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Full Length Article

Cellular responses to radical propagation from ion-implanted plasma polymer surfaces

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ABSTRACT

Biomolecule-functionalization, through the presentation of biological motifs that promote optimal cellular responses, has the capacity to improve the tissue integration of biomedical devices and hence patients' quality of life. Radical-functionalized plasma polymer films (rPPFs) readily immobilize bioactive molecules on exposure to a biomolecule-containing aqueous solution without the need for chemical reagents. However, the potential for damage to cells and tissues due to the high local concentration of radicals in freshly deposited radical-functionalized plasma polymer films is of concern. In this study, we compared a fresh (4 h post-deposition) rPPF with one that had been aged for 11 days to explore the effect of the different radical fluxes on cellular responses. Primary osteoblasts and MG63 bone osteosarcoma cells were used to determine whether rPPFs at early aging times exhibited radical-induced cytotoxicity. The aging behavior of the rPPFs demonstrated a connection between the radical decay kinetics and the surface chemistry and wettability. Significant increases in cell attachment and spreading compared to bare Ti were observed for both cell lineages on the rPPF surfaces. The proliferation assays showed equivalent proliferation rates on both the fresh and aged surfaces, and no evidence of cytotoxicity was observed. Overall, we demonstrated that the high flux of radicals emerging to the surface has minimal influence on the biocompatibility of radical-functionalized plasma polymer films.

1. Introduction

Biomolecule-functionalization has the potential to vastly improve the biocompatibility and longevity of implanted medical devices, with significant improvements to patients' quality of life [1,2]. Plasma polymers represent a promising method to bio-functionalize devices on the manufacturing scale [3]. Plasma polymer films (PPFs) are produced through plasma enhanced chemical vapor deposition (PECVD), a process that utilizes plasma discharges to partially ionize monomer gasses and deposit the resulting compounds on the surface of a substrate [4,5]. PPFs have been proven to possess reproducible surface chemical composition regardless of the substrate chemistry, geometry, and size [6,7] allowing for the biomolecule-functionalization of any underlying surface. PPFs have been applied to biologically functionalize cardiovascular stents [8–11], with recent attention directed towards other long-term medical devices, such as titanium (Ti) alloy orthopedics [12–15].

Two approaches have been developed to functionalize PPFs. In the first case, specific chemical groups contained in the monomers are used to facilitate a specific coupling reaction, while the second approach relies on non-specific reactions with radicals embedded in the PPF. Chemically functionalized PPFs (cPPFs) incorporate specific chemical groups, or moieties, according to the chemistry of the precursor monomers used. For example, surface functionalities of amine, carboxyl, aldehyde, and epoxy are obtained by using alkylamines [16], acrylic acids [16], allyl aldehydes [17], and allyl glycidyl ether [18], respectively. Alternatively, PPFs with a high concentration of radicals embedded through energetic ion impacts can be used to immobilize biomolecules in the absence of specific chemical groups [19]. The enhanced energetic ion bombardment during deposition results in a great degree of crosslinking and the retention of fewer specific chemical

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functional groups from the precursor gases [20]. The radicals embedded in the bulk of the film migrate towards the surface where they form covalent bonds with adsorbed biomolecules.

Both classes of PPFs are affected by a process known as aging in which the surface chemistry changes over time [21,22]. PPF aging occurs due to the oxidation of carbon-centered radicals (referred to as autoxidation) [5,22], chemical group oxidation [23,24], and polymer chain diffusion (hydrophobic recovery) [25,26]. When relying on surface chemical groups for functionalization, chemical instability may limit the applications in practice. Aging in cPPFs results in the oxidation of amines into amides and NO_x groups [21,27,28], carboxyl into aldehydes, alcohols, and CO_x groups [23,29], and epoxy groups undergoing ring opening reactions [24,30,31]. Chemical groups are also lost through hydrophobic recovery, where the polar chemical groups at the surface diffuse and re-orient towards the bulk of the PPF [25,26]. The changing surface chemistry and hydrophobic recovery brought about by plasma polymer aging can greatly impact the long-term functionality of cPPFs and cellular interactions [32]. Although strategies have been developed to mitigate hydrophobic recovery [33], cPPFs should be used immediately after deposition for best performance of biomolecule functionalization.

In the case of radical-functionalized plasma polymer films (rPPFs), aging does not directly affect the ability to immobilize biomolecules providing that sufficient reactive radicals are available in the coating structure. Embedded radicals diffuse to the surface and facilitate non-specific covalent coupling of macromolecules, such as proteins and peptides, contacting the surface [15,19,34,35]. Such ease of functionalization provides a great opportunity to create bio-functional interfaces. In the context of bone implants, for example, the application of these coatings opens the potential to reduce infection and increase osseointegration through the immobilization of multiple ligands, e.g. antimicrobials together with osteogenic agents. Biomolecule-functionalization can be performed immediately after deposition or after various aging periods (e.g. 15 days [14] or up to 4.5 months [36]) with only minimal changes in immobilization time to account for variations in the flux of radicals diffusing to the surface. Hydrophobic recovery occurs over time, initially due to the conversion of radicals to polar groups upon reaction with atmospheric constituents at the surface. The polar groups possess lower free energy than the radicals they replace, and as such, the surface becomes more hydrophobic but stabilizes in a mildly hydrophilic state [37]. In previous works, the rPPFs were aged to allow for the stabilization of surface chemistry prior to biomolecule functionalization and use in a biological context.

The higher concentration of radicals at short aging times, however, may have adverse biological consequences. Radical species are produced naturally as a consequence of cellular metabolism, and cells have antioxidative countermeasures to prevent oxidative damage [38,39]. However, high concentrations of radicals are known to be cytotoxic. An imbalance favoring radical species production compared to their removal can induce oxidative stress within the cell, which can lead to DNA, protein, and lipid damage, dysregulation of signaling cascades, and cell death through apoptosis or necrosis [40,41]. Radical-mediated cellular damage and dysfunction have been implicated in aging and the pathology of numerous diseases, including osteoarthritis and osteoporosis [41,42]. Therefore, there are concerns about the potential radical-induced cytotoxicity of fresh rPPFs. The high flux of radicals emerging from fresh rPPFs may induce oxidative stress in the attached cells, resulting in cell death and/or detrimental biological activity.

This study investigates the effects of radical flux and rPPF aging on cells by comparing cellular responses to fresh and aged rPPFs. The surface chemistry, surface energy, and radical density of rPPFs were characterized at a series of time points from 15 min to 2 weeks. Titanium was used as the substrate due to its extensive application in biomedical devices [43,44]. Bone lineage cells, i.e. primary mouse osteoblasts and the MG63 bone osteosarcoma cell line, were chosen to reflect the prominence of Ti in orthopedic devices [44–46]. The time points of 4 h (fresh) and 11 days (aged) post-deposition were selected for cell studies as they represent the earliest possible implant time following surface treatment and aging conditions for rPPFs used without inducing cytotoxicity in prior experiments, respectively [8,14,34].

2. Experimental section

2.1. Ion-assisted plasma polymerization

Ti-6Al-4 V foils (70 μm thickness, Firmetal, China) and polished Ti sheets (8 mm × 8 mm × 1 mm, Firmetal, China), hereby referred to as foils or sheets respectively, were cleaned with acetone, milliQ water, 35% nitric acid (v/v), water again, and ethanol. The Ti was cleaned in each solvent for 2× 10 min. with 1× 30 min. for the nitric acid. A custom-made plasma polymerization system, previously described in detail [6,8], was used to create rPPFs on titanium substrates. The chamber was pumped down to below 5×10^{-5} Torr using a screw (Ebara PDV250) and a turbomolecular (Edwards NEXT400) pump. The samples were initially cleaned using argon plasma (Ar flow rate = 40 sccm) for 10 min (RF input power = 75 W, substrate bias voltage = −500 V). A gaseous mixture of acetylene, N_2, and Ar was added at flow rates of 2.5, 12.5, and 15 standard cubic centimetre per minute (sccm), respectively, via a shower-head dispenser located at the top of the chamber. The chamber pressure was raised to 110 mTorr, the plasma RF input power was set to 50 W, and sample holder was biased to −500 V (pulse width = 20 μs, frequency = 3 kHz). The RF power supply was a 13.56 MHz Eni OEM-6, and the substrate bias pulses were delivered by a RUP 6 pulse generator (GBS-Electronik). Gas flow rates were individually controlled using Allicat mass flow controllers. The deposition time was 6.5 min, giving a film thickness of 60 ± 5 nm on silicon wafers as measured by variable angle spectroscopic ellipsometry.

2.2. X-ray photoelectron spectroscopy (XPS)

The surface chemistry of rPPF-coated foil samples (0.8 cm × 1 cm) were examined using a SPECS (FlexMode) spectrometer. The system was equipped with a monochromatic Al Kα ($h\nu$ = 1486.7 eV) radiation source, a hemispherical analyzer (PHOIBOS 150), and an MCD9 electron detector. The radiation source operated at 200 W (10 kV and 20 mA), and the electron take-off angle was 90° with respect to the sample surface. Measurements were performed at pressures below 5.0×10^{-8} mbar. The survey spectra were collected in an energy range of 0–1000 eV at a pass energy of 30 eV and a resolution of 0.5 eV. High-resolution (0.1 eV) C 1s spectra were collected at a pass energy of 20 eV. The resulting spectra were then analyzed with the CasaXPS software (version 2.3.18PR1.0).

2.3. Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)

The FTIR-ATR spectra of the uncoated and rPPF-coated Ti were recorded using a DigiLabFTS7000 spectrometer fitted with a multi-bounce ATR accessory and a trapezium germanium crystal at an incidence angle of 45°. The spectra of the uncoated and rPPF-coated foils (1.5 × 2 cm) were recorded at a resolution of 4 cm^{-1} within the range of 4000–850 cm^{-1} and averaged over 500 scans. The resulting spectrum underwent background spectral subtraction using the software Digilab Resolutions Pro 4.0.

2.4. Contact angle and surface energy

The surface energy of the rPPF was examined using a Krüss DS10 analyzer equipped with a CCD camera. The contact angles of H_2O (polar) and diiodomethane (non-polar) were measured (5 μL). The