occur theoretically at 1497 cm^{-1} [33]. Minitha et al. [23] found a similar band in phenothiazine at a slightly longer wavenumber (1501 cm^{-1}). Lastly, the fourth Raman band of pheomelanin corresponds to a zone of high Raman intensity around 2000 cm^{-1}. The latter is probably connected with overtone or combination bands that occur at this region (e.g., [34]). It is important to note that the high Raman intensity in the region around 1750–2500 cm^{-1} (Fig. 1) is very charactistic of pheomelanin, as the Raman spectrum of eumelanin is flat at this region ([4,18,19] and see below).

We found intense Raman signal in the powder sample of the synthetic trichochrome F in the wavenumber range of 1000–1700 cm^{-1}. Surprisingly, the Raman spectrum of trichochrome F resembled the Raman spectrum of eumelanin and not that of pheomelanin (Fig. 2). Indeed, the Raman spectrum of trichochrome F showed the D and G bands characteristic of eumelanin and disordered graphite [4,18,19] and absence of signal in the 1750–2500 cm^{-1} region. From four measurements at different sites of the trichochrome powder sample, we detected the D band at 1323, 1323, 1337 and 1360 cm^{-1}, and the G band at 1480, 1494, 1502 and 1502 cm^{-1} (Fig. 2). Therefore, the band assignments should be the same as for eumelanin: the stretching of the hexagonal carbon rings (G band) and the stretching of three of the six C–C bonds within the rings (D band; [4]). However, there is a difference, in quantitative terms, between the Raman spectra of eumelanin and trichochrome F: while in the former the Raman intensity of the D band seems to be always lower than [4,18], or similar to [19] the intensity of the G band, the Raman intensity of the D band of trichochrome F is considerably greater than the intensity of the

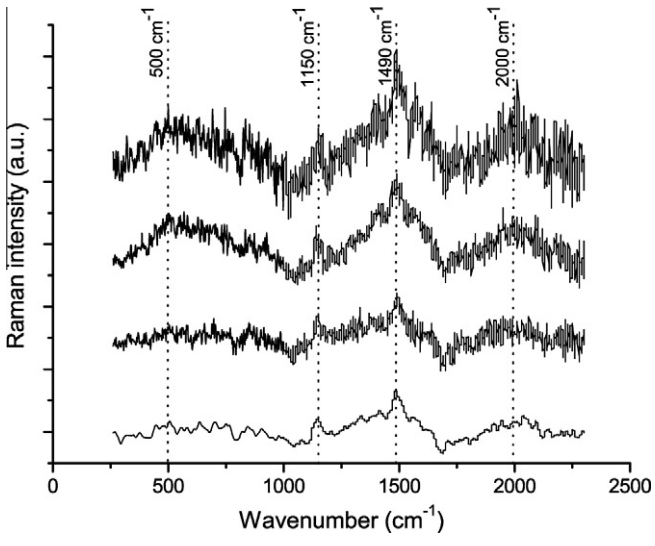

Fig. 1. Raman spectra of pheomelanin synthesized from 5-S-cysteinyldopa. The different spectra correspond to four Raman beams at different points of the pheomelanin powder.

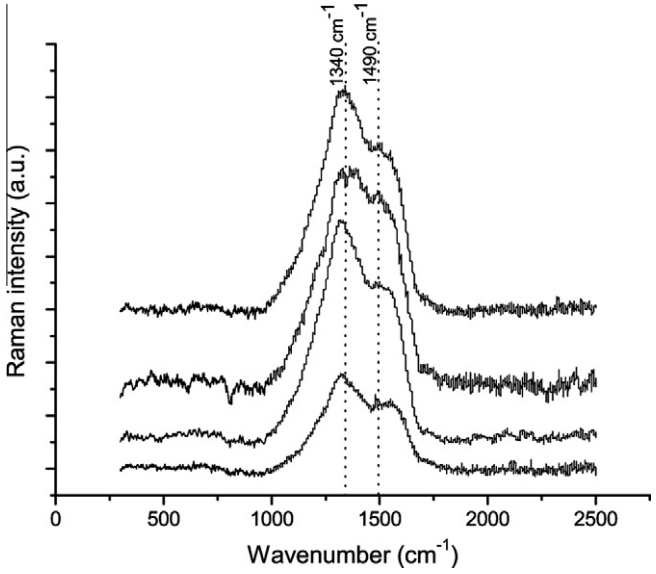

Fig. 2. Raman spectra of synthetic trichochrome F. The different spectra correspond to four Raman beams at different points of the trichochrome F powder.

G band (Fig. 2). This may be because eumelanin oligomers have four (if produced by oxidation of DHI) or three (if produced by oxidation of DHICA) hexagonal carbon rings [3] while trichochrome dimers only have two aromatic hexagonal carbon rings [28], which may lead to the lower Raman intensity of the G band observed in the trichochrome F. In any case, the difference in the shape of the Raman spectra of pheomelanin and trichochrome F is useful to distinguish pheomelanin from trichochromes (it is not likely that the Raman spectra of trichochromes B, C and E are markedly different from that of trichochrome F) in the tissues where they occur simultaneously such as feathers and hair [28]. The greater number of hexagonal carbon rings in the dimers of trichochromes than in the oligomers of pheomelanin [29] may be the cause of the resemblance of the Raman spectrum of trichochrome F to that of eumelanin and not to that of pheomelanin.

We detected Raman signal in all feathers and hairs. The same Raman spectrum obtained for synthetic pheomelanin (see above) was found in the orange breast feathers of chicken, in the orange flank feathers of zebra finches, in some barbs and barbules of the dorsal feathers of red-legged partridges, in the brownish portion of the hairs of wild boars and in the red human hairs (Fig. 3). As expected, there was much greater heterogeneity in the shape of the Raman spectra of *in vivo* samples than in synthetic pheomelanin, and the bands were slightly deviating from the wavenumbers where the bands of the synthetic pheomelanin were found (Fig. 3). We believe that the slight variation in the shape of the Raman curves observed in natural pheomelanin may help to understand possible differences in the structure of this pigment in biological species, an issue that should be considered by future studies. However, the 'three-humps' shape of the curves was the same than in synthetic pheomelanin.

By contrast, the black belly feathers of zebra finches, some barbs and barbules of the dorsal feathers of red-legged partridges, the black portion of the hairs of wild boars and the black human hairs produced the same Raman spectrum that have previously been reported for eumelanin [4,18,19] and for trichochrome F here (see above). In this case, the Raman bands were much closer to the D and G bands of eumelanin and disordered graphite expected at 1380 and 1580 cm^{-1} [4] than those of trichochrome F (Fig. 3). The 1750–2500 cm^{-1} region was flat, in contrast to the high Raman intensity found in the spectra from pheomelanin (Figs. 1 and 3). In some cases, we also found a band about 500 cm^{-1} that to our knowledge has never been discussed regarding the Raman spectrum of eumelanin (Fig. 3). However, this band can be found in the Raman spectrum of *Sepia* eumelanin extracted from the cuttlefish, but not in commercial (i.e., pure) *Sepia* eumelanin [19]. While the assignment of this band should be the same as for pheomelanin (i.e., the out-of-plane deformation of phenyl rings), its presence in only some non-pure eumelanin samples may indicate that it is produced not by the eumelanin polymers but by some compound that bonds to eumelanin. It could be speculated that this band about 500 cm^{-1} appears in eumelanin polymers that have sequestered Fe^{3+}, as this cation forms stable complexes with melanins through the action of ferritin [35], which in turn has a tyrosine residue, which may account for the vibration of the phenyl ring. This may be useful to distinguish pure (i.e., without proteins and metal ions) from non-pure eumelanins, a possibility that should be explored by future studies.

The shape of the Raman spectra of melanins in feathers and hair is very different from the Raman spectrum of keratin [36], and thus cannot be attributed to Raman signal of the keratin of these tissues. Therefore, it is possible to obtain the Raman spectrum of pheomelanin contained in feathers and hairs without the need of isolating the pigment. The orange flank feathers of zebra finches contain pheomelanin, but 75% of their melanin content is composed of eumelanin [30], and red-legged partridges also present both pigments (own unpublished data). We did not always obtain the Raman spectrum of pheomelanin when we focused the Raman beam at orange flank feathers of zebra finches or at dorsal feathers of red-legged partridges, but the spectrum of eumelanin was obtained in many of the trials. Similarly, red and black human hairs contain both pheomelanin and eumelanin, eumelanin at a lower proportion in the case of red hair and pheomelanin at a very low proportion in the case of black hair [32,37]. We also detected Raman signal from eumelanin in red human hair and from pheomelanin in black human hair. This indicates that eu- and pheomelanin show certain level of spatial segregation in the feathers and hairs and their polymers can be detected with the resolution of our

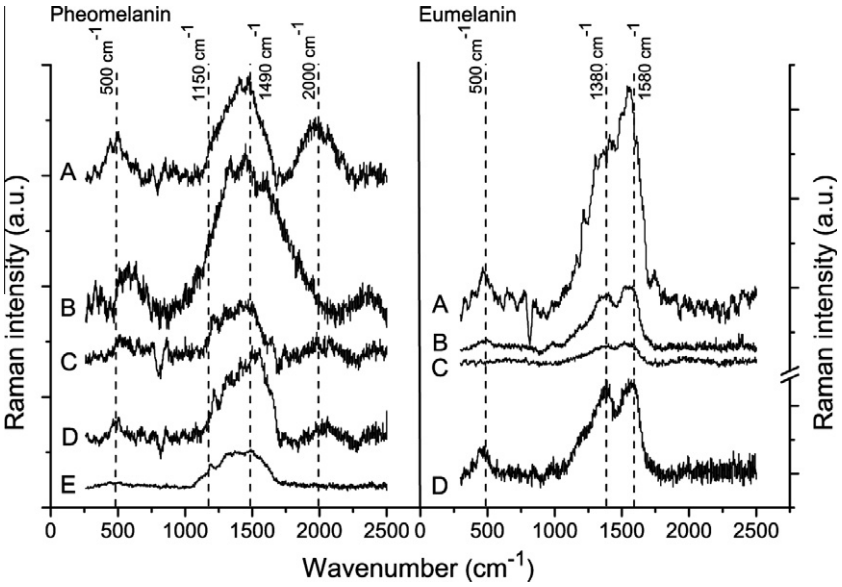

Fig. 3. Raman spectra of melanins in *in vivo* samples of feathers and hair. Left panel: Raman spectra of pheomelanin in: (A) a dorsal feather of a red-legged partridge, (B) a facial hair of a wild boar, (C) a belly feather of a chicken, (D) a red human hair, and (E) a flank feather of a zebra finch. Right panel: Raman spectra of eumelanin in (A) a black human hair, (B) a facial hair of a wild boar, (C) a breast feather of a zebra finch, and (D) a dorsal feather of a red-legged partridge. The zebra finch feathers used in left and right panels belong to the same individual bird.

Raman microscope (1 μm *x*,*y*). Thus, the Raman spectrum of pheomelanin can be obtained even when eumelanin simultaneously occurs in the same tissue.

Conclusion

In conclusion, we have detected Raman signals from pheomelanin, which produces a spectrum that is qualitatively different from those of trichochrome F and eumelanin, and it is probably caused by the out-of-plane deformation and the stretching vibration of the phenyl rings and by the stretching vibration of C–N bonds or the stretching and wagging vibration of CH_2. The Raman signal of pheomelanin can be obtained from feathers and hairs without the need of isolating the pigment. This can be used as a tool to detect and distinguish pheomelanin from eumelanin, trichochromes and other chromophores in different materials. Although we did not analyze skin samples, it should be possible to detect the Raman signal of pheomelanin in skin too, as previously shown for eumelanin [4]. The latter is especially relevant given the association between pheomelanin and skin diseases [11,12,24]. Future studies should investigate if variation in the Raman signal of pheomelanin can be used to quantify pigment content.

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