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Quantification of Primary Amine Groups Available for Subsequent Biofunctionalization of Polymer Surfaces

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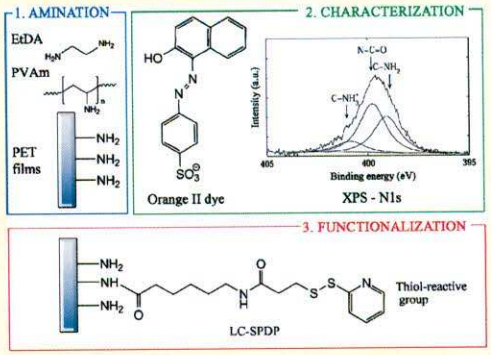
ABSTRACT: Biocompatible polymers are commonly functionalized with specific moieties such as amino groups to modify their surface properties and/or to attach bioactive compounds. A reliable method is usually required to characterize amino group surface densities. In this study, aminated polyethylene terephthalate (PET) films were generated via an aminolysis reaction involving either ethylenediamine molecules (EtDA), in order to vary easily the amino group density on PET surfaces, or 25 kDa polyvinylamine (PVAm) as an alternative reagent preventing bulk damages resulting from the aminolysis reaction. Among commonly used dyes for amino group quantification, Orange II and Coomassie Brillant Blue (CBB) were selected to quantify the extent of amine grafting resulting from these derivatization procedures. Rapid and convenient colorimetric assays were compared to surface atomic compositions obtained from X-ray photoelectron spectroscopy (XPS) measurements. Orange II was found to be the most appropriate dye for quantifying primary amine groups in a reliable and specific way. Due to its unique negative charge and low steric hindrance compared to CBB, the Orange II dye was very sensitive and provided reliable quantification over a wide range of amino group surface densities (ca. 5 to at least 200 pmol/mm^2). In order to further validate the use of the Orange II dye for amino group quantification, a heterobifunctional linker reacting with amino groups was then grafted on modified PET surfaces. Interestingly, the good correlation between the densities of adsorbed Orange II and covalently grafted linkers suggests that the Orange II method is a relevant, reliable, easy, and inexpensive method to predict the amount of amino groups available for subsequent functionalization of polymer surfaces.



INTRODUCTION

With respect to the development of synthetic biomedical implants, biocompatible materials are needed for the devices to perform a specific function and to prevent any adverse effect inside the host. As biological responses take place on the material surface exposed to outer environment, conventional materials usually need to be enhanced to get new specific surface properties.[1,2] With the bulk materials of interest being mostly organic polymers,[3,4] the introduction of amino groups is often the first step of surface modification.[5] Amine moieties per se can indeed modify the macroscopic surface properties, i.e., modulate cell adhesion and tissue compatibility[6] or hydrophilicity[2,7] or promote specific differentiation of stem cells.[8] For more specific and controlled surface properties, amino groups can also be used to chemically attach bioactive molecules[1,9] such as oligosaccharides,[10] enzymes,[11,12] peptides,[13−15] or other polymers, e.g., PEG[16] or dextran[17] providing the surface with antifouling properties. The surface properties after amine derivatization, as well as subsequent grafting, are nevertheless dependent on the amino group density that has to be quantified in order to control the biomaterial functionalization.[8,10,11]

Primary amine content can be determined by several methods,[18] among which fluorometry,[19,20] colorimetry,[21−26] and spectroscopy[27−29] have already been reported. However, most of those methods are either expensive (e.g., fluorometry with *o*-phthaldehyde (OPA)[20]), time-consuming (e.g., colorimetry with 2,4,6-trinitrobenzenesulfonic acid (TNBS)[20]), or semiquantitative (e.g., X-ray photoelectron spectroscopy (XPS)[29]). In contrast, colorimetric assays that take advantage of anionic dyes are quantitative, economical, and convenient to perform on various material geometries.[6,25,30−32] The reliability of colorimetric techniques may however be questioned because of possible nonspecific adsorption of the dye to the substrate surface.[33,34] To the best of our knowledge, primary amine contents that are determined with anionic dyes are either used without any validation or only qualitatively supported by Fourier transformed infrared spectroscopy (FTIR) or XPS analyses.[7,10,14,21,24,33,35−37] Furthermore, correlations between the amine densities and the

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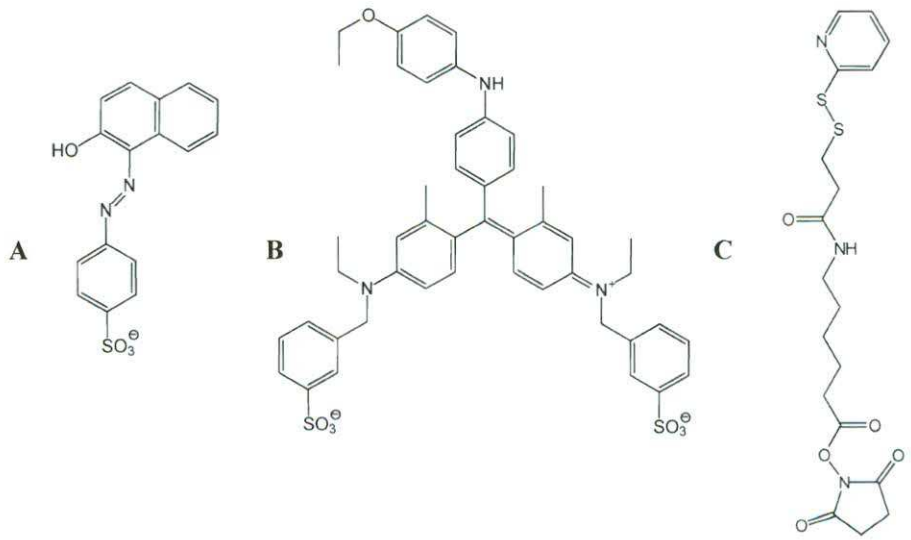

Figure 1. Molecules used for primary amine group quantification: Anionic dyes ((A) Orange II and (B) Coomassie Brillant Blue (CBB)) and (C) LC-SPDP heterobifunctional linker.

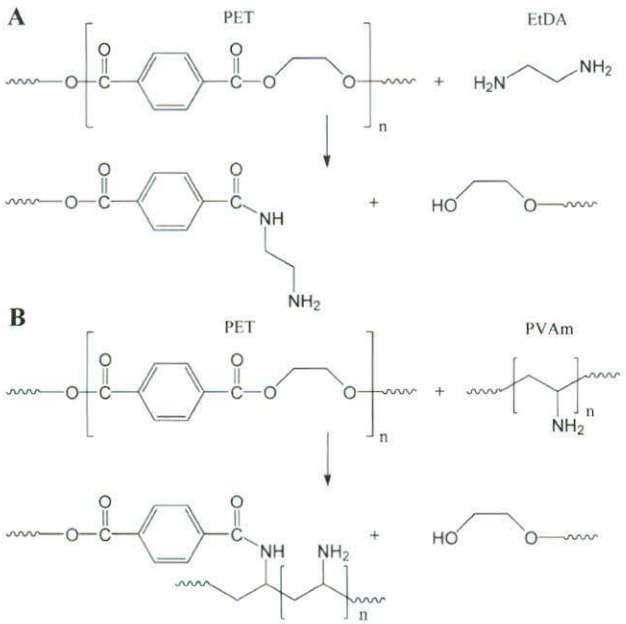

Figure 2. Aminolysis reaction scheme between PET and (A) EtDA or (B) PVAm.

amount of subsequently grafted molecules have never been clearly established.[10,11]

In this work, quantitative values of amino group density were determined via colorimetric methods based on two commonly used dyes, i.e., Orange II (Figure 1A)[7,10,14,23,33,35−37] and Coomassie Brilliant Blue (Figure 1B),[24,25,38,39] and were compared to atomic percent measurements made by XPS, as a reference. Those assays were performed on surfaces made of polyethylene terephthalate (PET), as this synthetic polymer is widely employed for its unique physical, chemical, and mechanical properties.[4] Due to its hydrophobic character and low adhesion properties, PET shows good stability in biological environments but moderate biocompatibility.[40] Therefore, this linear polyester is a relevant example of materials requiring surface functionalization, including bioactive molecules tethering,[13] for biomedical applications of PET-based devices. In this article, PET films were functionalized with primary amines via two different chemical treatments, involving a widely used small diamine molecule, namely, ethylenediamine (EtDA, Figure 2A),[10,11] or through the grafting of an aminated polymer (Figure 2B).[16] The various aminated films were then functionalized with an amino-reactive molecule (LC-SPDP linker, Figure 1C), which is of particular interest in the route of oriented immobilization of cysteine-tagged proteins.[13,41] The density of the grafted linker was further used to validate amine densities derived from Orange II assays.

MATERIALS AND METHODS

Chemicals and Reagents. Milli-Q quality water (18.2 MΩ. cm; total organic compounds (TOC) = 4 ppb) was generated with a Millipore Gradient A 10 purification system. Sodium hydroxide (NaOH, 99.3% purity), cysteine (99+% purity), acetic acid glacial (99.7% purity), ethanol (EtOH, 99.9% purity), methanol (MeOH, 99.9% purity), dioxane (99+% purity), and hydrochloric acid (HCl, 37.7% v/v) were obtained from VWR International, Ltd. (Mont-Royal, QC). Orange II sodium salt (92% purity), Coomassie Brillant Blue (100% purity), dimethylsulfoxide (DMSO, 99.7+% purity), 2-pyridinethiol (99% purity), phosphate buffered saline (10 mM PBS, NaCl − 0.138 M, KCl − 0.027 M, pH 7.4), potassium carbonate (K_2CO_3, 99% purity), and potassium chloride (KCl, 99+% purity) were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON). Succinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexanoate (LC-SPDP, 95+% purity) was obtained from Pierce Biotechnology, Inc. (Rockford, IL). Ethylenediamine (EtDA, 99+% purity) was purchased from Fisher Scientific, Ltd. (Nepean, ON). The linear aminated polymer polyvinylamine (PVAm) was obtained from Polysciences, Inc. (Warrington, PA). PET (Traytuf 9506 from M&G Group, Italy) films, 100 μm in thickness, were obtained by melt-extrusion using a Randcastle extrusion line.

PET Films Aminolysis. *Surface Preparation.* All glassware was carefully cleaned by overnight immersion in a bath of

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KOH-saturated isopropyl alcohol, followed by intensive rinsing with Milli-Q water. The polyethylene terephthalate (PET) films (12 mm × 5 mm) were cleaned in an ultrasonic bath for 15 min in ethanol and then dried in a laminar flow cabinet at room temperature.

Ethylenediamine (EtDA) Functionalization. The protocol was adapted from those previously described in the literature.[10,11] Briefly, PET films were immersed in a reactive EtDA/methanol solution for 30 min at 50 °C. Six concentrations of EtDA (ranging from 30% to 40% v/v, by 2% steps) were tested to generate a wide range of amino group densities. At the end of the reaction, the films were intensively washed with Milli-Q water (at least 3 times) and dried in air. Untreated and EtDA-treated PET films were then observed under a scanning electron microscope (SEM; JSM-6100 Scanning Microscope, JEOL, Japan) at an accelerating voltage of 5.0 kV, to qualitatively assess the roughness of the films.

Polyvinylamine (PVAm) Functionalization. PVAm was grafted on PET films as previously described.[16] Briefly, PET films were immersed in a 4 g/L solution of PVAm dissolved in Milli-Q water and dioxane (73:27 v/v, pH 4). NaOH and KCl (1:1, 67 mM) was added to adjust pH and ionic strength (pH 10). The reaction was carried out for 24 h at 70 °C. At the end of the reaction, the films were rinsed with Milli-Q water and ethanol, washed again with Milli-Q water, and finally dried in air.

X-ray Photoelectron Spectroscopy. Functionalized PET surfaces were characterized with respect to their elemental chemical compositions (in at. %) and chemical bond types by X-ray photoelectron spectroscopy (XPS) analyses (surveys, as well as C, N, and O high-resolution spectra, respectively). XPS spectra were obtained using a VG ESCALAB 3MkII instrument using non-monochromatic Mg Kα radiation (12 kV, 18 mA). All samples were extensively washed and immersed for 20 min in an ultrasonic bath prior to analysis in order to remove any possible traces of unreacted chemicals that could affect XPS measurements. None of the samples exhibited any evidence of X-ray-induced damage during room-temperature XPS measurements. XPS spectra were acquired at 0° emission angle, normal to the sample surface, and possible charging was corrected by referencing all peaks to the carbon (C 1s) peak at binding energy BE = 284.7 eV in accordance with the literature.[42] Quantification of the constituent elements was performed using broad-scan spectra and *Avantage v4.12* software (Thermo Electron Corp., Waltham, MA) by integrating the areas under relevant peaks after a Shirley-type background subtraction, and using sensitivity factors from Wagner table. Deconvoluted C 1s peak areas of relevant chemical bonds (C═C, C═O, and C−O) in pristine PET were kept proportional for each subsequent analysis, in order to identify any new functional groups added after surface modification. Peak fitting to experimental data was carried out using a minimum number of peaks, consistent with a reasonable fit to the raw data and the molecular structure of modified polymer. Binding energies from XPS analysis are reported as mean ± standard deviation.

Colorimetric Assays on Surfaces. The areas of EtDA- and PVAm-coated films were determined prior to colorimetric assays. Two colorimetric methods were used to quantify the amount of exposed primary amine groups on the PET surfaces, i.e., the Orange II method and the Coomassie Brillant Blue method, based on the work of Uchida et al.[23] and Coussot et al.,[24,38] respectively.

Orange II Method. The amino-covered films were immersed in 1.5 mL of dye solution (14 mg/mL) in acidic solution (Milli-Q water adjusted to pH 3 with 1 M HCl) for 30 min at 40 °C. The samples were then intensively rinsed 3 times using the acidic solution (pH 3) to remove unbound dye. Once air-dried, the colored films were immersed in 1 mL of alkaline solution (Milli-Q water mixture adjusted to pH 12 with a 1 M NaOH solution). The pH of the solution containing the desorbed dye was adjusted to pH 3 by adding 1% v/v of 12.3 M HCl. The absorbance of the solution was then measured at 484 nm.

Coomassie Brillant Blue (CBB) Method. The modified PET films were immersed in 1.5 mL of dye solution (0.5 mg/mL) in acidic solution (pH 2.2, 85:10:5 v/v Milli-Q water/methanol/acetic acid) for 5 min at room temperature. The samples were then intensively rinsed with the acidic solution, to remove unbound dye. Once air-dried, the films were immersed in alkaline solution (pH 11.25, 0.125 M K_2CO_3 in 50:50 v/v Milli-Q water/methanol). The pH of the solution containing the desorbed dye was adjusted to pH 3 by adding 7.5% v/v of 3 M HCl. The absorbance of the solutions containing the desorbed dye was finally measured at 620 nm.

Linear correlation between Orange II densities and XPS atomic contents, as well as between Orange II and CBB densities, are described by the square of Pearson coefficient (R^2) and the associated p-value. Values for dye densities are reported as mean ± standard deviation.

Functionalization of Aminated Surfaces using LC-SPDP. EtDA- and PVAm-modified PET films were reacted with LC-SPDP molecules, as previously described.[43] Briefly, amino-modified films were placed in test tubes containing 1.0 mL of 1 mM LC-SPDP in 10 mM phosphate buffer (10% v/v DMSO) for 150 min at 40 °C, unless mentioned otherwise. The films were rinsed by soaking them in DMSO, in 10 mM phosphate buffer (pH 7.4), and in Milli-Q water. In a second step, the dried films were incubated in a 25 mM cysteine solution (1 M NaCl in 0.1 M sodium acetate, pH 4.0) for 1 h at room temperature in order to release 2-pyridinethiol groups. The absorbance of the solution was finally measured at 343 nm.

Stability of LC-SPDP Thiol-Reactive Groups. Absorbance measurements at 343 nm were performed using a stopped-flow apparatus (Bio-Logic SFM-4, Claix, France) for the release of 2-pyridinethiol groups from LC-SPDP to be evaluated all along the 300 min experiments. The sample temperature (20 to 50 °C) was kept constant using a Peltier cell device. The measurements were initiated by mixing a 1 mM LC-SPDP solution in DMSO with PBS to yield a final solution of 0.1 mM LC-SPDP.

The absorbance measurements were normalized to the plateau value (reached after 10 min) corresponding to the addition of 10% v/v of the previous cysteine solution. From that final absorbance value, it is possible to calculate the 2-pyridinethiol concentration using the reported extinction coefficient (ε = 8.08 $mM^{-1}.cm^{-1}$).[44] Of interest, we found that this concentration was equal to the initial LC-SPDP concentration, hence further validating that all 2-pyridinethiol groups had been released in the mixture due to cysteine addition.

■ RESULTS AND DISCUSSION

Primary amine groups were introduced on PET films via an aminolysis reaction occurring in the presence of a short molecule, i.e., ethylenediamine (EtDA), or a 25 kDa aminated polymer, i.e., polyvinylamine (PVAm) as presented in Figure 2. The aminolysis process involves an acyl group substitution in which the PET carbonyl group is used as an electrophile and is attacked by a

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Table 1. Atomic Percentages Derived from XPS Analysis of EtDA-Treated PET Films

% v/v EtDA in MeOH (%)[a]	0	30	32	34	36	38	40
			C1s Peak Proportion (at. %)				
C=C	60.97	60.40	60.17	59.70	57.86	54.48	51.91
C−O	21.43	20.97	20.87	20.71	19.49	18.91	18.02
C=O, O=C−N	16.43	15.88	15.24	14.94	13.96	15.63	16.58
"Shake-up"[b]	1.16	2.33	2.24	1.11	1.37	1.58	1.81
C−N	0.00	0.42	1.48	3.55	7.32	9.40	11.69
			N1s Peak Proportion (at. %)				
NH_2	-	40.66	43.04	40.74	29.87	42.28	38.22
NH_3^+	-	3.30	8.23	6.17	19.48	6.50	11.46
O=C−N	-	56.04	48.73	53.09	50.64	51.22	50.32

[a] EtDA grafting was carried out as described in the Material and Methods section. ($n = 1$). [b] The "shake-up" peak, separated by 6.7 eV from C=C peak, is related to the excitations taking place in the π orbitals on the benzene rings.

Table 2. Atomic Percentages Derived from XPS Analysis of PVAm-Treated PET Films

	experimental conditions[a]		
	untreated	PVAm-treated	
	-	pH 4	pH 10
	C 1s Peak Proportion (% at)		
C=C	60.29	53.82	43.00
C−O	21.74	14.54	14.63
C=O, O=C—N	15.83	14.56	14.95
"Shake-up"[b]	1.61	1.85	1.31
C−C	-	7.61	13.05
C−N	-	7.61	13.07
	N 1s peak proportion (% at.)		
NH_2	-	37.50	33.86
NH_3^+	-	23.71	23.62
O=C—N	-	38.79	42.52

[a] PVAm grafting was carried out as described in the Material and Methods section ($n = 1$). [b] The "shake-up" peak, separated by 6.7 eV from C=C peak, is related to the excitations taking place in the π orbitals on the benzene rings.

nucleophilic amine group. As a result of the aminolysis reaction, an ester link (O=C—O) of the PET backbone is cleaved and a covalent amide bond (O=C—N) between the amino-based (macro)molecule and the PET surface is created.

XPS Characterization of Aminated PET Surfaces. XPS analysis was carried out to characterize EtDA and PVAm grafting on PET films as well as to quantify primary amine atomic percentage.

XPS spectrum of pristine PET (Tables 1 and 2, Figure 3A1) showed three main peaks in the C 1s region corresponding to C=C (284.7 eV), C−O (1.40 ± 0.12 eV shift), and C=O (3.68 ± 0.02 eV shift) environments, for which the positions and areas are in good agreement with values reported in the literature.[42] The satellite peak, corresponding to a shift of 6.7 eV, was most likely due to "shake-up" excitations taking place in the π orbitals on the benzene rings.[42]

For all EtDA-treated PET films (Table 1), the best envelope fit of C 1s spectra was obtained by adding one peak compared to untreated films (Figure 3B1); this additional peak was attributed to a C−N bond (0.61 ± 0.12 eV shift). The C−N peak indicated the presence of amino groups on PET surfaces but could not, on its own, confirm the covalent grafting of the EtDA molecule as O=C—N were not distinguishable from O=C—O (3.68 ± 0.02 eV shift). However, the high-resolution N 1s region revealed three components (Figure 3B2) that were attributed to C−NH_2 (398.76 ± 0.11 eV), O=C—N (399.74 ± 0.11 eV), and C−NH_3^+ (400.57 ± 0.11 eV), confirming the presence of both primary amines and covalent grafting. Altogether, as expected, the atomic percentages of C−N increased with EtDA concentration while the atomic percentages of C−O decreased (Table 1), due to EtDA grafting and chain scissions, respectively (Figure 2A). Interestingly, with the exception of the 30% EtDA concentration (for which some cross-linking is expected to be favored), about the same O=C—N/N content was obtained for all EtDA-modified films (ca. 50%). This suggests that, for each EtDA molecule, one of the two primary amine groups was involved in a covalent link to the PET matrix, while the other amino group was exposed to the surface, as already observed.[45]

For C 1s spectra of PVAm-treated PET films (Table 2), in addition to the three peaks assigned to the underlying PET layer, two new peaks were observed (Figure 3C1), i.e., C−N (0.93 ± 0 eV shift) and C−C (0 ± 0 eV shift), indicating the presence of PVAm on the surface. For all PVAm-treated films, the high-resolution N 1s spectra were deconvoluted into three peaks (Figure 3C2) that were attributed to C−NH_2 (398.76 ± 0.12 eV), O=C—N (399.74 ± 0.12 eV), and C−NH_3^+ (400.57 ± 0.12 eV). Once again, the presence of amide bonds suggested that PVAm chains were covalently grafted to the PET matrix.

For both series of functionalized films (Tables 1 and 2), it was observed that the higher the amount of C−N (at. %), the lower the amount of C=C (at. %). Given that the depth probed by XPS (ca. 50 Å) and the number of C=C bonds were constant, such a trend confirmed that the thicker the grafted layer (C−N) was, the thinner the probed underlying PET layer (C=C) was. Interestingly, the amount of C=C (at. %) for PVAm-treated films was found to be slightly lower than for EtDA-treated films, suggesting that the layer of grafted PVAm was thicker than the one obtained with EtDA, in good agreement with the size of the involved molecules.

Amino Group Quantification by Orange II Method. A wide range of amino group density was first generated on PET films by varying the concentration of EtDA in methanol (from 30% to

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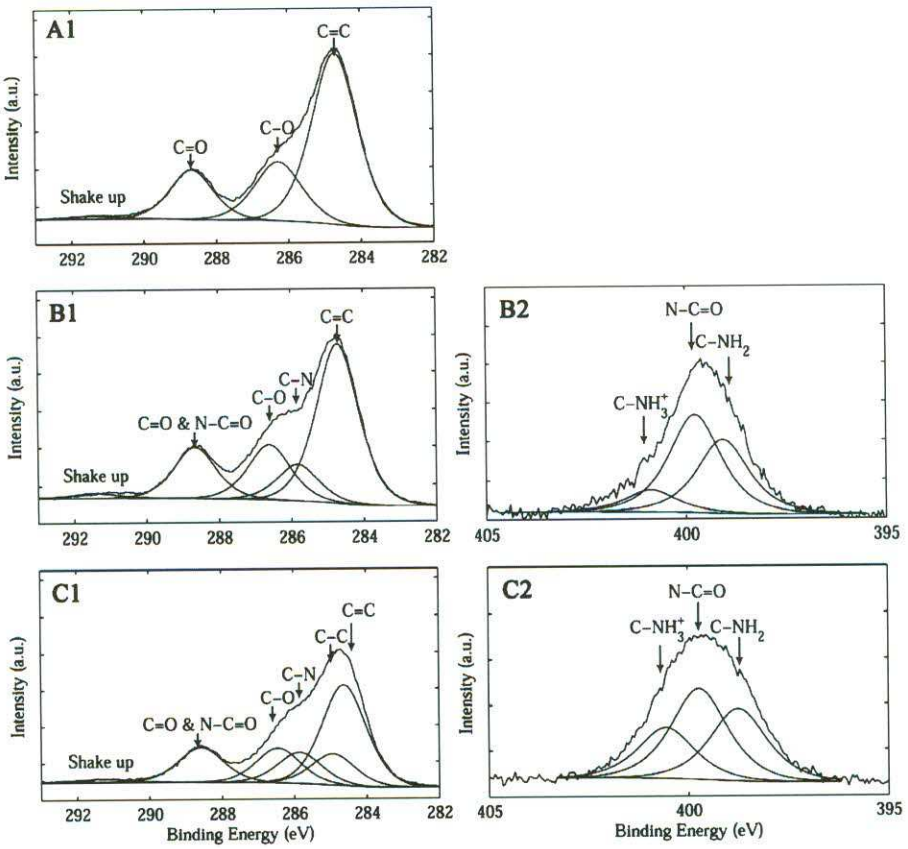

Figure 3. Characterization of EtDA and PVAm grafting by XPS. High-resolution XPS (1) C 1s and (2) N 1s spectra of (A) pristine, (B) EtDA-treated, and (C) PVAm-treated PET films. EtDA concentration was 40% v/v and PVAm grafting was carried out at pH 10.

40% v/v), according to previously described reaction parameters.[10,11] The amount of exposed primary amine groups was then quantified using the Orange II method, the latter being based on the reversible electrostatic interactions between the negatively charged sulfonated dye (Figure 1A) and the positive charge of protonated amino groups in acidic solution. Under alkaline conditions (pH 12), the neutral amino groups induced Orange II release into the solution, which absorbance was measured to evaluate the desorbed dye quantity.[37] The Orange II binding reaction onto protonated amino groups was complete after 30 min at 40 °C at pH 3 (data not shown), as previously observed.[23] Besides, the adsorbed dye content reached a plateau for pH values below 4.5 (data not shown), suggesting that all available amino groups were protonated under such conditions.

For pristine PET surfaces, a nonsignificant Orange II concentration (0.51 ± 0.04 pmol/mm^2) was obtained, suggesting that nonspecific interactions between the PET substrate and the dye were negligible. For EtDA-modified films, the concentration of desorbed dye in the solution, and therefore the density of EtDA grafted on the PET films, increased with the amount of EtDA used for derivatization (Figure 4A), validating a correlation between the amino group density and the Orange II concentration. The range of dye density was found to be between 6.0 ± 1.9 to 159.2 ± 24.8 pmol/mm^2 for EtDA concentration varying from 30% to 40% v/v in methanol, respectively. As previously reported for reaction time,[10] the Orange II density was observed to rise exponentially with EtDA concentration (Figure 4A). Such an exponential behavior is most likely attributable to an increase of surface roughness, as highlighted from SEM images (Figure 4B). That is, the specific polymer area accessible to the dye does increase due to a nonhomogeneous chain scission within the PET layer during the aminolysis reaction.

As an alternative to EtDA-based surface derivatization, we recently proposed the use of PVAm for PET surface modification,[16] as this method may preserve PET mechanical properties while introducing amino functionalities (unpublished results). Two experimental conditions, adapted from previous work,[16] were also explored in order to modulate the amino group density. More specifically, the chemical reactions were performed at pH 10 and 4 (see Materials and Methods section), giving rise to Orange II densities of 82.6 ± 2.4 and 5.1 ± 0.6 pmol/mm^2, respectively (data not shown). This difference was expected, since pH drastically influences the ratio of protonated versus unprotonated amino groups of PVAm ($pK_a \approx 9.25$)[46] and since the aminolysis reaction requires nucleophilic unprotonated amines (Figure 2B).

Comparison between Orange II Densities and XPS Measurements. Quantitative densities of Orange II adsorbed on aminated PET films were compared with the atomic content of C-NH_2 derived from XPS analysis (Figure 5). The C-NH_2 data corresponded to the product of %C−N by (%NH_2 + %NH_3^+), resulting from deconvolution of C 1s peak and N 1s peak, respectively (Tables 1 and 2). For EtDA-treated films, a linear correlation was obtained for EtDA concentration ranging from 0% to 38% v/v (R^2 = 0.987, p < 0.001), validating the Orange II method for primary amine quantification. The extreme experimental

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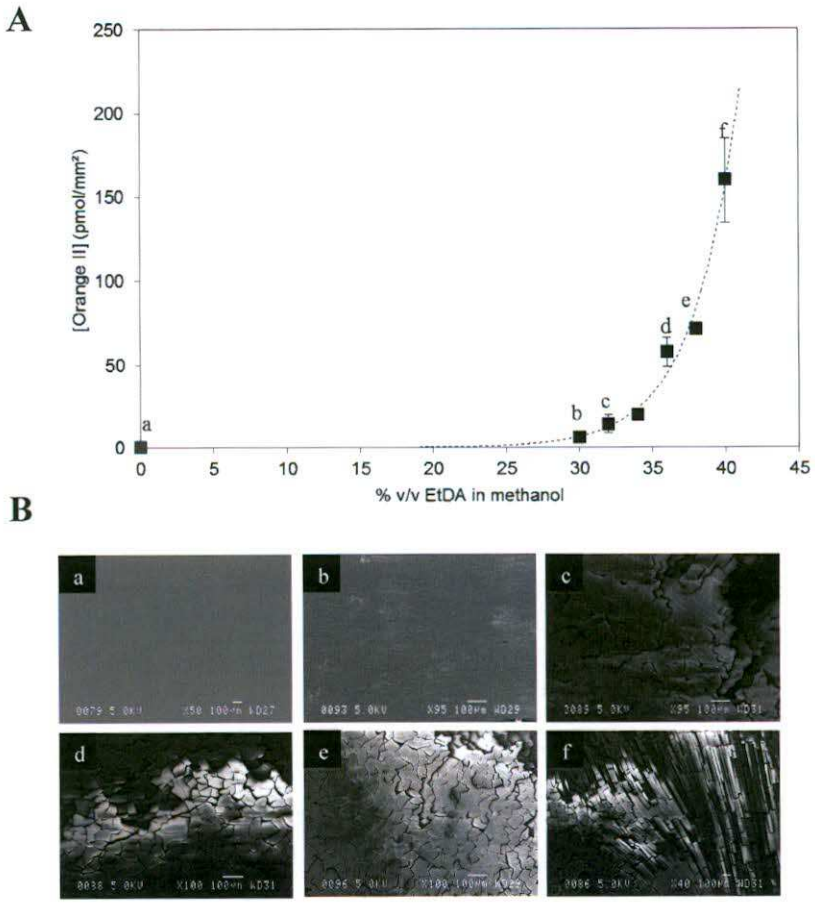

Figure 4. Characterization of EtDA-modified PET films by (A) the Orange II assay (n = 3) and by (B) SEM imaging for EtDA concentrations in methanol ranging from 30% to 40% v/v (a, 0%; b, 30%; c, 32%; d, 36%; e, 38%; f, 40%). The dashed line is only a guide for the eyes.

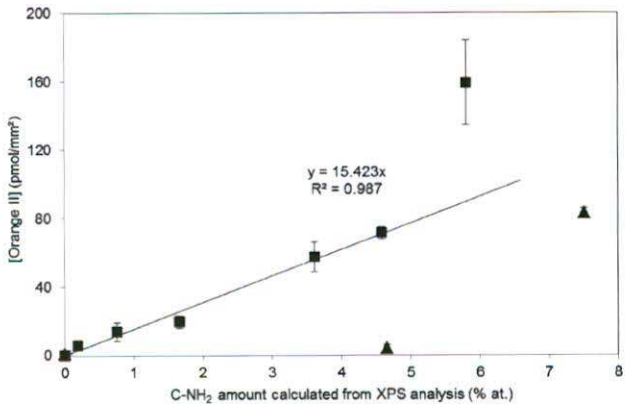

Figure 5. Correlation between amino group quantification from XPS analysis and adsorbed Orange II concentration for (■) EtDA-treated and (▲) PVAm-treated PET films (n = 3).

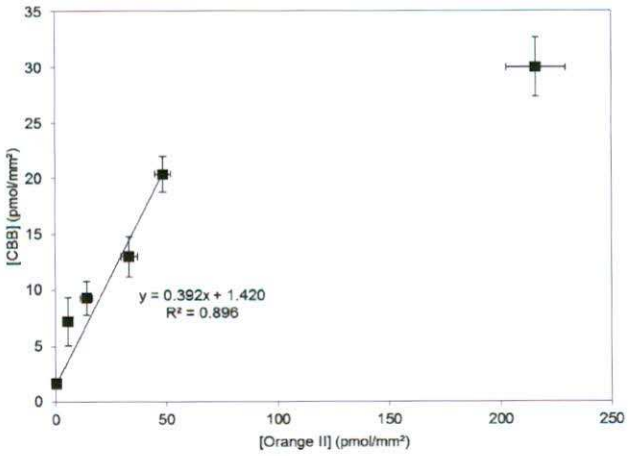

Figure 6. Correlation between Coomassie Brilliant Blue (CBB) and Orange II colorimetric methods for EtDA-treated PET films (n = 3). The highest point was excluded from the linear regression.

condition (i.e., 40% EtDA v/v) was characterized by relatively low $C-NH_2$ content, as determined from XPS measurements, and high Orange II value; this experimental condition was not properly described by the linear correlation. This outlier could be explained by the extreme roughness of the corresponding surfaces (Figure 4B, image f). That is, an excessive surface roughness could have indeed induced an overestimation of Orange II concentrations (due to a non-negligible increase of specific area) as well as undervalued $C-NH_2$ content from XPS measurements (since the analysis depth of XPS samples was kept constant, ca. 50 Å).[34]

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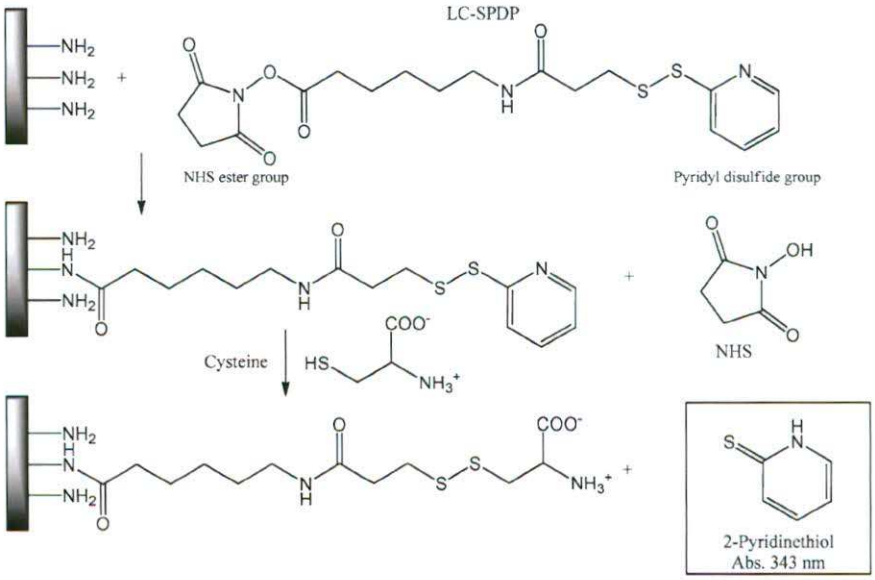

Figure 7. Schematic illustration of functionalization of amino-modified PET surfaces with heterobifunctional LC-SPDP molecules, followed by subsequent reaction with cysteine. The amount of released 2-pyridinethiol was measured at 343 nm.

The Orange II densities of PVAm-grafted films (i.e., 5.1 ± 0.6 and 82.6 ± 2.4 pmol/mm^2) were found to be in the same range as those obtained for EtDA-coated films (i.e., from 0.51 ± 0.04 to 159.2 ± 24.8 pmol/mm^2). However, the densities appeared to be underestimated when compared with the linear trend we previously determined for EtDA grafting (Figure 5), suggesting that all the PVAm amino groups detected by the XPS analysis were not able to interact with the dye. Such a difference between the two quantification techniques is most probably related to the polymer conformation at the PET surface, making some amino groups of the inner PVAm layer inaccessible to the dye. If our hypothesis is true, the Orange II method should provide a better quantitative prediction of the amount of primary amine groups that are readily accessible for subsequent chemical functionalization.

Comparison between Orange II and Coomassie Brilliant Blue Method. Many other dyes are commonly used to quantify primary amine groups onto solid surfaces.[23,38,39] Among these, the ability of Coomassie Brilliant Blue (a large divalent dye, Figure 1B) to quantify surface amino groups was compared to that of the small monovalent Orange II (Figure 1A) in terms of adsorbed densities onto aminated surfaces.

For both EtDA- and PVAm-treated PET films, dye densities obtained by the Coomassie Brilliant Blue (CBB) method were plotted as a function of those determined by the Orange II method (Figure 6). Interestingly, the CBB density measured on pristine PET (1.62 ± 0.19 pmol/mm^2) was found to be higher than that of Orange II (0.51 ± 0.04 pmol/mm^2), the former thus exhibiting higher nonspecific adsorption onto PET surfaces. As the experimental conditions (pH, time, temperature, solvent) were already optimized in the literature for the two colorimetric methods,[23,24] both dyes were supposed to detect accessible amino groups. However, for all the aminated PET films, densities determined by the CBB method were lower than those determined by the Orange II method (Figure 6). Such a result strongly suggests that steric hindrance between CBB molecules prevented them from binding to some amine moieties, inducing lower surface binding when compared to that of small Orange II molecules. Of interest, for Orange II densities below 50 pmol/mm^2, a linear correlation was found between the two dye densities (R^2 = 0.896, p < 0.01), showing a slope of 0.39 (Figure 6). This result suggests that, in this range, the CBB dye is indeed likely to bind to amino groups with a stoichiometry of 2:1 on account of its two charges per molecule, as previously proposed by Uchida et al.[23] As a consequence, Orange II dye appeared appropriate for amino group detection in a wide range of densities, in a quantitative and specific manner.

Functionalization of Aminated Surfaces with LC-SPDP. In order to confirm the ability of the Orange II dye to detect only available amino groups, a commonly used heterobifunctional linker (LC-SPDP, Figure 1C) was chemically grafted on both EtDA- and PVAm-modified PET films. The amine reactive N-hydroxysuccinimide (NHS) ester group of LC-SPDP reacted with the free primary amines on the modified PET surfaces at pH 7.4, to form a stable amide bond (Figure 7). The pyridyl disulfide group, located by the other end of the LC-SPDP linker, reacts specifically with thiol groups. Of interest, such a linker enables an oriented immobilization of cysteine-tagged protein. Proteins or other biomolecules grafted on LC-SPDP-covered surfaces can be quantified by UV readout thanks to the 2-pyridinethiol release induced by the reaction (Figure 7).[47] In order to eliminate any potential bias related to the size of the protein to be grafted, cysteine (i.e., the smallest thiol-containing proteinaceous unit) was reacted with the LC-SPDP-modified PET films. Under our conditions (i.e., large excess of cysteine and 60 min reaction), the reaction was observed to be complete in solution (1:1 stoichiometry between LC-SPDP and released groups) and thus allowed for the direct quantification of linker density.

The concentration of added LC-SPDP was optimized for a 15 min reaction at 20 °C (Figure 8A). The grafted linker density, assessed by released 2-pyridinethiol concentration upon cysteine addition, reached a plateau for LC-SPDP concentrations higher than 1 mM. Accordingly, 1 mM LC-SPDP solutions were used for subsequent grafting experiments.

LC-SPDP grafting kinetics were then studied by varying reaction time and temperature (Figure 8B) in order to get the

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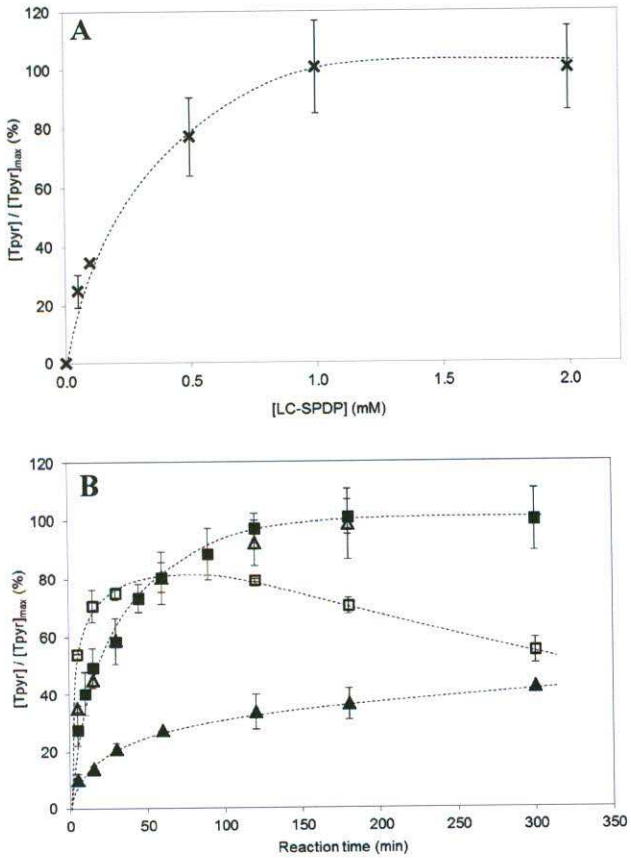

Figure 8. LC-SPDP grafting optimization on 38% EtDA-treated PET films (n = 3). (A) Influence of the LC-SPDP concentration in the reaction mixture for a reaction performed for 15 min at 20 °C. (B) Time course of the LC-SPDP grafting performed at (▲) 20 °C, (Δ) 30 °C, (■) 40 °C, and (□) 50 °C in a 1 mM linker solution. Grafted LC-SPDP densities were quantified thanks to 2-pyridinethiol release ([Tpyr]) determined by UV-readout, as described in the Materials and Methods section. The [Tpyr] values were normalized by the asymptotic value ($[Tpyr]_{max}$) obtained using a 2 mM solution and at 40 °C for (A) and (B), respectively. Dashed lines are guides for the eyes.

best surface coverage. As expected, the initial rate of the grafting reaction increased with temperature. After few hours (ca. 150 min), the same plateau value was reached at 30 and 40 °C (247 ± 15 pmol/mm^2), strongly suggesting that this maximum value could be accounted for a complete surface coverage. Quite different behaviors were observed for reactions carried out at both 20 and 50 °C. For the latter, the amount of released 2-pyridinethiol was found to decrease after a 60 min reaction, due to the instability of the thiol-reactive (pyridyl disulfide) groups at 50 °C (Figure 9). At 20 °C, the reaction was slower after 120 min of reaction, most likely due to the limited half-life of the NHS ester group of LC-SPDP at pH 7.4.[44] Accordingly, the grafted LC-SPDP density was found to be the best for a 150 min reaction at 40 °C in a 1 mM linker solution. Note that, for these experimental conditions, a loss of 7% of pyridyl disulfide groups was observed prior to cysteine grafting (Figure 9).

Comparison between Orange II Densities and Grafted LC-SPDP Densities. EtDA- and PVAm-covered PET films were reacted with LC-SPDP under our optimized conditions, i.e., at 40 °C for 150 min in a 1 mM solution. Linker densities on PET

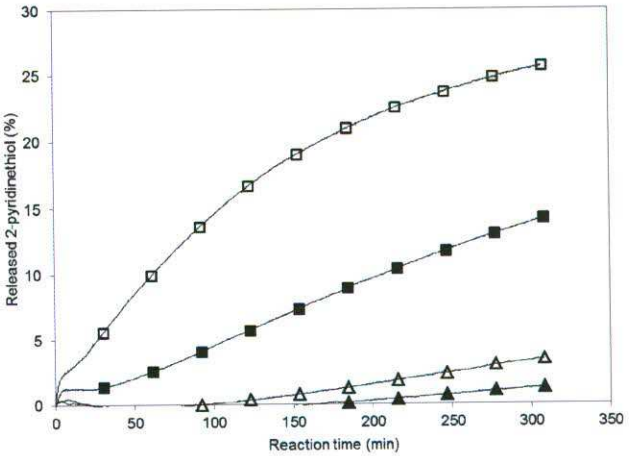

Figure 9. Stability of thiol-reactive groups of LC-SPDP. The release of 2-pyridinethiol was measured by stopped-flow absorbance at 343 nm in a LC-SPDP solution (0.1 mM, 90:10 v/v PBS/DMSO) at (—▲—) 20 °C, (—Δ—) 30 °C, (—■—) 40 °C, and (—□—) 50 °C. Absorbance values were normalized by the one measured after cysteine addition, as described in the Materials and Methods section.

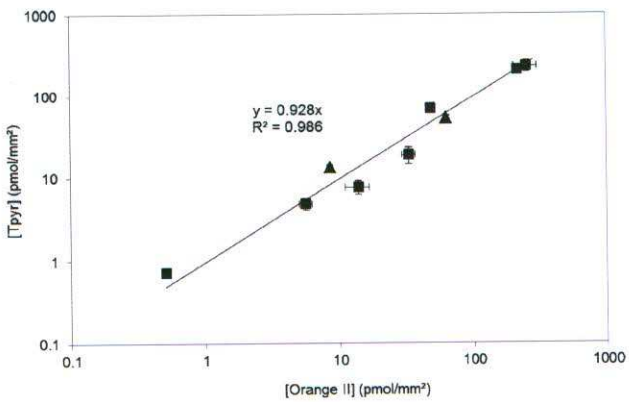

Figure 10. Correlation between released 2-pyridinethiol density and Orange II density assayed for (■) EtDA-treated and (▲) PVAm-treated PET films (n = 3). Treated PET films were reacted with a 1 mM LC-SPDP solution for 150 min at 40 °C. The 2-pyridinethiol concentration ([Tpyr]) corresponds to the grafted LC-SPDP density, as described in the Results and Discussion section.

films, thereby reaching maximized LC-SPDP surface coverage, were then determined thanks to the 2-pyridinethiol release induced by cysteine grafting, as described earlier (Figure 7). Quantification of LC-SPDP-modified amino groups was compared with Orange II densities (Figure 10), showing a linear correlation for all EtDA- and PVAm-covered films (R^2 = 0.986, p < 0.001), with a slope close to 1 (0.928). Such an excellent correlation thus demonstrates that all amino groups that were available for Orange II binding were also available for LC-SPDP grafting on EtDA- and PVAm-modified films. Primary amine density as determined by the Orange II colorimetric method being so closely correlated to the amount of grafted LC-SPDP unambiguously confirmed Orange II as an appropriate method for the quantification of amino groups available for subsequent biofunctionalization with a 1:1 stoichiometry.

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CONCLUSION

In this article, a rapid and convenient method using the Orange II dye was validated to quantify the amount of primary amine groups grafted on PET surfaces and available for subsequent functionalization.

Aminated surfaces were first generated via the PET chain scission-based aminolysis reaction involving diamine molecules (EtDA). Results given by the Orange II assays were found to be reliable, quantitative, and specific for a wide range of amino group surface densities (ca. 5 to at least 200 pmol/mm^2). SEM images revealed that the PET chemical etching was significant for high EtDA concentrations (ca. 40% v/v). Induced PET surface roughness was found to strongly interfere with conclusions drawn from XPS measurements but not from those derived from Orange II measurements.

As an alternative to surface-damaging EtDA functionalization, PET films were aminolyzed with polyamine chains (PVAm). For PVAm-treated films, the correlation between Orange II densities and XPS measurements was significantly different from the linear behavior observed with EtDA-treated films, most likely due to the fact that amino groups of the inner PVAm layer were not available for Orange II binding. Interestingly, subsequent LC-SPDP grafting revealed that those unavailable amino groups were not accessible to the linker either. Therefore, as opposed to Coomassie Brilliant Blue assays or XPS measurements, the Orange II-based quantification of amino groups was shown to be appropriate to quantitatively predict subsequent functionalization of both EtDA- and PVAm-treated PET surfaces by LC-SPDP, and potentially other types of molecules.

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