Chapter 26
Exploring the Mechanism of Biomolecule Immobilization on Plasma-Treated Polymer Substrates

Biomolecule Immobilization on Plasma-Treated Polymer

E.H. Lock, S.H. North, J. Wojciechowski, C.R. Taitt, and S.G. Walton

Abstract An electron beam-generated plasma source developed at NRL was used to modify polystyrene microtitre plates. A combination of complementary surface analytical and biochemical techniques was applied to evaluate the relationship between the biotic and abiotic layers, with particular emphasis on the efficiency of the preparation of polymer surfaces and its effectiveness for bioimmobilization. We conclude that the development of novel interface materials with superior transducing capabilities is dependent on the deeper understanding of the complex physicochemical, nanoscale interactions between the substrate surface and the biological components attached to it.

Keywords Polystyrene • Silanization • Characterization • Functionalization

26.1 Introduction

Microtitre plates are widely used in clinical diagnostics, biotech and pharmaceutical research and development. They are manufactured using a variety of polymeric materials, but polystyrene is most commonly employed because it readily adsorbs proteins, has excellent optical as well as mechanical properties, and is cost-effective.

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Typically, biomolecules are non-covalently immobilized through physisorption to the polymer substrate. However, this type of biomolecule attachment is often unstable and may cause protein denaturation, desorption and/or loss of biomolecule activity [1]. Thus, covalent biomolecule immobilization schemes are often preferred because they provide more stable attachment and functional display of the biomolecule of interest [2].

Plasmas are widely used as a preparation step of polymer substrates prior to bioimmobilization as signified by numerous review articles on this subject [3, 4]. Plasmas are effective in polymer surface functionalization, thin film deposition, and grafting of different functionalities onto the polymer surface. However, even though plasmas can produce the desired chemical modifications, in most cases, they also cause morphological modifications. The latter has been shown to affect the activity of the immobilized biomolecules [2], which is dependent on biomolecules presentation and orientation [5]. Therefore, we sought to develop a method that allows for precise control over polymer surface characteristics and thus allowing for better control over the biomolecule attachment. We use a combination of dry and wet chemistry to enhance selectivity in biomolecule immobilization. As a first step we use an electron beam-generated plasma characterized by its ability to introduce chemical modification without surface roughening [6] to activate the polymer surfaces. After microtitre plates functionalization, they were silanized and then crosslinkers were used to immobilize biomolecules of interest. We explored a broad range of experimental parameters to determine the optimal conditions for successful attachment of the biological recognition elements on a transducing polystyrene substrate surface.

26.2 Materials and Methods

26.2.1 Materials

26.2.1.1 Preparation of Fluorescently Labeled IgG

Rabbit IgG was conjugated with Cy3 mono-reactive dye following manufacturer's instructions. The labeled IgG was purified from unincorporated dye by gel filtration on BioGel P-10.

26.2.1.2 Surface Characterization

Contact angle measurements were performed with an automated goniometer (AST Products, Inc.) and surface energy estimations were performed as previously described [7]. *Surface elemental and chemical state analyses* were performed on K-Alpha X-ray photoelectron spectroscopy as discussed previously [8]. To produce

Table 26.1 Summary of experimental conditions

Exp. #	Pressure (mTorr)	Time (min)	Duty Factor (%)
1	60	0.5	10
2	90	2	10
3	95	2	10
4	90	1	10
5	90	5	10
6	90	2	20

consistent fits of minor C 1s components, their widths were constrained to the FMHW of the first peak (285 eV) and their positions were assigned as follows C-CO_2 285.7 eV, C-O 286.6 ± 0.2 eV, C=O 287.6 ± 0.2 eV, O-C=O 289 ± 0.2 eV, π-π^* 291.7 ± 0.2 eV. A linear combination of Shirley and linear functions with consistent parameters was used to model the background. *The polymer surface morphology* was examined using an atomic force microscope (Nanoscope III, Veeco Metrology, Santa Barbara, CA) operated in tapping mode. Surface images were obtained from $5 \times 5\ \mu m^2$ scans at resolution of 256 × 256 pixels and scan rate of 1 Hz. For a quantitative evaluation of the topography changes the root-mean square (RMS) roughness was calculated as previously shown [8].

26.2.1.3 Plasma Treatment of Microtitre Plates

The experimental apparatus has been discussed previously [9]. The microtitre plates were placed on a 10.2 cm diameter stage located at 2.5 cm from the nominal edge of the electron beam. The stage was held at ground potential and room temperature. The total gas flow rate was held constant at 50 sccm. The experimental conditions are summarized in Table 26.1.

26.2.1.4 Microtitre Plate Silanization and Immobilization of IgG

Plasma-treated or untreated NUNC Microfluor I microtitre plates (Thermo Fisher Scientific) were treated for 30 min with 2% (3-mercaptopropyl)triethoxysilane (MPTES) in acidic methanol (pH 4). Plates were treated for 30 min with 1 mM 4-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS, Amersham-Pharmacia). Cy3-labeled antibody (1, 3, or 10 μg/mL in PBS) was added into each microwell and incubated for 2 h at room temperature with gentle agitation; each concentration was patterned in quadruplicate. The plate was read on a Tecan Safire microplate reader.

26.3 Results and Discussion

26.3.1 Effects of Process Parameters on Reactive Species Generation

It is important to note that plasma production by electron beams differs from conventional discharges. When high electron energy beam is injected in the background gas approximately half of its energy goes into gas ionization and dissociation and only a small fraction into gas excitation. Because there is no electric field to heat the secondary electrons (those produced during electron-impact ionization), they quickly cool to produce a large population of low energy electrons. These electrons determine the plasma density, electron temperature, and thus plasma potential. The latter is particularly important to the polymer modification process, since it will determine the kinetic energy of the ions as they impact the polymer surface.

We have already shown that the electron temperature in argon is about 1 eV [10, 11] and is largely independent of process parameters. As shown in Fig. 26.1a, the ions bombarding the surface have energies in the range of about 3 eV. In conventional discharges, ion energies could be greater by an order of magnitude or more. Energies of a few eV are comparable to the bond strengths in polystyrene; C–C (π-bond) energies are 2.6 eV, while C–C and C–H are higher (~4 eV). Thus, the mechanism of hydrogen abstraction might differ from the well-accepted physical sputtering mechanism in conventional discharge processing.

Metastables and photons are important species to consider in polymer processing. Metastables carry approximately 11 eV of energy, which is substantial and can cause hydrogen abstraction, radical formation, local heating and thus chain scission and/or crosslinking. Photons are important because they can penetrate deep into the polymer structure and cause crosslinking and further surface rearrangement as well. In conventional discharges, metastables and photons are often the dominant species. However, their influence in this work is limited because their production rates are low in electron beam-plasmas. Based on theoretical predictions, in argon the amount of metastables is an order of magnitude lower than the amount of ions produced [6].

A summary of experimental conditions is shown in Table 26.1. The influence of process parameters, e.g. duty factor, treatment time, and pressure, on the generation of species and the flux on the surface of polystyrene has been explored [12]. Variations in the duty factor and treatment time are directly correlated with the flux of species. Increasing the duty factor increases the time the beam is on, i.e. the time between beam pulses is reduced, while increasing the treatment time simply increases the total plasma exposure time. However, changing the operating pressure affects both the electron beam intensity [12] and plasma density [10] in this system. Figure 26.1b shows the increase in beam current density with increasing argon pressure. While ion and electron production is increased, their destruction via ambipolar diffusion to the walls is decreased (D_a~1/p), which leads to enhanced ion flux bombarding the polymer surface. Thus, changing the pressure significantly influences polymer processing.

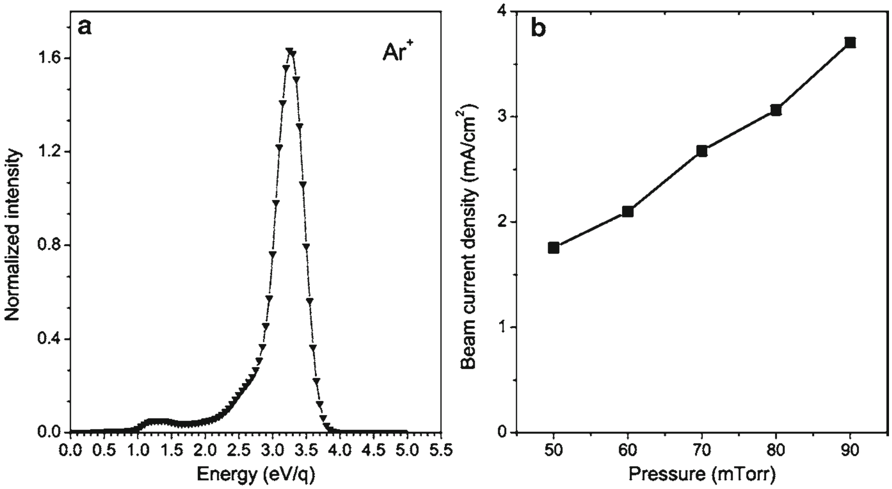

Fig. 26.1 Ion energy distribution at a pressure 50 mTorr (**a**) and beam current density as a function of pressure (**b**)

26.3.2 Polymer Surface Characterization

As variation in pressure has the greatest influence on the production of plasma species – charged particles, metastables and photons, it is expected to produce the most pronounced difference in oxygen incorporation. The highest degree of oxygen incorporation was observed at a pressure of 90 mTorr (exp #2, Tables 26.1 and 26.2) ($O/C = 0.3$) resulting in the highest polar component of the surface energy as well. The most likely mechanism of polystyrene modification in argon is the formation of carbon-centered polymer free radicals (P•), which preferentially crosslink. However, chain scission and formation of polyenes are also possible [13]. Higher pressures (exp # 3, Table 26.1) did not improve the surface chemical composition. In fact, the O/C ratio, C–O group concentration and γ^p decreased. This might be due to an excessive ion flux, which may cause an increase in etching and favor chain scission over crosslinking as the dominant mechanism. The surface roughness decreased at all pressures, which might be caused by ablation of low molecular weight fragments residing on the polymer surface.

Variations in both treatment time and duty factor at a fixed pressure led to similar conclusions. Increase in treatment time from 1 to 2 min (exp # 4 and 2, Tables 26.1 and 26.2) resulted in higher O/C ratios, increased amount of oxygen functionalities (C–O, C=O, COO groups) and improved polar component of the surface energy with no change in surface roughness. However, when the polystyrene substrate was treated for 5 min (exp # 5, Tables 26.1 and 26.2) the O/C ratio decreased and surface roughness increased. These results suggested that prolonged plasma exposure increases etching, increases surface roughness and promotes chain scission and not crosslinking. Increase in duty factor (exp #2 and #6) did not increase

Table 26.2 Summary of polymer surface characterization and bioimmobilization results

Exp. #	O/C	C–C(H)[a], C–CO$_2$[a]	C–O[a]	C=O[a]	COO[a]	π–π^*[a]	γ_p[b]	γ_d[b]	γ_{tot}[b]	RMS[c]	Bio efficacy
Ref	0.03	92.61	1.58	0	0	5.81	0	40	40	19	−
1	0.27	89.84	5.12	2.94	1.08	1.01	24	32	56	11	−
2	0.30	86.07	8.24	4.04	1.65	0	26	38	64	12	+
3	0.20	73.03	5.56	3.24	1.05	0	10	43	53	12	−
4	0.22	77.74	3.14	0.33	0	0.9	20	36	56	11	−
5	0.23	87.53	7.8	2.85	1.02	0.8	19	39	58	16	−
6	0.14	81.32	6.17	1.56	0	0	13	40	53	ND	−

[a] Concentration of functional groups was measured in at. %

[b] Surface energy was measured in mJ/m^2

[c] RMS roughness was measured in nanometers (nm)

[d] ND means not determined

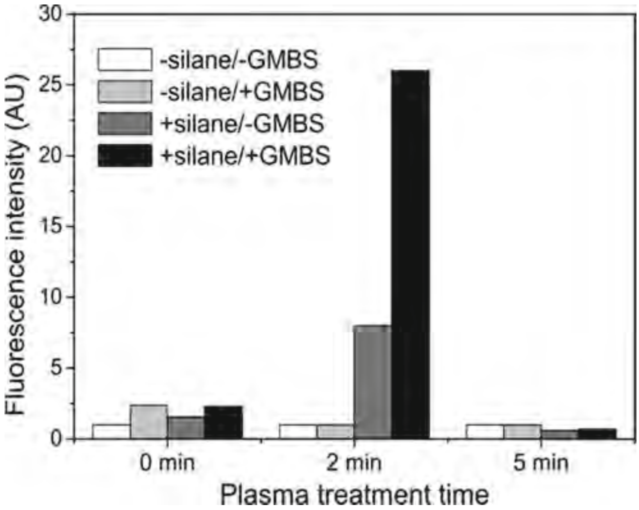

Fig. 26.2 Biomolecule immobilization efficacy as a function of plasma treatment time

the oxygen incorporation. In fact, it resulted in decrease of O/C ratio as well as a decrease of the polar component of the surface energy by a factor of two. Although the reasons are not clear, it appears that reducing the time between pulses does not promote the incorporation of OH-groups.

26.3.3 Biomolecule Immobilization

Covalent bioimmobilization was observed only at a pressure of 90 mTorr, treatment time of 2 min and 10% duty factor (exp. #2 Table 26.2, Fig. 26.2). At this condition the increase in fluorescent signal, signifying specific biomolecule attachment, was 26-fold above the background levels. These results are in direct correlation to the presence of oxygen functional groups on the polystyrene surface. That is, when the oxygen functionalities concentration on the polymer surface was optimized, it allowed for efficient silanization and thus *specific* biomolecule immobilization occurs.

26.4 Conclusions

The relationship between the plasma generated reactive species and photons, the chemical and morphological characteristics of the plasma treated surface and the following bio-immobilization were explored. High flux of low energy ions, low amount of metastables and photons are needed for successful hydroxylation of the polystyrene surface without increase in surface roughness. Covalent bio-immobilization was achieved at narrowly defined conditions characterized by the highest oxygen functionalities concentration and by the highest surface energy.

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