

IN VITRO BIOCOMPATIBILITY OF AMORPHOUS CARBON BASED COATINGS BY VARYING OF SURFACE CHEMISTRY AND NITROGEN CONCENTRATIONS

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The study of a-C:N coatings at different concentration of nitrogen, their surface chemistry and wettability effect on cell/material response in vitro test was performed. The surface structure of deposited coatings was investigated by means of scanning electron microscopy (SEM) and atomic force microscopy (AFM) methods. The coatings were characterized with respect to their bonding structure by photoelectron spectroscopy (XPS) analysis. The wettability was analysed by means of advanced water contact angle method and the surface free energy (SFE) was calculated according to Robertson equation. The biocompatibility was estimated by standard protocols. The best results were obtained in the case of coatings with the greater parameters of SFE and the minimal values of ratio $N_2 : C_7H_8$.

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INTRODUCTION

Amorphous carbon based coatings have a great potential for biomedical applications due to its high hardness, low frictional coefficient, chemical inertness, high wear and corrosion resistance. These properties match well with the criteria of a good biomaterial for applications in orthopedic, cardiovascular and dentistry. In vitro tests of cell adhesion on material and coating surfaces are the basic tools to determine the material surface/cell response on a cellular level [1, 2]. The effects of materials composition, surface chemistry and surface topography on cell adhesion and proliferation have been largely studied [3, 4]. The surface energy is also the fundamental material property that can influence on cell behavior [5]. Changing of surface chemistry enables to control wettability of amorphous carbon based coatings and future biological response.

1. MATERIALS AND METHODS

The investigations of a-C:N coatings surface properties effect on cell adhesion in vitro test were made. The coatings with different concentration of nitrogen were formed on glass substrates. The deposition process was carried out by adding steam-to-gas mixture in glow discharge plasma generated by DC ion source with different ratio $N_2:C_7H_8$ at chamber. The main parameters of deposition process were presented: the ion source power 150 W, bias voltage in the range 80...160 V, substrate temperature and nitrogen concentration in mixture in the ratio $N_2:C_7H_8$ 10:90, 15:85, 20:80, 25:75. Adjustment of deposition conditions has an important influence on surface chemistry and wettability of amorphous carbon based coatings.

The surface structure and morphology of deposited coatings were investigated by means of scanning electron microscopy (SEM) and atomic force microscopy (AFM) methods. X-ray photoelectron measurements have been carried out on the ESCALAB MkII (VG Scientific) electron spectrometer at a base pressure in the analysis chamber of 5×10^{-8} Pa using anode AlK α X-ray source with excitation energies of

1486.6 eV (160 W). The spectra are recorded at the total instrumental resolution (as it was measured with the FWHM of Ag3d_{5/2} photoelectron line) of 1.18 eV for AlK α excitation source. The energy scale has been calibrated by normalizing the C1s line of adsorbed adventitious hydrocarbons to 285.0 eV. The processing of the measured spectra includes a subtraction of X-ray satellites and Shirley-type background [6]. The peak positions and areas are evaluated by a symmetrical Gaussian-Lorentzian curve fitting. The relative concentrations of the different chemical species are determined based on normalization of the peak areas to their photo ionization cross-sections, calculated by Scofield [7].

The wettability was investigated by means of sessile-drop method of dynamic contact angle measurement of distilled water at temperature 20°C. The surface free energy (SFE) was calculated according to Robertson equation. The cytotoxicity and cyto compatibility were estimated in vitro tests by standard protocols. In the process of cell cultivation (fibroblasts) with amorphous carbon coated and control samples the cell cytology, morphology and vital capacity were determined after 24h and 3 days cultivation. Rat hypodermic cellular tissue was extracted to obtain an initial fibroblast culture. The suspension of extracted cells was centrifuged at 750 r.p.m. for 15 min. The cell density was 3×10^5 cells/ml. The seeded area was 0.5 cm². The fibroblasts were cultivated as a monolayer in 3 ml of Dulbecco Modified Eagle's Medium (DMEM, Sigma) at thermostat condition (37°C) for 5 days. The cells were stained by hematoxylin and eosin for further characterization of structural organization of cultured cells on coated and uncoated substrates. Cell structure and morphology were analyzed by optical microscopy (Micros-50). The experiments were run in triplicate. Cell viability was tested on amorphous carbon based coatings at different concentrations of nitrogen substrates. The qualitative and quantitative analysis were made. The number of detached cells was determined by quantitative assessment. Statistical processing of experimental results using the software

package with a preliminary estimation of the normal distribution was conducted. Statistically significant differences were determined at a significance level $P_1 > 0.05$.

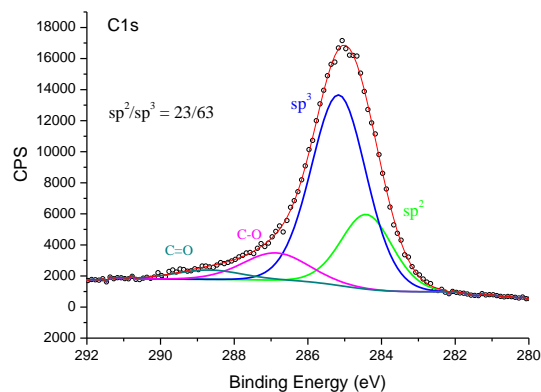
2. RESULTS AND DISCUSSION

The surface topography and morphology of deposited coatings were observed by AFM (Fig. 1)

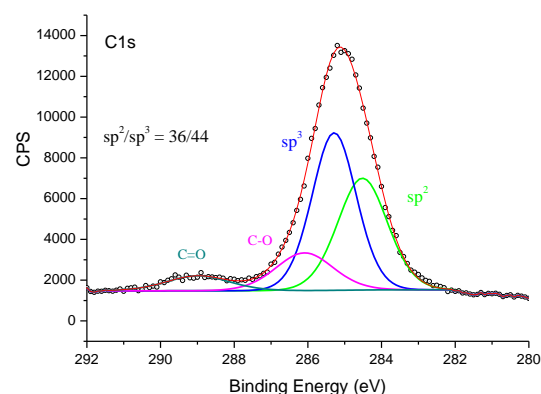


Fig. 1. The surface topography of the of the amorphous carbon based coatings by AFM

The coatings are characterized with respect to their bonding structure at different stoichiometric compositions by photoelectron spectroscopy (XPS) analysis. The ratio of sp^2/sp^3 carbon atoms is one of the most important factors governing the quality of the amorphous carbon based coatings. The fittings for the samples deposited at the different concentration of nitrogen are presented in Fig. 2. The first peak (see Fig. 2,a) is found at 284.5 eV and corresponds to sp^2 carbon atoms, while the second at 285.4 eV corresponds to sp^3 carbon atoms. A third peak of much smaller intensity at 286.8 eV has also been added and is attributed to some C–O contamination formed at the surface of the samples due to air exposure.



a



b

Fig. 2. Deconvolution of the XPS C 1s peaks and surface atomic concentrations of the amorphous carbon based coatings deposited at different nitrogen concentrations a) $N_2 : C_7H_8$ 10:90; b) $N_2 : C_7H_8$ 25:75

From the deconvoluted spectra, the sp^3 content in the films is evaluated. The binding energy values found for the sp^2 and sp^3 components of the amorphous carbon based coatings C 1s spectra are consistent with the binding energies of 284.4 and 285.2 eV detected for C 1s peaks of standard graphite and diamond XPS spectra [8, 9]. The analysis of the C 1s peak is a direct method to evaluate the sp^3 content in amorphous carbon based coatings at different concentration of nitrogen. The surface atomic concentrations of the amorphous carbon based coatings deposited at different nitrogen concentrations are presented in Table 1.

Table 1. Surface atomic concentrations of the amorphous carbon based coatings deposited at different nitrogen concentrations: $N_2 : C_7H_8$ 10:90, $N_2 : C_7H_8$ 25:75

Ratio: $N_2 : C_7H_8$	Surface Atomic concentrations, at. %		
	C1s	O1s	O1s/C1s
10:90	89	11	0.12
25:75	87	13	0.15

The values of surface free energy were calculated according to Robertson equation [10] from water contact angle measurements at 20°C (Table 2).

Table 2. The values of water contact angle and surface free energy of the amorphous carbon based coatings deposited at different nitrogen concentrations

Ratio: N ₂ : C ₇ H ₈	Water contact angle, θ	Surface free energy (mN/m)
10:90	76.7	89.62
15:85	80.1	85.38
20:80	88.3	75.02
25:75	89.7	73.24

The modification of coatings properties by changing plasma chemistry by adding nitrogen into the plasma mixture in the ratio N₂:C₇H₈ 10:90, 15:85, 20:80, 25:75 leads to distilled water contact angles varying in the range of 77...88° and SFE parameters in the range of 90...70 mN/m, respectively. Furthermore, an additional nitrogen concentration leads to an increase of contact angles and decrease of surface free energy. Generally, the obtained results show that the surface properties are strongly influenced by the coating's deposition conditions and a combination of deposition

parameters with optimized plasma chemistry allows to tailor surface free energy parameters.

Cell cytotoxicity was estimated during *in vitro* tests. After 3 days immersion in DMEM culture medium fibroblast cells were well-spread on all substrates. The cell structural organization corresponded to that of the initial fibroblast with strongly expressed phenotype. The meaningful differences in cell viability on substrate surfaces were observed. The maximal number of detached cells after 3 days cultivation demonstrate coatings with maximal ratio N₂:C₇H₈ composition in comparison with control samples (Table 3).

The data demonstrate that cell adhesive potential and phenotypical characteristics were different on the amorphous carbon based coatings at nitrogen concentration varying.

The best results were obtained in the case of coatings with the minimum values of distilled water contact angle and the greater parameters of SFE with the ratio N₂:C₇H₈ 10:90 and 15:85. The deposition process controlling allows to control the surface chemistry and wettability of amorphous carbon based coatings and the next biological response.

Table 3. The total and detached cells number on control and coated glass substrates after 3 days cultivation

Samples	Number of cells after 3 days staying in fibroblast culture	
	Total cells number	Detached cells number
Glass substrate (control)	3.77x10 ⁴ ±2.97x10 ³ P ₁ > 0.05	3.06x10 ³ ±3.15 x10 ² 8%
N ₂ : C ₇ H ₈ 10:90	3.96x10 ⁴ ± 3.57 x10 ³ P ₁ > 0,05	3.45x10 ³ ±3.17 x10 ² 8.7%
N ₂ : C ₇ H ₈ 15:85	3.85x10 ⁴ ±2.94x10 ³ P ₁ > 0,05	3.65x10 ³ ±2.82x10 ² 9%
N ₂ : C ₇ H ₈ 20:80	3.72x10 ⁴ +3.24x10 ³ P ₁ > 0.05	3.98x10 ³ +3.68x10 ² 10.6%
N ₂ : C ₇ H ₈ 25:75	3.88x10 ⁴ +3.49x10 ³ P ₁ > 0.05	4.46x10 ³ +3.83x10 ² 11.5%

CONCLUSIONS

The results show that the surface properties of deposited coatings are strongly influenced by the deposition conditions. The properties, and subsequently the quality of the amorphous carbon based coatings, strongly depend on their microstructure, which is commonly considered as an amorphous mixture of sp² and sp³ carbon atoms. The analysis of the X-ray photoelectron spectra of the C 1s core level of amorphous carbon based coatings, obtained at different concentration of nitrogen is presented. These spectra are deconvoluted into two different contributions, at 284.5 and 285.4 eV, which are respectively attributed to sp² and sp³ carbon atoms. The *in vitro* tests demonstrate the good biocompatibility of amorphous carbon based coatings. The additional nitrogen concentration leads to an increase of contact angles and decrease of surface free energy. Replacing the surface bounds with nitrogen by plasma treatment results in shear of surface parameters at more hydrophobic region. The best biological response parameters (cell number, viability, cell morphology) were obtained in the case of amorphous carbon based coatings with the most

parameters of SFE. The possibility of controlled cell adhesion on the material surfaces may propose novel methods of surface modifications for further tissue engineering applications.

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IN VITRO БИОСОВМЕСТИМОСТЬ АМОΡФНЫХ УГЛЕВОДОРОДНЫХ ПОКРЫТИЙ ПРИ ВАРЬИРОВАНИИ ПОВЕРХНОСТНОЙ ХИМИИ И КОНЦЕНТРАЦИИ АЗОТА

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Рассматривается влияние поверхностной химии и смачиваемости на клеточную адгезию *in vitro* для аморфных углеводородных покрытий, содержащих различные концентрации азота. Структура поверхности покрытий исследовалась методами сканирующей электронной и атомно-силовой микроскопии. Энергии связи были характеризованы методом фотоэлектронной спектроскопии. Смачиваемость поверхности анализировалась методом контактного угла, а поверхностная энергия рассчитывалась согласно уравнению Робертсона. Биосовместимость оценивалась по стандартным методикам. Лучшие параметры были получены для покрытий с наибольшими значениями поверхностной энергии и минимальным соотношением $N_2 : C_7H_8$.

IN VITRO БІОСУМІСНІСТЬ АМОΡФНИХ ВУГЛЕВОДНИХ ПОКРИТТІВ ПРИ ВАРІЮВАННІ ПОВЕРХНЕВОЇ ХІМІЇ ТА КОНЦЕНТРАЦІЇ АЗОТУ

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Досліджено вплив поверхневої хімії та змочування на клітинну адгезію *in vitro* для аморфних вуглеводних покриттів, що мають різні концентрації азоту. Структура поверхні покриттів була досліджена методами скануючої електронної та атомно-силової микроскопії. Енергії зв'язку були характеризовані методом фотоелектронної спектроскопії. Змочування поверхні було проаналізовано методом контактного кута, а поверхнева енергія розраховувалась згідно з рівнянням Робертсона. Біосумісність оцінювалась згідно з стандартними методиками. Найкращі параметри були отримані для покриттів з найбільшими значеннями поверхневої енергії та найменшим співвідношенням $N_2 : C_7H_8$.