



Direct immobilization and hybridization of DNA on group III nitride semiconductors

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ABSTRACT

A key concern for group III-nitride high electron mobility transistor (HEMT) biosensors is the anchoring of specific capture molecules onto the gate surface. To this end, a direct immobilization strategy was developed to attach single-stranded DNA (ssDNA) to AlGa_N surfaces using simple printing techniques without the need for cross-linking agents or complex surface pre-functionalization procedures. Immobilized DNA molecules were stably attached to the AlGa_N surfaces and were able to withstand a range of pH and ionic strength conditions. The biological activity of surface-immobilized probe DNA was also retained, as demonstrated by sequence-specific hybridization experiments. Probe hybridization with target ssDNA could be detected by PicoGreen fluorescent dye labeling with a minimum detection limit of 2 nM. These experiments demonstrate a simple and effective immobilization approach for attaching nucleic acids to AlGa_N surfaces which can further be used for the development of HEMT-based DNA biosensors.

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1. Introduction

Group III-nitride semiconductors, such as AlGa_N and Ga_N, are attractive for their chemical and biological sensing applications due to their low toxicity to living cells, high chemical and temperature resistance, high electron mobility, and simple integration with other electronic devices [1–3]. Schottky diode devices based on Ga_N and AlGa_N have been successfully utilized to detect variety of gases and the pH of liquid solutions [4–7]. In addition, AlGa_N/Ga_N heterostructures have been developed as highly sensitive electrical sensing elements [7–22]. In AlGa_N/Ga_N heterostructures, the band discontinuity, as well as discontinuities in polarization fields, leads to high density of two-dimensional electron gas (2DEG) at the interface. The 2DEG is a highly conductive channel that functions similarly to the doped region of standard metal oxide semiconductor field effect transistors (MOSFETs). Normally, changing the gate voltage to positive bias depletes the 2DEG at the AlGa_N/Ga_N interface, which in turn affects the source–drain current and forms the basis for a high electron mobility transistor (HEMT). The 2DEG is sensitive to minute changes in the local physical/chemical environment on top of the gate area which could be utilized to detect selected chemical and biological species. A key concern for

HEMT-based biosensors is the anchoring of specific capture molecules onto the gate surface. In AlGa_N/Ga_N HEMT biosensors, the AlGa_N layer is typically “capped” with additional material such as gold, silicon oxide or additional Ga_N. Common biomolecular immobilization strategies employ reactive organosilane [23,24] or alkanethiol reagents [25–27], on silicon oxide or gold surfaces, respectively. Previous studies have utilized these immobilization reagents to develop HEMT-based gas, pH, ion, and hydrogen sensors [7,11–22,28,29]. For example, Ren et al. [11] immobilized 5′ thiolated DNA probes onto gold-coated AlGa_N/Ga_N HEMT gates, and could detect 1 μM target DNA hybridized with probe DNA at a density of 2.0×10^{12} molecules/cm². The use of gold–thiol interactions or organosilane linkage methods, however, complicates fabrication and immobilization procedures. Replacing the gate material with biological molecules, such as proteins or nucleic acids, could create “gateless” HEMT biosensors where the change in source–drain current flow is associated with binding of a specific target. We have developed a direct immobilization method that couples nucleic acids directly to the AlGa_N surface. Directly interfacing capture molecules onto the AlGa_N surface has multiple advantages, including (1) fewer steps in device fabrication, (2) retention of optical qualities for subsequent optical analysis, and (3) potentially increased sensitivity resulting from binding/recognition occurring closer to the 2DEG.

Phosphonates and DNA 5′ phosphate groups show strong attachment to a number of III–V metal oxides including aluminum,

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titanium and zirconium oxides. At this time, however, it is not known whether electrostatic or covalent interactions are primarily responsible for the observed attachment [9]. DNA is also known to electrostatically bind to positively charged surfaces due to its negatively charged phosphate-rich backbone. It has also been shown that AlGa_N forms a nanometer thin layer of surface oxide when exposed to ambient atmosphere [8]. This thin oxide layer could therefore be exploited for biomolecular immobilization through phosphate–metal oxide attachment and/or through electrostatic interactions. In this work, we have utilized these interactions to develop a simple and direct strategy to link DNA to AlGa_N surfaces, most likely on the native surface oxide. The stability of DNA on these surfaces was evaluated by exposing the DNA to a range of buffer conditions (pH and ionic strength). Immobilized DNA was also evaluated through hybridization experiments to demonstrate the utility of this technique for biosensor applications.

2. Materials and methods

AlGa_N (Al_{0.5}Ga_{0.5}N) surfaces were prepared by metalorganic chemical vapor deposition (MOCVD) in a Veeco D180 system using tri-ethyl aluminum, tri-ethyl gallium, tri-methyl gallium and NH₃ as precursors. Deionized water (dH₂O) was prepared by ion exchange to a resistivity of 18.2 MΩ-cm. All chemicals were commercially available and used as received, unless noted otherwise. Tris-EDTA (TE) buffer, pH 8.0 was obtained from National Diagnostics (Atlanta, GA). Hydrochloric acid (HCl), boric acid, sodium tetraborate, HEPES buffer, sulfuric acid, hydrogen peroxide (30%), and 20× SSC buffer (0.3 M sodium citrate, 3 M NaCl, pH 7.0) were obtained from Sigma–Aldrich (St. Louis, MO). PicoGreen fluorescent dye was purchased from Invitrogen (Carlsbad, CA) and diluted into TE buffer 1:400 (v/v) prior to use. Single-stranded (ss) DNA oligonucleotides which target a gene encoding an adhesion protein from *Staphylococcus aureus* were purchased from Integrated DNA Technologies (Coralville, IA) having the following sequences: probe DNA (invaR), 5' TCA CCA TTA GTA CCA GAA TCA GTA ATT C; complementary target DNA (invaR comp), 5' GAA TTA CTG ATT CTG GTA CTA ATG GTG A. Upon receipt, all oligonucleotides were reconstituted to 500 μM with filter sterilized dH₂O and stored at –20 °C.

2.1. DNA patterning onto AlGa_N surfaces

AlGa_N samples were immersed in piranha solution (30% H₂O₂:98% H₂SO₄, 1:3 (v/v)) for 5 min and rinsed three times with 50 ml dH₂O. Samples were then immersed into boiling RCA2 cleaning solution (concentrated HCl:dH₂O:30% H₂O₂, 6:1:1 (v/v)) for 10 min, rinsed with dH₂O as above, followed by a second piranha clean for 5 min and final rinse with three times of 50 ml dH₂O. Samples were finally immersed in dH₂O, and treated in an ultrasonic bath for 5 min and subsequently blown dry with nitrogen gas.

Immediately following sample cleaning, the ssDNA invaR oligonucleotide was diluted in TE to 2 μM, and deposited onto the AlGa_N surface using a BioForce Nano eNabler (NeN) (BioForce Nanoscience, Ames, IA) surface patterning instrument. In short, BioForce surface patterning tools (SPTs—10 μm wide) were pre-treated in a BioForce UV/Ozone ProCleaner for 45 min prior to printing, after which the SPTs reservoirs were loaded with 0.5 μl of DNA solution and printed directly onto AlGa_N surfaces to give an average spot size of ~50 μm in diameter. The NeN chamber was kept at a relative humidity of 70% during the printing process. Printed AlGa_N surfaces were immediately rinsed by 0.5 ml TE buffer three times.

2.2. DNA hybridization

Patterned AlGa_N surfaces were immersed either in TE buffer, 2 μM non-complementary DNA solution or 2 μM complementary DNA (invaR comp) solution in 37 °C incubator for 30 min and cooled down to room temperature (RT), respectively. Each sample were rinsed three times with TE buffer 0.5 ml immersed in PicoGreen solution for 15 min, and finally rinsed with TE buffer three times as above. The surfaces were covered with glass cover slips and imaged using epifluorescence microscopy on a Nikon 80i fluorescence microscope (Nikon USA, Melville, NY). Fluorescence intensity measurements were performed with the ImageJ software [30]. In addition, some surfaces were blocked using bovine serum albumin (BSA) prior to DNA hybridization. After the initial printing, these samples were immersed in 0.1 mg/ml BSA for 15 min, rinsed three times with 0.5 ml TE buffer, and immersed in 2 μM complementary DNA (invaR comp) solution in 37 °C incubator for 30 min and cooled down to RT. These surfaces were then stained, washed and imaged as described above. In order to test the sensitivity, patterned surfaces were immersed in 200, 20 and 2 nM complementary DNA (invaR comp) solutions in TE buffer, respectively. Solutions were incubated at 37 °C for 30 min and cooled to room temperature. Following hybridization, these samples were washed, stained, and imaged as described above.

2.3. Fluorescence intensity measurements

Surface fluorescence intensity analysis was performed using the freely accessible ImageJ software from the National Institutes of Health (NIH) [30]. For each sample, nine printed spot areas and nine equivalent background areas next to the spots were measured and averaged, respectively. Average background intensities were subtracted from the average printed spot intensities to obtain normalized values. The standard deviations of all averaged intensities were calculated using Microsoft Excel.

2.4. DNA surface stability experiments

The stability of the DNA interaction with the AlGa_N surfaces was evaluated by exposing printed surfaces to a range of pH and ionic strength conditions. AlGa_N surfaces printed with invaR DNA were immersed in either 800 μl 10 mM HCl (pH 1.1), 10 mM HEPES (pH 7.0), 5× SSC (pH 7.4), TE (pH 8.0), or 10 mM borate buffer (pH 9.4) for 30 min at 25 °C. Most buffers were used at 10 mM concentration where 5× SSC was the only buffer with higher ionic strength (0.75 M NaCl). Following incubation in the various buffers, samples were rinsed with TE buffer three times and immersed into 20 nM complementary DNA (invaR comp) solution, incubated at 37 °C for 30 min and then cooled to RT. Samples were then rinsed with TE buffer three times as above, immersed in PicoGreen solution for 15 min, followed by additional three rinses with TE buffer. Glass cover slips were immediately placed over the samples which were imaged with epifluorescence microscopy, as described above.

3. Results and discussion

Immobilization of oligonucleotide probes to AlGa_N semiconductor surfaces were evaluated by printing ssDNA directly onto cleaned AlGa_N substrates using the BioForce NeN molecular printing system. In initial experiments, oligonucleotide probes were mixed with 10% glycerol solution in TE buffer (as per BioForce printing protocols) to reduce sample evaporation during printing. However, this resulted in non-uniform spotting and inconsistent hybridization, primarily due to glycerol that remained after air drying and persisted after multiple washing steps. To avoid

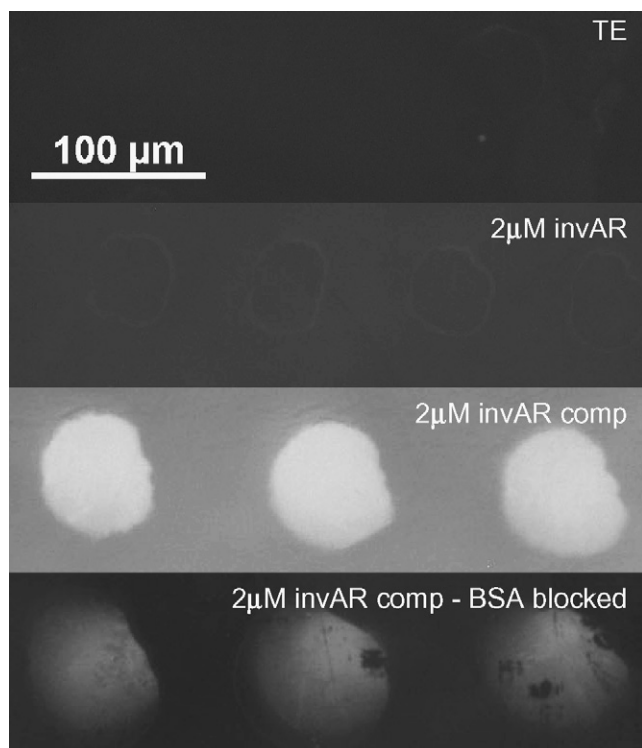


Fig. 1. ssDNA printing and hybridization on AlGaIn surfaces. Single-stranded invAR DNA probe was printed onto AlGaIn surfaces and hybridized with TE buffer (negative control), 2 μM invAR (non-complementary target), 2 μM ss invAR comp (complementary target), and 2 μM invAR comp (with BSA surface blocking). Following hybridization, samples were stained with PicoGreen dye and imaged via epifluorescence microscopy.

artifacts from residual glycerol, probe DNA was suspended in TE buffer and printed under high humidity conditions (70% relative humidity) maintained in the NeN environmental chamber. Under these conditions, DNA probes (invAR) were printed onto AlGaIn surfaces and immediately washed in TE, preventing drying artifacts and salt build-up on the surface. This treatment ensured that only a thin layer of immobilized DNA was present on the surface. Following this washing step, the printed arrays were hybridized with either TE buffer, non-complementary or complementary ssDNA to demonstrate that immobilized probes were functional on the surface and capable of specific binding. Hybridized DNA was stained with PicoGreen dye, a fluorescent nucleic stain that exhibits a 1000-fold increase in fluorescence when bound to double-stranded DNA. The results from this hybridization study are shown in Figs. 1 and 2. Fluorescence micrographs (Fig. 1) illustrate that printed DNA probes were firmly immobilized onto the AlGaIn surface and capable of hybridizing to complementary single-stranded target DNA (invAR comp). The hybridization shows that probe DNA was sequence specific, since non-complementary DNA and buffer-only controls (TE) did not stain with PicoGreen (Fig. 1). Quantitative image analyses with ImageJ software (Fig. 2) confirm that hybridization to complementary target DNA results in a ninefold increase in fluorescence intensity. These results indicate that single-stranded probe DNA can successfully be immobilized directly onto AlGaIn surfaces and further demonstrate that hybridization-based discriminatory assays can be performed on the immobilized DNA.

In an attempt to reduce the fluorescence background intensity; printed AlGaIn surfaces were incubated with a blocking solution with BSA prior to hybridization with complementary target. Since ssDNA bound firmly to the substrate during printing, it was hypothesized that any target DNA could interact with the AlGaIn

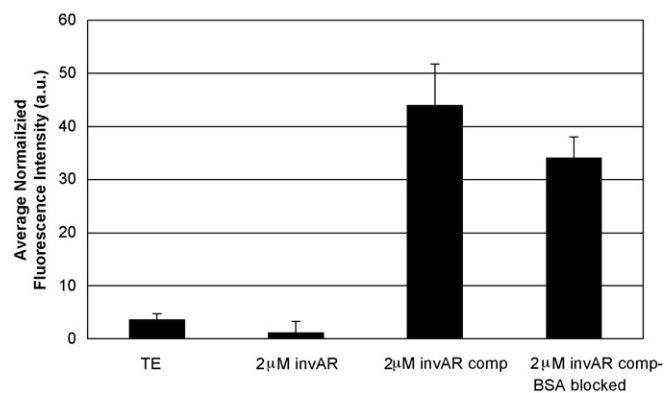


Fig. 2. Fluorescence intensity of PicoGreen stained, hybridized DNA on AlGaIn surfaces. Nine printed spots and nine background areas from each sample were analyzed using ImageJ. Average normalized fluorescence intensities (background subtracted) are shown for each sample type (error bars represent one standard deviation).

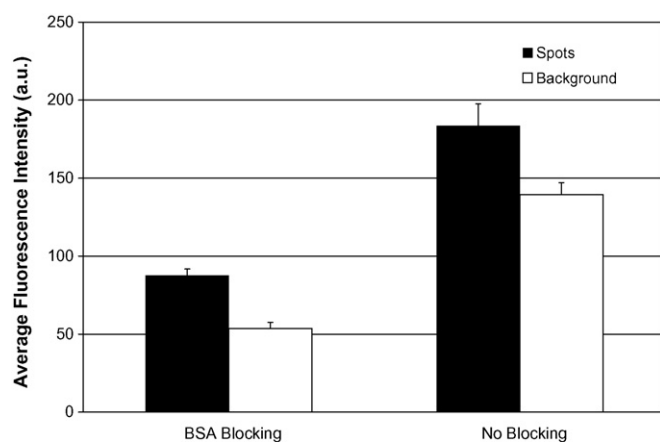


Fig. 3. Effect of surface blocking with BSA on background fluorescence intensity. The average fluorescence intensity of nine hybridized invAR spots and nine background areas are shown for BSA blocked and non-blocked AlGaIn surfaces (error bars represent one standard deviation).

surface during the hybridization and raise the background signal. As can be seen in Figs. 1 and 3, BSA blocking significantly reduced the background fluorescence. In addition to measuring average spot intensity, the ratio of average spot intensity to the average background intensity was calculated. These data are shown in Table 1. For a target concentration of 2 μM , the signal to background ratio for a non-blocked surface was 1.32:1. Pre-blocking with BSA prior to hybridization resulted in an increase of the signal to background ratio to 1.64:1. Clearly, pre-blocking the surface with BSA leads to an improvement in the signal to background ratio, likely by reducing the non-specific interactions of target DNA with the AlGaIn surface. At low concentrations of

Table 1

Signal to background ratios for varying target DNA concentrations and surface blocking procedures. For each concentration and surface treatment, the average fluorescence intensity for nine probe spots and nine background spots were used to calculate signal to background ratios.

Target concentration	Signal to background ratio
2 μM (with BSA blocking)	1.64:1
2 μM	1.32:1
200 nM	1.32:1
20 nM	1.35:1
2 nM	1.59:1
No target control	1.07:1

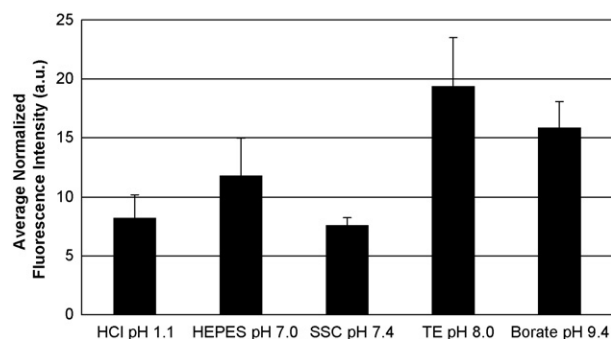


Fig. 4. Stability of printed DNA under varying pH conditions. AlGaIn surfaces were printed with invAR DNA and exposed to solutions ranging from pH 1.1 to pH 9.4, as well as varying ionic strength (up to 750 mM NaCl in 5× SSC). Following exposure, samples were hybridized with complementary target DNA, stained with PicoGreen dye and imaged via epifluorescence microscopy. Average normalized fluorescence intensity (background subtracted) is shown for each condition.

target (2 nM), an improved signal to background ratio was observed (1.59:1), further supporting this hypothesis. It should be noted that blocking also reduced the fluorescence intensity observed for the printed DNA spots. The reduction in spot intensity may well be due to BSA binding in this region, which to some extent could prevent target DNA from interacting with the probe.

The stability of probe DNA immobilized to AlGaIn surfaces was evaluated by exposing printed surfaces to a range of pH and ionic strength conditions. AlGaIn surfaces printed with invAR DNA were immersed in 10 mM buffer solutions ranging from pH 1.1 to pH 9.4. One solution, 5× SSC, had an elevated ionic strength, with a NaCl concentration of 0.75 M. Following immersion in respective buffer, the samples were washed three times with 0.5 ml TE buffer and then hybridized with 2 μM complementary target DNA (invAR comp). Samples were stained with PicoGreen dye and imaged via epifluorescence microscopy (Fig. 4). The results show that probe DNA was firmly attached to AlGaIn surfaces under a wide range of pH conditions and in higher ionic strength solution indicating a strong surface interaction. Fluorescence intensity data suggest that the probe DNA may be more stable under low ionic strength in neutral and alkaline conditions, as compared to higher ionic strength or low pH (pH 1.1) conditions. However, probe DNA remained attached to the surface and available for hybridization after exposure to each of the buffer conditions. This indicates that probe DNA is bound to the AlGaIn surface by complex interactions which may include charge–charge interaction, hydrogen bonding and Lewis acid–base coordination. Charged ions (such as NaCl in 5× SSC) would be expected to disrupt electrostatic interactions and although incubation samples with higher ionic strength solution show reduced signal, there is still significant amount of DNA bound to the surface to be readily detected upon hybridization with target DNA.

To further characterize the direct immobilization method, hybridization sensitivity testing was performed. For this study, AlGaIn surfaces were printed with invAR probe DNA and then exposed to solution of complementary DNA ranging in concentration from 2 nM to 2 μM. Similar to previous studies, samples were stained with PicoGreen dye and analyzed via epifluorescence microscopy and fluorescence image analysis (Fig. 5). Our results show that hybridization could be evidenced for target concentrations as low as 2 nM. Hybridization and staining conditions could likely be further optimized to increase fluorescent detection sensitivity. However, the future goals of this effort will be to utilize AlGaIn HEMT devices to perform electrical-based detection of DNA hybridization events. When this immobilization strategy is integrated with functional HEMT devices, we expect that the

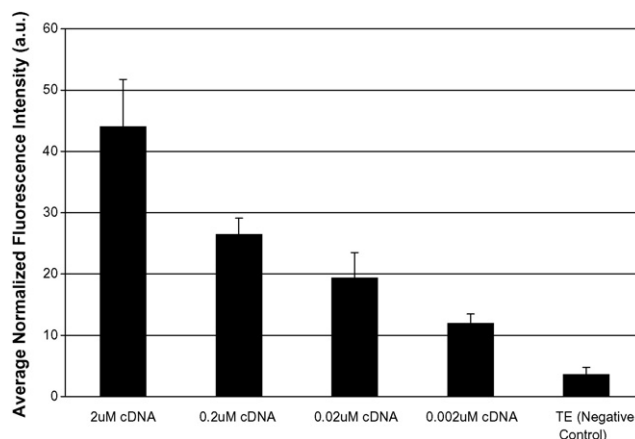


Fig. 5. Hybridization of target DNA with printed AlGaIn surfaces. AlGaIn surfaces printed with invAR ssDNA were hybridized with complementary single-stranded target DNA ranging from 2 nM to 2 μM, stained in PicoGreen dye and imaged via epifluorescence microscopy. Average normalized spot intensity (background subtracted) is shown with error bars representing one standard deviation.

detection sensitivity will further improve beyond the sensitivity obtained in this study, due to DNA charge-based interactions with the AlGaIn/GaN 2DEG. There have been limited studies demonstrating DNA detection with AlGaIn/GaN HEMT devices, but initial work indicates that detection at sub-micromolar levels is possible [10]. By optimizing HEMT architectures to promote a more charge interaction with the 2DEG, we expect that detection limits into the sub-nanomolar range could be possible.

The methods developed in this effort will significantly reduce the number of fabrication and functionalization steps needed for typical HEMT biosensors with metallic gate contacts, such as gold. Furthermore, the direct immobilization method will likely increase HEMT sensitivity for detection of nucleic acid hybridization, since no additional cross-linking agents are required. Alternative immobilization strategies rely upon chemical modification of the probe DNA, or extended linker molecules such as organosilanes and thiol-based self-assembled monolayers. The direct immobilization of unmodified probe DNA on the AlGaIn surface makes it possible to bring both probe and target DNA into closer proximity to the 2DEG, which should significantly enhance changes in the source–drain current for HEMT-based sensors. For this effort, no electrical measurements were performed and therefore a comparison with devices with and without metal gate contacts is not possible. Future efforts will focus on fabrication of HEMT devices for comparison of direct immobilization strategies with those utilizing metal gate contacts.

4. Conclusions

Single-stranded oligonucleotide DNA can be directly deposited onto AlGaIn semiconductor surfaces using simple printing techniques without the addition of chemical cross-linkers or other complex surface modifications. Our results demonstrate that printed DNA probes are firmly bound to the AlGaIn surface over different ionic strengths and a wide range of pH conditions and that immobilized probes are available for sequence-specific hybridization to single-stranded target DNA. Surface-bound DNA–DNA hybrids can be observed via fluorescent staining where background fluorescence can be reduced by blocking surfaces with BSA prior to hybridization. Hybridization reactions were observed for target DNA concentrations ranging from 2 μM to 2 nM, allowing this effective immobilization approach to be used for biosensing applications. DNA probe immobilized on the AlGaIn substrate treated with varying pH and ionic strength conditions

suggests complex DNA–AlGa_N interactions. Direct coordination interaction between phosphate groups and the thin surface layer of aluminum/gallium oxide could play a role in the attachment mechanism. Determination of the precise binding interactions will require future studies on the surface chemistry of AlGa_N and detailed analysis of its chemical and electrostatic interactions with nucleic acids.

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