Cytocompatibility of aH-CN$_x$ films deposited by CH$_4$/N$_2$ dielectric barrier discharge plasmas: HEK and PC 12 cell lines

Abhijit Majumdar and Rainer Hippler

*Institut for Physics, Ernst-Moritz-Arndt-University Greifswald, Felix-Hausdorff-Str. 6, 17489 Greifswald, Germany

Abstract: Amorphous hydrogenated carbon nitride (aH-CN$_x$) films have been prepared on glass substrates for the investigation of adhesion and proliferation of different mammalian and cancer cell lines. Human Embryonic Kidney (HEK) and Rat Adrenal Pheochromocytoma (PC12) suspensions have been exposed on the surface of aH-CN$_x$ film to investigate the cytocompatibility of these surfaces. The cells did not adhere to the surfaces, and most of the cells died after certain time spans. The microscopic images and results from the MTS assays indicated that the deposited aH-CN$_x$ film is cytotoxic to cancer cell lines.

Keywords: Amorphous Carbon-nitride (aH-CN$_x$) film, DBD plasma, HEK and PC 12 Cell culture, Cytotoxicity.

I. Introduction

Over the last five years, enormous developments have been reported in biomedical plasma research and technology [1, 2, 3 and 4]. Different plasmas are being successfully used in biomedical applications such as sterilization of plastics, controlling or modifying cell surfaces, decontamination of bacteria etc. The film deposition by CH$_4$/N$_2$ DBD plasma has several interesting features. It offers attractive prospect for the coating deposition and surface funtionalization as it provides an easily applicable system in the industrial/commercial process [5, 6 and 7]. Carbon nitride (CN$_x$) films have been attracting great interest in biomedical applications mainly because of their excellent mechanical and tribological properties, chemical inertia; optical properties [8, 9 and 10]. In 2000, Cui and Li summarized the previous investigated work on biocompatibilities of DLC and CN$_x$ coatings [11]. CN$_x$ films have been prepared by magnetron sputtering [12], electron cyclotron resonance plasma-assisted vapor deposition [13], plasma enhanced chemical vapor deposition (PECVD) [14] and DBD plasma CVD [5, 6 and 7]. Only one contradictory result has been reported regarding cytocompatibility (with respect to HEK and PC 12 cell lines) of aH-CN$_x$ film deposited by dielectric barrier discharge plasma [15]. 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. 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 structur.

In the current study we have investigated the effect of the DBD assisted plasma deposited film (CH$_4$/N$_2$=1:1) on HEK and PC12 cell lines to understand cytotoxicity of these films. The chemical properties/composition of the deposited film has been analyzed by photoelectron spectroscopy (XPS) and Fourier Transformed infrared spectroscopy (FTIR).

II. Experimental

A. Film preparation

Three films were deposited with varying CH$_4$;N$_2$ gas mixture ratios as 1:1, 3:1 and 1:3 renamed as BIOCN1, BIOCN2 and BIOCN3 respectively. The experimental set up of the dielectric barrier discharge has been explained in details elsewhere [15 and 16]. Experiments were performed at 10.5 kV (peak-to-peak) and at 4 kHz. The electrical power under these conditions was of 4 W.

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 vapour, 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 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 aH-CNₓ films were performed on a multi-technique 100 mm hemispherical electron analyser (CLAM2: VG Microtech), using Mg Kα radiation (photon energy 1253.6 eV) as the excitation source and the binding energy (BE) of Au (Au 4f⁷/₂: 84.00 eV) as the reference. The XPS spectra were collected in a constant analyser energy mode, at a chamber pressure of 10⁻⁸ mbar and pass energy of 23.5 eV at 0.125 eV/step.

D. Cell culture

Human embryonic kidney (HEK) and rat adrenal pheochromocytoma (PC12) cell lines were cultivated on three independent aH-CNₓ films (BIOCN1, BIOCN2 and BIOCN3) samples. The cells were seeded in vessels created on the coated glass with the help of silicon rings. For parallel experiments, 4 wells were investigated per coating. 1 ml cell suspension was dosed in 1 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% foetal calf serum (FCS). HEK cells were cultured in Dulbecco’s modified eagles’s medium (DMEM) 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 hours. The dishes were taken out at different times to take some pictures [15]

III. Results and Discussion

A. Chemical and Physical Properties:

Fourier transforms infrared spectroscopy (FTIR)

FTIR absorption measures the vibrational, stretching, symmetric/antisymmetric bands configuration (range 4000-500 cm⁻¹) present in the deposited polymer film. Two aH-CNₓ films (BIOCN1 and BIOCN3) have been analyzed by FTIR technique. Spectroscopic properties of the polymerized films deposited at different mixture concentrations of the reactive gases CH₄:N₂ are presented in figure 1, where the ratios are 1:1 and 1:3 respectively [7, 15]. The band between 3100 cm⁻¹ and 3700 cm⁻¹ is attributed to stretching vibrations of NH and OH functionally groups [7 and 15]. The second interval (3010 cm⁻¹ - 2810 cm⁻¹) is the characteristic for CH₂ and CH₃ groups [7 and 15]. The broad absorption single peak at 2176 cm⁻¹ is attributed to C≡N triple bond stretching vibration (so called nitrile group). The broad band from 1645 cm⁻¹ to 1665 cm⁻¹ is attributed to C=C and C=N stretching mode [7 and 15]. The absorption band observed in the interval 1350 cm⁻¹ - 1480 cm⁻¹ corresponds to the C-N single bond stretch [7 and 15].

X-ray photo electron spectroscopy (XPS)

X-ray photoelectron spectroscopy measures the chemical composition and the bond structure of the polymer-like film (aH-CNₓ) layer. Two CNₓ films (BIOCN1 and BIOCN3) have been analyzed by XPS technique. As the proportion of N₂ in the CH₄:N₂ gas mixture is increased, (Figure 3) so does the C≡N group also increase in the BIOCN3 film. Figure 3 (a) shows, the C-1s spectrum (CH₄:N₂ = 1:1) exhibits peaks at 284.65 eV, 285.65 eV, 286.68 eV and 287.98 eV, which are attributed to C=C, C=N, C-N or C≡N and C-O bonds, respectively [6, 7 and 15]. Similarly, from figure 3 (b), the C-1s the spectrum for CH₄:N₂ = 1:3 (dotted lines)
which are attributed to C≡C, C≡N, C-N or C≡N and C-O bonds, respectively [6, 7 and 15]. In some cases, a peak appears at 286.4 eV and is attributed to the nitrile group (C≡N) [7 and 15].

Fig 2. Typical C 1s XPS spectra of aH-CN$_x$ films when (a) CH$_4$:N$_2$ = 1:1 and (b) CH$_4$:N$_2$ = 1:3 (f = 4 kHz and P = 400 mbar)

B. Cell Culture

Cell Culture:

Glass is a suboptimal substrate for cell cultivation, however less sensitive cells like HEK cells adhere quite well (Fig 3a). PC12 cells are a more challenging cell line, they adhere in a small density (Fig. 3b) and a noticeable amount swims death in solution (out of focus). For comparison, HEK and PC12 were grown on BIOCNI 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. 4a. The cells are round up, their diameter is drastically reduced. PC12 cell adhesion is completely inhibited (Fig 4b). While a similar cell behavior was obtained on the BIOCNI2 film (not shown here), the cell adhesion on BIOCNI3 film is even worse (Fig 5). HEK cell adhesion is also drastically suppressed on this film, compared to BIOCNI1 film. The reason could be the higher N$_2$ dose applied in film preparation and higher N-content obtained in XPS [15]. 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.

Fig 4. Microscopic images of (a) HEK and (b) PC12 cells both on BIOCNI aH-CN$_x$ film. The film has been deposited at ratio of CH$_4$:N$_2$ = 1:1. Time duration of the deposition is 9 hours

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 aH-CN$_x$ coated substrate. Still there are some arguments regarding this behavior. aH-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 aH-CN$_x$ film could break the peptide configuration and induce apoptotic signals. This way aH-CN$_x$ films could be highly active to live cells. The cell death is very important to examine, what kind of poly-carbon 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 seen to be
damaged and causes the aggressive and invasive growth in future which turns to move towards cancer cells growth. In such a case we can use the coating of CN\textsubscript{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.

Fig 5. Microscopic images of (a) HEK and (b) PC12 cells on BIOCN3 aH-CN\textsubscript{x} film. The film has been deposited at ratio of CH\textsubscript{4}:N\textsubscript{2} = 1:3. Time duration of the deposition is 17 hours.

Reference