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Full Length Article

Cytocompatibility of polyethylene grafted with triethylenetetramine functionalized carbon nanoparticles

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ABSTRACT

Various carbon nanostructures are widely researched as scaffolds for tissue engineering. We evaluated the surface properties and cell-substrate interactions of carbon nanoparticles functionalized with triethylenetetramine (CNPs) grafted polymer film. Two forms of polyethylene (HDPE, LDPE) were treated in an inert argon plasma discharge and, subsequently, grafted with CNPs. The surface properties were studied using multiple methods, including Raman spectroscopy, goniometry, atomic force microscopy, X-ray photoelectron spectroscopy and electrokinetic analysis. Cell-substrate interactions were determined *in vitro* by studying adhesion, proliferation and viability of vascular smooth muscle cells (VSMCs) from the aorta of a rat. Cell-substrate interactions on pristine and modified substrates were compared to standard tissue culture polystyrene. Our results show that CNPs affect surface morphology and wettability and therefore adhesion, proliferation and viability of cultured muscle cells.

1. Introduction

Between crucial features for materials application in medical sciences, and mainly for tissue engineering, belong cell-substrate interactions [1]. These interactions play an important role in determining the cell growth, differentiation and organization [2,3]. A key factor in cell/material or cell-to-cell communication is extracellular matrix (ECM). ECM provides biochemical and mostly structural support to surrounding cells. ECM is secreted naturally by cells and is made of a collection of extracellular molecules. This collection of molecules (e.g. various proteins and non-protein substances, metabolites or ions) is unique in composition for different types of cellular structures [4,5]. In tissue engineering, cells adhere and proliferate to artificial scaffolds. Materials used for such purposes are called synthetic extracellular matrix, because they provide the necessary structural support [6].

A wide variety of polymers that are of natural or synthetic origin can be used for medical applications (e.g. tissue engineering). For such applications the materials need to be not only non-toxic but they must evince certain characteristics depending on the target application e.g. nerve tissue [7], artificial skin [8], bone grafts [9], cartilage [10] etc. The desired characteristics may include mechanical or electrical properties which are an essential part of the materials that are to perform a given function. To enhance their biological performance those materials need to be further adapted. One of the possibilities is reinforcing the polymers with nanoparticles [11,12].

Carbon based nanomaterials, including carbon nanotubes, graphene, nanodiamond and carbon nanoparticles, have emerged as potential candidates for a wide variety of applications because of their unusual electrical, mechanical, thermal and optical properties [13–15]. However, carbon nanostructures have a tendency to aggregate. To improve their limited solubility and aforementioned tendency to aggregate they can be functionalized with hydrophilic groups [16]. Such hydrophilization can be done by introducing different amine groups onto the surface of carbon nanoparticle [17,18].

This work is focused on the study of plasma modified and subsequently amine-functionalized carbon nanoparticles grafted polyethylene (high and low density). Such modified substrates were studied by multiple analytical methods such as goniometry, atomic force microscopy and X-ray photoelectron spectroscopy. Cytocompatibility of selected samples was tested *in vitro* using vascular smooth muscle cells from the aorta of a rat (our work was focused on adhesion, proliferation and viability of seeded cells).

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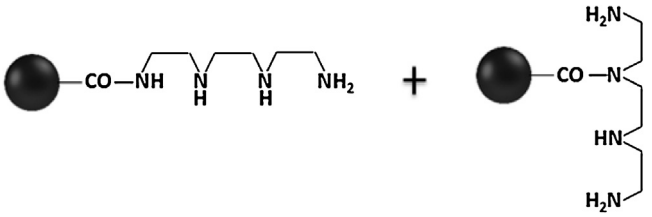

Fig. 1. Scheme of CNPs functionalized with triethylenetetramine.

2. Experimental

2.1. Material, plasma treatment, carbon nanoparticles activation and their grafting

The experiments were carried out on high and low density polyethylene (HDPE, LDPE) in the form of foils: (i) HDPE (thickness 40 mm, 0.941 g cm^{-3}, Granitol, CZ) and (ii) LDPE (50 μm, density 0.920 g cm^{-3}, Goodfellow Ltd., UK). The polymers were treated by Ar^+ plasma in Balzers SCD 050 under the following conditions: gas purity 99.997 %, pressure 10 Pa, electrode distance 50 mm, power 8 W, treatment times 60, 120 and 300 s.

The carbon nanoparticles (Activated charcoal-DARCO® KB-G, Sigma Aldrich, D, size 20–40 nm) were used in this study. The carbon nanoparticles were modified with triethylenetetramine in a three-step synthesis [18,19]. Such modified carbon nanoparticles (CNPs, molecular structure see Fig. 1), were activated in 1 mol l^{-1} water solution of HCl (1 h, room temperature (RT)). The plasma treated polymers' surfaces were grafted from activated CNPs suspension (24 h, RT, constant stirring).

In this work we studied several types of polymeric substrates samples: (i) pristine, (ii) plasma treated, (iii) plasma treated and etched in water solution of hydrochloric acid (environment from which the CNPs are grafted) and (iv) plasma treated, etched and CNPs grafted polymers.

2.2. Used analytical methods

The properties of pristine, plasma treated and CNPs grafted samples were studied using multiple analytical methods.

Raman spectroscopy was measured using a Thermo Scientific Raman Dispersion Spectrometer – a DXR Microscope model equipped with an Olympus confocal microscope. The excitation source was a diode Nd: YAG laser with a wavelength of 532 nm and an input power of 10 mW. A multichannel thermoelectric cooled CCD camera was used as a detector. Samples were measured at 50× magnification with a measurement trace of approx. 1 μm^2. To avoid thermal degradation of the sample, measurements were carried out at a power of 1 mW, a measurement time of 10 s and 10 spectra accumulations. Raman spectra were evaluated as difference spectra between Raman spectra of CNPs grafted and etched substrates.

Surface contact angle (CA, wettability) was measured using the static (sessile) water drop contact angle method. The measurements and evaluation was performed using the See System (Advex Instruments, CZ). The CA was measured at RT on 10 different positions on the sample surface using water (8 μl). The contact angles of all modified samples were measured 30 days after their complete modification. It is known that after such aging time the CA achieves saturation and remains constant [19].

Surface morphology and roughness of the pristine and modified samples were determined using a VEECO CP II AFM device (tapping mode) equipped with Si probe, RTESPA-CP with a spring constant 20–80 N m^{-1}. By repeated measurements in the same region (2 × 2 μm^2) it was shown that the surface morphology did not change after three consecutive scans. The roughness value (R_a) represents the arithmetic average of the deviations from the central plane of the sample.

An Omicron Nanotechnology ESCAProbeP spectrometer was used to measure ARXPS spectra of modified polymer surfaces. The 2 × 3 mm^2 area was analyzed. The X-ray source provided monochromatic radiation of 1486.7 eV. The spectra were measured stepwise with a step in the binding energy of 0.05 eV at each of the six different sample positions. The O(1s), C(1s) and N(1s) peaks were studied. The spectra evaluation was carried out by using CasaXPS software. The concentrations of the elements (with only exception of hydrogen which is not observed by XPS) are given in at.%.

Electrokinetic analysis (zeta potential) of all samples was determined by SurPASS Instrument (Anton Paar GmbH, AT). Samples were studied inside the adjustable gap cell with an electrolyte of 0.001 M KCl in water. All samples were measured four times at constant pH = 6.7 and at RT. For zeta potential determination the streaming current method and Helmholtz-Smoluchowski equation were employed with experimental error of 5% [20].

2.3. Cell culture, adhesion and proliferation and viability

The adhesion and proliferation of vascular smooth muscle cells (VSMC) on pristine and modified polymers were studied *in vitro*. The samples were sterilized for 1 h in 70 % ethanol in a Petri dish. After that they were inserted to 12-well plates (TPP, CH, diameter 2.14 cm) and fixed to the well bottom with plastic rings. VSMC (7.pasage) were seeded on the samples with the density of 50 000 cells/well (i.e. 17000 cells cm^{-2}) into 3 ml of Dulbeccoís Modified Eagle Minimum Essential Medium (DMEM, Sigma, USA, Cat. No. D5648) containing 10% fetal bovine serum (FS, Sebak GmbH, Aidenbach, Germany) and 40 μm/ml of gentamicin (LEK, Ljubljana, Slovenia). The cells were cultivated on the samples for 24, 72 and 144 h at temperature 37 °C, humidity 85% and air atmosphere containing 5% CO_2.

After the cultivation times, the samples were rinsed in phosphate-buffered saline (PBS) and fixed in a deep-frozen 70 % ethanol (−20 °C) for 45 min. The samples were then stained for 1 h at room temperature with the following combinations of two fluorescent dyes: (i) Texas Red C_2-maleimide (Molecular Probes, Invitrogen, No.T6008; concentration 20 ng ml^{-1} in PBS) which stains the membrane and cytoplasmic proteins (red color) and (ii) Hoechst # 33342 (Sigma, USA, 5 μg ml^{-1} PBS) which stains the cell nuclei (blue color). Before taking photographs, the samples were rinsed in PBS. The number and morphology of cells on the sample surface were then evaluated in photographs taken under an Olympus IX 51 microscope (objective 20×, 20 photos for each sample, visualized area of 0.136 mm^2), equipped with an Olympus DP 70 digital camera. The number of cells was determined using the image analysis software NIS-Elements AR 3.0.

The viability of cultivated cells was measured on Vi-Cell XR (Beckman Coulter) using the trypan blue dye exclusion method. The samples were rinsed in PBS, inserted to 12-well plates with trypsin and placed in the thermostat for 7 min. After that DMEM was added to the samples in well plates. The cells in suspension (1 ml) were then transferred to cuvettes and placed in the automated Vi-Cell device.

3. Results and discussion

3.1. Characterization of CNPs grafted polymers

Difference Raman spectra presented in Fig. 2 show the successful grafting of CNPs onto the polymeric surfaces of HDPE and LDPE. In