AMINE FUNCTIONALIZED CARBON NANOPARTICLES GRAFTED BIOPOLYMER FOR CELL ADHESION AND PROLIFERATION

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Abstract

Various carbon nanostructures are widely researched for use in a number of medical applications. We study the surface properties and cell-substrate interactions of amine functionalized carbon nanoparticles (CNPs) grafted on biopolymer film. Poly-L-lactic acid, in form of polymer film, was treated in an inert argon plasma discharge and, subsequently, grafted with functionalized carbon nanoparticles. Selected samples were thermally stressed during or following the grafting procedure. The surface properties were studied using multiple methods (goniometry, X-ray photoelectron spectroscopy). 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 (TCPS). Our results show that CNPs affect surface morphology and wettability and therefore adhesion, proliferation and viability of cultured cells.

Keywords: Carbon nanoparticles, Polymer film, Surface plasma and thermal treatment, Surface properties, Cytocompatibility

1. INTRODUCTION

New possibilities for cell adhesion and proliferation are widely researched in order to find the best possible materials for numerous applications in tissue engineering. Among others, various forms of carbon nanostructures are researched because of their unique properties [1]. Enhancing required properties such as cyto- and bio-compatibility for medical usage (e.g. drug carriers, scaffolds etc.) by nanoparticle functionalization is nowadays a common approach [2]. However research is mainly focused on graphene [2], carbon nanotubes [3], carbon nanodiamonds [4] and diamond-like-carbon [5]. Those structures are functionalized by other types of nanoparticle (e.g. metal nanoparticles) [6] or by variety of other chemical compounds [7]. This creates an opportunity to study spherical forms of carbon nanoparticles and functionalize them by different types of compounds (e.g. amines) in order to enhance their desired properties [8].

Cell adhesion can be divided in two major groups - receptor enhanced interactions and interactions without receptor intervention. Mechanism of cell interactions using receptor intervention is arranged through molecules of extracellular matrix (ECM). Cell adhesion to artificial substrates is mediated by ECM proteins (e.g. fibronectin, laminin, collagen, vitronectin etc.) that are adsorbed to the material surface from biological fluids (*in vivo*) or from cultivation media (*in vitro*). Range and strength of cell adhesion is important factor for regulation of cell proliferation and differentiation. Therefore is important for the material to produce “the right signals” which will lead the cell towards desired behaviour [9-11].

In this study we prepared three types of amine functionalized spherical carbon nanoparticles grafted biopolymer films. The samples were characterized by goniometry and X-ray photoelectron spectroscopy. Cytocompatibility of these samples was determined in vitro, by studying adhesion, proliferation and viability of vascular smooth muscle cells (VSMCs).

2. EXPERIMENTAL

2.1. Material, plasma treatment, grafting

The experiments were carried out on poly-L-lactic acid (PLLA). Polymer was in the form of foil (Goodfellow Ltd., UK): thickness 50 μm, 1.25 g cm^{-3}. The polymer was treated in Ar^+ plasma (Balzers SCD 050) at room

temperature and under the following conditions: gas purity 99.997 %, flow rate 0.3 l s^{-1}, pressure 10 Pa, electrode distance 50 mm, its area 48 cm^2, chamber volume approx. 1000 cm^3, plasma volume 240 cm^3, power 8 W, treatment time 120 s.

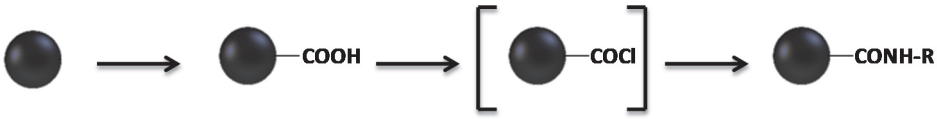

Fig. 1 Scheme of the three-step synthesis of functionalized carbon nanoparticles

The carbon nano-particles (Activated charcoal-DARCO® KB-G, Sigma Aldrich, D, size 20-40 nm) were used in this study. The carbon nanoparticles were modified with "amine" structure in a three-step synthesis (see **Fig. 1**) [12]. Such modified carbon nanoparticles (TET) (see **Fig. 2**) were activated in 1 mol l^{-1} HCl (1 hour, room temperature (RT)). The plasma treated polymers' surfaces were grafted from activated CNPs suspension in 1 mol l^{-1} HCl for 24h under constatnt stirring at (i) RT, (ii) 60°C, (iii) RT + 60°C (1h). "Blind sample" was prepared by etching the plasma treated substrate in water solution hydrochloric acid.

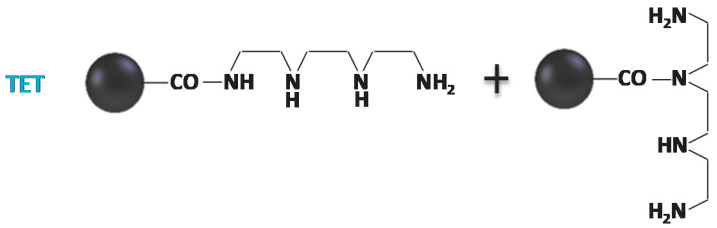

Fig. 2 Triethylenetetramine-functionalized carbon nanoparticles (TET)

2.2. Characterization methods

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

Surface contact angle (CA, wettability) was determined by goniometry, i.e. the static (sessile) water drop contact angle method. In this experiment, the contact angles of all modified samples were measured 30 days after the plasma modification. Advancing water (error ± 5 %) angles were measured at 10 different positions at room temperature using the Surface Energy Evaluation System (Advex Instruments, CR).

An Omicron Nanotechnology ESCAProbeP spectrometer was used to measure ARXPS spectra of modified polymer surfaces. 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 with respect to the detector axis (0° - perpendicularly to sample). The O(1s), C(1s) and N(1s) peaks were studied.

2.3. Study of cytocompatibility

The adhesion, proliferation and viability of vascular smooth muscle cells (VSMC) on pristine and modified polymers were studied *in vitro* as described in [12]. The number and morphology of cells on the sample surface were then evaluated in photographs taken under an Olympus IX 51 microscope. The number of cells was determined using the image analysis software NIS-Elements AR 3.0.

3. RESULTS AND DISCUSSION

3.1. Characterization of CNPs grafted polymer

Between factors that significantly affect cytocompatibility and therefore cell adhesion and proliferation belongs wettability. For this reason surface water contact angle was measured. After plasma treatment there is increase in CA values due to the formation of new groups and "removal" of polar hydroxyl group. Etching of the substrate causes a decrease of CA values due the removal of plasma degraded parts of the surface. TET grafting at

room temperature causes dramatic increase in the value of CA. This may be due to the heightened content of carbon in the layer. Reorientation of groups on the surface during grafting at glass transition temperature causes formation of more hydrophilic surface. Thermal stress of pristine, plasma treated and room temperature grafted substrate has no significant effect on surface wettability.

It is known, that free radicals, new bonds and subsequently new chemical groups arise after plasma treatment. This process is applied in surface chemical grafting, where different types of compounds can be covalently bonded to plasma modified surfaces [12]. Verification of TET bonded onto the biopolymer surface was studied by XPS and is summarized in **Table 1**. It is evident, that plasma treatment causes a decrease of oxygen content in the pristine polymer due to the ablation of surface layer containing polar hydroxyl group. After etching in water solution of hydrochloric acid, more hydroxyl groups are being removed from the degraded surface layer. Grafting of TET causes another decrease of oxygen level in the surface layer, due to the build-up of carbon and nitrogen. Thermal stress of plasma treated substrate causes an increase of oxygen concentration in the surface layer due to the possible reorientation of hydroxyl group to the surface. Thermal stress of room temperature grafted substrate causes slight increase in nitrogen concentration. Grafting of TET at the temperature of 60°C causes increase of nitrogen concentration and decrease in oxygen concentration in the surface layer due to the reorientation of groups on the surface at glass transition temperature.

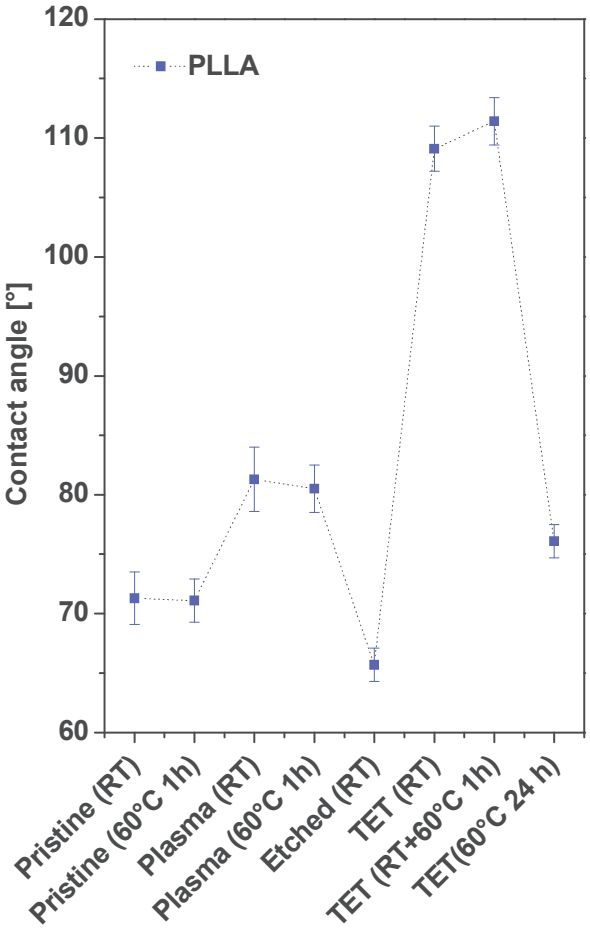

Fig. 3 Water contact angle measured on various PLLA samples (with and without thermal stress): pristine (pristine), plasma treated (plasma), etched in water solution of hydrochloric acid (etched) and CNPs grafted (TET)

Table 1 Element concentration (XPS) of different PLLA samples (with and without thermal stress): pristine (pristine), plasma treated (plasma), etched in water solution of HCl (etched) and CNPs grafted (TET)

Sample	Element concentration [at. %]		
	C (1s)	O (1s)	N (1s)
Pristine (RT)	60.9	39.1	--
Pristine (60°C 1h)	60.7	39.3	--
Plasma (RT)	63.0	35.6	1.4
Plasma (60°C 1h)	60.5	38.5	1.0
Etched (RT)	68.8	31.2	--
TET (RT)	67.6	31.8	0.6
TET (RT + 60°C 1h)	69.2	30.0	0.8
TET (60°C 24h)	70.1	28.7	1.2

3.2. Cytocompatibility of CNPs grafted polymer

Compared to the standard tissue culture polystyrene (TCPS), samples grafted with TET, with thermal stress achieve the same level of cell viability. Cells cultured on the substrate grafted with TET at room temperature achieve 98.2% viability, in comparison with TCPS with only 93.8%.

Table 2 Viability of VSMCs adhered and proliferated on different PLLA samples (with and without thermal stress): pristine (pristine), plasma treated (plasma), CNPs grafted (TET) and TCPS

Sample	Viability [%]		
	1st day	3rd day	6th day
Pristine (RT)	91.7	98.6	95.0
Pristine (60°C 1h)	76.5	98.3	97.1
Plasma (RT)	96.6	96.8	96.7
Plasma (60°C 1h)	93.5	97.6	97.0
TET (RT)	86.4	98.3	98.2
TET (RT + 60°C 1h)	87.5	99.0	93.5
TET (60°C 24h)	92.0	98.1	94.7
TCPS	76.2	97.9	93.8

Cell adhesion was determined by the number of VSMCs cultured on the samples 24 h after seeding. Cell proliferation was determined by the number of VSMCs cultured on the samples 3 and 6 days after seeding. Detailed counts of cultured cells can be seen in **Fig. 4**. In comparision with pristine substrate, both plasma treatment and grafting causes significant improvement of cell adhesion and proliferation. In case of TET grafted at room temperature, the number of cultured cells was greater then on the standard TCPS. Thermal stress of grafted substrates causes slight decrease in cell numbers. However, both thermal stressed surfaces were comparable with standard TCPS. Larger deviations are caused by the inhomogeneous coverage of cells on the substrate surface.

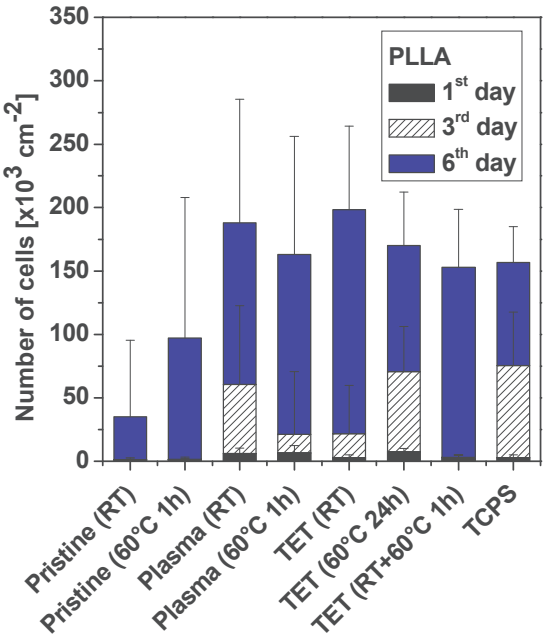

Fig. 4 Water contact angle measured on various PLLA samples (with and without thermal stress): pristine (pristine), plasma treated (plasma), etched in water solution of hydrochloric acid (etched) and CNPs grafted (TET)

4. CONCLUSION

To the best of our knowledge we are the first to prepare such modified biopolymer film. Conducted analyses confirmed successful grafting of TET onto the surface of PLLA. Significant changes in CA values between room temperature grafted substrate and substrate grafted at glass transforming temperature may be caused by reorientation of the surface layer. Cell adhesion and proliferation was improved by both plasma treatment and grafting. Grafting of TET, with and without thermal stress, has positive effect on adhesion, proliferation and viability of VSMCs. In all cases the cell numbers and viability was comparable or even higher on grafted samples then on TCPS.

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