

Cytocompatibility of amorphous hydrogenated carbon nitride films deposited by CH₄/N₂ dielectric barrier discharge plasmas with respect to cell lines

Abhijit Majumdar,^{1,a)} Karsten Schröder,² and Rainer Hippler¹

¹*Institut für Physik, Ernst-Moritz-Arndt-Universität Greifswald, Felix-Hausdorff-Str. 6, 17489 Greifswald, Germany*

²*Leibniz Institute for Plasma Science and Technology, Felix-Hausdorff-Str.2, 17489 Greifswald, Germany*

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Special amorphous hydrogenated carbon nitride (*a*-H-CN_x) films have been prepared on glass substrates for the investigation of adhesion and proliferation of different mammalian cell lines. CH₄/N₂ dielectric barrier discharge plasmas were applied to deposit *a*-H-CN_x coatings at half of the atmospheric pressure. Film quality was modified by varying the CH₄:N₂ ratio and deposition duration. Chemical composition was determined by x-ray photoelectron spectroscopy and Fourier transformed infrared spectroscopy. The N/C ratio was in the range of 0.20–0.55. A very small surface roughness was verified by atomic force microscopy. Human embryonic kidney (HEK) and rat adrenal pheochromocytoma (PC12) cells were cultivated on the *a*-H-CN_x films to investigate the cytocompatibility of these surfaces. The microscopic images show that both kinds of cells lines were unable to survive. The cells did not adhere to the surfaces, and most of the cells died after certain time spans. Increased amounts of nitrogen in the film induce an accelerated cell death. It is concluded, that the deposited CN_x film behaves cytotoxic to HEK and PC12 cell lines. © 2008 American Institute of Physics. [DOI: 10.1063/1.2990054]

I. INTRODUCTION

Carbon nitride (CN_x) films have been attracting great interest in biomedical applications mainly because of their excellent mechanical and tribological properties, chemical inertia, and optical properties.^{1–3} Numerous research works have been published to deposit CN_x films using different experimental methods. CN_x films have been prepared by magnetron sputtering,^{4,5} electron cyclotron resonance plasma-assisted vapor deposition,⁶ ion beam deposition,⁷ plasma enhanced chemical vapor deposition (CVD),^{8,9} and microwave plasma CVD.¹⁰ Diamondlike carbons (DLCs) have emerged as promising coatings for biomedical applications. It is generally accepted that DLC coatings do not induce any cytotoxicity or inflammation on usual adherent cells¹¹ including human embryonic kidney (HEK) cells.¹² Only some contradictory clinical results have been reported regarding cytocompatibility of DLC-coated femoral head compared to alumina-coated one.¹³

Therefore it is very important that the behavior of special carbon-based coatings should be discussed based on the detailed information of the coating such as atomic bond structure, composition, and/or electronic structure.¹¹ Comparing amorphous carbon (*a*-C) and amorphous carbon nitride (*a*-CN) coatings prepared by dc magnetron sputtering, osteoblast proliferation was higher on *a*-C compared to *a*-CN but differences to the stainless steel (SS) substrate were rather small. Osteoblast and fibroblast adhesion was improved with respect to SS and Ti coated SS.¹⁴ The nitrogen content can be controlled by nitrogen partial pressure. In-

creased nitrogen content was useful for the improvement of mouse fibroblasts and human endothelial cells.¹⁵ Radio frequency magnetron sputtering has been compared to plasma immersion ion implantation and deposition.^{16,17} A very interesting technique for the preparation of *a*-CN films is ion beam-assisted deposition. This technique allows the preparation of *a*-CN hard coatings near room temperature since about 1990. Allen *et al.*¹² investigated the behavior of osteoblasts on *a*-CN coatings compared to polystyrene. Cells grown on the coated substrates exhibited normal cellular growth and morphology films. Similarly, the growth of osteoblasts was examined on coated and uncoated silicon wafers. The spreading and proliferation is improved on amorphous C–N films without apparent impairment of cell physiology.¹⁸ Blood compatibility of these films was also found to be superior to that of NiTi alloy and 316L SS.¹⁹ In 2000, Cui and Li summarized the previous investigated work on biocompatibilities of DLC and CN_x coatings.²⁰

No cytotoxic effects of the coatings were found in any of these studies.^{12,17,18,20} However, these are studies on hard carbon layer, while there exists no research on cell behavior on rather polymerlike *a*-H-CN_x films prepared with dielectric barrier discharge (DBD) methods.

The film deposition by CH₄/N₂ DBD plasma has several interesting features. It offers attractive prospect for the coating deposition and surface functionalization as it provides an easily applicable system in the industrial/commercial process.^{21–23} The most important feature is that the plasma reaction zone is confined on the surface of the electrode or substrate area. Therefore a DBD of CH₄/N₂ gas mixture was implemented at half of the atmospheric pressure to deposit polycarbon nitride film (*a*-HCN_x) on glass substrate. This

^{a)}Author to whom correspondence should be addressed. Electronic mail: majumdar@physik.uni-greifswald.de. FAX: +493834864701.

TABLE I. Deposition conditions for a -H-CN_x films.

Number (a -H-CN _x)	CH ₄ :N ₂	Time duration (h)
BIOCNI	1:1	9
BIOCNI2	3:1	17
BIOCNI3	1:3	17

paper concentrates on chemical composition, chemical bond structure, and surface morphology of (a -H-CN_x) films and discusses these results with cell [HEK and rat adrenal pheochromocytoma (PC12)] culture experiments.

II. EXPERIMENTAL

A. Film preparation

Three films were deposited with varying CH₄:N₂ gas mixture ratios as 1:1, 3:1, and 1:3, respectively (see Table I). The experimental setup of the DBD has been explained in details elsewhere.²⁴ Both Ag electrodes are covered by dielectrics: the upper (powered) electrode is covered with aluminum oxide ($\epsilon \sim 10$); the lower (grounded) electrode with a glass plate ($\epsilon \sim 3.8$). The glass electrode was used as a dummy substrate. Both electrodes are separated by 0.15 cm from each other. The upper electrode is connected to a home-built high voltage power supply, while the lower electrode is grounded. The chamber is pumped by a membrane pump down to a base pressure of about 10 mbar. The pressure inside the plasma chamber was controlled by two gas flow controllers for methane and nitrogen and by an adjustable needle valve between the chamber and the membrane pump. The high voltage power supply consists of a frequency generator delivering a sinusoidal output that is fed into an audio amplifier. The amplifier can be operated at up to 500 W. Experiments were performed at 10.5 kV (peak to peak) and at 4 kHz. The electrical power under these conditions was of 4 W. Table I summarizes the deposition conditions.

B. Chemical surface analysis

Fourier transform infrared (FTIR) transmission spectra were obtained by means of FTIR spectrometer Bruker (Vector 22). The plain sample was placed in a vacuum chamber built inside the spectrometer in order to minimize the IR signal of water vapor, CO₂ content, and noise. The measuring signal passed the optical way with an aperture diameter of 3 mm with spectral resolution 4 cm⁻¹. For optimal signal-to-noise ratio of 50 scans were averaged per sample spectrum and apodized by applying of the Norton Beer apodization function for Fourier transformation. Interferograms were zero filled using a zero-filling factor of 2. The background spectrum was independently measured on a pure silicon substrate independently.

X-ray photoelectron spectroscopy (XPS) measurements of the a -H-CN_x films were performed on a multitechnique 100 mm hemispherical electron analyzer (CLAM2: VG Microtech), using Mg $K\alpha$ radiation (photon energy 1253.6 eV) as the excitation source and the binding energy (BE) of Au (Au 4f_{7/2}: 84.00 eV) as the reference. The XPS spectra were

collected in a constant analyzer energy mode, at a chamber pressure of 10⁻⁸ mbar and pass energy of 23.5 eV at 0.125 eV/step.

C. Surface morphology

Atomic force microscopy (AFM) is a powerful tool to characterize the surface morphology, and quantitative surface roughness in a nanometer scale can be measured. Surface topographies of the a -H-CN films were investigated by Nanoscope IV AFM (M/S Veeco, USA) in the tapping mode at ambient conditions using a silicon cantilever with a sharp silicon tip. The radius of curvature of the Si tip is around 10 nm. The scan areas were varied from 1 × 1 to 5 × 5 μm² with a resolution of 512 pixels. In order to avoid an overestimation of the surface roughness resulting from the presence of a tilted plane when examining the film surface by AFM, a line-by-line flattening was made in the fast direction using Nanoscope data processing software to remove such a tilted plane.

D. Cell culture

HEK and PC12 cell lines were cultivated on three independent a -H-CN_x films (BIOCNI, BIOCNI2, and BIOCNI3) samples. The cells were seeded in vessels created on the coated glass with the help of silicon rings. For parallel experiments, four wells were investigated per coating. 1 ml cell suspension was dosed in one vessel (1.000.00 PC12 cells and 500.000 HEK cells). The conditions are as follows: PC12 cells were cultured in RPMI-1640 medium with 10% horse serum and 5% fetal calf serum (FCS). HEK cells were cultured in Dulbecco's modified eagles's medium with 7% FCS. The cells were cultivated in an incubator under static conditions (5% CO₂, 37 °C) immediately after seeding. The cultivation usually lasted for 24 h. The dishes were taken out at different times to take some pictures.

III. RESULTS AND DISCUSSION

A. Chemical and physical properties

1. FTIRs

FTIR absorption measures the vibrational, stretching, and symmetric/antisymmetric band configuration (range 4000–500 cm⁻¹) present in the deposited polymer film. Two a -H-CN_x films (BIOCNI and BIOCNI3) have been analyzed by FTIR technique. Typical IR transmission spectra are shown in Fig. 1, within the range from 4000 to 500 cm⁻¹. Spectroscopic properties of the polymerized films deposited at different mixture concentrations of the reactive gases CH₄:N₂ are presented in Fig. 1, where the ratios are 1:1 and 1:3, respectively.²³ The band between 3100 and 3700 cm⁻¹ is attributed to stretching vibrations of NH and OH functionally groups.^{23,25} However, the separation of the overlapped bands is not possible due to intermolecular interactions as H-bridges, which are very intensive in this region and cause the broadening of the bands. The second interval 3010–2810 cm⁻¹ is the characteristic for CH₂ and CH₃ groups.^{25,26} The intensity of the bands is low (absorption up to 1%) and is related with the concentration of methane in

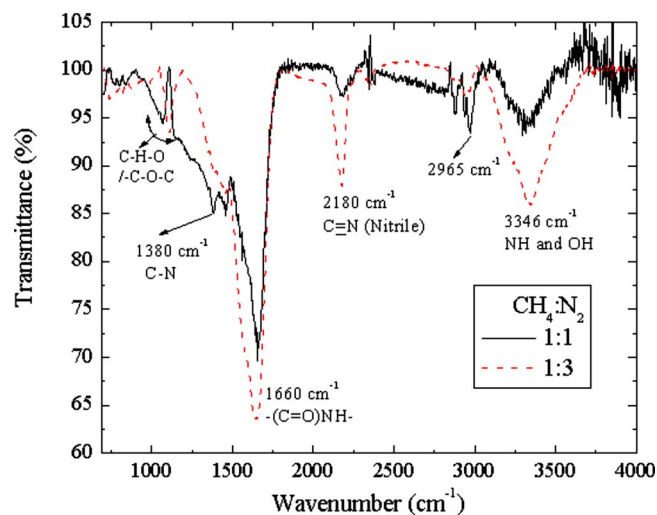


FIG. 1. (Color online) A typical FTIR transmission spectra of $a\text{-H-CN}_x$ film deposited in mixture of $\text{CH}_4:\text{N}_2=1:1$ (straight lines) and $\text{CH}_4:\text{N}_2=1:3$ (dotted lines).

the gas discharge. The broad absorption single peak at 2176 cm^{-1} is attributed to $\text{C}\equiv\text{N}$ triple bond stretching vibration (so-called nitrile group). The broad band from 1645 to 1665 cm^{-1} is attributed to $\text{C}=\text{C}$ and $\text{C}=\text{N}$ stretching mode.²⁷ There is an absorption band due to trace absorption of carbonyl of amide group— $(\text{C}=\text{O})\text{NH}$ —at $\sim 1670\text{ cm}^{-1}$. The absorption band observed in the interval $1350\text{--}1480\text{ cm}^{-1}$ corresponds to the C-N single bond stretch.²⁷ Finally, the absorption peak in the zone $1080\text{--}1160\text{ cm}^{-1}$ corresponds to the C-O stretching mode.²⁸ From Fig. 1, we see that the bands corresponding to the intervals to $1350\text{--}1480$, $1645\text{--}1665$, and $2260\text{--}2290\text{ cm}^{-1}$ indicate that the carbon and nitrogen atoms in the CN_x film are linked as C-N , C=N , and $\text{C}\equiv\text{N}$ bonds, respectively.

2. XPS

XPS measures the chemical composition and the bond structure of the polymerlike film ($a\text{-H-CN}_x$) layer. Two CN_x

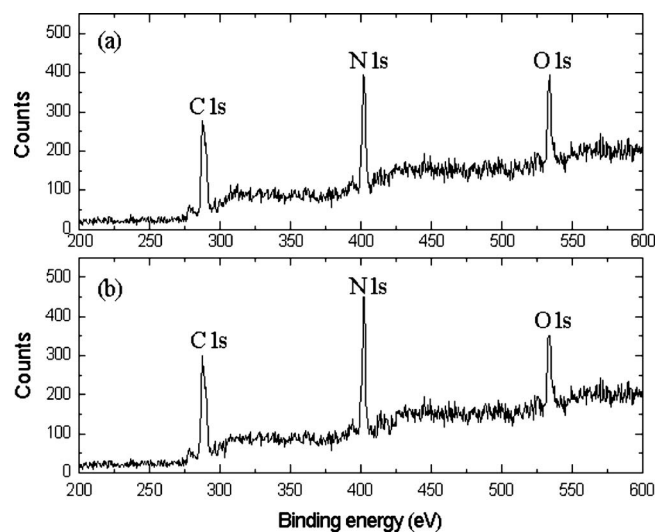


FIG. 2. Full scale XPS spectra of the deposited $a\text{-H-CN}_x$ films when (a) $\text{CH}_4:\text{N}_2=1:1$ and (b) $\text{CH}_4:\text{N}_2=1:3$ ($f=4\text{ kHz}$ and $P=400\text{ mbar}$).

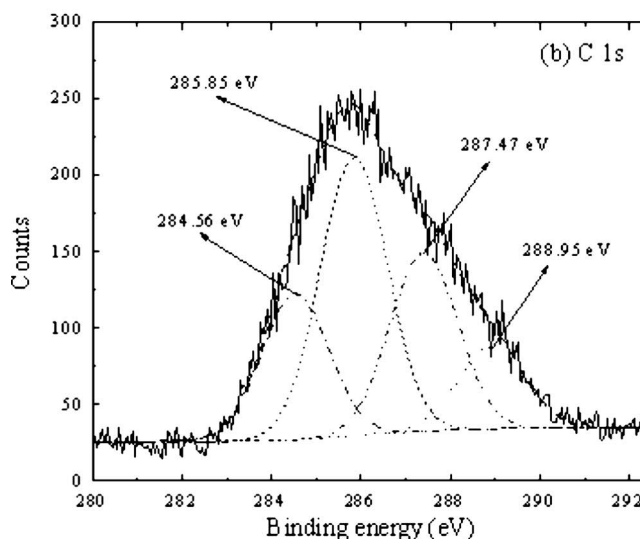
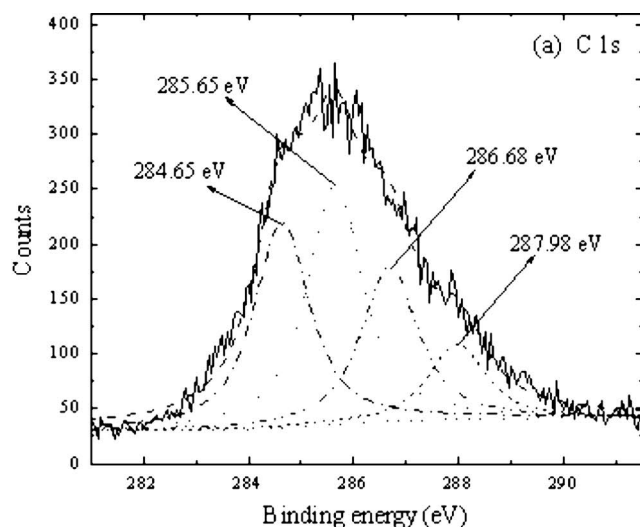


FIG. 3. Typical $\text{C } 1s$ XPS spectra of $a\text{-H-CN}_x$ films when (a) $\text{CH}_4:\text{N}_2=1:1$ and (b) $\text{CH}_4:\text{N}_2=1:3$ ($f=4\text{ kHz}$ and $P=400\text{ mbar}$).

films (BIOCN1 and BIOCN3) have been analyzed by XPS technique. As the proportion of N_2 in the $\text{CH}_4:\text{N}_2$ gas mixture is increased (Fig. 3), so does the $\text{C}\equiv\text{N}$ group increases in the BIOCN3 film.

Full scale XPS spectra of the deposited films are shown in Fig. 2, with BE from 200 to 600 eV for both $\text{CH}_4:\text{N}_2=1:1$ and $\text{CH}_4:\text{N}_2=1:3$, supporting the presence of $\text{C } 1s$, $\text{N } 1s$, and $\text{O } 1s$ bands in the deposited polymer film. The observed chemical shift have been calibrated by peak fitting program.²³ With the increase in nitrogen concentration, the $\text{C } 1s$ peak broadens and also becomes more asymmetric in nature, which indicate that the nitrogen is involved in chemical bonds with carbon in three possible chemical states: C-N , C=N , $\text{C}\equiv\text{N}$ and C-O bonds.²³ The best Gaussian fits to the XPS lines resulted in to four different peaks for the $\text{C } 1s$ line and three peaks for the $\text{N } 1s$ line.

Typical $\text{C } 1s$ spectra of the polymer films ($\text{CH}_4:\text{N}_2\sim 1:1$) are shown in Fig. 3(a). Figure 3(a) shows that the $\text{C } 1s$ spectrum ($\text{CH}_4:\text{N}_2\sim 1:1$) exhibits peaks at 284.65 , 285.65 , 286.68 , and 287.98 eV , which are attributed to C=C , C=N , C-N or $\text{C}\equiv\text{N}$, and C-O bonds, respectively.^{23,25,29} Similar to Fig. 3(b), the $\text{C } 1s$ spectrum

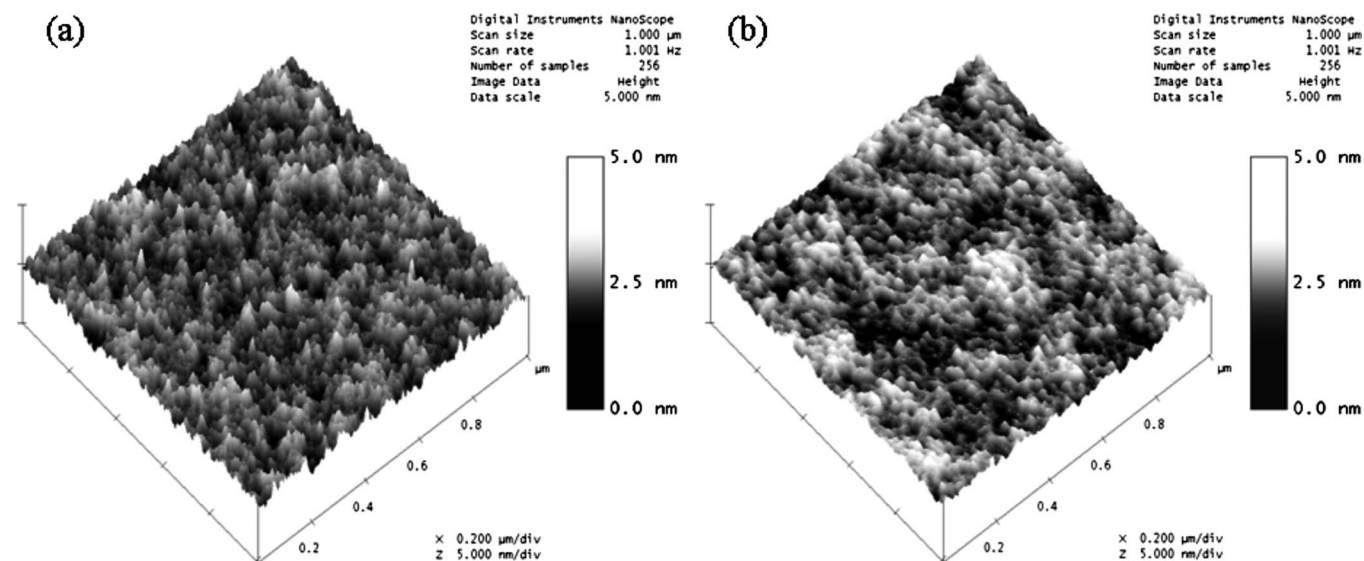


FIG. 4. Three-dimensional AFM images of a -H-CN $_x$ films on Si substrate when (a) CH $_4$:N $_2$ =1:1 and (b) CH $_4$:N $_2$ =1:3 (f =4 kHz and P =400 mbar).

for CH $_4$:N $_2$ ~1:3) is seen to exhibit peaks at 284.56, 285.85, 287.47, and 288.95 eV, which are attributed to C=C, C=N, C-N or C≡N, and C-O bonds, respectively.^{23,28–30} In some cases, a peak appears at 286.4 eV and is attributed to the nitrile group (C≡N).^{23,29} The energy of the sp^2 C-N peak falls within 285.5–285.9 eV and that of the sp^3 C-N peak is assigned in the range 287.0–287.8 eV. Due to these wide spectral ranges a clear understanding of the differences between C-N and C≡N bonds^{23,31} in the analysis of C 1s XPS spectra is quite difficult. It can be deduced from Fig. 3, those carbon-nitrogen bonds (C=N and C-N) irrespective of the nitrogen content of the CH $_4$:N $_2$ gas mixture at the expense of carbon-carbon and carbon-oxygen bonds.

3. AFM

AFM measures the surface morphology [root mean square roughness (rms)] of the film surface. Figures 4(a) and 4(b) present typical three-dimensional $2 \times 2 \mu\text{m}^2$ topographies of CN $_x$ polymer films with different CH $_4$:N $_2$ mixtures. For smaller ratios of the nitrogen contents, the film shows a three-dimensional island growth. The average size of the islands as measured from the sectional profile typically ranges between 30 and 40 nm for the CH $_4$:N $_2$ mixture at 1:1. As N $_2$ increases in CH $_4$:N $_2$ ratio, the islands gradually flatten down and the surface becomes smoother. The surface roughness of the underlying Si substrate is about 0.65 nm, whereas the roughness of the film in the plateau region is about 0.35–4.0 nm, which indicates that the present CN $_x$ film is much smoother compared to CN $_x$ films deposited by vapor deposition, such as radio-frequency plasma enhanced pulsed laser deposition (rms roughness below 1.0 nm).³² Apparently, more deposition occurs in the valleys of the Si surface than on the crests. It may also indicate that plasma ions injected into the growing films tend to grow atomically smooth films. Figure 4 shows the evolution of rms roughness for a $2 \times 2 \mu\text{m}^2$ sample area as a function of the N $_2$ ratio in

CH $_4$:N $_2$ mixture. It is seen that the surface roughness of the deposited CN film decreases with the increase in the N $_2$ ratio.

B. Cell culture

Adherent mammalian cells can be grown on glass slides. Glass is a suboptimal substrate for cell cultivation, however less sensitive cells like HEK cells adhere quite well [Fig. 5(a)]. PC12 cells are a more challenging cell line, they adhere in a small density [Fig. 5(b)] and a noticeable amount swims death in solution (out of focus).

For comparison, HEK and PC12 were grown on BIOCN1 CN $_x$ film. The optical quality of the microphotographies is impaired because of inhomogeneous and yellow CN $_x$ film coloring. The films partially dissolve in the cell culture medium. A drastically reduced density of HEK cells can be obtained in Fig. 6(a). The cells are round up, their diameter is drastically reduced. PC12 cell adhesion is completely inhibited [Fig. 6(b)].

While a similar cell behavior was obtained on the BIOCN2 film (not shown here), the cell adhesion on BIOCN3 film is even worse (Fig. 7). HEK cell adhesion is also drastically suppressed on this film compared to BIOCN1 film. The reason could be the higher N $_2$ dose applied in film preparation and higher N-content obtained in XPS.

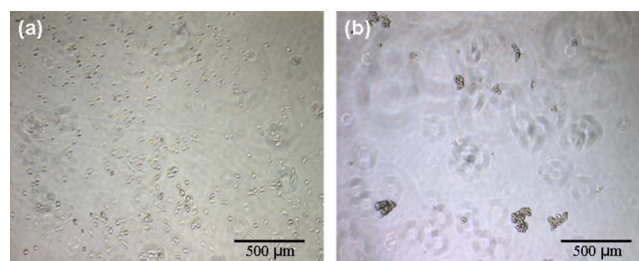


FIG. 5. (Color online) (a) HEK cells and (b) PC12 cells on untreated glass substrates.

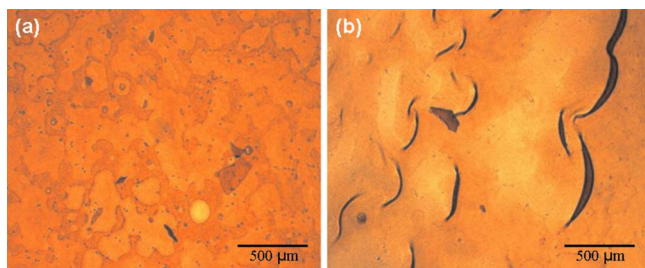


FIG. 6. (Color online) Microscopic images of (a) HEK and (b) PC12 cells both on BIOCNI $a\text{-H-CN}_x$ film. The film has been deposited at ratio of $\text{CH}_4:\text{N}_2=1:1$. Time duration of the deposition is 9 h.

This could induce a higher charge and more free radicals at the film surface. These surface properties could prevent cell adhesion and cause cytotoxic effects. Further investigation on atomic/molecular level surface interaction is necessary.

Starting from pure CH_4 and N_2 (with different ratios, at constant pressure) DBD plasma has been employed to deposit amorphous hydrogenated carbon nitride ($a\text{-H-CN}_x$) film at different time scales on glass substrate. The tendency of the film color extensively depend on the deposition time as well the amount of nitrogen contained in the film. It has been observed that the more nitrogen contain film exhibits darker color, i.e., yellowish brown film (Fig. 7, BIOCNI3) and comparatively less transparent than the low nitrogen contained film (Fig. 6, BIOCNI1). The chemical configuration and bond structure of the two $a\text{-H-CN}_x$ films (BIOCNI1 and BIOCNI3) have been analyzed by FTIR and XPS techniques. Both spectroscopic results exhibit the common feature of the increasing behavior of the nitrile ($\text{-C}\equiv\text{N}$) group in the deposited film. It reveals that as the proportion of N_2 in the $\text{CH}_4:\text{N}_2$ gas mixture increases, the absorption by the nitrile ($\text{C}\equiv\text{N}$) group as well as NH group are also increased (Fig. 1), while the intensity of the absorption band of CH_2/CH_3 decreases, which supports the experimental results that are statistically significant.²³ The film exhibits a pronounced cross-linking and nitrile and amides functionalities. The detailed quantitative analysis of $\text{C}=\text{C}$, $\text{C}=\text{N}$, $\text{C}-\text{N}/\text{C}\equiv\text{N}$, and $\text{C}-\text{O}$ in the $\text{C } 1s$ XPS spectra has been analyzed in our previous work.²³ It shows that the amount of $\text{C}=\text{N}$ and $\text{C}-\text{N}$ increases and that of $\text{C}=\text{C}$ decreases as the $\text{CH}_4:\text{N}_2$ ratio is changed from 1:1 to 1:3. This interpretation has been derived from a comparison of the measured BEs of nitrogen-rich organic polymers. FTIR measurement reveals that as the proportion of N_2 in the $\text{CH}_4:\text{N}_2$ gas mixture increases (Fig. 1)

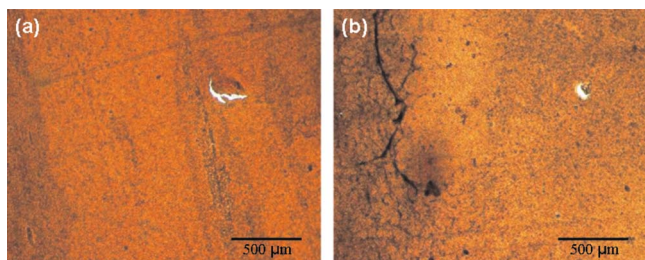


FIG. 7. (Color online) Microscopic images of (a) HEK and (b) PC12 cells on BIOCNI3 $a\text{-H-CN}_x$ film. The film has been deposited at ratio of $\text{CH}_4:\text{N}_2=1:3$. Time duration of the deposition is 17 h.

(BIOCNI1 and BIOCNI3), the absorption by the nitrile ($\text{C}\equiv\text{N}$) group as well as NH group are also increased. The increase in $\text{C}\equiv\text{N}$ and NH/OH overlapping group has a strong impact on BIOCNI3 film, which exhibits relatively higher cytotoxicity toward HEK and PC12 cell lines. XPS reveals that as the proportion of N_2 in the $\text{CH}_4:\text{N}_2$ gas mixture is increased (Figs. 3 and 4), the $\text{C}\equiv\text{N}$ group also increased in the BIOCNI3 film, which pronounced the effectively higher cytotoxicity toward HEK and PC12 cell lines rather than other deposited polymer films. Surface roughness was carried out by AFM, in which it has been observed that the higher rms roughness of the corresponding polymer films (in case of BIOCNI3) exhibits relatively higher cytotoxicity toward both HEK and PC12 cells lines.

As per as the present research work is concerned, it is difficult to explain in the physical point of view about the actual mechanism of the cells to growth on such a $a\text{-H-CN}_x$ coated substrate. Still there are some arguments regarding this behavior. $a\text{-H-CN}_x$ films seem to have a tendency to impair the protein-based cell adhesion mechanism and to stop the cell growth process. Strong homo- and heteronuclear dipolar coupling properties of $a\text{-H-CN}_x$ film could break the peptide configuration and induce apoptotic signals. This way $a\text{-H-CN}_x$ films could be highly active to live cells. The cell death is very important to examine: What kind of polycarbon nitride film has the potential to reduce or stop the growth of cells? Such surfaces could be very interesting for the suppression of unwanted cell growth on biomedical devices inside the human body. In some cases, after the surgery few cells surrounded by the operated area are seen to be damaged and causes the aggressive and invasive growth in future, which turns to move toward cancer cells' growth. In such a case we can use the coating of CN_x film to stop the growth of such cell lines in or outside the human body. To support this assumption, a further study of those films in cell culture is necessary.

IV. CONCLUSION

Polycarbon nitride films ($a\text{-H-CN}_x$) have been successfully deposited on glass substrates by CH_4/N_2 gas mixtures in DBD plasmas. FTIR spectroscopy reveals that the proportion of N_2 in the $\text{CH}_4:\text{N}_2$ gas mixture increases, the absorption by the nitrile ($\text{C}\equiv\text{N}$) group as well as NH group are also increased. $\text{C}=\text{C}$, $\text{C}=\text{N}$, $\text{C}-\text{N}/\text{C}\equiv\text{N}$, and $\text{C}-\text{O}$ bonds are observed in the $\text{C } 1s$ XPS spectra including with increasing behavior of nitrile ($\text{-C}\equiv\text{N}$) group, as the proportion of N_2 in the $\text{CH}_4:\text{N}_2$ gas mixture is increased. Higher rms roughness of the corresponding polymer films (in case of BIOCNI3) exhibits relatively higher cytotoxicity toward both HEK and PC12 cells lines, obtained from AFM.

Most of the cells died in the film which has higher concentration of nitrogen (BIOCNI3, $\text{CH}_4:\text{N}_2=1:3$). Over all cell growth performance of PC12 on CN_x film is not promising, as most of the cells are dying after a certain time spans. The cell growth are stopped and most of the cell died in the medium of $a\text{-H-CN}_x$ film, which can turn to develop a novel method to kill or stop the growth of unwanted/invasive cells. Further experimental processes are necessary for the quanti-

fication of the living cells on the film substrate after the incubation. The statistical significance (number of cell) of the cell death and the cytotoxicity toward the HEK and PC12 cell lines will be the next prospect of this work and in future, it will be the aim to do all treatment on cancer cell lines.

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