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Bioelectronics communication: encoding yeast regulatory responses using nanostructured gallium nitride thin films

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Baker's yeast, *S. cerevisiae*, is a model organism that is used in synthetic biology. The work demonstrates how GaN nanostructured thin films can encode physiological responses in *S. cerevisiae* yeast. The Ga-polar, n-type, GaN thin films are characterized *via* Photocurrent Measurements, Atomic Force Microscopy and Kelvin Probe Force Microscopy. UV light is used to induce persistent photoconductivity that results in charge accumulation on the surface. The morphological, chemical and electronic properties of the nanostructured films are utilized to activate the cell wall integrity pathway and alter the amount of chitin produced by the yeast. The encoded cell responses are induced by the semiconductor interfacial properties associated with nanoscale topography and the accumulation of charge on the surface that promotes the build-up of oxygen species and in turn cause a hyperoxia related change in the yeast. The thin films can also alter the membrane voltage of yeast. The observed modulation of the membrane voltage of the yeast exposed to different GaN samples supports the notion that the semiconductor material can cause cell polarization. The results thus define a strategy for bioelectronics communication where the roughness, surface chemistry and charge of the wide band gap semiconductor's thin film surface initiate the encoding of the yeast response.

Introduction

Information is transmitted through the process of communication which relies on suitable encoding-decoding schemes.[1] The encoding part of the communication progression transfers an instruction into a specific format, where the decoding part deciphers the meaning of the instruction. Societal needs have pushed the development of different routes of communication starting from physical/verbal to digital. Digital communication relies on electronic components and devices that can participate in encoding and decoding. Increasing demand on amount and long-term storage of data during communication has led to looking into molecule-based (*i.e.* molecular) communication *via* a suitable biological host such as bacteria.[2] Thus, bioelectronics[3] is rapidly evolving around two essential components: an interface that can be integrated into processing electrical signals and a living interface that can generate and respond to molecular information. Bioelectronic living interfaces explored so far include *in vitro* and *in vivo* neural based interfaces[4] along with ones utilizing various microorganisms.[5] Finding a suitable interface that can receive and transmit electrical signals, while being able to efficiently integrate with current infrastructure that processes digital information, is the key to advancing bioelectronics.

Nanostructured semiconductor thin films offer several advantages for bioelectronics. The nanoscale roughness and surface chemistry that is created during fabrication can be used for directed placement of biomolecules and/or cells.[6,7] A vast amount of materials research has generated knowledge to tune their optical, electronic and mechanical properties to make them suitable for device integration.[8] Further research into the processing of nanostructured semiconductor thin films has resulted in the development of chemical functionalization methods[9,10] as well as the utilization of a wealth of microscopy and spectroscopy techniques to understand the changes in many of their interfacial properties after modification.[11] However, not all nanostructured semiconductor thin films are suitable for bioelectronics. Leaching of toxic ions and lack of biocompatibility can hinder the integration of certain materials compositions into bioelectronics. With these

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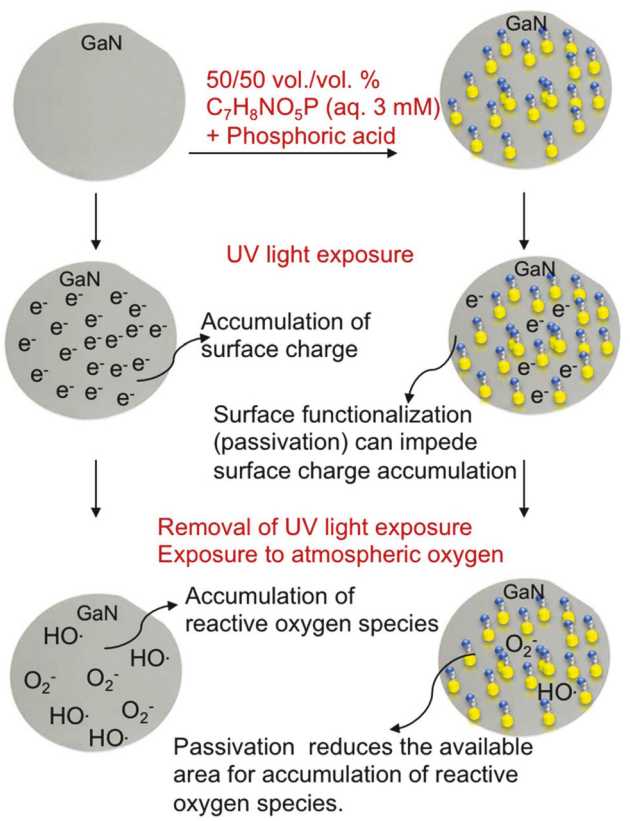
requirements in mind, III-nitrides[12] have recently been identified as a promising platform for bioelectronics because they can function as sensing, signaling and integration optoelectronic components.[13,14]

Here, we demonstrate the incorporation of gallium nitride (GaN) nanostructured films into a bioelectronic interface for the purpose of encoding yeast regulatory responses. We describe experiments to take advantage of the surface roughness and chemistry of the GaN films along with our ability to vary its surface charge by inducing persistent photoconductivity (PPC) after exposure to UV light, as shown in Scheme 1. The first row of Scheme 1 identifies the conditions used to functionalize the surface using *in situ* modification with phosphoric acid and a phosphonic acid derivative ($C_7H_8NO_5P$). The second row of Scheme 1 pictorially represents the accumulation of surface charge after exposure to UV light and compares and contrasts the difference when the surface is either passivated or not. The third row of Scheme 1 depicts changes after the removal of UV light and shows the transformation of the surface upon contact with atmospheric oxygen. Possible types of reactive oxygen species are shown as well as the qualitative difference in their accumulation on clean *vs.* passivated GaN material. We monitor adhesion of *S. cerevisiae* strains onto the GaN thin films and examine changes in the physiology of the yeast due to extrinsic cues generated by the semiconductor interface.



Scheme 1 Representation of the processes used to change the properties of the semiconductor thin films.

Results and discussion

Ga-polar, n-type, GaN thin films were grown on c-plane sapphire with two levels of Si doping: low Si doping yielding a carrier concentration $n = 8 \times 10^{17}$ cm^{-1} and high Si doping with carrier concentration of $n = 2 \times 10^{19}$ cm^{-1}. Further details on growth process are found elsewhere.[15,16] Atomic Force Microscopy (AFM) analysis, Fig. 1, revealed similar morphology and rms values below 3 nm for both the high and low doped samples. It has to be noted that, at higher doping, the roughness slightly increases likely as a consequence of increased tensile stress[17] in comparison to the atomic smooth spiral/step flow growth morphology in low doped GaN. GaN, along with other wide bandgap materials such as ZnO, exhibits PPC (increased conductivity under above bandgap illumination *i.e.* photoconductivity that persists after the removal of illumination) characterized by excess majority carriers (in this case, electrons) with very long lifetimes. Interestingly, in GaN, the PPC is accompanied by a similarly persistent surface charge or surface potential.[18] We recently reported on the use of PPC in biointerface studies with neurotypic cells.[19] We characterized n-type Ga-polar GaN before and after UV light exposure to assess any changes in surface chemistry. Treatment with UV light resulted in significant reduction of carbon species on the surface,[20] making the surface more hydrophilic. In this work, we quantified the amount of adsorbates present on the surface after exposure to growth media utilized in yeast proliferation studies. The quantification was done by measuring changes in surface roughness, Fig. 1a. Two types of growth media (Yeast Extract/Peptone/Dextrose medium (YPD) and Yeast Extract/Peptone medium (YP)) were used in adsorption experiments where the semiconductor films were submerged in solution for 24 h, rinsed and examined by AFM. YP media type had no glucose and the other (YPD) contained glucose. The AFM data in Fig. 1a reveals that the composition of the growth media as well as the UV light treatment can lead to variable amount of adsorption. The changes in roughness, though quantifiable on both types of semiconductor films after UV exposure, were statistically significant on the material with high doping. Assessing the attachment of biomolecules to surfaces from solution media is essential since a number of studies have shown that initial surface adhesion of proteins not only facilitates cell attachment but also subsequent behavior.[21,22] Exposure to UV light of the n-type Ga-polar GaN samples results in accumulation of charge on the surface. The charged semiconductor surfaces promote an increased adsorption of biomolecules and salts present in the yeast broth. In addition to AFM we performed X-ray Photoelectron Spectroscopy (XPS) analysis after the adsorption of YP and YPD media on the GaN samples with different doping which were either exposed or not exposed to UV light, Fig. 1b–d. In the N 1s spectrum we consistently recorded the presence of C-NH_2 and N–C=O

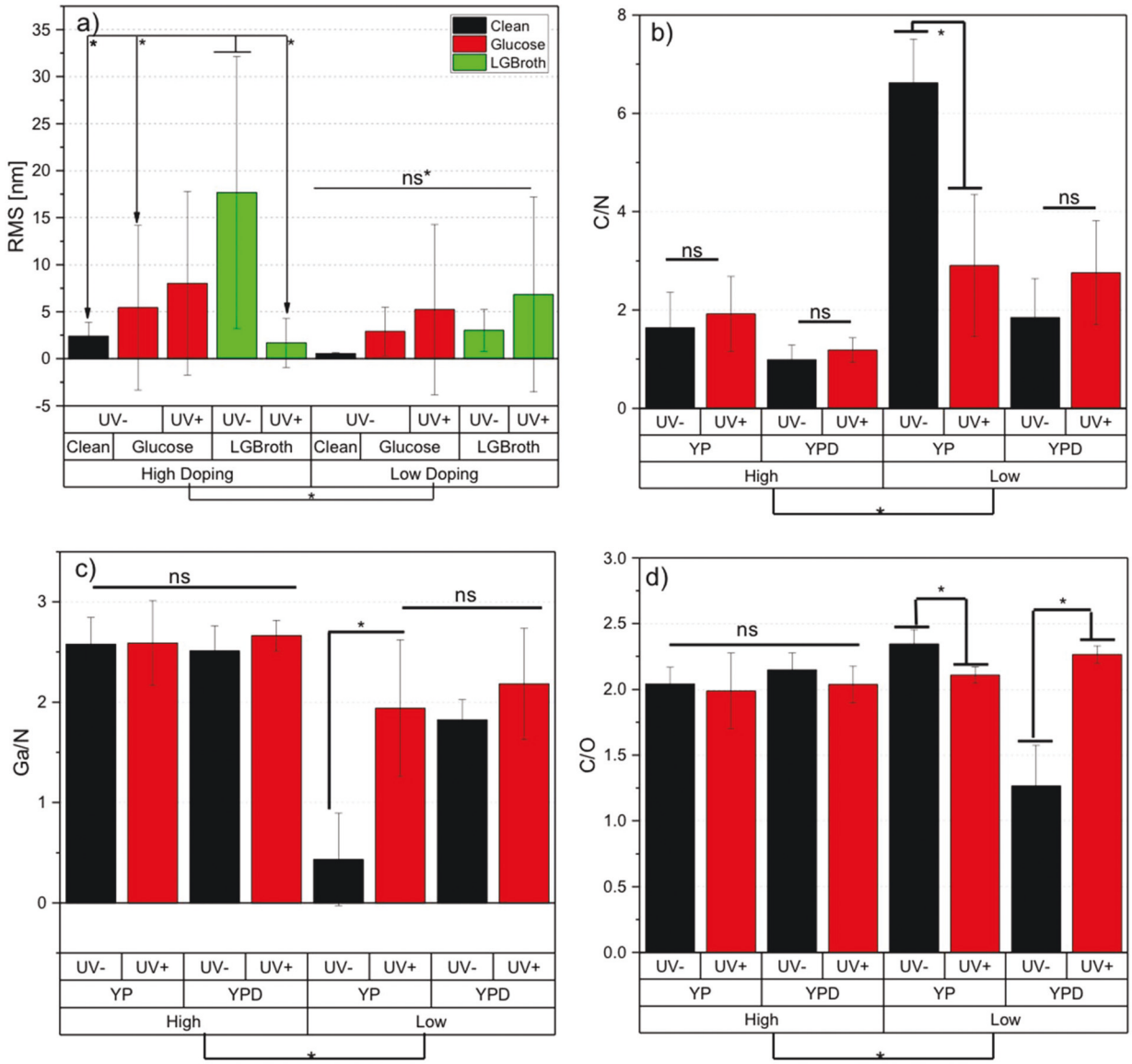


Fig. 1 (a) Surface roughness changes of the different GaN samples before and after UV treatment as well as incubation in growth media with two compositions (YPD and YP medium); (b) C/N ratios extracted from XPS analysis of the different GaN samples before and after UV treatment as well as incubation in growth media with two compositions (YPD and YP medium); (c) Ga/N ratios extracted from XPS analysis of the different GaN samples before and after UV treatment as well as incubation in growth media with two compositions (YPD and YP medium); (d) C/O ratios extracted from XPS analysis of the different GaN samples before and after UV treatment as well as incubation in growth media with two compositions (YPD and YP medium).

(399.51 eV). Prior publications have discussed the origin of the peak at a binding energy of ~400 eV and have attributed it to amide and amine groups.[23] In the C 1s high resolution spectrum we detected evidence for C–C, C–N, C–O, amide and C═O with the carbonyl species appearing at the highest binding energy of ~288.5 eV. The C/N ratio was only significantly changed on the low doping samples when the UV light exposure conditions were compared with the YP media, Fig. 1b. The same significant change was also observed with respect to the Ga/N ratios, Fig. 1c. In addition, the C/O ratios were statistically different with and without UV treatment when both YP and YPD media was adsorbed onto the low doping GaN samples, Fig. 1d. Furthermore, based on all the elemental ratios we recorded by XPS, Fig. 1b–d, there was a uniform significant variation in the ratios when the data from the low *vs.* high doping n-type GaN samples was compared. Taken in sum, the XPS data supports the AFM analysis that adsorption of molecules from the growth media does occur, and can alter the surface chemistry prior to yeast attachment. We note that on polar materials, such as GaN and lithium niobite, we[24] and others[25] have shown that the difference in adsorption behavior of biomolecules play a small role in cell

adhesion, and a more significant role in cell migration. Our observations in this study are consistent with prior work done by us[20] and others[26] that shows that surface charge on GaN can be used to drive the assembly of charged species on the material interface.

Chemical functionalization has been widely used to improve the optical and electrical properties of semiconductors.[27] One has a choice among various adsorbates with different terminal groups and affinities for the semiconductor surface. In this study we used a simple chemical functionalization using phosphoric acid along with a water soluble phosphonic acid derivative terminated on a nitro group, $C_7H_8NO_5P$, in order to modulate the surface chemistry as well as the PPC of the GaN. We and others have shown that phosphonic acids can be used for the functionalization of semiconductors.[28,29] The photocurrent *vs.* time measurements we collected, Fig. 2, indicate that functionalization changes the type of PPC behavior observed. Prior work with wideband gap materials has also demonstrated that chemical modification can change the photoconductivity and alter the photocurrent decay.[30] Chemical functionalization resulted in a slower current decay compared to the curve recorded for the clean samples. The results summarized on Fig. 2 indicate that chemical functionalization can be used to change the PPC of n-type Ga-polar GaN. This indicates that the PPC in GaN is partly or completely due to surface band bending and the associated electron–hole separating electric fields. However, the increased roughness due to strain relaxation may introduce increased surface recombination centers and hence rough surfaces are more sensitive to passivation mechanisms. Further, one expects to observe variable chemical passivation on surfaces with heterogeneous nanoscale topography when a small adsorbate that does not form well-ordered monolayers is used.[31] The data on Fig. 2 supports the notion that the adsorbate passivates better the samples that are rougher resulting in less accessible surfaces to reactive oxygen species, as shown in Scheme 1, leading to hindrance of the carrier recombination process at the surface and slower photocurrent decay.[32]

It should be noted that in addition to passivating the surface, the chemical functionalization may induce a charge transfer or an interface dipole at the surface. This charge plays a role in cell adhesion and therefore we quantitatively compared the amount of surface charge before and after functionalization as well as UV exposure by collecting Kelvin Probe Force Microscopy (KPFM) data, Fig. 3. Chemical functionalization did not lead to statistically different surface potential values when the high doping samples were examined. In addition, as expected the surface potential increased after exposure to UV light. In the case of the low doping samples the surface potential was altered both as a result of UV light exposure as well as chemical functionalization. KPFM agrees with the adsorption experiments where we observed higher amount of adsorbates on samples exposed to UV light and can be explained by electrostatic interactions between the charged semiconductor and ions and protonated biomolecules in solution. Taken in sum, the materials characterization we performed and described in Fig. 1–3 indicates that we have a tunable electronic interface that can be modulated to have different chemical functional groups, facilitate variable biomolecular adsorption *via* its nanoscale topography, and carry alterable surface charge.

In this work we aimed to use the ability to induce surface charge *via* UV illumination to encode a regulatory response in

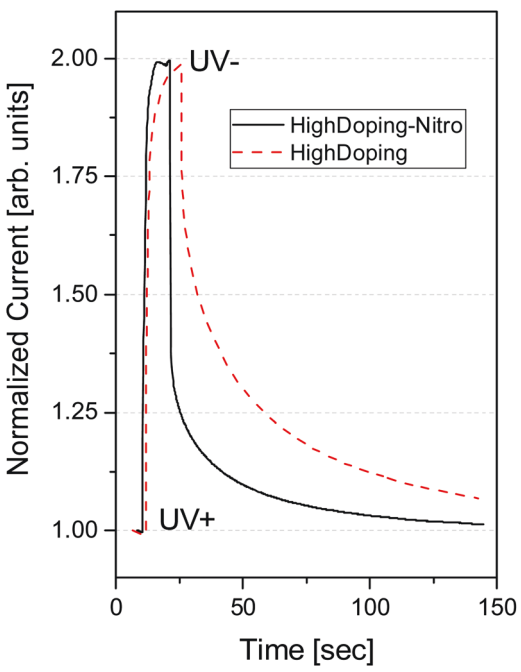


Fig. 2 Photoresponse of clean and functionalized n-type Ga-polar samples. The symbol UV+ indicates the point when the UV light was turned on and the symbol UV– indicates the point when the UV light was turned off.

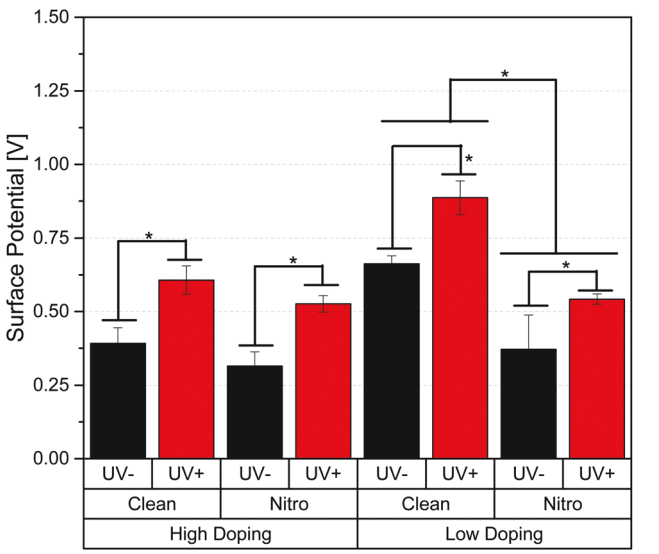


Fig. 3 Summary of surface potential changes on the different substrates extracted from KPFM measurements.

Table 1 Adhesion and clustering of TBR1 yeast on different n-type Ga-polar GaN samples

Substrate	Cells/cluster (n)	Cells/FOV (n)
Glass	6.7 ± 2.0 (34)	NA
Low doping	3.1 ± 1.1 (63)	24 ± 7 (11)
Low doping +UV	6.2 ± 1.8** (52)	34 ± 16* (11)
High doping	4.2 ± 1.4 (39)	20 ± 4 (10)
High doping +UV	5.3 ± 2.0* (61)	31 ± 8* (11)
Low doping-nitro	5.3 ± 2.7 (29)	37 ± 16 (8)
Low doping-nitro +UV	7.9 ± 2.4** (41)	78 ± 14* (7)
High doping-nitro	7.1 ± 2.5 (22)	20 ± 8 (7)
High doping-nitro +UV	7.9 ± 2.8* (44)	45 ± 15* (7)

*P < 0.5 between control (non-UV) and UV exposed. **P < 0.001 between control (non-UV) and UV exposed.

yeast. We note that no yeast cells were ever exposed to the UV illumination. To determine the effects of induced surface charge on the behavior of *S. cerevisiae*, we exposed cells of the TBR1 yeast, a wildtype haploid strain of *Saccharomyces cerevisiae*[33,34] to GaN (low and high doping) surfaces that have been exposed to UV illumination. As controls we also examined these same cells on glass surfaces and the same GaN substrates which were not illuminated with a UV lamp prior to their contact with the yeast. We recorded significance in both cell–cell clustering as well as adhesion to the GaN substrate after exposure to UV radiation (Table 1). Yeast on all non-activated GaN substrates have smaller cell clusters than those incubated on GaN substrates that have been activated by UV exposure (Table 1). For instance, on low doping substrate we observed the clusters of yeast cells to contain 3–4 cells on average, while on low doping substrates that have been exposed to UV light we observe almost twice this number. We also detected significantly more cells on GaN surfaces that have been activated by UV illumination (Table 1), which supports the notion that GaN samples with induced surface charge have a higher binding affinity for yeast cells. Differences were observed based on the doping of the samples indicating that it is an important control parameter in addition to UV illumination and surface roughness.

To determine whether charged GaN surfaces experiencing PPC alter the physiology of yeast, we labeled the TBR1 yeast with Calcofluor White (CFW), a dye that binds to the polysaccharide chitin.[35,36] The levels of chitin within the cell wall directly correlates to the activation of the Cell Wall Integrity Pathway and is indicative of the amount of mechanical stress applied to yeast cell.[36,37] The levels of chitin expression as demonstrated by CFW staining is roughly equal among all yeast cells on all GaN surfaces that have not been photo-activated and are similar to the levels of yeast cells cultured on a glass substrate (Fig. 4A). However, in all cases TBR1 yeast cells express reduced levels of chitin when cultured on a GaN surface that has been photo-activated (Fig. 4A; compare Fig. 7A–D). The accumulation of surface charge or increase in surface potential after UV illumination encodes a behavior response in yeast that causes a decrease in chitin and we hypothesize a possible build-up of oxygen species on the surface. The oxygen adsorption has also been hypothesized for ZnO.[30,32] The diminution in chitin can be viewed as a semiconductor induced hyperoxia response of yeast[38] in contact with a photo-activated GaN surface. This hyperoxia due to an external cue from the environment is manifested in the mechanical properties change in the cell wall but has been studied extensively and shown to lead to a number of changes in gene expression.[39] Thus the altered chitin levels are a direct

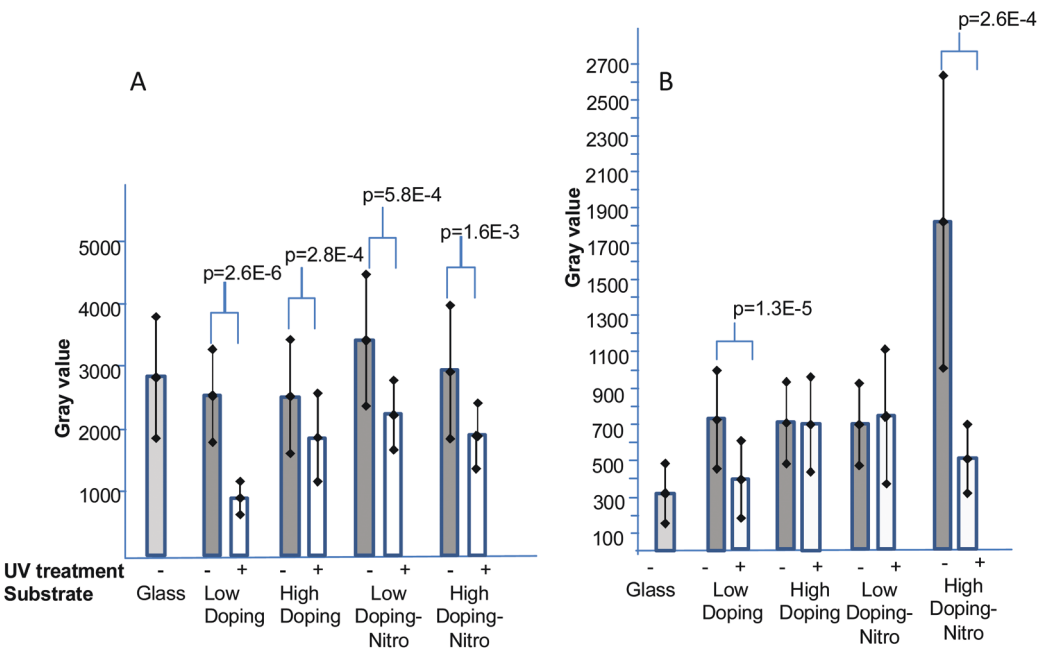


Fig. 4 (A) Dosimetry of the CFW/chitin signal; (B) Dosimetry of the $DiBac_4(3)$/membrane voltage.

indication of encoding an yeast behavioral change caused by the electronic properties of the nanostructured semiconductor surface due to photo-activation.

We also examined the membrane potential of the yeast plasma membrane using the anionic membrane potential-sensitive probe Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol (DiBAc4(3)).[40] Compared to glass, the membrane potential increased when yeast was exposed to the different GaN samples, Fig. 4B. DiBAc4(3) is an anionic dye and its intensity increases as the membrane potential becomes more positive.[41] Changes in membrane potential lead to altered fluxes of ions across the cell membrane. The observed modulation of the membrane potential of the yeast exposed to different GaN samples supports the notion that the semiconductor material can cause cell polarization.[42] The membrane potential results were variable when one compares samples before and after UV illumination. TBR1 yeasts demonstrated a significant reduction in DiBAc4(3) fluorescence when cultured on GaN surfaces with low doping that have been photo-activated (Fig. 4B, compare Fig. 7D and E). UV illumination caused no alteration to the DiBAc4(3) signal on clean high doping samples and on low doping samples functionalized with the nitro group. However, the modified high doping GaN substrate showed a trend similar to the low doping GaN substrate; *i.e.* a reduction of the DiBac4(3) signal, however the control signal from the non-photo-induced nitro-modified high doping GaN substrates is higher than all other conditions and may be a product of the high background signal from these samples. The variability of the membrane potential changes are not surprising since yeast cells polarization is very sensitive to external and internal cues.[43] Moreover, the membrane potential changes in yeast can be very localized as it has been demonstrated by optogenetics studies and experiments with application of external electrical fields in different directions.[42,44] Our results demonstrate that the heterogeneous nanostructured thin films can also encode a variable membrane potential change.

It is important to determine whether internal cellular processes can also be changed in yeast by exposure to photoactivated GaN. For this purpose we examined the mitochondrial membrane potential (MMP) in live yeast using MitoTracker Red CMX ROS dye, which labels mitochondria in a MMP dependent fashion.[45] Yeast cells incubated on the low and high doped UV illuminated GaN substrates demonstrated a significant reduction in MMP when compared to control substrates that were not exposed to light (Fig. 5). Reduction of MMP in yeast has been demonstrated to be controlled by extrinsic and intrinsic factors including increased levels of ROS as well as age and is known to initiate a complex intracellular signaling pathway, called the retrograde response.[46–48] In the context of this work we note that cell behavior such as clustering, changes to cell wall content and the membrane potential of the plasma membrane represent alterations to the external state/condition of the cell, but changes to the MMP within the cells demonstrate that the response by the yeast is biological when placed in contact with GaN that experiences PPC. The reduction of MMP has been demonstrated by others

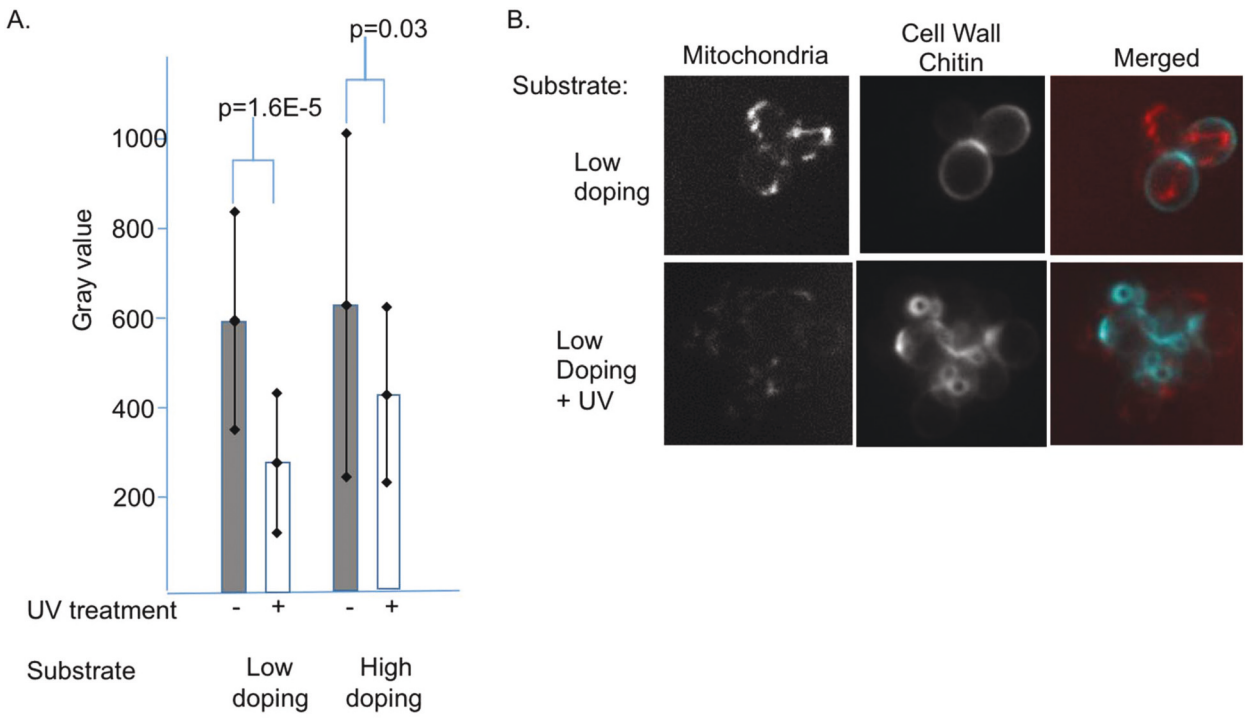


Fig. 5 PPC-induced reduction of yeast mitochondrial membrane potential. (A) Dosimetry of the Mitochondria/Mitotracker Red CMXRos signal. (B) Confocal micrographs of TBR1 yeast cells stained with CFW and Mitotracker Red CMXRos; top row low dope GaN substrate not exposed to UV; bottom row TBR1 yeast on GaN substrates exposed to UV; merged images, mitochondria (red) and cell wall chitin (blue).

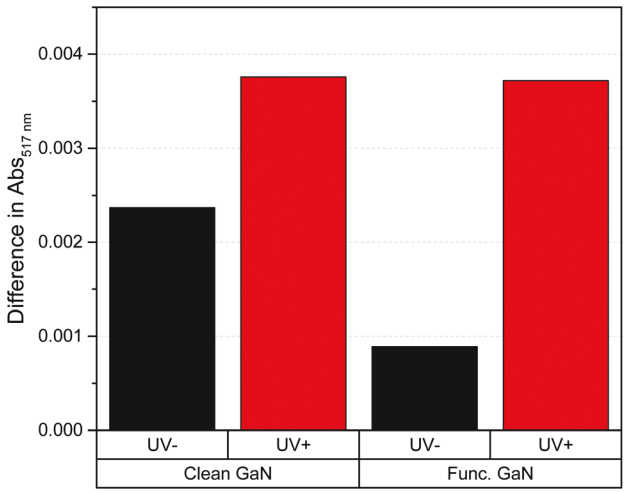


Fig. 6 Changes in the absorbance of C.I. Reactive Red 198 dye in the presence of clean and functionalized GaN before and after exposure to UV light.

to be the one response to excess reactive oxygen species.[49,50] However, the MMP we observed in this study in conjunction with the other phenotypes (*i.e.* clustering, change in cell wall content and plasma membrane polarization) is novel and suggests a unique cellular response.

We also gathered additional data to support our conclusion regarding which property of the surface triggered the cell responses we observed. We performed a dye quenching experiment to validate Scheme 1. Azo dyes are commonly used to detect hydroxyl radicals ($OH^{\bullet}$) and other reactive oxygen species such as $O_2^{\bullet -}$.[51] We used C.I. Reactive Red 198 dye ($C_{27}H_{18}ClN_7Na_4O_{15}S_5$) because of its characteristic λ_{max} at 517 nm.[52] We measured changes in the dye UV absorbance spectrum in the presence of different GaN samples, Fig. 6. The clean and modified GaN samples were immersed in the dye solution before and after UV illumination. All UV illumination was done outside of the solution with dry samples. The changes in absorbance were measured using a protocol reported by others.[53] We observed that we can decrease the dye absorbance in the presence of samples exposed to UV light. This experiment verified the presence of reactive oxygen species on the surface after the removal of UV light. The results are in agreement with the mechanism we propose in Scheme 1 and support the notion that the observed yeast behavior changes are encoded through the surface present radicals. The reactive oxygen species we generated on the photoactive semiconductor surface did not kill the yeast but were responsible for a very specific deviation from its regular behavior.

It is important to address the possibility of yeast physiological changes due to the production of reactive oxygen species (ROS) induced by metal ions leached by the semiconductor

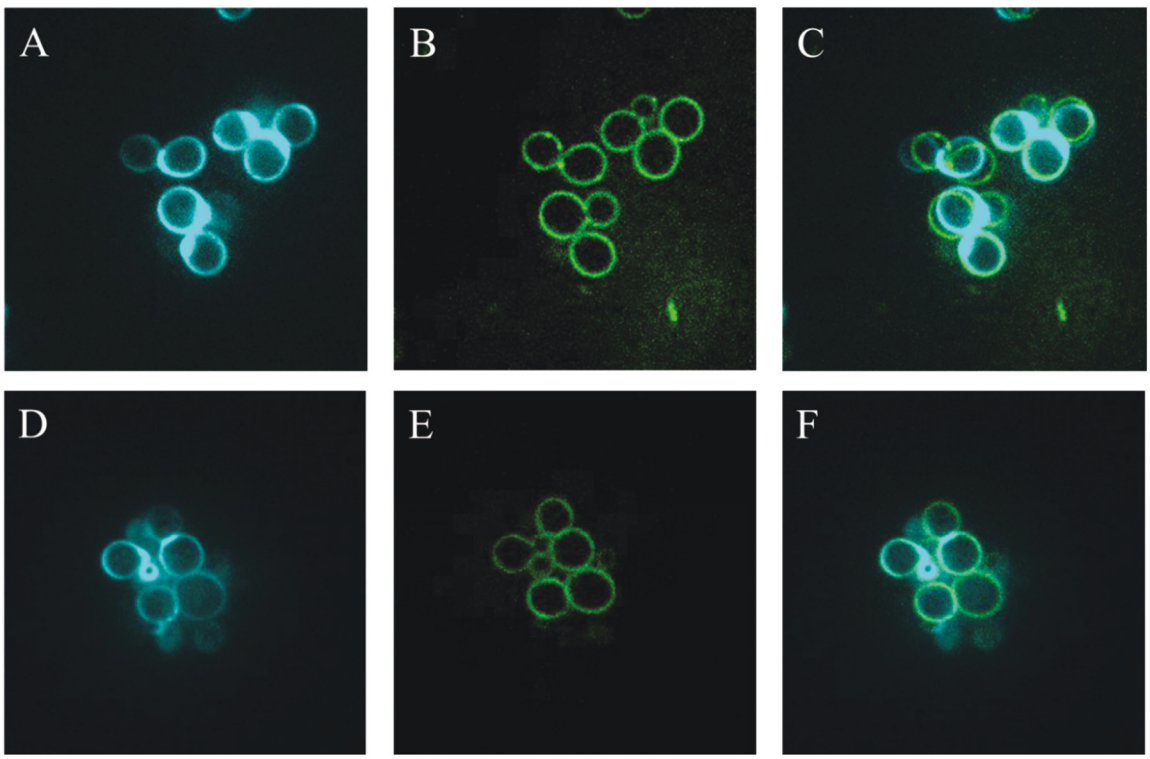


Fig. 7 *S. cerevisiae* TBR1 cells on GaN surfaces. (A–C) Clusters of TBR1 yeast cells that were cultured on GaN substrates with low doping and no UV treatment, two of the clusters have three cells and one has two. (A) CFW signal; (B) $DiBac_4(3)$ signal; (C) merge of the two signals. (D–F) One cluster containing five TBR1 yeast cells that were cultured on GaN substrates with low doping after UV treatment; (D) CFW signal; (E) $DiBac_4(3)$ signal; (F) merge of the two signals.

surface. A number of toxicity studies with nanostructured materials have established that elevated metal ions in solution can lead to variable responses and production of ROS which are dependent on specific cell type.[54] We have evaluated the release of gallium ions from our semiconductor thin films and have quantitatively confirmed that significant amount of gallium leaches only after days in contact with water solutions.[20,24,55] All cell experiments were done where yeast was exposed to the semiconductor thin films for 1 hour prior to any imaging and assay quantification. Literature reports also indicate that in the case of yeast, release of ions only slightly contributes to the recorded toxicity, compared to other factors that cause adverse changes such as cytoplasm leakage, crushed cell wall and irregular cell shapes.[56] We observed no evidence of such abnormalities in our microscopy studies, Fig. 7. The biological assays we performed demonstrated that the n-type Ga-polar GaN thin films can transduce external stimuli in the form of nanoscale topography and/or surface chemistry and charge into a cellular reaction associated with a physiological change. The nanostructured thin films can participate in bioelectronics communication because their morphological and electronic properties encoded the observed yeast response.

Conclusions

In summary, we described how one can integrate many of the unique properties of nanostructured n-type Ga-polar GaN thin films for the purpose of encoding regulatory responses in *S. cerevisiae* yeast. The number of cells and the size of the yeast clusters was dependent on surface chemistry and charge of the semiconductor surface. UV illumination of the thin films resulted in accumulation of surface charge that triggered a cell wall integrity pathway response which yielded a decrease in chitin. Compared to control experiments with glass surfaces the membrane voltage was also altered due to the presence of the semiconductor interface. In this work we focused on demonstrating that one can encode a response in yeast, which is a very well-studied model system. One needs to be able to decode a response in order to complete a communication. The responses we induced *via* the use of n-type Ga-polar GaN thin films are known to result in changes in signaling pathways which can be decoded by monitoring change in ion fluxes for instance. In future work we plan to explore the second part of the bioelectronics communication and pursue decoding using the nanostructured thin film rather than traditional fluorescence based biological assays.

Experimental methods

Semiconductor thin growth and characterization *via* photocurrent measurements, AFM and KPFM

All sample growth was done using metalorganic chemical vapor deposition and has been detailed in prior work.[15] A cleaning procedure was done for all semiconductor samples which included 20 min sonication in acetone and 20 min sonication in methanol and drying of the samples using compressed N_2 gas. Chemical functionalization of some samples was conducted after the cleaning procedure and a 100 °C soak in a 50/50 vol% H_2O/45% HCl for 10 min. After rinsing with DI water, the samples were soaked in a 50/50 vol% in a 3 mM of a 4-nitrobenzyl phosphonic acid ($C_7H_8NO_5P$)/97% phosphoric acid for 2.5 hours. The conditions for the photocurrent measurements and all microscopy using AFM and KPFM were identical to the ones reported in our recent publication.[20]

Yeast strains and culture

S. cerevisiae strains used in this study were TBR1(*MAT*α*ura3-52 leu2*::*hisG his3*::*hisG*).[33,34] Frozen stocks were maintained at −80 °C. For each experiment, colonies from freshly streaked YPD plates were used. Liquid YPD (Yeast extract, Peptone, Dextrose, Water) media was inoculated with one colony per 10 ml. Liquid cultures were grown with shaking ~ 200 rpm at 25 °C to an OD_{600} ~0.5 indicative of mid-log phase growth.[57] One culture was used to seed each surface for one hour prior to imaging.

Vital staining and confocal microscopy imaging of yeast on different GaN substrates

Yeast culture of the appropriate optical density (OD600 ~0.5) were cultured on GaN substrates for one hour. Twenty minutes prior to the end of this culture period dyes were loaded into the YPD culture media at the following final concentrations: CFW (Sigma),1 μg ml^{-1}; $DIBAC_4(3)$, 1.7 μM; Mitotracker Red CMXRos, 250nM (Invitrogen). After one hour, samples were washed twice in fresh YPD and then imaged in a petri dish. All images were collected using Zeiss Observer Z.01 spinning disc confocal with Axiovision software. All images were collected using the same exposure times and laser settings. Densitometry data was collected from individual frames using the Interactive Measurement application within Axiovision. Data for both the CFW and DiBac staining was collect on similar portions of the cell; specifically avoiding areas of cell–cell contacts, which have higher levels of both signals, and avoid bud scars for the same rationale. Densitometry data was statistically analyzed using Microsoft Excel software specifically the *t*-test function.

Conflicts of interest

There are no conflicts of interest to declare.

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