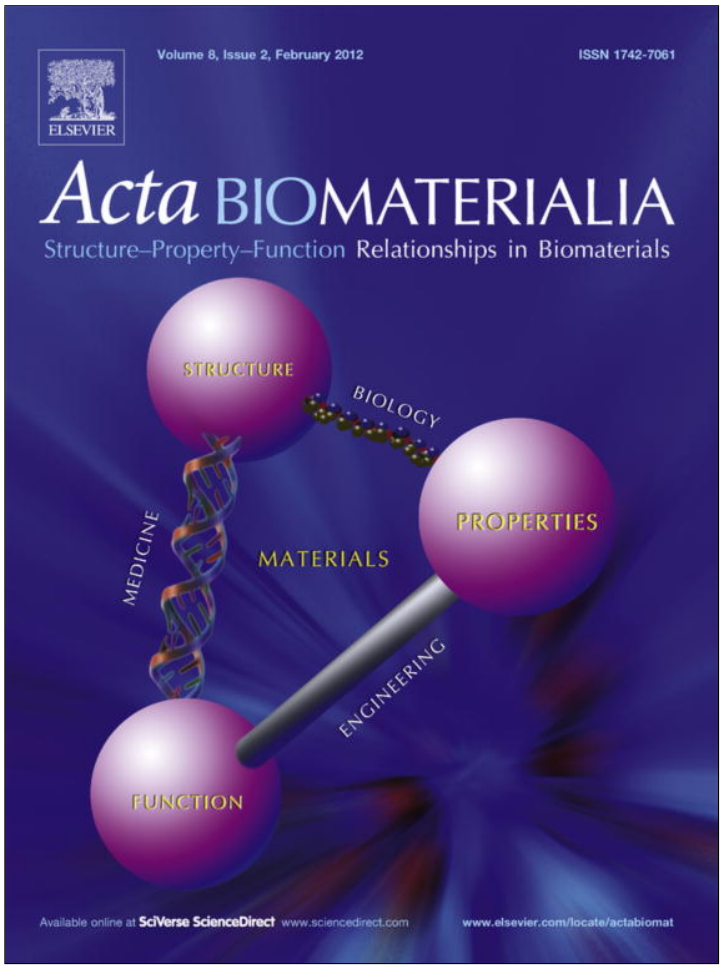

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Brief communication

Gallium nitride is biocompatible and non-toxic before and after functionalization with peptides

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ABSTRACT

The toxicity of semiconductor materials can significantly hinder their use for in vitro and in vivo applications. Gallium nitride (GaN) is a material with remarkable properties, including excellent chemical stability. This work demonstrated that functionalized and etched GaN surfaces were stable in aqueous environments and leached a negligible amount of Ga in solution even in the presence of hydrogen peroxide. Also, GaN surfaces in cell culture did not interfere with nearby cell growth, and etched GaN promoted the adhesion of cells compared to etched silicon surfaces. A model peptide, "IKVAV", covalently attached to GaN and silicon surfaces increased the adhesion of PC12 cells. Peptide terminated GaN promoted greater cell spreading and extension of neurites. The results suggest that peptide modified GaN is a biocompatible and non-toxic material that can be used to probe chemical and electrical stimuli associated with neural interfaces.

1. Introduction

Identifying interfaces that are suitable to probe neuronal processes is of interest to chemists, biologists, engineers and clinicians [1]. Neurons are highly specialized cells, with properties and functions governed by both chemical and electrical mechanisms. Many groups have explored questions associated with what kinds of chemical cues are responsible for processes such as growth, apoptosis and regeneration [2]. The literature is equally rich in studies centered on fundamental and applied aspects of using electrical stimuli to probe and guide neuronal function [3]. Reviewing this field, one can identify the need for a material that will simultaneously probe chemical and electrical mechanisms associated with neuron function. Limited evidence suggests that gallium nitride (GaN) is an ideal material for this purpose. Young and Chen [4] demonstrated that rat cerebellar granule neurons cultured for 7 days resulted in greater cellular growth on GaN than on silicon or tissue culture polystyrene. GaN has also been used to culture aging granule cells and neural stem/precursor cells [5,6]. Additional advantages of GaN include a wide bandgap of 3.4 eV [7], availability of Ga bonds for covalent surface modifications [8], biocompatibility [9], chemical stability [10] and low electrical drift of GaN biosensors in ionic solutions [11]. However, there are no published studies that have examined the biocompatibility and/or toxicity of this material after chemical functionalization, which can significantly increase its utility.

Surface functionalization is important in tailoring surface properties for a desired application. One can significantly change the electronic properties of GaN by chemisorption or physisorption of molecular species to the surface due to this material's high spontaneous polarization and piezoelectric constants [10]. Chemisorption on GaN of the following adsorbates has been reported: ammonia, octanethiol, water, aniline, 3-pyrroline and pyridine [12–19]. In addition, photochemical functionalization has been used to modify GaN [20], and phosphonic acid linkages have been successfully attached to oxidized GaN [21]. Recently, we reported functionalization of GaN with organic molecules using olefin metathesis [22]. This versatile route has also enabled us to covalently attach peptides to GaN [23]. The placement of peptides on surfaces has been extensively utilized to guide the behavior of cells [24]. In this study we use a well-studied peptide, the IKVAV sequence derived from laminin [25], which has been shown to have a high affinity for PC12 cells and to promote cellular adhesion to surfaces [26]. The PC12 cell line was chosen for this study due to its common usage in modeling neural

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differentiation [27]. We report the remarkable lack of toxicity and excellent biocompatibility of GaN before and after functionalization with a peptide.

2. Materials and methods

2.1. Materials

Ammonium fluoride, phosphorus pentachloride, benzoyl peroxide, chlorobenzene, 2 M allylmagnesium chloride in tetrahydrofuran (THF), Grubbs first generation catalyst, 6-heptenoic acid, 5-hexen-1-ol, dimethyl sulfoxide, phosphate-buffered saline (PBS), horse serum, bovine serum and penicillin/streptomycin were purchased from Sigma–Aldrich. Dichloromethane was purchased from Mallinckrodt. Dulbecco's modified Eagle's medium (DMEM) powder and murine 2.5S nerve growth factor (NGF) were obtained from Invitrogen. *p*-Maleimidophenyl isocyanate (PMPI) was purchased from Thermo Scientific. The peptide of sequence CSRARKQAASIKVAVSADR-NH_2 (IKVAV) was purchased from Anaspec. Note that the peptide has an amine group added to the C terminus.

2.2. Surface functionalization

All experiments described here were performed with GaN films grown on sapphire substrates by hydride vapor-phase epitaxy. The films were unintentionally doped with typical residual n-type carrier concentrations in the low 10^{16} cm^{-3} range and exhibited a smooth surface morphology. The surface functionalization of GaN using a chlorination step, followed by terminating with an alkene group and binding an alkene-terminated molecule through olefin cross-metathesis proceeded as discussed previously [22], and was based on a scheme performed on silicon [28,29].

The GaN wafer was diced into 3 mm × 3 mm squares with a Disco DAD-2H/6 dicing saw. These wafers were rinsed sequentially with acetone, ethanol and deionized (DI) water. The GaN wafers were next cleaned in a piranha solution of 3:1 concentrated H_2SO_4 to 30% H_2O_2 for 10 min. The native surface oxide on the GaN surfaces was removed by a 5–6.5 min etch in HCl at room temperature. Surfaces were hydrogen terminated by a 15 min soak in 40% NH_4F. Silicon surfaces were prepared similarly, but with a 15 s etch in 48% HF to remove the native oxide.

For the chlorination, alkene termination and olefin cross-metathesis surface functionalization procedures, the GaN or Si wafers were loaded into a fluidic system that maintained a continuous flow of reagents across the wafer surface. Briefly, the system was enclosed in a nitrogen-purged glovebox, was composed of polyetheretherketone, had a heater affixed to the bottom and was connected to a syringe pump to cycle the reagents through the system. Further details of this fluidic system are provided elsewhere [22,23]. GaN and Si wafer surfaces were terminated with chlorine by a saturated solution of PCl_5 in chlorobenzene with the addition of several grains of benzoyl peroxide. The solution was pumped over the wafer surfaces and the solution temperature was maintained at 90 °C for 30 min. While the wafers and fluidic system cooled, as-received chlorobenzene was pumped over the wafer surfaces for 30 min to remove any unbound PCl_5. The wafers were then rinsed with ethanol and dried with N_2 gas. Alkene termination of the GaN and Si wafer surfaces proceeded by pumping 2 M allylmagnesium chloride in THF over the wafer surfaces with the temperature maintained at 60 °C for 30 min. The wafers were soaked in THF overnight to remove any precipitate that had spotted the surfaces during the reaction. Following the overnight soak, the wafers were rinsed with ethanol and dried with N_2 gas. 5-Hexen-1-ol was bound to the GaN and Si wafer surfaces through olefin cross-metathesis to terminate the surfaces with a hydroxyl group. The surfaces were primed with Grubbs first generation catalyst by pumping a 13.3 mM solution of the catalyst in dichloromethane over the surfaces for 30 min while maintaining a temperature of 40 °C. The wafers were rinsed with as-received dichloromethane to remove unbound catalyst. A 0.1 M solution of 5-hexen-1-ol in dichloromethane was then pumped across the wafer surfaces and maintained at 40 °C for 120 min. As-received dichloromethane was pumped across the surfaces for 30 min to remove unbound reagents. The wafers were rinsed with ethanol and dried with N_2 gas.

To promote PC12 cell adhesion, the hydroxyl-terminated surfaces were functionalized with the IKVAV peptide. This peptide has a single cysteine residue that is located at the N-terminus. PMPI is a heterobifunctional crosslinker that couples hydroxyls to thiols [30]. PMPI was used to link the peptide at the thiol in the cysteine residue to the terminal hydroxyl on the GaN or Si surface. The hydroxyl-terminated wafers were placed in microcentrifuge tubes filled with 2 mM PMPI in dimethyl sulfoxide for 2.5 h, situated on a rocking platform for continuous agitation. The wafers were rinsed with DI water and dried with N_2 gas. Next, 10 μl of a 0.1 mM solution of IKVAV peptide in 10 mM PBS was spotted onto each wafer and placed in a humidified chamber that was kept in a refrigerator for about 5.5 h. Following incubation with the peptide, the wafers were rinsed with PBS and dried with N_2 gas.

2.3. Cell culture

The PC12 cell culture medium consisted of DMEM containing 12.5% horse serum, 2.5% bovine serum and 1% penicillin/streptomycin, and cells were maintained in this at 37° C in 5% CO_2. The substrates for the cell culture experiments consisted of HCl-etched GaN, HF-etched silicon, IKVAV-functionalized GaN and IKVAV-functionalized silicon. Prior to culture, all substrates were sterilized in UV light for 30 min. Previous studies suggest that exposure to a 400 μW UV lamp for $\geqslant$1 h induces damage to proteins in a hydrogel matrix [31]. However, in this experiment, both the power (100 μW) and the duration (30 min) of the UV exposure were lower, making it unlikely that UV exposure had a major detrimental effect on peptide structure or function. Three substrates of each type were then placed in collagen-coated 24-well plates, with one substrate per well. One milliliter of PC12 suspension containing 4×10^4 cells ml^{-1} was added to each well, and the cells were allowed to adhere for 10 h. The medium was then replaced with DMEM containing 1% horse serum and 50 ng ml^{-1} NGF. This cell medium was replaced with fresh medium containing NGF on day 3. Before imaging, substrates were rinsed twice by immersing in fresh cell medium to remove any loosely bound cells. Substrates were imaged after 1, 3 and 6 days of NGF stimulation with an Olympus BX51 microscope using ×5, ×10 and ×50 objectives. Cells were quantified by counting the number of cells per image with the ×10 objective at a minimum of four random spots on each substrate. At times, the PC12 cells formed tightly packed clusters on the surfaces, making quantification difficult in certain images. When this occurred, the size of the cluster was used to approximate the number of PC12 cells contained within it. The number of cells was counted three separate times for each image, then averaged to obtain the final cell count.

2.4. Atomic force microscopy

Root-mean-square (RMS) roughness data was collected on a Multimode Nanoscope IIIa scanning probe microscope (Veeco, Plainview, NY, USA). A total of 5×5 μm^2 images at a resolution of 512 × 512 pixels were collected in tapping mode at a scan rate of 2 Hz. Roughness measurements were collected on two wafers from each surface treatment. The mean roughness of five images on each wafer was used to calculate the mean and standard deviation of the roughness for each treatment.

Table 1
Ga concentration measured by ICP-MS following soaking of GaN wafers in 1.5 ml solutions for 7 days.

Solution	Gallium concentration[a] (ppb)
DI water	3.68 ± 0.54
10% H_2O_2 in saline	3.52 ± 1.65
0.1 M sodium acetate pH 5	4.10 ± 2.52
0.1 M Tris base pH 9	45.19 ± 31.41

[a] Three replicates were tested for each solution. Means and standard deviations are shown.

3. Results and discussion

Lack of stability of III–V semiconductor surfaces under ambient or aqueous conditions can result in many limitations [32]. For example, GaAs is notoriously hard to work with under aqueous conditions, and the prevention of the release of gallium and arsenic oxides in aqueous solutions has proven difficult [33]. The stability of another III–V material, GaP, has been reported by Linsmeier et al. [34], and their findings support the notion that uncoated GaP is toxic and not biocompatible. Kim and co-workers [21] assessed the stability of organic monolayers on oxidized GaN by X-ray photoelectron spectroscopy, but to the best of our knowledge no one has previously measured the amount of Ga released from this semiconductor surface in various aqueous solutions.

Here we performed a rigorous aqueous stability study by soaking 3×3 mm^2 GaN wafers for 7 days in 1.5 ml of DI water, 10% H_2O_2 in physiological saline, a pH 5 solution of 0.1 M sodium acetate or a pH 9 solution of 0.1 M Tris base. Hydrogen peroxide was added to saline solutions to mimic the in vivo conditions when macrophages release H_2O_2 during the oxidative burst [34]. The gallium concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS). The results summarized in Table 1 show that GaN is remarkably robust in aqueous solutions. Our findings indicate that the mean gallium concentration after soaking the GaN wafers in DI water, 10% H_2O_2 in saline and 0.1 M sodium acetate is less than 5 ppb. For comparison, Pozo et al. [35] has shown that the gallium concentration in drinking water is <1 ppb. The pH 5 and pH 9 solutions gave a wide pH window around the physiological pH of 7.4. Even at pH 9, the average gallium concentration was less than 50 ppb. Because gallium nitrate is approved by the FDA, there have been considerable efforts to understand the toxicity of gallium; these were recently reviewed by Chitambar [36]. Some data on gallium toxicity is conflicting and therefore it is difficult to quantify our findings in terms of possible clinical toxicity of GaN surfaces. Note that the supporting information gives additional data on comparing the stabilities of functionalized and bare GaN.

In this study, we used the PC12 cell line to assess the toxicity and biocompatibility of GaN substrates vs. silicon substrates. As seen in Fig. 1, for the 1, 3 and 6 day treatments, the etched GaN surfaces had substantially more cells present compared to etched silicon surfaces. These results agree with previous studies suggesting that cells grow better on GaN than on silicon surfaces [4]. On etched silicon surfaces there is a steady decline in the number of cells from 1 to 6 days, which we attribute to the cells detaching from the surface because of weak adherence. PC12 cells are notorious for their inability to adhere to untreated surfaces, including glass and standard well plates [37]. This is in agreement with the low number of cells seen absorbed to silicon, which, with a thin surface oxide, has a similar chemical composition to glass. This trend is also seen between days 1 and 3 for GaN, but seems to level out after thereafter, which suggests that the PC12 cells remain adhered to etched GaN.

The PC12 cells on GaN appeared viable and were able to form neurites in the presence of NGF, as seen in Fig. 2. NGF induces differentiation and neurite growth in PC12 cells [38]. We have previously confirmed that PC12 cells on GaN show little or no differentiation without NGF. Interestingly, the few cells that were attached to bare silicon substrates were not healthy, as evidenced by the blebbing that occurred in a large percentage of cells (Fig. 2B). Furthermore, all PC12 cells on etched silicon exhibited no evidence of morphological differentiation since there were very few neurite-producing cells. On the other hand, cells on GaN were morphologically fully differentiated because they exhibited processes with length greater than the diameter of their cell bodies. We note that the 3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide assay commonly used to estimate the number of live cells was unable to distinguish differences in cell viability between materials or surface functionalizations. It is also important to note that upon exposure to an aqueous environment the semiconductor surfaces will tend to form an oxide layer. In the case of GaN and silicon, the degree of oxidation will differ. Others have shown that biomolecules clearly bind differently to planar surfaces based on the degree of surface oxidation [39]. Differential physisorption of biomolecules present in the cell culture media may contribute to differences in cellular adhesion across surfaces.

To increase PC12 cell adhesion, the hydroxyl-terminated surfaces were functionalized with the IKVAV peptide (Scheme 1). This peptide has a single cysteine residue that is located at the N-terminus. PMPI was used to link the peptide at the thiol in the cysteine residue to the terminal hydroxyl on the GaN or Si surface. It is remarkable that PC12 cells adhere well to etched GaN, but certain

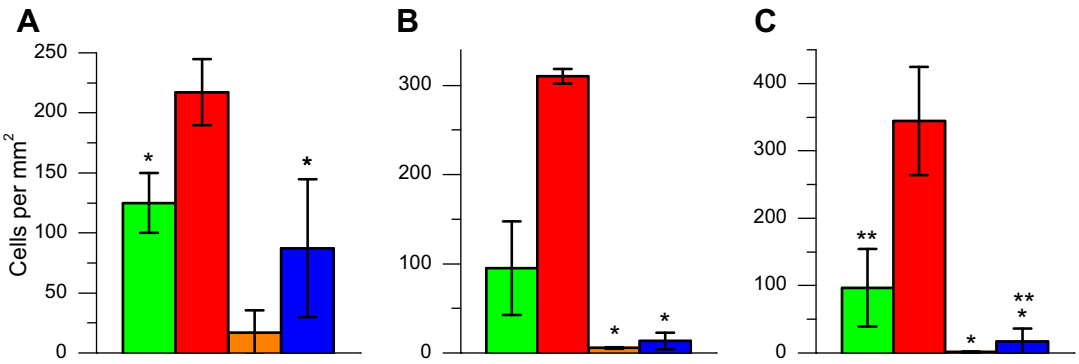


Fig. 1. PC12 cells present on GaN and silicon surfaces at 1 (left side), 3 (middle) and 6 (right side) days with NGF treatments. The bars on each graph represent etched GaN (green), IKVAV-functionalized GaN (red), etched silicon (orange) and IKVAV-functionalized silicon (blue). Symbols represent no statistical difference between groups (analysis of variance, least squares difference, $\alpha = 0.05$).

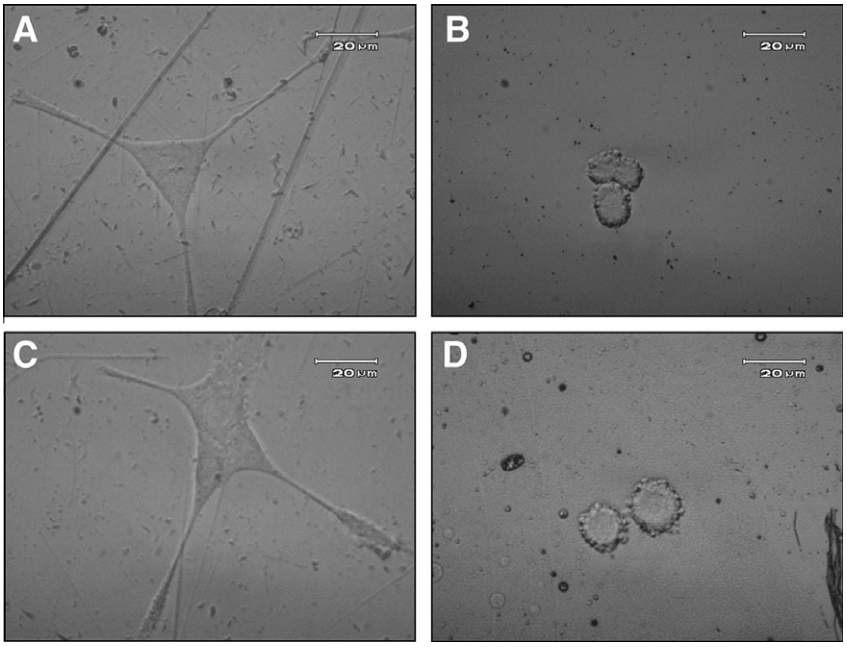
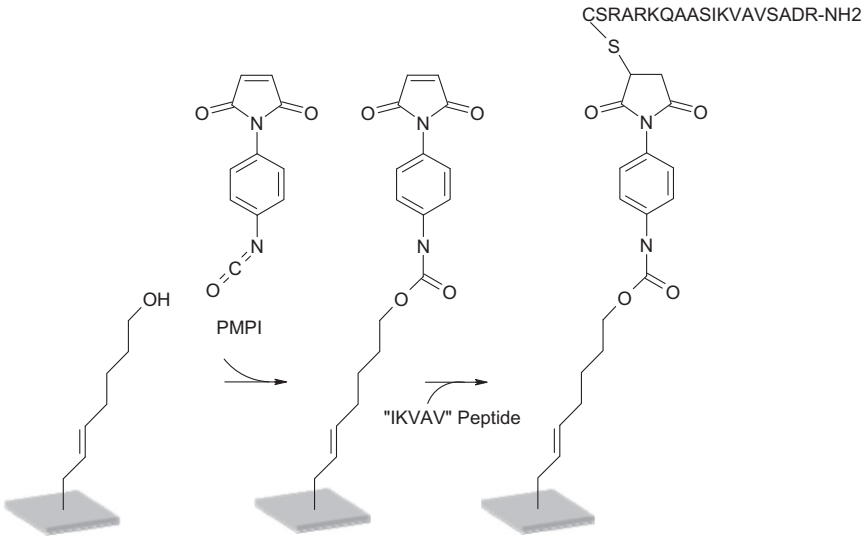


Fig. 2. Representative images of PC12 cells on (A) etched GaN, (B) etched silicon, (C) IKVAV-terminated GaN and (D) IKVAV-terminated silicon. All images shown were taken after 3 days of NGF treatment.



Scheme 1. Binding the IKVAV peptide to the hydroxyl-terminated surface with the PMPI crosslinker.

applications may require a higher density of cells on the substrate. The laminin-derived IKVAV peptide has been shown to have a high affinity for PC12 cells, promote differentiation in two- and three-dimensional matrices [27], and promote adhesion [40]. As seen in Fig. 1, the presence of the peptide increased cellular adhesion on both GaN and silicon for the 1, 3 and 6 day treatments. In general, the number of cells for each substrate is similar for each day, with slight decreases that are attributable to the detachment of cells over time. As shown previously using X-ray photoelectron spectroscopy, peptide density plays an important role in the adhesion of cells onto planar surfaces [41]. This study did not assess peptide density. However, we have previously shown successful peptide functionalization on GaN using olefin cross-metathesis to bind a linker molecule with peptide coverage of around 0.5–1 monolayers [23].

Atomic force microscopy was used to determine surface roughness for each surface treatment. The literature has shown that increased surface roughness at the nanometer scale can improve cellular adhesion [42]. The means and standard deviations of the RMS surface roughness are 0.99 ± 0.10, 0.68 ± 0.03, 5.32 ± 1.32 and 21.74 ± 1.67 nm for etched GaN, etched Si, IKVAV-terminated GaN and IKVAV-terminated Si, respectively. While this data does not rule out the contribution of increased surface roughness to greater cellular adhesion, the contribution is likely much lower for the IKVAV-terminated GaN than for the IKVAV-terminated Si due to the smaller change in surface roughness between GaN treatments. We conclude that the increased roughness on the IKVAV-terminated GaN has a minimal effect on cell adhesion.

There is also evidence that the IKVAV peptide can improve the differentiation of PC12 cells by increasing the number and length

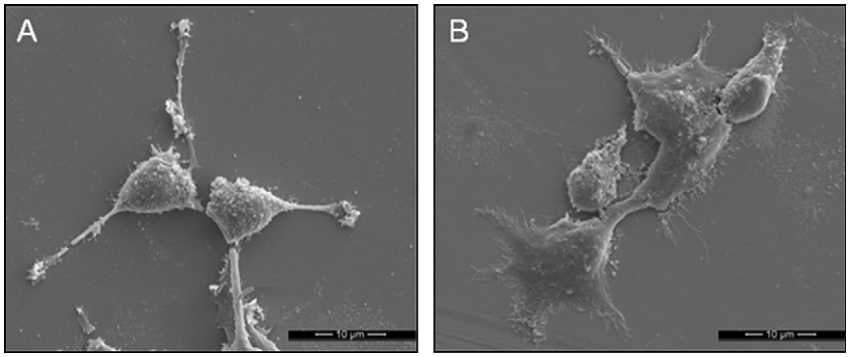


Fig. 3. Representative SEM images of PC12 cells on (A) etched GaN and (B) IKVAV-terminated GaN. The images were taken after 6 days of NGF treatment.

of neurites [27]. We were able to observe neurites on the substrates using light microscopy (Fig. 2). Qualitatively, there were obvious neurite-producing cells on both the etched and IKVAV-terminated GaN surfaces, in drastic contrast to the etched and IKVAV-terminated silicon surfaces. However, with light microscopy, it was difficult to determine if there were any morphological differences between IKVAV-terminated and bare GaN substrates. Despite the presence of the IKVAV peptide, the majority of the cells present on the IKVAV-terminated silicon surface had blebbing and did not produce neurites (Fig. 2D).

High-resolution scanning electron microscopy (SEM) was used to contrast neurite extension on etched and IVVAV-terminated GaN. We note that it was difficult to locate any cells on the etched and IKVAV-terminated silicon surfaces during SEM imaging. The few cells that were found on silicon were circular, and no evidence of neurite extensions was seen (see Supplementary data for additional SEM images). The SEM data showed that on the IKVAV-terminated GaN surfaces the degree of cell spreading was greater than on the etched GaN (Fig. 3). On the etched GaN surfaces we observed long neurite extensions (Fig. 3A), similar to the behavior of PC12 cells grown on collagen-coated tissue culture plates [43]. On the IKVAV-terminated GaN surfaces, the cells appeared flatter and had a greater density of microvilli extending from the cell bodies and neurites (Fig. 3B). This type of behavior is similar to the behavior of PC12 cells on cellulosic filters, with extensive anchoring of the PC12 cells to the substrate by neurite extensions and microvilli [44].

In summary, the SEM imaging revealed that the PC12 cells on IKVAV-terminated GaN exhibited a flattening of the cell body and increased microvilli growth that likely improved cellular adhesion, as expected from this laminin-derived peptide sequence, in comparison to etched GaN or silicon.

4. Conclusion

This work has demonstrated that GaN is remarkably stable in aqueous solutions, and a covalently bound peptide increased cell adhesion and spreading. The Ga levels detected by ICP-MS in aqueous solutions were less than 50 ppb. The increased neurite outgrowth on peptide terminated GaN can be used in future studies to develop better network formation assays [45].

Acknowledgements

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figure 1, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.09.038.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2011.09.038.

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