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Genotoxicity Study of Carbon Nanoforms using a Comet Assay

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Carbon nanoforms due to their unique properties can be applied in many areas also in medicine. This article presents preliminary genotoxicity studies of electrospun carbon nanofibers (ECNF). This material, apart from its numerous applications, may also be a candidate for use in medical therapy and diagnostics. Polyacrylonitrile (PAN) nanofibers received in the electrospinning process were carbonized and thereafter subjected to oxidation treatment (ECNF-F). Both types of fibres were analyzed with regard to genotoxic influence on the fibroblast line cells using comet assay. Additionally, comet assay experiments were conducted on biocompatible carbon nanotubes with a hydrophilic surface. The results indicate the key role of the oxidation process in the functionalization of carbon nanoparticles intended for medical purposes.

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1. Introduction

Carbon nanotubes and nanofibers are materials of high utility potential in numerous fields, including medicine. On the basis of carbon nanotubes, nanodrug and genes delivery systems are designed, as well as various kinds of biosensors with properties vastly exceeding current diagnostic tools [1, 2]. Carbon nanotubes are a material able to stimulate bone and nerve tissue regeneration. Therefore, beside carbon nanotubes, thanks to their electric, thermal, and mechanical properties, as well as vast possibilities of chemical modification, carbon nanofibers can also be used in medicine, particularly in systems designed to aid tissue regeneration [3]. On the basis of nanofibers, 3D systems can be designed for tissue engineering applications or as nanocomposite biomaterials in which nanofibers constitute one of the phases and influence the properties crucial for medical uses [4, 5]. Nonetheless, despite the numerous documented, extraordinarily beneficial properties of carbon nanotubes, there are concerns related to their toxicity [6, 7]. Currently, it is assumed that carbon nanotubes require specific functionalization in order to be used in medicine, while nanotubes not subjected to treatment, particularly oxidation treatment, are toxic for cells and tissues. Carbon nanofibers received in carbonization of polymer precursors produced through the electrospinning process are a material significantly easier to functionalize in comparison to carbon nanotubes. The structure of carbon nanofibers results in the phenomenon that the edges of graphene layers, exposed on their surface, become spots of high activity,

easily reacting with substances used for their functionalization [8].

The purpose of the paper was genotoxicity assessment of two types of nanofibers: carbon nanofibers (ECNF), and carbon nanofibers subjected to oxidation treatment (ECNF-F). Additionally, modified carbon nanotubes (MWCNT-F) were tested. Genotoxicity of investigated nanomaterials was evaluated by the comet assay. This test, which can detect DNA double and single-strand breaks as initial damage and those developed from alkali-labile sites under alkaline condition, is a rapid and sensitive procedure for detecting genotoxicity in mammalian cells [9, 10].

2. Materials and methods

Carbon nanofibers were manufactured through carbonization of a polymer precursor. The precursor nanofibers were created from polyacrylonitrile (PAN) copolymer composed of 93–94% w/w of acrylonitrile mer units, 5–6% w/w of methyl methacrylate mer units, and 1% w/w of sodium allyl sulfonate mer units (Zoltek, Hungary). As solvent, *N,N*-dimethylformamide (DMF) with a molar mass of $M = 73.10$ g/mol (POCH SA, Poland) was used. Polymer nanofibers were subjected to a two-phase processing into carbon fibers. The first phase was conducted at an oxidation temperature of 250–300 °C and the second phase consisted of carbonization in a nitrogen atmosphere up to 1000 °C. Thereafter, a portion of the fibers was subjected to oxidation treatment in a concentrated nitric acid at 60 °C for 1 h. Each group of nanomaterials was fragmented in conditions safe from environmental influence. MWCNT (Nano-Amor USA) were modified in acid mixture (H₂SO₄, HNO₃). The oxygen on the surface of the MWCNTs creates single and double oxygen bonds with the graphene layer [11]. Scanning

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electron microscopy (SEM) images of carbon nanofibers are presented in Fig. 1, while data on type of oxygen group, received from measurements obtained from X-ray photoelectron spectroscopy (XPS) are presented in Table I. Microscopic observations were performed using a scanning microscope Nova-Nano-SEM, FEI Europe Company. Evaluation of the oxygen groups on the surface of carbon nanoforms was conducted by XPS (Vacuum System Workshop Ltd. England).

TABLE I

Oxygen groups O 1s (binding energy E_b [eV] and atomic concentration n [%]) on surfaces of carbon nanofibers — calculated from XPS studies.

Functional group	ECNF		ECNF-F	
	E_b	n	E_b	n
carbonyl	531.47	8.78	530.95	6.92
hydroxyl, ethers	532.50	82.11	532.27	76.04
lactone, carboxylic	534.17	9.91	533.92	17.04

To estimation of genotoxicity of studied nanomaterials the normal human skin fibroblasts from cell line CCL-110 (American Type Culture Collection, ATCC) was used. Cells were cultured in MEM medium supplemented with 20% FBS, 1% penicillin-streptomycin and 2 mM L-glutamine at the temperature of 37°C and 5% CO₂. The cells were seeded on the 6-wells plates. The studied nanomaterials (7.5 mg) were suspended in 4 ml PBS and mixed with use of ultrasonic probe (PALMER INSTRUMENTS, Model: CP 130 PB) for 2 min. Nanomaterial's suspension (500 μ l) was added to well containing cells in 2 ml culture medium. Then fibroblasts were chemically treated with nanomaterials (MWCNT-F, ECNF and ECNF-F) at 37°C for 1 h or 24 h. After chemical treatment, the cells were washed in PBS, and analysed by comet assay procedure. The analysis of DNA damage levels after *in vitro* treatment was performed using the alkaline version of the comet assay [10, 11]. The suspension of *in vitro* treated fibroblasts (50 μ l) in agar LMA (150 μ l) was accumulated in layers on a microscopic slide covered with 1% agar NMA. These materials were left to gelation at 4°C. The slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 1% sodium sarcosinate, 100 mM TRIS) with 10% dimethyl sulfoxide and 1% Triton X-100 added immediately before use. After 1 h cell lysis, the slides were transferred to an electrophoretic apparatus and immersed in freshly made, cold electrophoretic buffer (1 mM EDTA, 300 mM NaOH) of pH>13 and left for 20 min to allow the DNA to unwind. Electrophoresis was carried out for 30 min at 4°C (30 V, 300 mA). After electrophoresis, the slides were washed in 0.4 M TRIS buffer of pH 7.5. After neutralization, the slides were stained with 20 μ l of ethidium bromide (17 μ g/ml). The stained slides were analyzed for the presence of DNA damage in the comets by means of an Olympus BX 50 epifluorescence microscope. For the analysis of DNA in the comets, DNA percentage in the comet tail parameter (tDNA) from the Komet

3.0 software (Kinetic Imaging Company, Liverpool, UK) was used. Additionally, for all samples the dead cells were manually counted. Two independent experimental replicates were performed for each aliquot: 200 cells were analyzed for each data point (2 slides per each dose, 100 cells analyzed from each slide). The data show mean value and standard error. The statistical analysis was performed using *t*-Student test from Excel software. The *p*-values equal to or less than 0.05 were considered significant.

3. Results and discussion

Materials examined in the research are significantly different; the diameter of carbon nanofibers is larger in comparison to the diameter and length of MWCNTs. SEM-images of the carbon nanofibers indicate large difference in size of the particles, however, images of both types of nanofibers are similar to one another (Fig. 1). Differences between carbon nanotubes and ECNF nanofibers are first and foremost in their structure. Nanotubes have well-defined structure, consisting of coiled coaxial

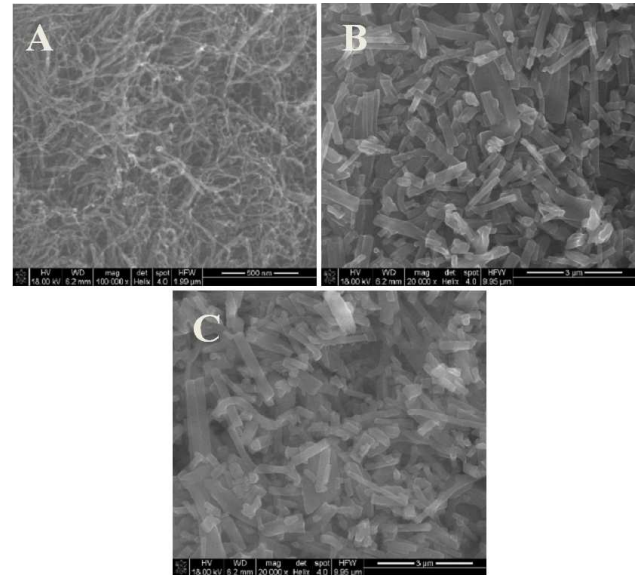


Fig. 1. SEM images of functionalized carbon nanotubes (A), carbon nanofibers before (B) and after oxidation treatment (C).

Genotoxicity of nanomaterials in fibroblasts cell line estimated by use t-DNA comet assay parameter for time of chemical incubation τ . TABLE II

τ	Control	MWCNT-F	ECNF	ECNF-F
1 h	3.59±0.40	4.03±0.23	8.77±0.81	5.31±0.18
significance/control	3% dead cells	4% dead cells	28% dead cells	12% dead cells
		0.46	0.00	0.00
24 h	3.92±0.60	4.74±0.65	7.85±0.64	4.51±0.39
significance/control	4% dead cells	5% dead cells	32% dead cells	6% dead cells
		0.04	0.00	0.20

graphene layers. The oxygen groups created as a result of MWCNTs modification appear on the surface of the graphene layer, as well as on its edges [7]. Carbon nanofibers are materials with a structure difficult to define, similar to turbostratic carbon [8]. The data on character of chemical groups present in both types of nanofibers indicate that their number and type change as a result of the oxidation treatment. On the surface of oxidized nanofibers, an increase in carboxyl groups concentration is noted with simultaneous decrease in carbonyl and hydroxyl groups in comparison to the surface of non-oxidized fibers. Oxidation of carbon nanofibers increases especially the concentration of carboxylic groups, which gives to the hydrophobic carbon surface a hydrophilic character (Table I). Genotoxicity of the carbon nanoforms has been assessed using the analysis of comets, in terms of the tail DNA, as shown in Table II. In comparison to control, for ECNF significantly higher level of DNA damage level (8.77) was observed. These results and additionally higher percent of dead cells (30%) indicate that the ECNF do not provide favorable conditions for cells adhesion and growth. Findings obtained for non-fictionalized carbon nanofibers suggested their genotoxicity, which is confirmed by Kisin et al. research performed on lung fibroblast (V79) cell line by the use comet assay and micronucleus (MN) test [12]. However, after functionalization these nanomaterials (ECNF-F) are better tolerated by the cells (12% dead cells) and have lower genotoxicity (5.31). Furthermore, after 24 h incubation the DNA damage level (4.51) was comparable to control. A positive effect of functionalization was observed also in case of carbon nanotubes, which indicated good cyto-compatibility.

4. Conclusion

The oxidation treatment of the carbon nanofibers does not cause significant changes in the morphology of the material however it influences the surface chemical state. Genotoxicity assessment determined that the least tolerable material for the cells is ECNF and its toxicity level is not influenced by the time of incubation in the nutrient media in contact with cells. On the other hand, ECNF-F is a material of much better properties in the context of reactions with cells — the results obtained in this case indicate the cyto-compatibility character of ECNF-F. The results presented in the paper unambiguously indicate that oxidation treatment necessary for nanotubes for medical use is also crucial in the case of carbon fibers. It has already been determined that subjecting carbon fibers, received through nanometric precursor carbonization, to even short-term treatment with nitric acid changes their character from toxic to non-toxic.

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