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Solid lipid nanoparticles as potential tools for gene therapy: *In vivo* protein expression after intravenous administration

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ABSTRACT

Naked plasmid DNA is a powerful tool for gene therapy, but it is rapidly eliminated from the circulation after intravenous administration. Therefore, the development of optimized DNA delivery systems is necessary for its successful clinical use. Solid lipid nanoparticles (SLNs) have demonstrated transfection capacity *in vitro*, but their application for gene delivery has not been conveniently investigated *in vivo*. We aimed to evaluate the capacity of SLN–DNA vectors to transfect *in vivo* after intravenous administration to mice. The SLNs, composed of Precirol® ATO 5, DOTAP and Tween 80 were complexed with the plasmid pCMS-EGFP which encodes the enhanced green fluorescent protein (EGFP). The resulting systems were characterized *in vitro* showing a mean particle size of 276 nm, superficial charge of +28 mV, the ability to protect the plasmid and transfection capacity in culture cells. The intravenous administration in mice led to transfection in hepatic tissue and spleen. Protein expression was detected from the third day after administration, and it was maintained for at least 1 week. This work shows for the first time the capacity of SLN–DNA vectors to induce the expression of a foreign protein after intravenous administration, supporting the potential of SLNs for gene therapy.

1. Introduction

Naked plasmid DNA (pDNA) is a powerful tool for gene therapy because of its easy preparation, great safety and stability (Liu et al., 2007). However, naked DNA is rapidly eliminated from the circulation after intravenous administration, due to the digestion by nucleases and to the hepatic uptake clearance, which occurs preferentially by non-parenchymal cells in a specific manner for polyanions (Liu et al., 2007). Consequently, the therapeutic application of these pharmaceuticals is seriously limited. Therefore, the development of optimized pDNA delivery systems is necessary for its successful and conventional clinical use.

Gene delivery systems include viral and non-viral vectors. Viral vectors are the most effective, but their application is limited by their immunogenicity, oncogenicity and the small size of the DNA they can load. Non-viral vectors, however, are safer, of low cost, more reproducible and do not present DNA size limit. The main limitation of non-viral systems is their low transfection efficiency. Non-viral transfection systems are usually composed of cationic peptides, cationic polymers or cationic lipids, although the combination of some of them is also possible (Hyndman et al., 2004; Tokunaga et al., 2004; del Pozo-Rodríguez et al., 2009).

Gene delivery using cationic lipids has gained increasing interest due to a variety of functions that helps to introduce genes into cells. The molecular architecture of cationic transfection lipids consists of a positive charged polar headgroup, and a hydrophobic moiety (Gascón and Pedraz, 2008). Cationic lipids can be used to form DNA–lipid complexes by direct mixing the positively charged lipids at the physiological pH with the negatively charged DNA. The formulation of cationic lipids has been widely applied for *in vitro* nucleic acid transfection, and more than 30 products are commercially available for this purpose, including Lipofectin® (a 1:1 mixture of DOTMA and DOPE), Transfectam®, LipofeACE™, LipofectAMINE™, and LipoTAXI™. However, their use *in vivo* is limited by the toxicity. Therefore, the development of more effective and safer systems useful for *in vivo* transfection is needed in order to develop medicines for gene therapy. Lipoplex structures such as liposomes or solid lipid nanoparticles (SLNs) are widely investigated for transfection. Several authors have administered *in vivo* liposomes for gene therapy (Yamashita et al., 2007; Wang et al., 2008; Inoh et al., 2009); however, the use of SLNs for gene delivery has not been conveniently investigated *in vivo*.

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The aim of the present work was to evaluate for the first time the expression of a foreign protein after intravenous administration of SLN–DNA vectors to mice. Vectors were previously characterized *in vitro* in terms of size, superficial charge, nuclease protection, transfection capacity and cell viability.

2. Materials and methods

2.1. Materials

Precirol® ATO 5 (glyceryl palmitostearate) was provided by Gattefossé (Madrid, Spain). Antibiotic/antimycotic, and Triton® X-100 were purchased from Sigma–Aldrich (Madrid, Spain). Tween 80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain). 1,2-Dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) was acquired from Avanti Polar Lipids, Inc., and DOTAP Liposomal Transfection Reagent from Roche Applied Science (Germany).

Plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was purchased from BD Biosciences Clontech (Palo Alto, U.S.) and amplified by Dro Biosystems S.L. (San Sebastián, Spain).

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain).

Female Balb/c nude mice weighing 18–22 g (5 weeks of age) were purchased from Harlam Interfauna Ibérica S.L. (Barcelona, Spain).

Primary antibody (polyclonal anti-GFP, IgG fraction) and secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG) were provided by Invitrogen (Barcelona, Spain), and the normal goat serum (NGS) from Chemicon International, Inc. (Temecula, CA, USA).

BD Viaprobe kit was provided by BD Biosciences (Belgium). Fluoromount G was purchased from SouthernBiotech (Coultek, España).

2.2. Production of SLN–DNA vectors

The SLNs were produced by a solvent emulsification–evaporation technique, previously described by del Pozo-Rodríguez et al. (2007). Briefly, Precirol® ATO 5 was dissolved in dichloromethane (5%, w/v), and then emulsified in an aqueous phase containing DOTAP (0.4%, w/v) and Tween 80 (0.1%, w/v). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) for 30 s at 50 W, and after the evaporation of the organic solvent an SLN suspension was formed upon solidification of the Precirol® ATO 5 in the aqueous medium. Finally, the SLNs were washed by centrifugation (3000 rpm, 20 min, ×3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

SLN–DNA vectors were obtained by mixing the pCMS-EGFP plasmid with an aqueous suspension of SLNs under agitation for 30 min, which allows the formation of electrostatic interactions between the positive charges of SLNs and the negative charges of DNA. The SLN to DNA ratio, expressed as the ratio DOTAP to DNA (w/w), was fixed at 5:1, which has previously demonstrated to fully condense DNA (del Pozo-Rodríguez et al., 2007, 2008).

2.3. In vitro characterization

2.3.1. Size and ζ potential measurements

Size of SLN–DNA vectors was determined by photon correlation spectroscopy (PCS), given in volume distribution. ζ potential was measured by Laser Doppler Velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). Samples were diluted in 0.1 mM NaCl (aq.).

2.3.2. DNase I protection study

In order to show the capacity of the SLNs to protect DNA, SLN–DNA vectors were put in contact with DNase I, then decomplexed in presence of SDS 1% and further subjected to electrophoresis on a 0.8% agarose gel (containing 1% ethidium bromide for visualization) for 30 min at 120 V. The bands were observed with a Vilber-Lourmat TFX-20M transilluminator. Images were captured using a BioRad DigiDoc digital camera. The integrity of the DNA was compared with a control of untreated DNA.

2.3.3. In vitro transfection

In vitro assays were performed with the human embryonic kidney (HEK293) cell line obtained from the American Type Culture Collection (ATCC). For transfection, HEK293 cells were seeded at a density of 150,000 cells per well on 24 well plates containing culture medium supplemented with 10% serum and 1% antibiotic/antimycotic (complete medium) and allowed to adhere overnight.

The SLN–DNA vectors were diluted in HBS (Hepes Buffer Saline) and added to the cell cultures containing complete medium. As positive control DOTAP Liposomal Transfection Reagent was used. Complexes of DNA with DOTAP Liposomal Transfection Reagent were prepared accordingly to manufacture's instructions. In all cases, 2.5 μg of DNA per well was added.

At different times transfection efficacy was quantified in a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, USA) by measuring at 525 nm (FL1) the percentage of fluorescent cells due to EGFP. For cell viability measurements, the BD Via-Probe kit was employed, and the fluorescence corresponding to dead cells was measured at 650 nm (FL3).

2.4. Intravenous administration of SLN–DNA vectors

Animals were handled in accordance with the Principles of Laboratory Animal Care (http://www.history.nih.gov/laws). Mice were quarantined for approximately 1 week prior to the study. They were housed under standard conditions and had ad libitum access to water and standard laboratory rodent diet. The SLN–DNA vectors were injected in standard way into the tail vein in a volume of 100 μL (60 μg of plasmid). Controls were employed by administering free DNA and SLNs without plasmid in the same way and volume. The treatment was administered to three mice in each group. Three and 7 days post-injection mice were sacrificed and the liver, lungs and spleen were removed, quick frozen in liquid nitrogen embedded in tissue freezing medium (Jung, Leica) and thin sectioned on a cryostat (Cryocut 3000, Leica).

2.5. Immunolabelling of EGFP in tissue sections

Cryostat sections (7–10 μm) were fixed with 4% paraformaldehyde during 10 min at room temperature. Following washing in PBS, sections were blocked and permeabilized in PBS 0.1 M, 0.1% Triton® X-100 and 2% normal goat serum (NGS) for 1 h at room temperature. Then, sections were incubated in primary antibody (polyclonal anti-GFP, IgG fraction) for 2 h at room temperature. Following adequate washing in PBS, sections were incubated in secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG) for 45 min at room temperature. Finally, sections were washed again in PBS and coverslipped with Fluoromount G.

Images of the immunolabelled sections were captured with an inverted microscopy equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon).

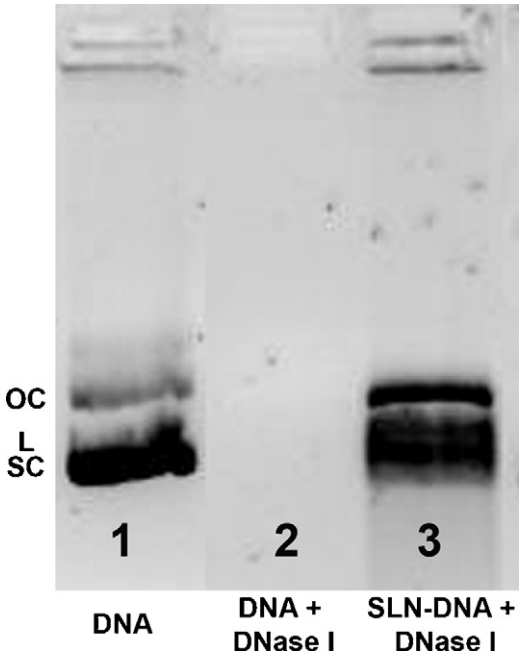

Fig. 1. Protection of DNA from DNase I digestion by SLNs, as visualized by agarose gel electrophoresis.

2.6. Statistical analysis

Results are reported as mean values (SD = standard deviation). Statistical analysis was performed with SPSS 14.0 (SPSS®, Chicago, IL, USA). Normal distribution of samples was assessed by the Shapiro–Wilk test, and homogeneity of variance, by the Levene test. The transfection levels and cell viability obtained with SLNs and DOTAP Liposomal Transfection Reagent were compared with the student's *t* test, whereby differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. In vitro characterization of SLN–DNA vectors

The size of SLN–DNA vectors was 276 ± 61 nm, and the ζ potential +28 ± 2 mV.

The agarose gel electrophoresis in Fig. 1 shows the bands corresponding to the DNA released from SLN–DNA vectors treated with DNase I (lane 3). Lane 1 corresponds to non-treated free DNA, and lane 2 to DNase I-treated free DNA. The absence of bands in this lane indicates that DNA was totally digested by the enzyme. Whilst the non-treated free DNA shows only two bands: supercoilled (SC) and open circular (OC), the DNA released from SLN–DNA vectors treated with DNase I (lane 3) shows a new band, corresponding to the lineal DNA (L). However, after the treatment with DNase I, the SC band, which is the most bioactive isoform (Remaut et al., 2006), still shows the greatest intensity.

The percentages of HEK293 cells expressing EGFP were measured at different times from 1 h to 7 days after the addition of the vectors. As observed in Fig. 2 green fluorescence was detected from 24 h, with 14% of the cells expressing EGFP, to 7 days, with 40% of EGFP positive cells (the same transfection efficacy observed at 72 h). The transfection efficacy of DOTAP Liposomal Transfection Reagent (positive control) was also measured at 72 h, and no differences ($p > 0.05$) were detected between the transfection capacity of SLNs and the positive control.

In order to evaluate the effect of the formulations on cell viability, the percentage of dead cells was also determined by flow cytometry. Cell viability was over 75% along time with both SLNs and DOTAP Liposomal Transfection Reagent ($p > 0.05$). In the cultures of non-treated cells the same percentage of viable cells was observed ($p > 0.05$).

3.2. Protein expression after intravenous administration of SLN–DNA vectors

For the *in vivo* assays, free DNA or SLN–DNA vectors were intravenously administered to mice. Furthermore, in order to ensure that the observed green fluorescence was not an artifact of the immunolabelling, we subjected samples of mice treated with empty SLNs to the same procedure with the primary and the secondary antibodies, and no green fluorescence was detected. No evidence of toxicity was detected.

The tissue sections of the mice treated with free DNA did not show fluorescence due to EGFP. However, the hepatic and splenic sections from the mice treated with the SLN–DNA vectors did show EGFP expression (Fig. 3). These sections were obtained from mice sacrificed 3 or 7 days after the intravenous administration. From each tissue, 12 sections representing the whole organ were analyzed. On day 3, all sections (100% of the sections) showed EGFP, while on day 7 green fluorescence was only detected in two sections of each tissue (17% of the sections). No transfection was observed in lung sections.

4. Discussion

This paper shows the capacity of SLNs containing the plasmid pCMS-EGFP to transfect after intravenous administration to mice. Actually, we were able to detect the green fluorescent protein in the liver and spleen of the animals. The lipid nanoparticles were previously characterized regarding size, superficial charge, nuclease protection, *in vitro* transfection activity and cell viability.

SLN–DNA vectors were prepared with a mean particle size of 276 nm, which is small enough to be intravenously administered. Typically the mean particle size for intravenously administered suspensions is in the submicron range (<1 μm) and the 99th percentile particle size is less than 5 μm (Wong et al., 2008). The complexation of DNA with cationic SLNs induced the formation of positively charged nanoplexes (ζ potential = +28 mV), which is advantageous because cationic charges facilitate the interaction with the negative charged cell surface and the cell entry (Elouahabi and Ruysschaert, 2005). Furthermore, the binding and condensation of DNA onto cationic carriers must prevent DNA degradation. The gel electrophoresis in Fig. 1 shows that SLNs were able to protect the plasmid from this enzyme. Although the presence of nucleases in cell cultures is reduced, when vectors are administered *in vivo* the genetic material is much more exposed to extra- and intracellular DNases which can damage DNA prior to the entry into the nucleus.

The SLN–DNA vectors also showed transfection capacity *in vitro* from 24 h to at least 7 days after their addition to culture cells, even in presence of serum, and cell viability was not affected. The presence of biological fluids such as serum during the transfection procedure has been reported to be a limiting barrier for transfection (Zhang and Anchordoquy, 2004), and most *in vitro* transfection protocols require that cells are not exposed to the cationic lipid–DNA complex in presence of serum (Kearns et al., 2008; Zhu et al., 2008). Serum has shown an inhibitory effect either by diminishing the amount of lipid–DNA complexes associated with the cells or via the binding of serum proteins to lipid–DNA complexes thereby diminishing their ability to deliver the plasmid (Nchinda et al., 2002).

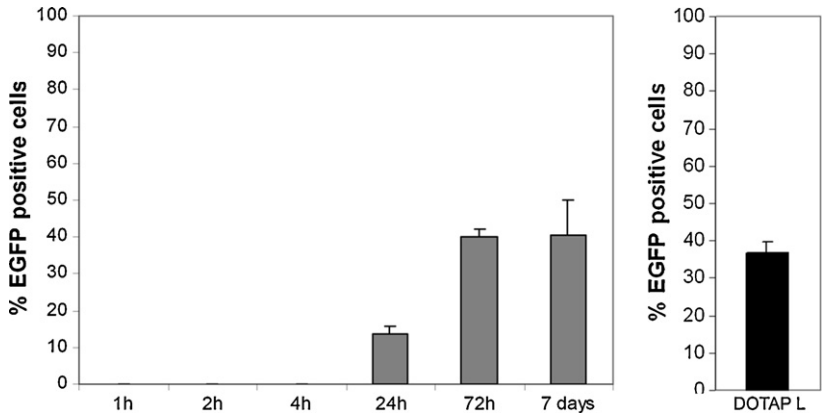

Fig. 2. Transfection levels in HEK293 culture cells over time with SLN–DNA vectors. The SLN to DNA ratio expressed as DOTAP to DNA ratio (w/w) was 5:1. As positive control the transfection levels in HEK293 at 72 h with DOTAP Liposomal Transfection Reagent (DOTAP L) is represented. Error bars represent SD ($n = 3$).

Although we have developed SLNs with appropriate characteristics for *in vitro* transfection, it is important to take into account that *in vitro–in vivo* correlation of nucleic acid carrier systems is difficult because of the biological, physiological barriers the systems need to overcome in a living animal, namely, circulation within the bloodstream, tissue distribution, cell uptake and intracellular trafficking. After intravenous administration to mice, the SLN–DNA vectors we prepared were able to transfect in liver and spleen. The anatomical and physiological characteristics of these organs, more specifically the discontinuous endothelium, facilitate the uptake of the nanoparticles. Nishikawa et al. (2005) have already described the uptake of lipoplexes by parenchymal cells due to the discontinuous endothelium. The transfection of liver and spleen is useful if these organs are the target tissue, for example, in vaccination (DNA vaccines), for the treatment of tumours in these organs or for the secretion into the bloodstream of lacking proteins in the organism. The spleen has more frequently been studied as target organ for DNA-based vaccines (Tupin et al., 2003; Nakamura et al., 2007) as it is a secondary lymphoid organ involved in the initiation of immune responses, bringing together antigen and naive lymphocytes in organized microenviroments that support the antigen-specific clonal expansion of these cells and their differentiation into memory-effectors subsets. The targeting to the liver is not only useful for DNA vaccines (Raska et al., 2008), but also for the treatment of diseases such as cancer (Tang et al., 2008) or hepatitis B (Zhang et al., 2007). Furthermore, it is also interesting the use of the liver as a depot organ, which produces large amounts of a therapeutic enzyme that is secreted to

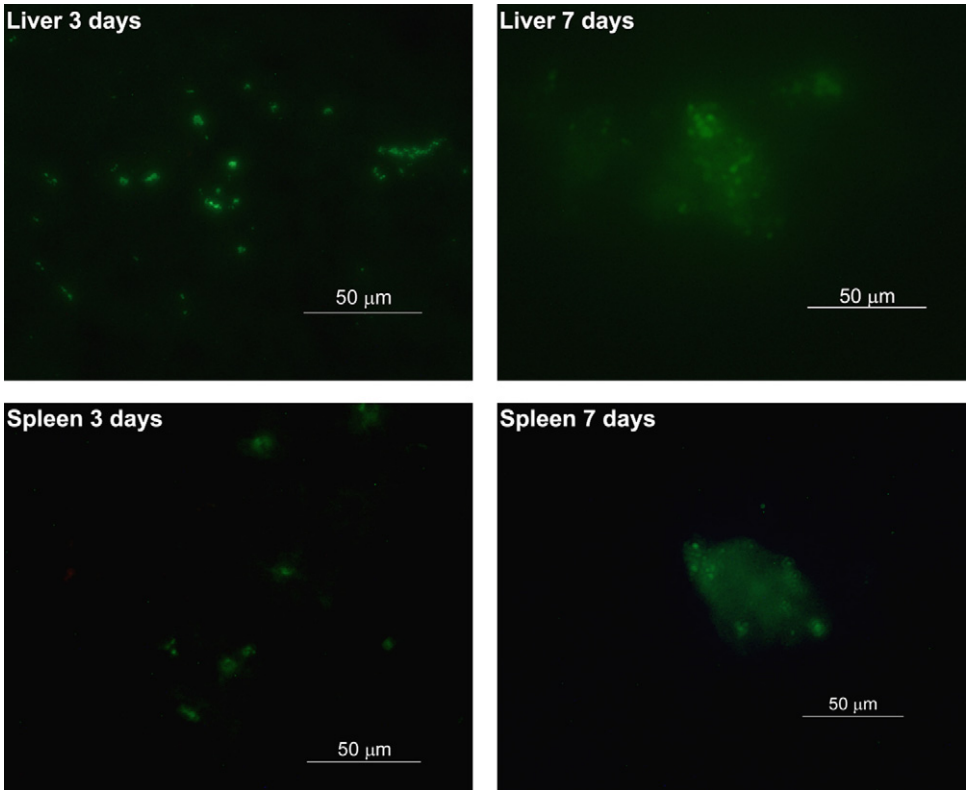

Fig. 3. Images of the sections of liver and spleen after the immunolabelling of EGFP (green). Liver and spleen were removed from the sacrificed animals 3 and 7 days after the intravenous injection of SLN–DNA vectors (dose: 60 μg of pCMS-EGFP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

the bloodstream and recaptured by target organs (Mango et al., 2004).

EGFP was not detected in lungs, although it is well known that when DNA delivery systems based on cationic compounds are intravenously administered, up to 80% of the dose may accumulate in the lungs and substantial gene expression is achieved in that tissue (Bragonzi et al., 2000; Ishiwata et al., 2000; Sakurai et al., 2001; Eliyahu et al., 2002; Kichler, 2004). These authors attribute the accumulation in lungs to the positive superficial charge of non-viral vectors, which form large aggregates when interact with blood components and keep retained in that organ. Despite the cationic superficial charge of our vectors, we did not detect transfection in lungs, but it has to be taken into account that other factors than superficial charge condition those interactions, such as intrinsic properties of the cationic compound and the non-viral vector to DNA ratio (Schiffelers et al., 2007). Furthermore, shielding of positive surface charge is a commonly used strategy to avoid the formation of aggregates when cationic lipid–DNA vectors are systematically administered (Tranchant et al., 2004; Harvie et al., 2006). This strategy is based in the addition of water-soluble, neutral, flexible polymers, such as poly(ethylene glycol) (PEG), which display low interfacial energy and high flexibility (Schiffelers et al., 2007). In our opinion, the PEG chains contained in the Tween 80 we used as surfactant could have a beneficial effect on the absence of aggregates in the bloodstream, and could justify in part, the lack of transfection in lungs.

Scarce studies about the use of SLNs for the *in vivo* transfection have been reported. Rudolph et al. (2004) demonstrated transfection locally, but the expression of a protein encoded by a plasmid delivered systemically in SLNs has not been reported. Choi et al. (2008) used the intravenous route in order to evaluate the capacity of SLNs to transfer the p53 gene to lungs, and reported the detection of DNA and the expression of mRNA. However, the presence of DNA or RNA does not necessary imply the expression of the protein, which is the objective of gene therapy with DNA. In our study we show, for the first time, the capacity of SLN–DNA vectors to induce protein expression after the injection into the tail vein of mice in standard way.

This preliminary study shows the capacity of SLN–DNA to transfect in liver and spleen, but further studies are needed in order to know if other organs are also transfected. Moreover, other administration routes should be explored. The inclusion of target ligands on the formulations can be useful to direct the vectors to different organs, depending on the disease to be treated; e.g. RGD sequences for neurons, tumour or endothelial cells, transferrin or folic acid for tumour cells, lactoferrin for brain, or sugars for hepatocytes. The incorporation in the vectors of molecules which have demonstrated improvement of transfection efficacy *in vitro*, such as cell penetrating peptides (CPP) (del Pozo-Rodríguez et al., 2009) or nuclear localization signals (NLS) (Zhang et al., 2009), may result in more relevant benefits.

On the basis of these results we can conclude that SLN-based vectors are potential gene delivery systems, able to induce the expression of foreign proteins in the spleen and the liver, which is maintained for at least 7 days.

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