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Enhanced proliferation of PC12 neural cells on untreated, nanotextured glass coverslips

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Abstract

Traumatic injury to the central nervous system is a significant health problem. There is no effective treatment available partly because of the complexity of the system. Implementation of multifunctional micro- and nano-device based combinatorial therapeutics can provide biocompatible and tunable approaches to perform on-demand release of specific drugs. This can help the damaged cells to improve neuronal survival, regeneration of axons, and their reconnection to appropriate targets. Nano-topological features induced rapid cell growth is especially important towards the design of effective platforms to facilitate damaged neural circuit reconstruction. In this study, for the first time, feasibility of neuron-like PC12 cell growth on untreated and easy to prepare nanotextured surfaces has been carried out. The PC12 neuron-like cells were cultured on micro reactive ion etched nanotextured glass coverslips. The effect of nanotextured topology as physical cue for the growth of PC12 cells was observed exclusively, eliminating the possible influence(s) of the enhanced concentration of coated materials on the surface. The cell density was observed to increase by almost 200% on nanotextured coverslips compared to plain coverslips. The morphology study indicated that PC12 cell attachment and growth on the nanotextured substrates did not launch any apoptotic machinery of the cell. Less than 5% cells deformed and depicted condensed nuclei with apoptotic bodies on nanotextured surfaces which is typical for the normal cell handling and culture. Enhanced PC12 cell proliferation by such novel and easy to prepare substrates is not only attractive for neurite outgrowth and guidance, but may be used to increase the affinity of similar cancerous cells (ex: B35 neuroblastoma) and rapid proliferation thereafter—towards the development of combinatorial theranostics to diagnose and treat aggressive cancers like neuroblastoma.

Keywords: PC12, enhanced cell growth, reactive ion etching, nanotextured coverslip, atomic force microscopy, central nervous system healing

(Some figures may appear in colour only in the online journal)

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1. Introduction

Traumatic injury to the central nervous system (CNS) is a significant health problem with injuries to the spinal cord and brain accounting for approximately 265 000 and 1.5 million new injuries each year, respectively [1]. Regeneration of axons, guided by mechanical, electrical, magnetic, chemical and/or physical cues has been explored for neural circuit reconstruction following CNS related injuries [2–5]. The functional recovery from CNS injury requires improved neuronal survival, regeneration of axons, and their reconnection to appropriate targets [6]. However, the efficacies of the existing strategies are limited since the CNS is refractory to axonal regeneration and relatively inaccessible to many pharmacological treatments [1]. For example, to promote the regeneration of axons, inhibitory molecules need to be destroyed to create a growth-permissive environment or the response of axons contacting these inhibitors must be decreased [7], which requires superfine precision in the intracellular environment. Contemporary treatment strategies used for CNS related injury include surgical, thermal, and pharmacological interventions largely targeted at decreasing neuronal loss and the inflammatory response initiated after acute injury. Stem cell therapies provide fascinating alternatives for treating chronic CNS injuries and in improving nervous system regeneration [1]. However, the particular cells used in the pre-clinical studies are oligodendrocyte precursors [8]. Pre-clinical studies using undifferentiated stem cells indicate that these cells do not form neurons in the CNS and may lead to the development of tumors [9, 10]. Considering the complexity of CNS injuries and recovery responses, the development of novel combinatorial therapeutic strategies that trigger axon regeneration and facilitate connectivity need to be explored [11].

Recent progress in nanoscale engineering offers exciting alternatives for designing biocompatible micro- and nano-vectors for controlled release of therapeutic and diagnostic agents to targeted cells to improve treatment efficacy and evaluation [1, 12]. It has been observed that cell capture, growth, adhesion, translocation behavior, and orientation are influenced by nanoscale topography of the substrates [13–19]. In the areas of tissue engineering, recent investigations have reported that nanostructured scaffolds can significantly increase densities of certain cells [20, 21], and the nano-topological features have various impacts on cell functions [21–27] by offering biomimetic cell-stimulating cues. It has been also observed that the basement membranes of most tissues are composed of complex mixtures of nanoscale structures [27]. Cells sense nanotopography and react by bridging or conforming in a selective manner. Moreover, nanoscale patterning significantly impacts the organization and type of focal adhesions either by disrupting their formation or by inducing specific integrin recruitment [21, 27]. Since integrins are directly linked to the nuclei, gene expression is indeed affected due to the cellular response to nano-topological features.

Despite being an attractive subject of investigation, interactions between neurons or neuron-like cells and nano-textured features have remained largely unexplored thus far. Some recent studies have observed promising outcomes on cell behavior and neurite outgrowth [28–32]. It has been observed that neuronal network morphology appears to be more preferential on the nitrogen rich titanium nitride (TiN) films and also with reduced nano-topographical features [28]. Nano-grooved surface patterns [29], and nanotextured gallium nitride (GaN) surfaces [30] have been reported to enhance adhesion/growth/orientation of PC 12 neuron model cells. Guided neurite outgrowth is also possible by meticulously designed nano-patterned substrates [2, 32], which minimizes the complications of confinement and offer an efficient model to investigate the underlying mechanisms of topological neurite guidance. It has been observed that the overexpression or down-regulation of specific biomolecules in nano-patterned regions may be inducing and directing the neurite growth [2].

Although the aforementioned reports describing neuron or neuron-like cell response to nano-topological features are indeed promising, choice of substrate materials, coating procedures (for example, silicon wafers [28], TiN film [27]) and lack of biocompatibility (e.g. GaN nanowires) add substantial complexities for potential biomedical applications. Although neuronal alignment on glass substrates has been reported [32], these substrates were modified with polymer deposition, and further poly-D-lysine coating. This paper presents neuron-like cell growth on un-treated, easy to prepare nanotextured surfaces. In this work, the bare glass coverslips were selected as the substrate material. These are extensively used for cell culture related biomedical applications and are common substrates in fluorescence/confocal/light microscopy. The high light transmission capability also makes these attractive for applications requiring optical irradiation, an option which is rapidly gaining traction in the area of neuro-science research. Thus, the PC12 neuron-like cells were cultured on untreated and nanotextured coverslips, and for the first time, the effect of nanoscale random topology was observed exclusively as physical cue for the growth of PC12 cells. This eliminated the possible influences, which may originate from the enhanced concentration of proteins and materials from the media on the nanotextured surface, polymer surface chain bonds, surface free energy changes, etc. In this work, it is established that micro reactive ion etched (micro-RIE) glass coverslips enhanced the PC12 cell proliferation substantially, when compared to plain coverslips. The enhancements were even higher than the ECM treated cell culture substrates. Enhanced PC12 cell proliferation is especially promising, since this further bolsters the potential of neurite outgrowth and connectivity. The PC12 cells provide an excellent neuronal model. These results are major advancement towards the design of an effective nano-platform to achieve enhanced and controlled neural cell growth and differentiation, which will be beneficial to treat various neuro-degenerative disorders.

2. Materials and methods

2.1. Preparation of nanotextured glass substrates

The cover glasses were cleaned with isopropyl alcohol (IPA), rinsed in deionized (DI) water and dried in nitrogen. A reactive ion etching (RIE) system (Technics Micro-RIE Series 800 Plasma System) was used to create nanotexture on the substrates. The etching was performed using the mixture of oxygen (O_2) and carbon tetrafluoride (CF_4) for 45 min (10 sccm O_2 and CF_4, 250 Watt, 250 mTorr). After etching, each substrate was cleaned in sonicated IPA followed by cleaning in piranha solution (H_2O_2:H_2SO_4, 1:3) and DI water.

2.2. Surface characterization using atomic force microscope (AFM)

Surface texture of the coverslips was evaluated qualitatively, and quantitatively with a Dimension 5000 AFM. The root mean square surface roughness was measured for both plain and nanotextured coverslips. Micrographs of the samples were captured in the ambient air with 15%–20% humidity at a tapping frequency of approximately 300 kHz. The field measured was 5 μm × 5 μm at a scan rate of 1 Hz with 256 scan lines.

2.3. Scanning electron microscope (SEM) and elemental analysis

SEM was also used to examine surface texture. The samples were coated with 5 nm of gold to avoid charging before taking SEM micrographs. The micrographs were taken at 12 kV accelerating voltage and 30 μm aperture. Energy dispersive x-ray spectroscopy (EDS) was also done to identify and quantify the surface elements of plain and nanotextured coverslips. An EDS detector (EDAX, Genesis) was attached to the SEM. The SEM was set at a 15 mm working distance with 20 kV accelerating voltage and data were recorded followed by mapping analysis.

2.4. Contact angle measurements

Contact angles for plain and nanotextured coverslips were measured using a contact angle goniometer (NRL-100; Rame-Hart, Washington, DC). A 10 μl water droplet was placed on the sample and the contact angle of the water-substrate interface was recorded by visual observation through a microscope. On average, 5 measurements were taken for each substrate.

2.5. Cell culture and treatment

PC12 cells, which are derived from rat pheochromocytoma, were purchased from ATCC (Manassas, VA). These were cultured at 37 °C in 5% CO_2 in F-12 nutrient mixture with Kaighn's modification (F12K) containing 2.5% fetal bovine serum and 15% horse serum (both from Invitrogen, Carlsbad, CA). A hemocytometer was used to count cells in the stock solution and volumes of the seeding solutions (5000 cells cm^{-2}) were calculated. To obtain a homogeneous suspension, cells were micro pipetted several times before transfer. The cells were then plated on the plain coverslips, nanotextured coverslips and tissue culture treated membranes. These three samples were placed in 24-well tissue culture plates (TCPs). The cells were allowed to grow for 48 h for surface attachment. All the experiments were performed in triplicates.

After 48 h of cell growth and attachment, cultures were washed twice with PBS and placed into serum-free F-12K with or without 100 ng ml^{-1} nerve growth factor β subunit (β-NGF, Sigma-Aldrich, St. Louis, MO), added daily for 96 h. Cultures were fixed in 4% paraformaldehyde and 2% gluteraldehyde in PBS and washed twice in PBS. For cell density assessment, bright-field images (4–5 images/condition/experiment) were captured through a 10× objective on a Vee Gee Vanguard 1491 INi inverted microscope. Nuclear morphology was assessed using confocal images captured through a 64× objective from cells labeled with 4′,6-diamidino-2-phenylindole (DAPI, Ex = 405 nm, Em = 450/35 nm), following treatment containing normal and nanotextured glass coverslips and tissue culture treated membranes in 24-well TCPs as growth surfaces.

3. Results and discussions

3.1. Surface topography of nanotextured substrates

Micro-RIE has been reported to create uniform nanotextured glass and polymer substrates previously [17–19]. Micro-RIE also provided reasonably uniform nanotexture on glass coverslips as well. The measured average roughness of plain and nanotextured coverslips were 11.34 ± 2.25 nm and 105.06 ± 17.87 nm, respectively. The AFM micrographs of plain and nanotextured surfaces are shown in figure 1.

3.2. Elemental analysis and compositional mapping

AFM provided both qualitative and quantitative information of the roughness of the surfaces. SEM was used for qualitative assessment of nanotextured glass coverslips. The SEM micrograph of nanotextured coverslip is shown in figure 2(a). EDS was performed on the sample surfaces to observe the compositional mapping [17, 18]. This analysis showed that there was no chemical effect of RIE on elemental composition of coverslips. The change in elemental composition of glass coverslips could have affected the growth of cells. EDS elemental analysis of plain coverslips showed the predominant presence of oxygen and silicon (figure 2(b)) which was identical to nanotextured coverslips figure 2(c)). Therefore, etching did not make any change in the chemical nature of coverslips.

3.3. Contact angle measurement analysis

Contact angle from a water droplet provides the measurement of hydrophobicity or hydrophilicity of a surface [17, 18]. The hydrophilicity of the substrate is necessary to obtain better attachment and proliferation of cells. It is known that contact

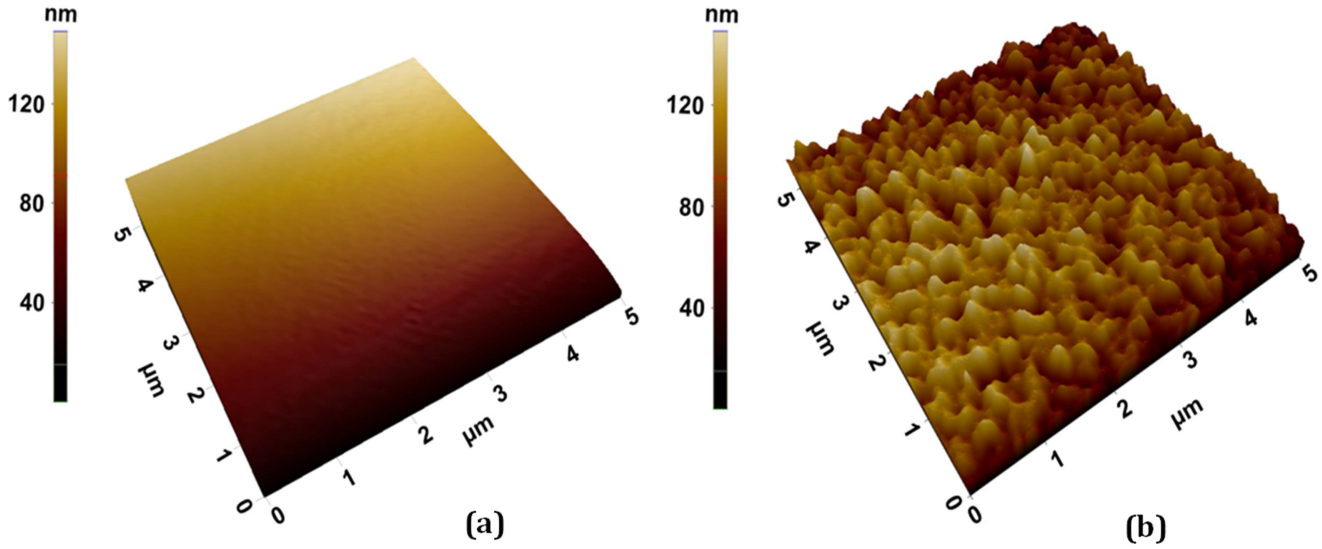

Figure 1. AFM micrographs of (a) plain and (b) nanotextured coverslips.

angle is more than 90° for hydrophobic surfaces and less than that for hydrophilic surfaces [17, 18]. Nanotexturing augments the hydrophobicity of hydrophobic surfaces and hydrophilicity of hydrophilic surfaces. The contact angles of all experimental surfaces were measured and their average values with standard deviations are given in table 1. Glass is hydrophilic by nature and nanotexturing enhanced its hydrophilicity in this case.

Table 1. Contact angles measurement on plain and nanotextured coverslip surfaces.

Surface type	Plain	Nanotextured
Contact angle	25.4 ± 1.14°	12.60 ± 1.52°

3.4. PC12 cell growth on nanotextured surfaces

The PC12 cell growth was observed in the presence and absence of β-NGF in the culture media on the three substrate types. In all cases, the cell densities were calculated by analyzing micrographs using *ImageJ*.

In the absence of β-NGF in the culture media, the density of the cells was observed to attain the values of 475 ± 86 and 310 ± 76 per mm^2 for the TCPs and the plain coverslips substrates, respectively. On the other hand, for nanotextured coverslips the cell density value surged to 612 ± 95 per mm^2, demonstrating ~200% increase compared to plain coverslips, and significantly higher than the TCP substrates (figure 3). Next, the cell growth was observed in the presence of β-NGF in the culture media, which is biologically active and advocates axon growth [1]. In this case, cell densities on TCP, plain, and nanotextured coverslips attained the values of 437 ± 69, 344 ± 89, and 649 ± 49 per mm^2, respectively (figure 3). Observed PC12 cell growth was significantly higher in both cases (±β-NGF) on nanotextured coverslips compared to plain surfaces (figure 3).

One-way analysis of variance depicted statistically significant differences (p-value < 0.01) in the cell density values between plain and nanotextured coverslips for both the cases. The presence of β-NGF did not significantly impact the cell growth. It should be noted that during these experiments, the substrates were not subjected to any kind of ECM treatment, such as collagen [33, 34], laminin [35–37], or fibronectin [30, 38, 39] etc, each of which possesses the potential to provide better surface attachment and subsequently neurite elongation from the neuron or neuron-like cells. Additionally, the laminin treatment would undermine or overstate the effects of nanotextured features. Since nanotexture offer larger effective surface area, these can lead to higher surface density of laminin, consequently resulting in faster cell growth. In essence, only the effect of nanoscale random texture was responsible for the growth of PC12. This eliminated the possible influences which may have originated from the enhanced concentration of coated materials on the nanotextured surface.

Proper cell attachment is imperative for the growth, differentiation, and survival of cells. Nanotexture provide a biomimetic cell-stimulating cue as cells *in vivo* contact many nanotextures and not the plain interfaces because of the presence of complex nanoscale structures in the basement membrane of various tissues, making the interaction natural and easier [21]. Moreover, nanotextured features significantly influence the interfacial forces, focal adhesion, cytoskeletal, and membrane receptor organization [24], and as a result, regulate cell function in a noninvasive and non-biological manner [21]. The cell morphologies were thus modulated by nano-engineering the surface topology, making substantial local biomechanical deformations to activate specific signaling cascades that eventually regulated cellular growth. This cell growth was independent of the ECM agents.

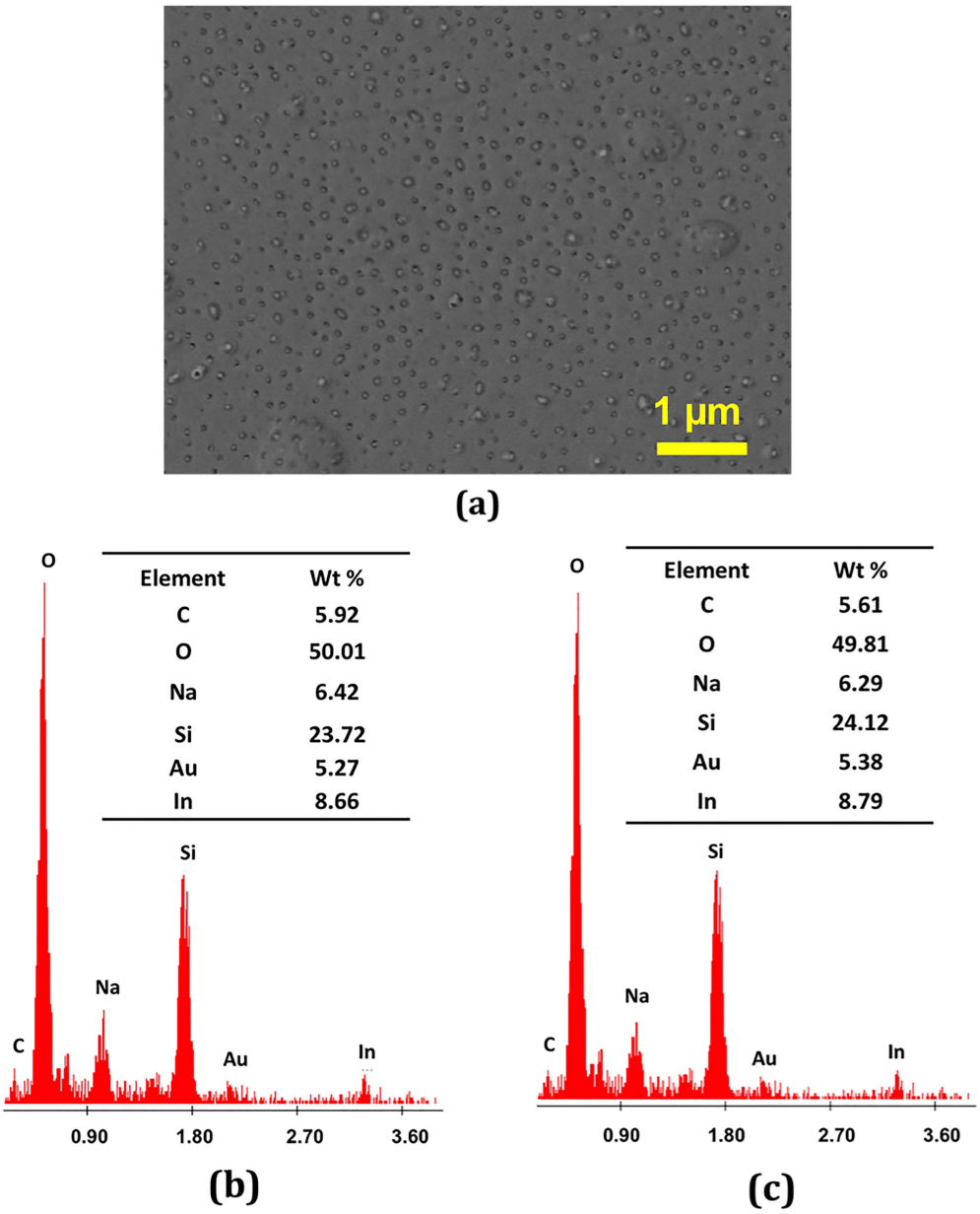

Figure 2. (a) SEM micrograph of a nanotextured glass substrate; EDS elemental composition of (b) plain and (c) nanotextured glass coverslips.

The nanotexturing not only provided larger effective surface area, it offered biomimic surface and enhanced the adhesion of cells by augmenting cell-surface interactions. Our prior work [12] on assessment of cytoskeletal microtubules and actin filaments arrangement for PC12 cells indicates that nanotextured features enhance the level of mRNA expression of β3-tubulin in PC12 cells to facilitate better cell attachment, as has been observed by others as well [40].

3.5. Nuclear morphology of cells

Nuclear morphology was assessed by observing the DAPI stained cell nuclei. This showed the induction of apoptosis as a result of the presence of nanotexture on the coverslip substrates. Very little fragmentation, blebbing or DNA condensation were seen in the cells grown on the nanotextured surfaces, when compared to the cells from normal glass coverslips or TCP membranes. Most of the cells had round and homogeneous nuclei as can be seen in figures 4(a)–(c) (negative β-NGF data not shown). Quantification of pyknotic nuclei, which is indicative of cell death [41], is displayed in figure 4(d). It was observed that between 4% and 8% cells did have deformed and condensed nuclei with apoptotic bodies, which is typical for the normal handling and culture processes. Just like the data of figure 3, cytoplasmic blebbing and irregularities in shape were absent in cells grown on the nanotextured coverslips in both cases of positive β-NGF and

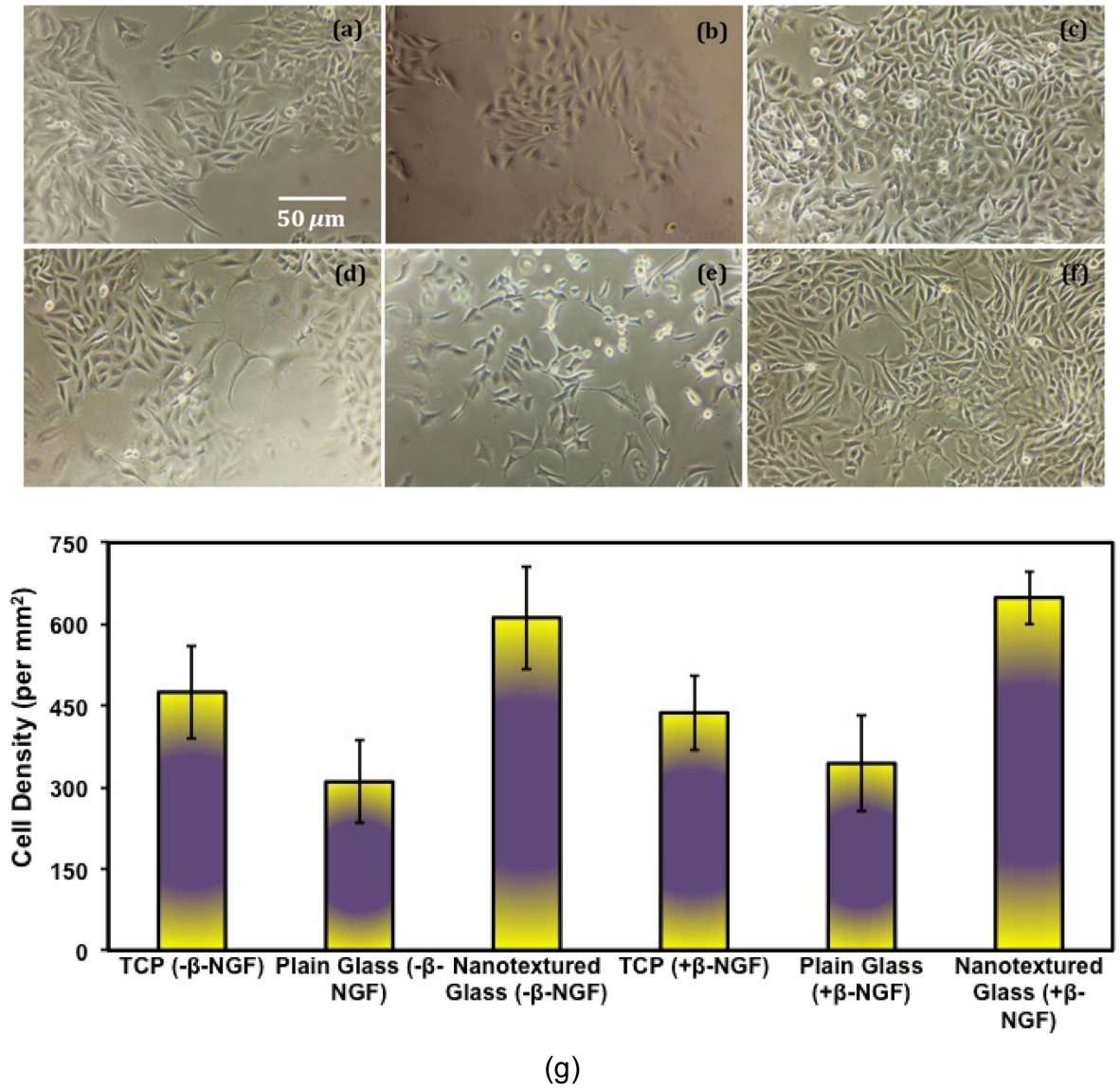

Figure 3. Micrographs of PC12 cells grown on (a) TCP; (b) plain cover glass; (c) nanotextured coverslip. (a)–(c) Were treated with 100 ng ml^{-1} β-NGF. PC12 cell micrographs with no β-NGF on (d) TCP; (e) plain cover glass and (f) nanotextured coverslip. (g) Density of PC12 cells on these surfaces.

negative β-NGF. All these results indicate that PC12 cell attachment and growth on the nanotextured substrates did not incur cellular apoptosis.

4. Conclusions

Micro-RIE provided a rapid and cost-effective way of fabricating nanotextured glass coverslips that were used as substrates for neuron-like cell culture. Nanonanotexture was found to stimulate the growth of PC12 cells in culture even in the absence of any ECM treatments such as collagen, laminin etc. The cell density was observed to increase by almost 200% on nanotextured glass coverslips compared to plain ones. The effect of nanotexture as a physical cue on neuron-like cell growth has been investigated for the first time. The morphology study indicated that PC12 cell attachment and growth on the nanotextured substrates did not launch any apoptotic machinery of the cell. Coupled with the capability of inducing enhanced proliferation, these substrates carry excellent potential to be used for neural cell attachment, which is the precursor of enhanced differentiation that can be used to manipulate axon regeneration and guidance to facilitate neural circuit reconstruction. Finally, since the PC12 neuron-like cell attachment and proliferation behavior is very similar to that of the B35 neuroblastoma cells (in many cases), these micro-RIE glass coverslips are expected to increase the affinity of B35 cancer cell attachment as well and result into better B35 cell capture and rapid proliferation thereafter. This opens an interesting front of possibilities of developing combinatorial theranostics approaches to diagnose and treat aggressive cancers like neuroblastoma.

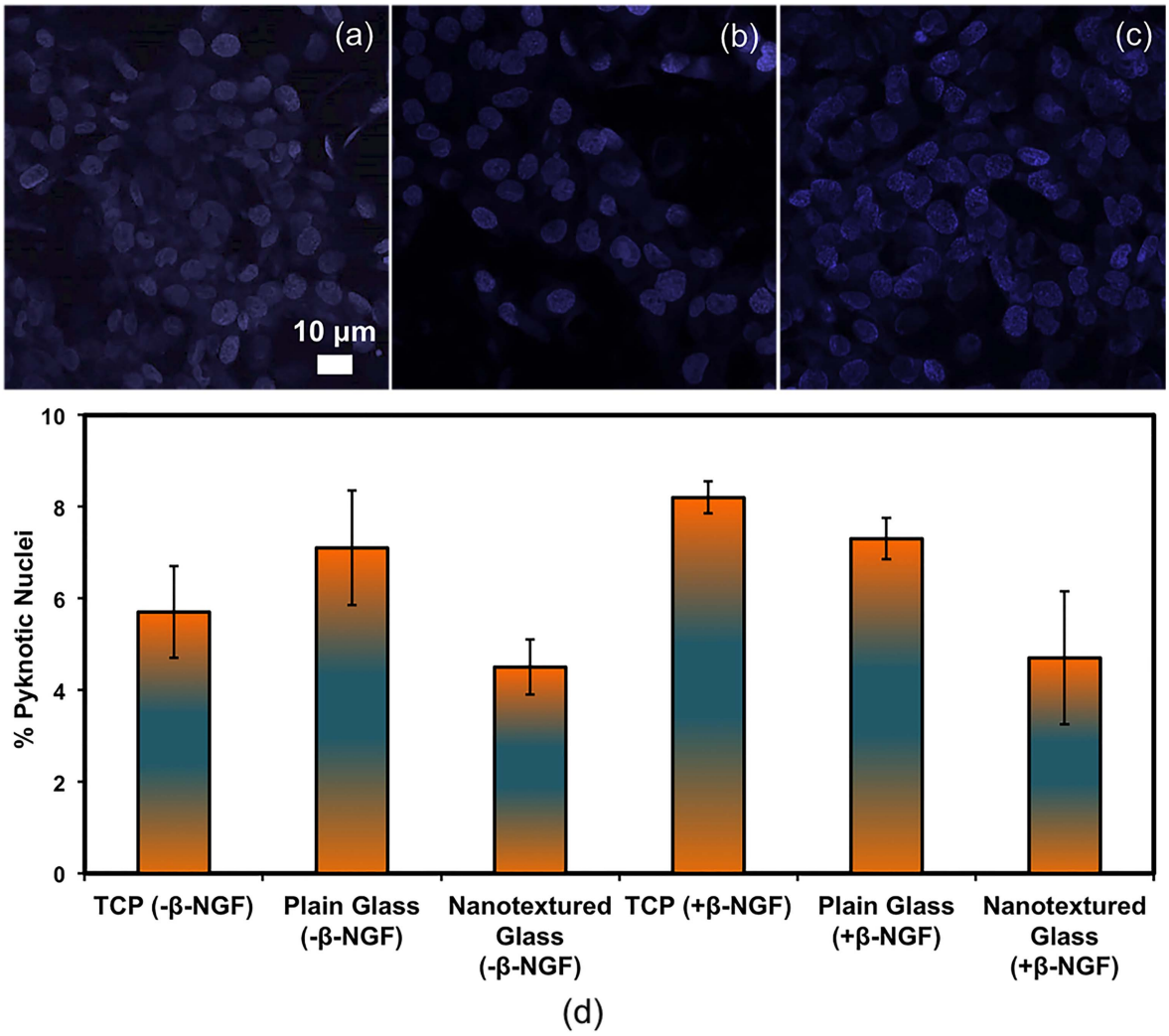

Figure 4. Fluorescence micrographs of DAPI stained cell nuclei of PC12 cells under the treatment of β-NGF on (a) TCP; (b) plain coverglass; and (c) nanotextured coverglass. (d) Quantification of pyknotic nuclei on various surfaces, with and without β-NGF.

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MI, SMI and SG designed the experiments. MI fabricated and characterized the nanotextured coverslip glasses. RA and SM performed PC12 cell related experiments. SG and SMI directed the project and supervised this work. All authors have read and approved the final manuscript.

The authors declare no conflict of interests.

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