ADHESION AND MORPHOLOGY OF FIBROBLASTIC CELLS CULTURED ON DIAMOND-LIKE CARBON (DLC)-COATED AND UNCOATED Ti-13Nb-13Zr

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Abstract. *Diamond-like carbon (DLC) coatings were deposited on Ti alloy (Ti-13Nb-13Zr) substrates using the plasma immersion process. The plasma immersion process, unlike the conventional techniques, allows the deposition of DLC on three-dimensional workpieces with high adhesion. We quantitatively evaluated the adhesion of fibroblasts on DLC-coated and uncoated Ti alloy at 2 h. Comparisons between sample groups were made using one-factor ANOVA and the Tukey test was used to determine statistical significance at $p<0.05$. After incubation of fibroblasts (Vero cells) on DLC-coated and uncoated Ti alloy for 24 h, the cells were observed with scanning electron microscopy (SEM). Our results suggest that cells adhere better in DLC-coated Ti alloy than in uncoated Ti alloy.*
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Keywords*: diamond-like carbon, Ti-13Nb-13Zr alloy, cell adhesion, in vitro, orthopaedic implants*

1. Introduction

Ti-6Al-4V alloy has been extensively used for many years as an implantable material mainly in the application of orthopaedic prostheses. However, the toxicity of Vanadium and neurological disorders associated with Aluminium, have also created problems for biological applications, and new types of alloys have been developed. Ti-13Nb-13Zr alloy has been proposed as an alternative to the Ti-6Al-4V because of its superior corrosion resistance and biocompatibility (Long and Rack, 1998; Wang, 1996).

However, titanium alloys have relatively poor resistance against wear and a high friction coefficient. A diamond-like carbon (DLC) film could be used to protect titanium-based implants. Studies have indicated excellent tribological properties of DLC and its biocompatibility (Thomson et al, 1991; Dowling et al, 1997; Cui and Li, 2000).

DLC coatings can be deposited using various techniques, and recently the plasma immersion process, a technique developed for surface modification of three-dimensional components, was used to deposit DLC films with adhesion properties superior to those prepared with conventional techniques (Walter, Nastasi and Munson, 1997; Miyagawa et al, 2000).

In this study, DLC coatings were deposited on Ti-13Nb-13Zr alloy substrates using the plasma immersion process. We evaluated the adhesion and morphology of fibroblastic cells on DLC-coated and uncoated Ti-13Nb-13Zr.

2. Materials and Methods

The DLC coatings have been deposited on Ti-13Nb-13Zr substrates using the plasma immersion process (Uzumaki et al, 2004). Before the coating deposition, the substrates were ultrasonically cleaned in acetone for 20 minutes. The substrates were ultrasonically cleaned in acetone for 20 minutes. After Ar sputtering, to remove surface contamination, DLC deposition was performed by immersion in a methane plasma. Our deposition equipment possesses a vacuum system, a power source (pulsed DC negative output) and a reactor.

The thickness of the coatings ranged between 0.7 and 1 μm. The Ti alloy substrates were machined from Ti-13Nb-13Zr ASTM F 1713-96. Mean surface roughness of the processed substrates measured by atomic force microscopy (AFM) ranged between 41 and 51 nm. The Raman spectrum showed structures typical of DLC films. AFM shows that the DLC-coated Ti alloy presents a surface without any defect.

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*), were obtained from the Adolfo Lutz Institute, São Paulo, Brazil. Vero cells are recommended for studies of cytotoxicity and cell-substratum interactions in biomaterial research (Kirkpatrick, 1992; ISO 10993-5, 1992). The cells were cultured in Ham-F10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, from Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C.

In the adhesion assay, Fibroblast cells (Vero) were cultured on uncoated and DLC-coated discs for a period of 2 h. The culture plate itself (polystyrene) was used as a positive control (substrate that is optimum for adhesion) and Teflon disks as a negative control. All controls are in agreement the ISO 10993-5. Six repetitions were made of all experiments. Adhesion was evaluated by MTT (Sigma Chemical Co.) colorimetric assay (Mosmann, 1983). Briefly, the substrates were incubated in 96 well plates (Corning/Costar Corporation, Cambridge, MA, USA) with culture medium (Ham-F10 medium with 10% FCS) for 24 h at 37°C. After this incubation, 100µL of a cell suspension (2.5×10^5 cell/mL) in Ham-F10 medium (Sigma) with 10% FCS (Nutricell) was added to the wells containing the different samples. The cells were cultured for 2h at 37°C, and then the medium was replaced by 50µL of MTT (5mg/mL in HamF-12). After 4h incubation, 100µL/well of isopropanoic acid (Merck) was added. Blanks without cells were also run for the MTT reaction in all experimental conditions. The plate was read in a microplate reader (Multiskan Bichromatic Version 1.06) at 540 nm. Then comparisons between sample groups were made using one-factor ANOVA and the Tukey test was used to determine statistical significance at $p<0.05$.

Morphology of fibroblast on control (glass coverslips), DLC-coated glass, DLC-coated Ti-13Nb-13Zr and uncoated Ti-13Nb-13Zr were analyzed by scanning electron microscopy (SEM). After 24h of incubation, the samples were fixed in 4% paraformaldehyde / 2.5% glutaraldehyde in phosphate buffer pH 7.2, for 45 min at room temperature, and postfixed with 1% OsO_4 (Sigma) in the same buffer for 15 min, at 4°C. The specimens were then dehydrated through an ethanol series and critical point (Balzers CPD030) dried and gold sputtered (Balzers SCD050). The samples were examined in a JEOL JXA 840 scanning electron microscope.

3. Results and discussion

Figure 1 shows the fibroblast adhesion after an incubation period of 2 h (Figure 1). The adhesion of fibroblastic cells on DLC-coated Ti alloy was similar to the positive control (polystyrene - substrate that is optimum for adhesion), and the adhesion was greater in the DLC-coated Ti alloy than in uncoated Ti alloy ($p<0.05$).

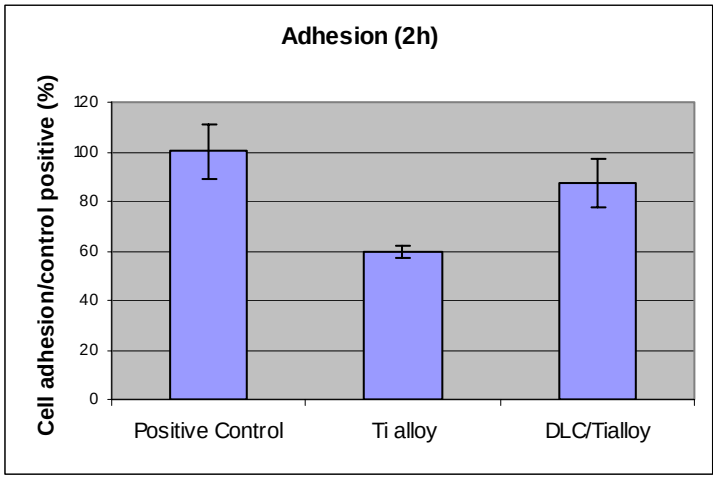

Figure 1. Cellular adhesion assay for 2h. Columns represent means of six absorbance readings, vertical bars indicate standard deviation interval.

Fibroblasts formed a semi-confluent layer on glass coverslips (Figure 2), DLC-coated glass coverslips (Figure 3) and DLC-coated Ti alloy (Figure 4). These cells are often elongated, much flattened with microvilli and/or cell prolongations on their surface.

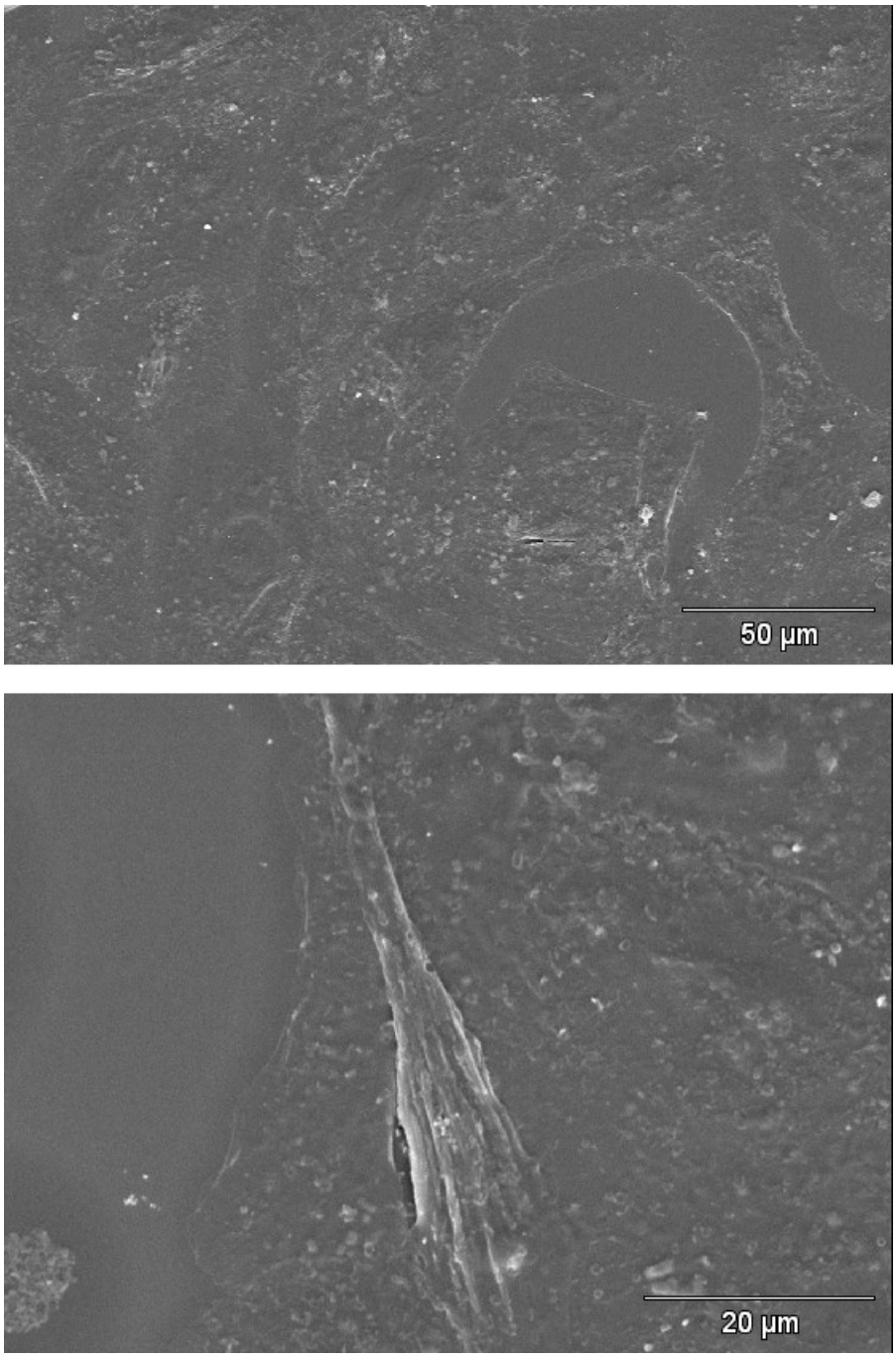

Figure 2. SEM micrographs showing fibroblastic cells grew on control (glass coverslips) after 24h.

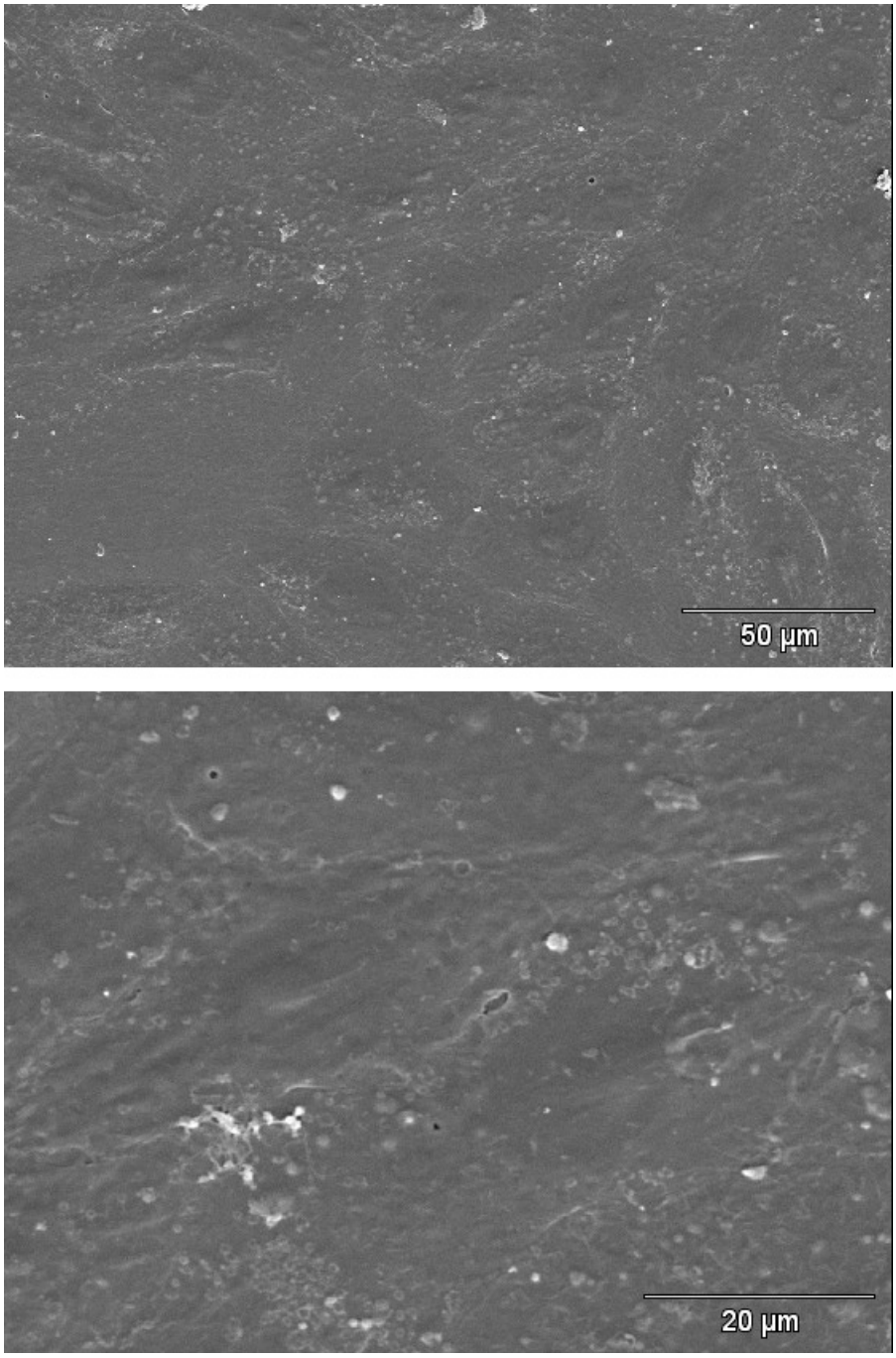

Figure 3. SEM micrographs showing fibroblastic cells grew on DLC-coated glass coverslips after 24h.

Scanning electron microscopic observations of fibroblasts on DLC coatings showed high densities of fibroblasts (Fig 3 and 4) with well-developed attachment systems in the form of cytoplasmic projections. The cells show a round to oval centrally located nucleus.

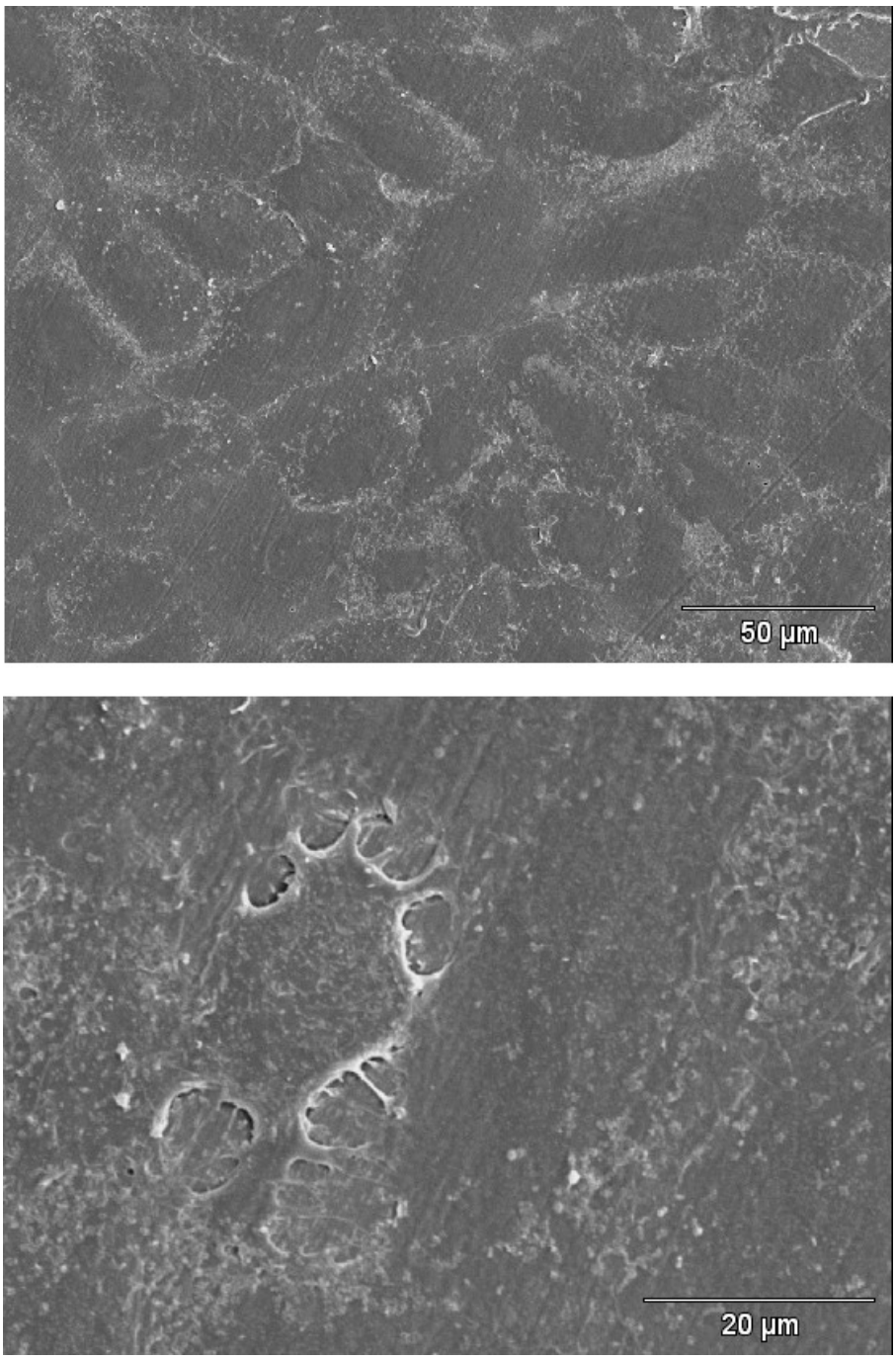

Figure 4. SEM micrographs showing fibroblastic cells grew on DLC-coated Ti alloy after 24h.

The titanium alloy surface exhibited a semi-confluent layer with good cellular morphology (Figure 5), although fewer cells could be seen on its surface compared to DLC coatings and control. Besides, cells cultured on Ti alloy were less flattened, but it still showed some microvilli and/or vesicles on their surface.

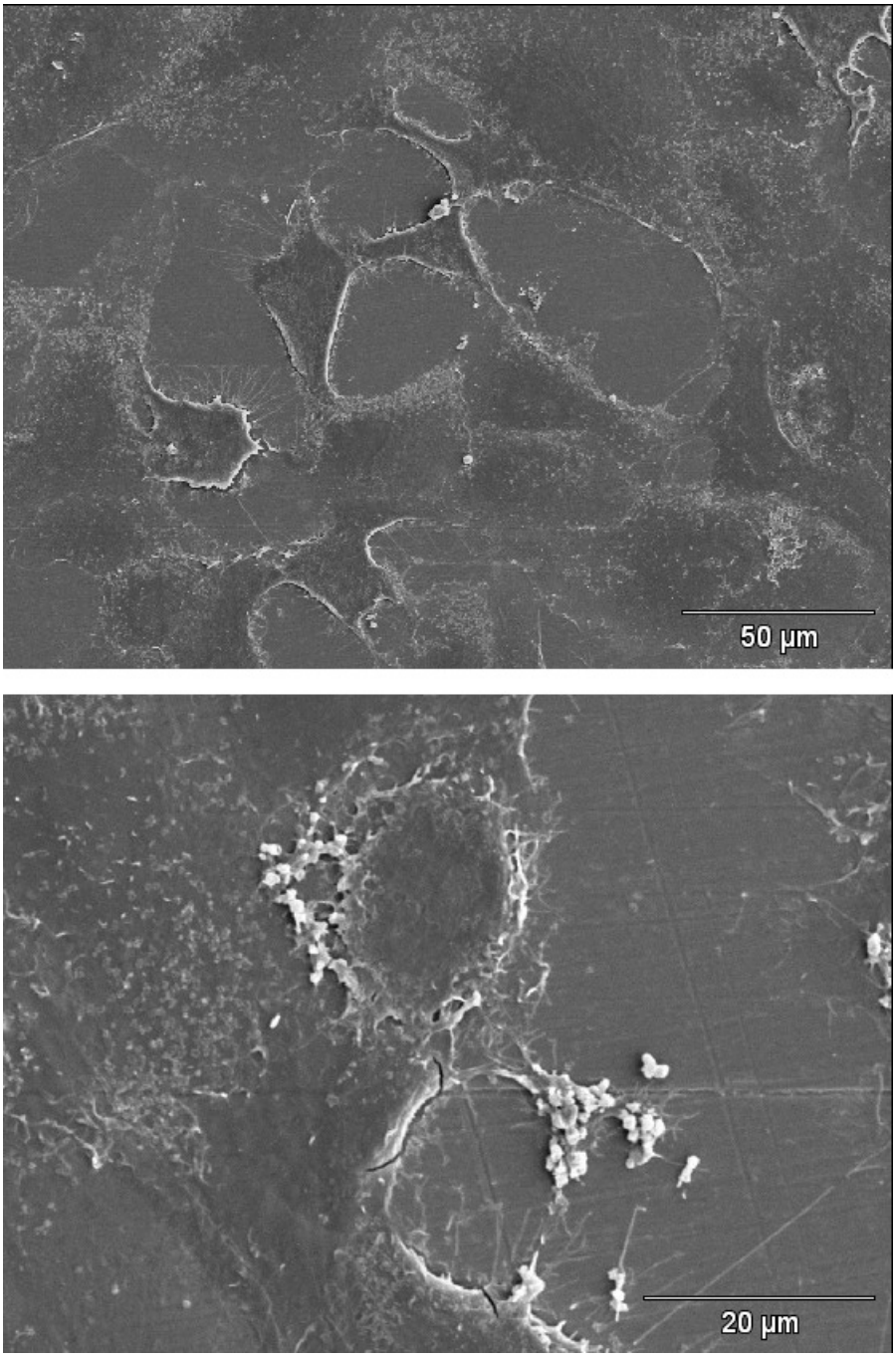

Figure 5. SEM micrographs showing fibroblastic cells grew on DLC-coated Ti alloy after 24h.

4. Conclusions

The presence of a semi-confluent layer of elongated and adherent cells demonstrated very good adhesion and good spreading of the fibroblasts cells on DLC coatings. This behaviour was similar to that of cells cultured on glass surface (control). Besides, the adherence of fibroblasts was significantly enhanced when Ti alloy was coated with DLC from the uncoated.

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