Biocompatible Surface Preparation Using Amino-Functionalized Amylose

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Aminopropyl amyloses with various degrees of substitution (DS) were prepared and investigated with respect to their surface modification properties. Poly(acrylic acid) was grafted to plasma-activated PVDF films, and the functional amylose was bound via amide linkage formation. Layer formation was confirmed by X-ray photoelectron spectroscopy. Contact angle measurements and surface MALDI-TOF mass spectrometry indicated a hydrophilic surface and minimization of protein adsorption.

Introduction

Amino-functionalized polysaccharides can be applied in bioanalysis, as nonviral gen carriers,[1] for enzyme stabilization,[2] and medical applications. Amino groups allow physisorption or covalent binding on surfaces of gold, silver, glass, and silicon, as well as on plasma-activated materials by reductive amination or amide formation. The remaining amino groups can be used to link biomolecules such as antibodies or enzymes.[3] The polysaccharide forms a three-dimensional hydrogel as an appropriate micromilieu for biological events, which protects biomolecules against shear stress in microfluidic systems. Chitosan, a 2-amino-2-deoxy β-1,4-glucan, has been used to form a monolayer on gold nanoparticles for an immunosensor.[4] Amino polymers have also been applied in microchip bioreactors to immobilize enzymes.[5] Amino dextrans prepared by oxidative sugar ring cleavage followed by reductive amination according to Piehler et al.[6] have found wide application. Investigation of structural parameters influencing protein adsorption have shown that density and distribution of functional groups are important for self-assembling of surface layers and therefore for their morphology.[7] Zacher and Wischerhoff used amino- and carboxy-modified dextrans to provide a functionalized hydrogel layer for binding studies by SPR.[8] Yagi and Nakamura coated iron oxide nanoparticles with functional polysaccharides for magnetic resonance imaging (MIR).[9] Biocompatibility of implant material mainly depends on its surface properties. Not complete inertness, but specific interactions to induce specific cell growth is required.[10] Therefore, preparation of hydrogel surface layers inhibiting unspecific protein adsorption, but with binding capacities for selected signal molecules, is of interest. Poly(vinylidene fluoride) (PVDF) has been used as biomaterial due to its nontoxicity, outstanding durability, and chemical stability. Ademovic et al. have shown how to minimize protein adsorption on PVDF by coating it with linear PEG chains, after creating carboxy groups and coupling with polyethylenimine (PEI) as interlayer. The protein-repellent properties depended on PEG chain length and the degree of coverage.[11] Recently, Groll et al. reported that star-shaped PEG prepolymers are appropriate to achieve the required high surface coverage.[12] We now report on surface layer formation of aminopropyl amyloses on a PVDF membrane and the investigation of its properties.

Materials and Methods

Materials. PVDF was purchased from Solvay Adv. Polym. (France). Acrylic acid (AAc) from Fluka (Germany) was distilled under nitrogen prior to use. Phosphate-buffered saline (PBS) was obtained from Merck (Germany). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (EDC), *N*-hydroxysuccinimide (NHS), and lysozyme were purchased from Sigma-Aldrich (Germany). Porcine insulin was obtained from Aventis (U.S.A.) and discharged from zinc by gel chromatography (Sephadex G 25). Amylose (potato) was purchased from Sigma. All reagents for APA preparations were of the highest purity available and purchased from Fluka, Aldrich, and Merck.[13]

***O*-(3-Aminopropyl) Amyloses.** *O*-(3-Aminopropyl) amyloses (APA) were synthesized according to a procedure described earlier.[13] Briefly, amylose was dissolved in DMSO, tetrabutylammonium bromide (TBAB) was added, and the polysaccharide was deprotonated by treating with Li-dimsyl, and etherified with *N*-3-bromopropyl-phthalimide. Products were isolated by dialysis. *N*-Deprotection was achieved by sodium borohydride reduction (10 equiv) in water/methanol (7:3, v/v) in silylated glassware, first at room temperature and then at 50 °C, and subsequent glacial acetic acid hydrolysis for 2 h under reflux. The degree of substitution (DS) was estimated from ^{1}H NMR and nitrogen content. Distribution in the glucosyl unit was determined after acid hydrolysis and trimethylsilylation by GLC and GLC-MS. APA with DS values of 0.10 (APA 12), 0.20 (APA 13), and 0.43 (APA 14) were applied in this study. For regioselectivity of substitution see Table 1. Only monosubstituted glucosyl units were detected.

Surface Layer Formation on PVDF. Plasma treatment was carried out with a microwave plasma unit from Eltro-Puls (Germany). PVDF films were argon plasma treated at a plasma power of 900 W for 30 s at a gas flow of 20 mL min^{-1} and a pressure of 20 Pa. Subsequently, the films were exposed to air for 20 min. The air-exposed PVDF films were immersed in an aqueous solution containing 20% (v/v) AAc and heated to 90 °C for 30 min. PAAc-grafted PVDF films were rinsed with distilled water and stored in distilled water until use. APA was grafted on the PVDF−PAAc surface by the EDC/NHS method. Carboxylic acid groups were treated with 0.1 M EDC/0.1 M NHS in water for 20 min at room temperature.[14] After rinsing with water, samples were incubated with APA (1 mg mL^{-1}) in a carbonate buffer at pH 8.4 for 1 h.

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Scheme 1. Immobilization of Aminopropyl Amylose to Polyvinylidene Fluoride after Plasma-Induced Grafting of Acrylic Acid

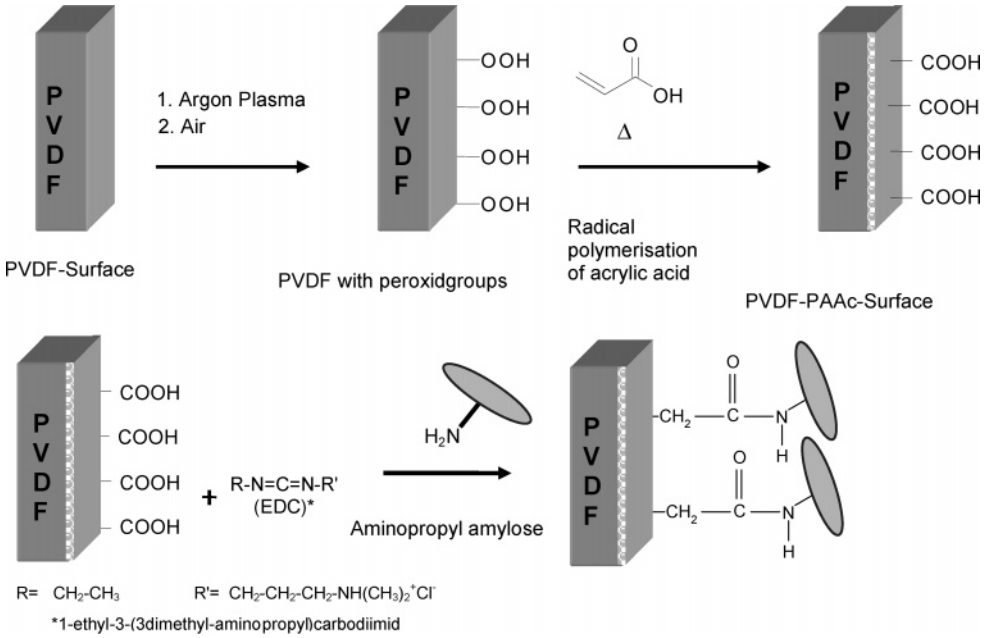


Table 1. Aminopropylamylose Applied for Surface Layer Formation on PVDF

	preparation		AP in position (%)		
sample	base/solvent	DS	2	3	6
APA 12	Li-dimsyl/DMSO/TBAB	0.10	100	n.d.	n.d.
APA 13	Li-dimsyl/DMSO/TBAB	0.20	89.4	2.2	8.4
APA 14	Li-dimsyl/DMSO/TBAB	0.43	94.0	2.5	3.5

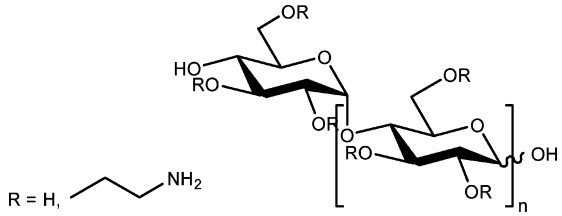
X-ray Photoelectron Spectroscopy (XPS). XPS measurements were performed by an Ultra Axis spectrometer (Kratos Analytical, Manchester U.K.). The samples were irradiated with monoenergetic Al $K\alpha_{1,2}$ radiation (1486.6 eV), and spectra were recorded at a power of 144 W (12 kV, 12 mA). The binding energies were referenced to aliphatic carbon (C−C, C−H) at 285.0 eV (C 1s photoline). Determination of the composition does not consider H and He, which cannot be detected by XPS. The information depth is up to 10 nm for polymers, depending on the emission angle of electrons.

Contact Angle. Contact angle measurements were performed with a goniometer microscope (Krüss GmbH, Germany) by the captive bubble method. Ten positions per sample were measured.

Surface MALDI-TOF-MS. Surface MALDI-TOF-MS were recorded in positive mode with a BRUKER BIFLEX III mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) equipped with a nitrogen laser (337 nm, 3 ns pulse width). APA-modified PVDF was immersed in the protein solution (porcine insulin and lysozyme (1 mg mL^{-1}) in PBS buffer, pH 7.4 (1 mg mL^{-1}), at 37 °C for 1 h, and subsequently washed twice with buffer and twice with water. A small piece of the modified PVDF was placed on the sample holder. Sinapinic acid as a matrix dissolved in a 0.1% solution of TFA in acetonitrile/water was applied onto the sample surface, and the solvent was left to evaporate before the sample holder was inserted into the spectrometer.

Results and Discussion

Synthesis and Characterization of *O*-(3-Aminopropyl) Amyloses. *O*-(3-Aminopropyl) amyloses (APA) with various DS values (DS = average number of substituents/glucosyl unit) were synthesized by a two-step polymer analogous reaction.[13] Amylose was etherified with *N*-phthalimido-protected aminopropyl bromides. The reaction efficiency could be improved by addition of TBAB as a phase transfer catalyst. The N-protecting group was quantitatively removed by adapting the method reported by Karas and Hillenkamp[15] and Leize et al.[16] to the different solution and stability requirements of the polymer. Thus, a hydrophilic polysaccharide backbone to which functional groups are linked via a C_3 spacer is obtained (**1**). In



(**1**)

comparison to amino dextrans these compounds are more acid resistant due to intact glucosyl moieties, while periodate oxidation of the 1,6-linked dextrans forms more acid-sensitive noncyclic acetals with loss of C-3 of the original glucosyl residues. The propyl linker between the carbohydrate core and the amino function should enhance the availability of amino groups for grafting reactions to the surface and to biomolecules as well, thus also improving susceptibility of the latter for receptor molecules.

The aminopropyl pattern can be tuned by the reaction conditions as has been described.[13] It has been determined by GLC of the trimethylsilylated glucose derivatives obtained by hydrolysis and is summarized in Table 1. In this study, three APA with high regioselectivity of O-2 substitution and DS values of 0.10 (APA 12), 0.20 (APS 13), and 0.43 (APA 14) are applied.

Surface Layer Formation on PVDF. PVDF, a polymer widely used in biomedical applications, was used as the basis for surface layer formation. Because PVDF lacks the reactive groups needed for further modification of the surface, a surface pretreatment is required in order to create reactive sites. Therefore, PVDF film was activated using argon plasma. By contact with air, radicals that are formed on the surface react to form hydroperoxide groups. In the following step, the activated surface undergoes a thermal-induced graft-copolymerization of acrylic acid (AAc) without loss of bulk properties (Scheme 1).

Table 2. Elemental Composition, Binding States of Carbon, and Binding Energies of Untreated and Stepwise-Modified PVDF Determined by XPS Analysis

		carbon (C1s)								
		atom %					atom %			
surface	total atom %	285.0 eV *C*−H *C*−C	286.5 eV *C*H$_2$−CF$_2$ *C*−O *C*−N	288.3 eV HN−*C*═O O−*C*−O	289.1 eV O−*C*═O	290.9 eV *C*F$_2$−CH$_2$	O1s	N1s	F1s	other
PVDF	46.9	3.2	23.4			20.2	2.5	0.9	49.8	
PVDF−PAAc	79.3	68.8			10.5		20.0	0.5		0.2
PVDF−PAAc−APA 12	65.9	21.5	33.4	8.9	2.1		26.1	5.8	2.2	
PVDF−PAAc−APA 14	67.4	23.0	36.3	7.2	1.0		26.6	4.2	1.5	0.3
PVDF−PAAc−APA 13	65.6	18.5	38.0	8.3	0.9		29.1	3.6	1.6	

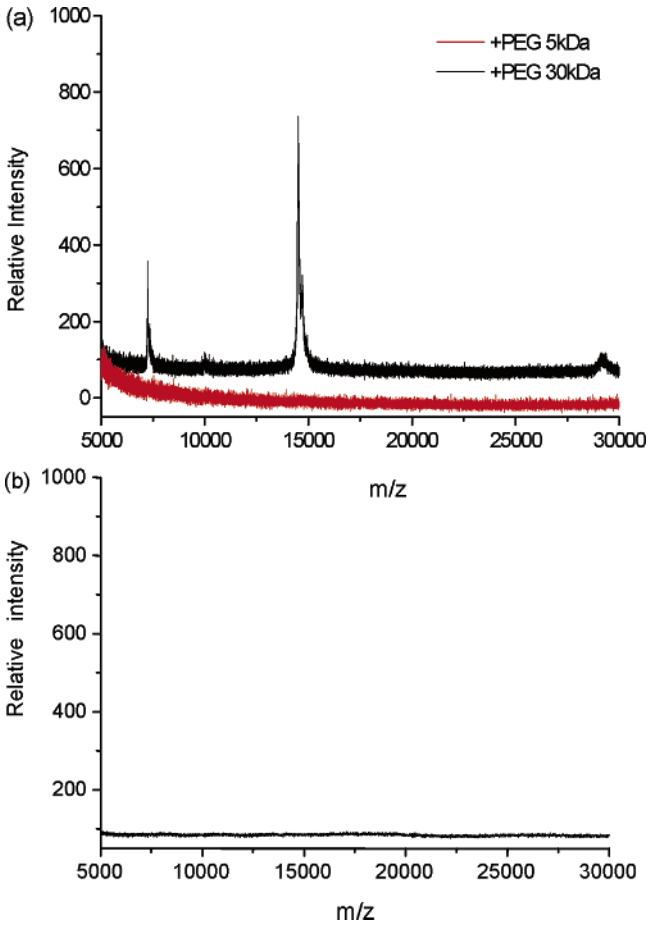


Figure 1. Surface MALDI-TOF mass spectra after adsorption of lysozyme (MW 14 228 Da) on PVDF−PAAc−PEI surfaces modified with linear mPEG-ald (M_W = 5000 and 30 000) (ref 11) (a) and on an APA-modified PVDF−PAAc surface (b).

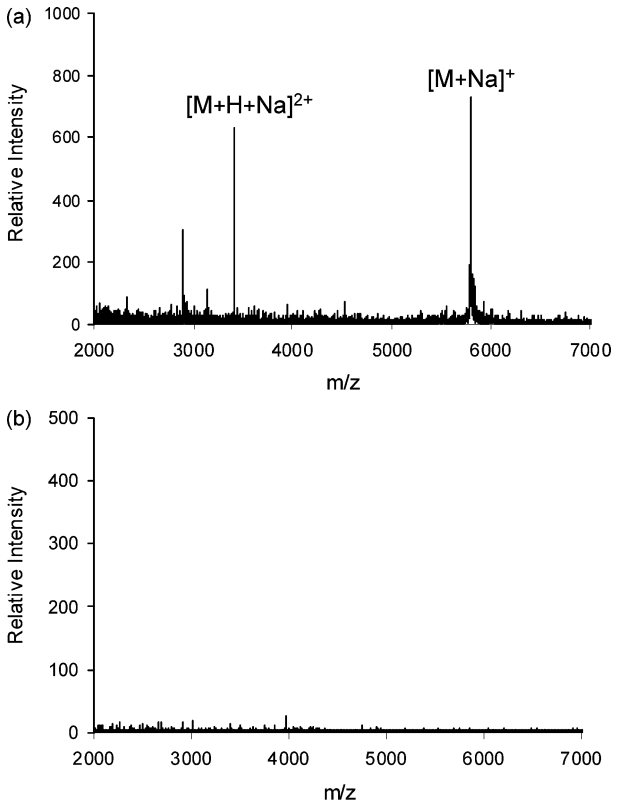


Figure 2. Surface MALDI-TOF mass spectra of PVDF (a) and PVDF−PAAc−APA (b) coating with APA 13 after exposure to insulin (MW 5778 Da).

Subsequently, the aminopropyl amyloses are covalently coupled via amide linkages by the ECD/NHS method. Chemical changes of the surfaces were characterized by XPS after each modification step. XPS determines the binding energy and the elemental composition of the surface with an information depth of about 10 nm. In Table 2 all XPS data are summarized. The data of C and F in the untreated PVDF are close to the theoretical 1:1 ratio with some additional oxygen and traces of nitrogen resulting from processing agents or atmospheric contaminations. After plasma activation and acrylic acid coupling (→ PVDF−PAAc), the content of oxygen and carbon atoms in the upper layer strongly increased. The signals of the original PVDF (CH_2−CF_2, and fluorine) cannot be detected anymore, indicating a complete covering of the surface with a layer of at least 10 nm thickness. The atomic ratio of 4:1 for C/O and the C−H/O−*C*═O of 7:1 does not correspond to pure PAAc (3:2 and 2:1, respectively), but show an over presentation of aliphatic carbon, probably due to side reactions on PVDF. XPS data after APA coupling (→ PVDF−PAAc−APA) clearly indicate the successful binding of the polysaccharide. Both nitrogen and oxygen increase, and a higher amount of C−N, C−O, and O−C−O binding sites present the new structural feature of the glycosyl units of the amylose derivative (**1**). The nitrogen content was between 3.60 and 5.82 atom %, compared to 12.58% on a similarly prepared PVDF−PAAc−PEI surface.[11] Some fluorine from the underlying substrate could now be detected again for all APA-coated samples. It should be mentioned that the polymer brushes of the hydrogel coating can collapse during the XPS measurement by high-vacuum conditions so that the bulk material (PVDF) can be seen by XPS, partially. Additionally some delamination could be caused during grafting of APA.

Properties of the APA-Coated PVDF−PAA Surface. To further characterize the surface properties of APA-coated PVDF−PAAc, contact angles were determined by the captive bubble method. Unmodified PVDF has a contact angle Θ of 78°. A contact angle Θ <20° was determined for all APA layers, indicating a strong increase in hydrophilicity and, therefore, wettability of the surface.

Unspecific protein adsorption is a problem in biosensing, and as a consequence acceptor-functionalized surfaces in biosensors are often blocked with BSA. Therefore, protein adsorption of APA surfaces was investigated. Matrix-assisted laser desorption/ionization (MALDI), introduced by Karas and Hillenkamp in 1988,[15] is widely used in combination with time-of-flight mass spectrometry (TOF-MS) in the analysis of macromolecules (genomics, proteomics, glycomics). It has also been used to investigate interactions of cells or proteins with biomaterial surfaces[16,17] and with respect to adsorption phenomena and gen expression as well.[18] To study the adsorption of proteins surface MALDI-TOF-MS is applied. Surface MALDI-TOF-MS measurements are difficult to quantify, and the method itself is semiquantitative. This is very difficult especially for a little amount of adsorbed protein. But the detection limits of the method are competitive, and surface MALDI-TOF methods are becoming more and more important.[18] In former examinations the protein adsorption of lysozyme and insulin on coatings from reactive star-shaped PEG-stat-PPG prepolymers (Star-PEG) and grafted linear methoxy-terminated PEG-aldehyde PEG (mPEG-ald, M_w = 5000 and 30 000) on silicon wafers was compared using the surface MALDI-TOF method.[12] No protein adsorption could be detected by Star-PEG layers and on mPEG-ald 5000, while mPEG-ald 30 000 layers only prevent the lysozyme adsorption but not the smaller insulin. Besides surface MALDI-TOF-MS, protein adsorption was monitored by fluorescence microscopy.[12] In this study, insulin with a molecular mass of 5778 Da and a pI of 5.3 is applied as a model compound with a net negative charge, and lysozyme with a mass of 14228 Da and a pI of 11.1 is used as a candidate with a net positive charge at the physiological pH 7.4, too. Figure 1 shows the results of protein adsorption on a PVDF−PAAc−APA surface (b) in comparison with mPEG-ald 5000 and 30 000 modified PVDF−PAAc−PEI surfaces (a) for lysozyme. The linear mPEG-ald 5000 and 30 000 were grafted under cloud point conditions (60 °C, 0.6 M K_2SO_4) to ensure optimal grafting density.[11] The signals that correspond to $[M + H]^+$ and $[M + 2H]^{2+}$ indicate lysozyme adsorption. Even at "cloud point" grafting condition with a highest PEG chain density, a small peak of lysozyme is detected for the mPEG-ald 30 000 coating on the PVDF−PAAc−PEI surface, while the mPEG-ald 5000 coating prevents lysozyme adsorption.[11] In contrast, APA-modified PVDF−PAAc (b) does not show any protein adsorption within experimental error. In Figure 2 surface MALDI-TOF mass spectra of insulin adsorbed on PVDF (a) and on PVDF−AAc−APA (b) are shown. While two signals corresponding to insulin are observed at *m*/*z* 5801 and 2901 on the PVDF (a), no adsorption of this more acidic and smaller protein could be detected after APA modification (b). This means that APA is a promising surface coating for example PVDF to prevent unspecific protein adsorption and to establish defined surface interactions after binding of specific biofunctional signal molecules.

Conclusion

Aminopropyl amyloses in the DS range of 0.10−0.43 have been shown to be suitable materials for nonadhesion hydrogel coating on biomaterial surfaces. In comparison to linear mPEG-ald modification of PVDF surfaces (PVDF−PAAc−PEI−mPEG-ald), one step less was necessary to build up a hydrogel with promising properties for biomedical applications. The density of the coverage was shown to be sufficient by XPS, the surface exhibited a hydrophilic character with a contact angle <20°, and protein adsorption could not be detected by surface MALDI-TOF-MS within experimental error.

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