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Considering the degradation effects of amino-functional plasma polymer coatings for biomedical application

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ABSTRACT

Materials for biomedical applications typically involve surface engineering. Scaffolds used for tissue engineering, for example, require a surface functionalization in order to support cell growth. The deposition of functional plasma polymer coatings seems to be an attractive approach to modify substrates for biomedical applications. Possible degradation of highly functional plasma polymers and the effect of its degradation products on cell growth, however, are not yet investigated in detail. Plasma polymer formation is governed by gas phase (mainly determining the chemical composition) and surface processes (inducing cross-linking) which both influence the incorporation of amino groups in a-C:H:N coatings deposited by NH_3/C_2H_4 discharges. Aging is studied in air and in aqueous conditions revealing the degradation of such plasma polymers (loss in thickness and loss of amino groups). Degradation products seem to influence viability and proliferation of mouse skeletal muscle cells on electrospun poly(ε-caprolactone) scaffolds. Thus, possible chemical changes as a function of time or exposure to different media must be taken into account in the design of functional plasma polymer coatings for biomedical applications in order to avoid possible adverse effects on cell growth.

1. Introduction

Biomedical applications require special conditions for functional plasma polymer films grown at the surface of scaffolds or membranes: while adding functional groups that have to be stable in aqueous environments, changes in the mechanical and topographical properties of the substrates need to be minimized. For this purpose, both gas phase and surface processes should be well controlled during plasma polymerization. While gas phase processes are governed by the energy invested per particle (plasma chemistry), surface processes also depend on the energy flux and on the momentum transfer during film growth (plasma physics). The latter are calculated by measuring mean ion energies and ion fluxes as well as deposition rates.

Investigating hydrocarbon discharges (mainly C_2H_4) mixed with reactive gases such as CO_2 and NH_3, we found that the film density is proportional to momentum transfer by ion bombardment during film growth, whereas the functional group density (carboxylic or amino groups) scales inversely with this deposition-controlling parameter [1]. Hence, the functional group density and permanence (by cross-linking) of the a-C:H:O and a-C:H:N plasma polymers can be optimized.

Plasma activation or polymerization processes have been utilized for several years to create optimal culture substrates for adhesion dependent mammalian cells and commercially available oxygen functionalized culture dishes (TCPS) present a gold standard. Further, amino-functionalized Primaria™ culture dishes were widely employed for cell types that grow only poorly under conventional conditions. During the last decade, plasma-functionalization has been used for the creation of enhanced biointerfaces on scaffolds for tissue engineering applications [2]. Particularly, oxygen-functional groups were demonstrated to enhance cell adhesion, proliferation and differentiation. Importantly, scaffolds must present stable functional groups, but should not release toxic degradation products upon storage or culture under aqueous conditions.

Electrospun scaffolds for tissue engineering and porous membranes for drug delivery were thus functionalized using plasma polymerization with the aim to maintain their submicrometer 3D architecture and their mechanical properties. Ultrathin plasma polymer layers (<10 nm) were deposited, since thicker coatings were found to affect substrate stiffness and degradability [3,4]. While CO_2/C_2H_4 gas discharges have already been proven to support cell growth on electrospun poly(ε-caprolactone) (PCL) scaffolds used for tissue engineering [5], we now investigate the ability of NH_3/C_2H_4 gas discharges for the same purpose. Such amino-functional plasma polymers were recently used as adhesion promoting layers in fiber-reinforced composites as well as on membranes to chemically attach thermoresponsive poly(oligo(ethylene glycol) methacrylate) (pOEGMA) copolymers forming brushes [6,7]. After the chemical reaction the amino-functionalized plasma layers were found to be permanent [8].

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Plasma polymer films with nitrogen-functional groups (a-C:H:N) are also frequently used for biomedical applications [2,3,9,10]. Degradation effects such as oxidation and leaching of low molecular weight fragments that are known to occur for amino-functional plasma polymers, however, require special handling [9,11–16]. Prior to cell attachment a-C:H:N-coated samples are therefore stored in air for several weeks, thermally treated (in vacuum) and/or washed with solvents, while the media is frequently exchanged during cell growth [17–19]. We could thus demonstrate that cell growth of mouse fibroblasts (cell line 3T3) is supported on amino-functionalized plasma polymer-coated Petri dishes after 1 day of pre-incubation in water [20]. In the current study, however, we investigate the effect of plasma polymer layer/coating degradation on cell growth of mouse skeletal muscle cells (cell line C2C12) cultured on a biodegradable, electrospun PCL scaffold without pre-incubation or further surface treatment.

2. Experimental

2.1. Plasma processing

Amino-functional plasma polymers were deposited from NH_3/C_2H_4 discharges using two different (almost symmetric) plasma reactors with 13.56 MHz radiofrequency (RF) excitation [21]. A lab-scale batch reactor with an electrode size of 700 cm^2 and a pilot-scale web coater with an electrode size of 12,000 cm^2 were used. RF was capacitively coupled to the substrate bearing electrode. The range of power input W per monomer gas flow rate F_m was kept between 60 and 350 J cm^{-3} for all experiments, while the NH_3/C_2H_4 gas flow ratio was varied between 0.8 and 4 and the pressure was fixed at 10 Pa. The deposited mass was measured by weighing thin glass slide substrates right before and after deposition. Electric characteristics of the discharge were observed by a V/I probe (ENI Model 1065) and electron densities were measured using microwave interferometry (JE PlasmaConsult MWI 2650) showing a linear behavior with power input [21]. Chemical composition was examined by XPS surface analysis (PHI 5600 LS), also using derivatization with 4-(trifluoromethyl)benzaldehyde (TFBA) vapor to determine the amino group density with respect to the carbon concentration (NH_2/C) [22]. Samples were stored in controlled environment (21 °C, 65% relative humidity (RH)) for 20 days and in deionized water for 2 h.

2.2. Scaffold production and characterization

Nanofibrous scaffolds were produced by electrospinning on a home-built setup. Briefly, a 15% w/v polymer solution of PCL in acetic acid/pyridine (all from Sigma–Aldrich, Switzerland) was introduced in an electrostatic field and processed at an electric field strength of 1 kV cm^{-1} and 30 μL min^{-1} flow rate [5,23]. As-spun substrates were plasma functionalized in the lab-scale batch reactor. Plasma process parameters were set to an energy input of 110 J cm^{-3}, an NH_3/C_2H_4 ratio of 2:1, and a process duration of 20 min at a pressure of 10 Pa. Chemical surface composition was assessed by XPS, fiber morphology by scanning electron microscopy (SEM, Hitachi, Canada).

2.3. Cell culture and analysis

All experiments were accomplished on NH_3/C_2H_4 plasma functionalized scaffolds (indicated as 'amino'), as-spun PCL scaffolds ('ctrl.') and tissue treated culture dishes ('TCPS'; TPP Omnilab, Switzerland). Different intervals between plasma coating and cell seeding were considered (freshly prepared or stored/aged for 9 months in air) without pre-incubation or washing. Cell seeding and culture on the scaffolds were accomplished as described in detail by Guex et al. [24]. Briefly, 6 mm diameter substrates were cut and sterilized under UV. Murine skeletal myoblasts (C2C12, ATCC, Manassas, USA) were seeded at a density of 0.25 10^6 cells per patch. Constructs were cultured under standard conditions in a humidified incubator at 37 °C and 5% CO_2. Myotube differentiation was initiated 7 days post seeding with a serum-deprived culture medium for an additional period of 14 days.

Cells were assessed for viability and substrate toxicity as previously reported [5]. In short, substrate toxicity and resulting cell death were monitored by lactate dehydrogenase (LDH) release into the culture medium on days 3, 5 and 7 (LDH toxicity test, Sigma–Aldrich, Switzerland); whereas cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test, 48 h after seeding (MTT, Sigma–Aldrich, Switzerland). Cell number was quantified on days 3, 5 and 7 post seeding using a commercially available cell proliferation assay kit (CyQuant; Invitrogen, USA). Myoblast differentiation and fusion into myotubes were assessed by immunohistochemistry and positive desmin staining (anti-desmin antibody, Abd Serotec, USA; and EnVision System-HRP Kit, Dako, USA).

3. Results and discussion

The deposition of plasma polymers is both governed by gas phase and surface processes, where the latter is different on flat and 3D structured substrates due to the interaction with energetic particles (mainly ions). While the ions incident on a 3D structured material mainly reach surfaces in the line of sight, the film-forming species as produced by gas phase processes are able to penetrate deeper into the material. The amino-functional plasma polymer is thus deposited under rather mild conditions on the electrospun PCL substrate, i.e. the deposition is mainly governed by plasma-chemical reactions. Therefore, (flat) samples used for surface analytics (Si wafers) were coated in the web coater which enables a low energy flux (product of mean ion energy and ion flux) to the surface, while the fibrous PCL substrates were treated in the smaller batch reactor. Both electron temperature T_e and electron density n_e are found to decrease with the reactor size, since electrons are kept for a longer time within the plasma volume [25]. Ion-induced etching effects that strongly reduce deposition rate are thus only found for high NH_3/C_2H_4 ratios, where a high energy density due to the overall low deposition rates is observed [21,26]. Note that energy density is defined as energy flux per deposition rate, i.e. the flux of depositing species [27]. Mass deposition rates for an NH_3/C_2H_4 gas ratio between 0.8 and 2, on the other hand, follow an Arrhenius behavior as a function of the energy input W/F_m (power input per monomer gas flow rate), which is indicative of a predominant plasma-chemical reaction pathway with an activation barrier E_a:

$$\frac{R_m}{F_m} = G\exp\left(-\frac{E_a}{W/F_m}\right), \tag{1}$$

where G is a reactor and process dependent factor related to the conversion of monomer into film growth [28]. Deposition on the PCL scaffolds (by choosing an NH_3/C_2H_4 ratio of 2) was performed in a smaller batch reactor that, nevertheless, enabled the same plasma-chemical reaction pathway [21]. The used range of W/F_m was kept above E_a as obtained for NH_3/C_2H_4 discharges in previous works. Following Eq. (1) the deposition rate thus increases slowly with power.

The stability and functional group density of the amino-functional plasma polymers were examined with respect to the momentum transfer during film growth, since we could recently show that cross-linking and densification scale with momentum transfer as given by

$$\pi_{surf} = \frac{n_0\sqrt{V_{sh}}}{R} f(T_e, p), \tag{2}$$

where n_0 is the plasma density (which equals n_e for electropositive discharges), V_{sh} is the sheath voltage in front of the sample, R is the (atomic) deposition rate, and f is a factor that depends on electron temperature T_e and pressure p [1,28]. Since the variation of T_e with power input can be neglected and p was kept constant at 10 Pa, the factor f is actually a