Surface Analysis of PP/EVA Blends Modified with Amino Acids for Biomedical Applications

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SYNOPSIS

The copolymers poly(propylene-*co*-ethylene) (PP/E) and poly(ethylene-*co*-vinyl acetate) (EVA) and blends of these were modified to develop an artificial matrix which promotes the growth of endothelial cells. Covalent immobilization of amino acids or sequences of adhesion glycoproteins should trigger the formation of an endothelial cell monolayer onto the polymeric surface. Reactive functional groups were generated by saponifying the ester groups of the EVA component. Esterification with oxalylic or malonic dichlorides in the gas phase yielded the required monoesters and gave the best results for further immobilization of amino acids, while reaction with α,ω-dicarboxylic acid dichlorides in solution led to diester formation. Subsequently, various protected amino acids were immobilized via the carbodiimide method. Surface analytical methods like infrared spectroscopy using attenuated total reflection (IR-ATR), X-ray photoelectron spectroscopy (XPS), and secondary ion mass spectrometry (SIMS) were used to prove the modification steps. The analytical results confirmed covalent side-chain generation in the upper surface region. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Biomedical materials interfaced with the blood stream trigger a series of processes that are more rapid and often more intense than reactions known for materials implanted into soft or hard tissue. The contact of polymeric material with blood starts the intrinsic clotting system via contact activation. The following thrombin generation and fibrin formation lead to the clinical consequence of thrombus and emboli formation. In particular, small-diameter vascular grafts fail early due to thrombotic occlusion.

Natural blood vessels are lined with a monolayer of endothelial cells, which play an important pharmacological role in inhibiting thrombus formation. One method to overcome thrombogenicity is to line the inner surface of artificial vascular grafts with endothelial cells.[1,2] Suitable surface properties for endothelial cell growth depend on the chemical composition of the outermost surface. Hence surface activation with adhesion molecules or amino acids and investigation of these covalently bonded molecules in the upper surface region with suitable surface-sensitive methods is important. A number of studies suggest that the chemical and morphological nature of surfaces directs the biological responses.[3,4]

In this article we describe the biomaterial side upon defined variation in surface composition and the analytical investigation by means of combined use of the surface-sensitive techniques IR-ATR, XPS, and SIMS. Data obtained from each analytical method will be interpreted with regard to information depth and kind of information. In addition, the morphology of the incompatible PP/EVA blend with an inhomogeneous distribution of vinyl acetate segments must be taken into account. This kind of multitechnique approach will give information that can be integrated to provide a more complete picture of the surface with respect to its possible biological interactions.

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Journal of Applied Polymer Science, Vol. 58, 699–710 (1995)
 CCC 0021-8995/95/040699-12

EXPERIMENTAL

Materials

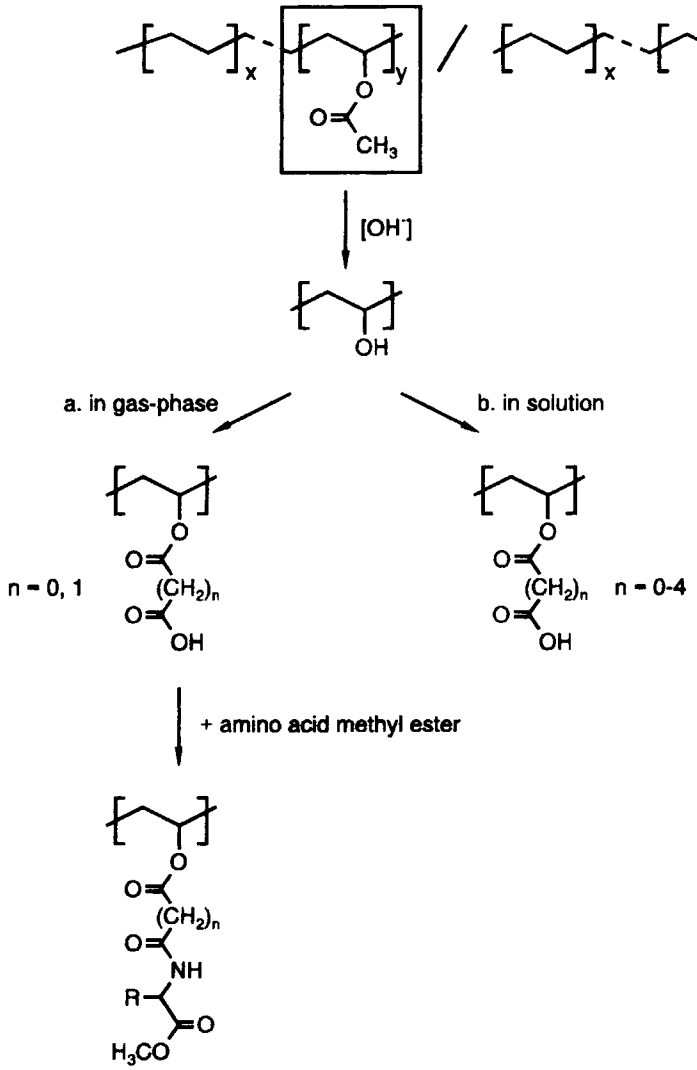
Poly(propylene-*co*-ethylene) and poly(ethylene-*co*-vinyl acetate) and blends of these copolymers in different compositions (Table I) were provided by Hüls AG (Marl, Germany). All polymers were obtained as 70-μm-thick films. Surface modifications were carried out with analytical grade chemicals. Protected amino acid hydrochlorides were supplied by Bachem (Heidelberg, Germany). Prior to analysis, the films were purified by Soxhlet extraction with analytical grade or distilled solvents and dried *in vacuo* at 30°C. Modification steps were carried out heterogeneously as solid phase reactions.

Table I Composition of Polymer Films

	EVA (%)	PP/E (%)
PP/E	0	100
Blend 30/70	30	70
Blend 50/50	50	50
Blend 70/30	70	30
EVA	100	0

Saponification of Vinyl Acetate Groups

Saponification of the ester groups of poly(ethylene-*co*-vinyl acetate) was carried out according to well-known methods.[5] Samples of untreated polymer film were heated under reflux in methanolic NaOH ($2M$)



Scheme 1 Surface modification on PP/EVA blends.

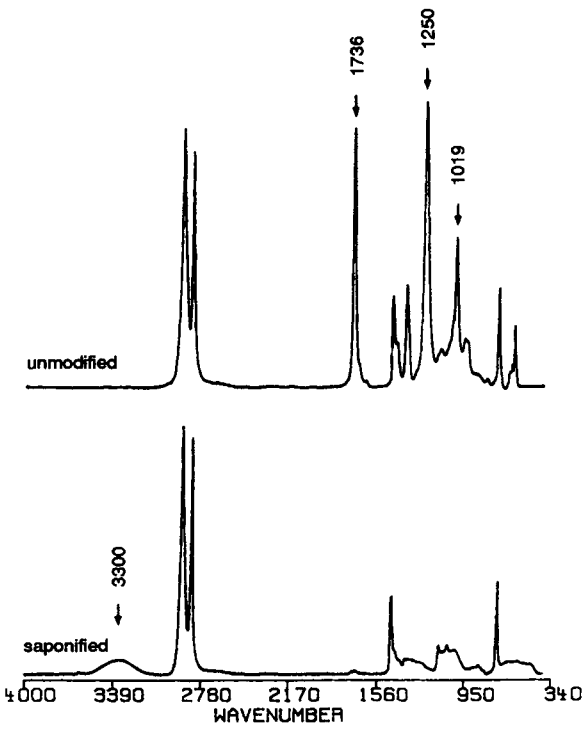


Figure 1 Superimposed IR spectra of unmodified and saponified EVA.

for 1 h. Subsequently, the films were washed with water overnight and dried *in vacuo* at 30°C, followed by Soxhlet extraction with methanol for 24 h and a second drying step.

Esterification with α,ω-Dicarboxylic Acid Dichlorides

In Solution

Several saponified polymer films were swollen for 15 min in 50 mL dry tetrahydrofurane in nitrogen atmosphere. Then 5 mmol α,ω-dicarboxylic acid dichloride and 5 mmol pyridine were added and the reaction mixture was stirred for 24 h at room temperature. The modified films were washed with tetrahydrofurane (THF) (100 mL, 1 h), a 1 : 1 (vol) mixture of tetrahydrofurane and water (100 mL, 4×, 0.5 h), and distilled water (100 mL, 4×, 0.5 h). Drying and extraction were carried out as described earlier.

In the Gas Phase

Five milliliters of oxalylic or malonic acid dichloride were placed in a 100-mL Schlenk flask. The flask was closed with a clip-containing stopper, cooled with liquid nitrogen, and evaporated. After closing the tap and warming to room temperature, the saponified polymer samples were exposed to the dicarboxylic acid dichloride atmosphere for 5 min. Polymer samples were stored in a desiccator at 10 hPa overnight. Subsequently, samples were washed for 30 min with water, 0.1*M* carbonate buffer (pH 8.5, 4×, 15 min), 0.1*M* acetate buffer (pH 4.0, 4×, 15 min), and water (4×, 15 min). The polymer films

Table II XPS Data of Unmodified and Saponified EVA

		(1)		(2)	
Element	BE (eV)	Theory (atom %)	Experiment (atom %)	Theory (atom %)	Experiment (atom %)
C 1s		90.8	87.5	94.7	92.5
C—C/C—H	285.0	81.6	76.0	89.4	85.6
C—O	286.5	4.6	7.3	5.3	6.9
CO—O	289.3	4.6	4.2	0	0
O 1s		9.2	10.0	5.3	6.7
C=O/C—OH	532.3	4.6	7.0	5.3	6.7
C—O—C	533.7	4.6	3.0	0	0
Others		0	2.5	0	0.8

BE: Binding energy.

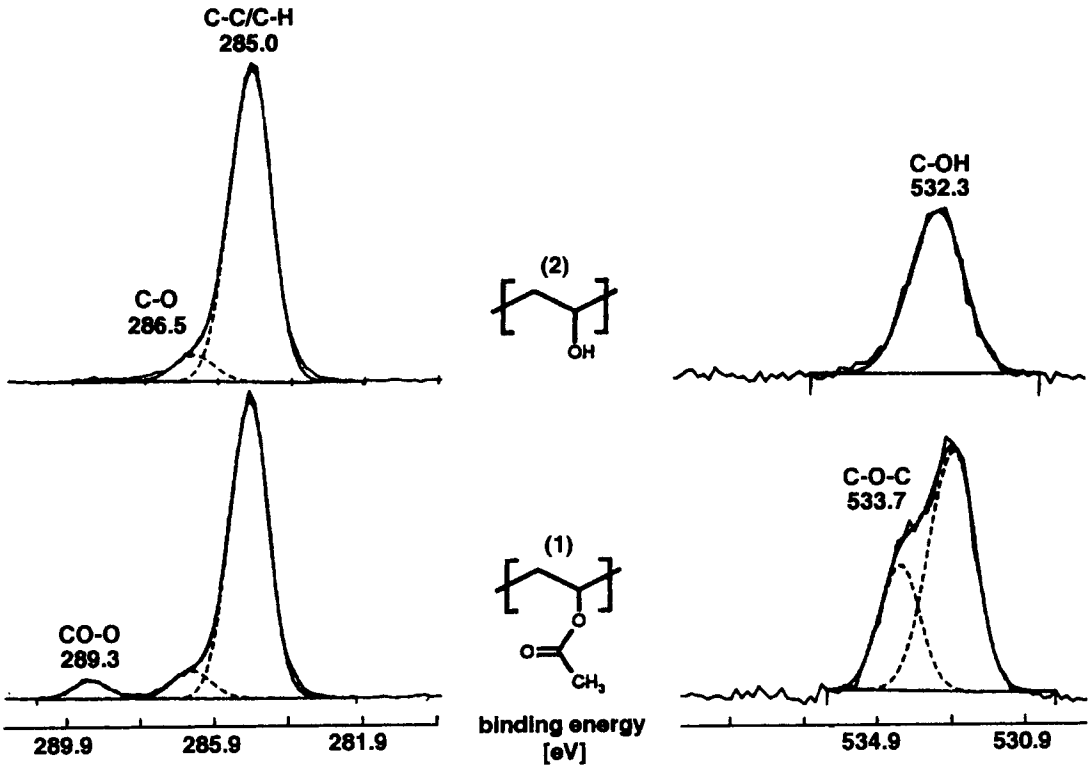


Figure 2 C ls and O ls X-ray photoelectron spectra of unmodified and saponified EVA.

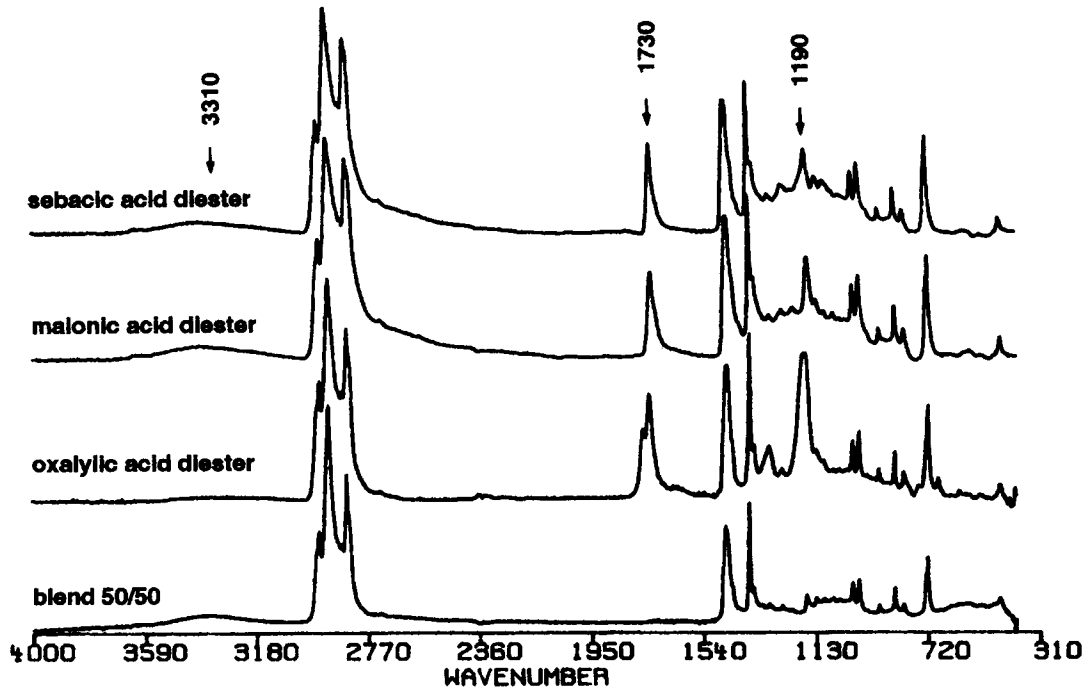


Figure 3 Superimposed IR spectra of saponified blend 50/50 after esterification with various α,ω-dicarboxylic acid dichlorides in THF.

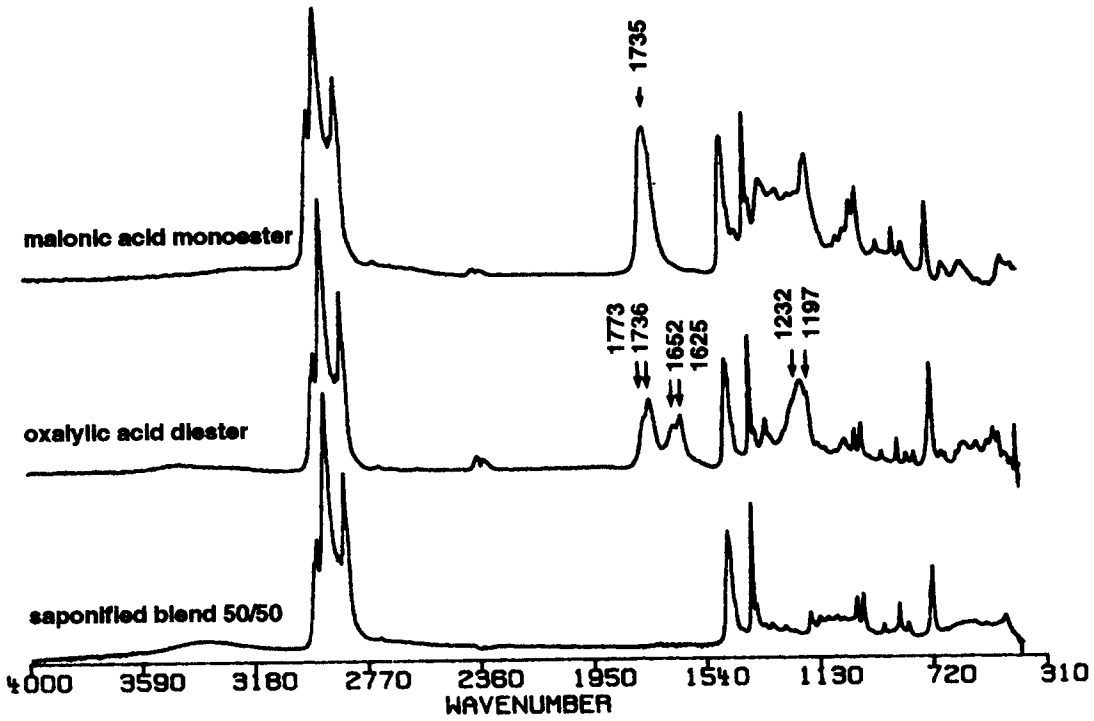


Figure 4 IR spectra of saponified blend 50/50 after esterification with oxalylic and malonic acid dichloride by gas-phase reaction.

were dried *in vacuo* at 30°C and purified by Soxhlet extraction with hexane.

Immobilization of Amino Acids

Immobilization of amino acids was carried out with the polymer films modified with oxalylic or malonic acid dichloride. These modified polymer films were swollen in 50 mL dimethylformamide for 15 min. Then 5 mmol amino acid ester hydrochloride, 0.58 g (5 mmol) *N*-hydroxysuccinimide, and 693 μL (5 mmol) triethylamine were added. The solution was stirred and cooled to −10°C during addition of dicyclohexylcarbodiimide (DCC) (1.03 g, 5 mmol). Subsequently, the reaction mixture was warmed to room temperature and stirred overnight. The modified polymer films were rinsed with dimethylformamide (50 mL, 24 h), a 1 : 1 mixture of dimethylformamide and water (4×, 50 mL, 0.5 h), and distilled water (4×, 50 mL, 0.5 h), followed by drying *in vacuo* at 30°C and Soxhlet extraction with hexane. Reference samples were treated in an analogous way without addition of DCC.

Analytical Methods

IR-ATR

FTIR spectra were obtained using a Nicolet FTIR spectrometer 60 SXR with ATR technique, with a KRS-5 crystal as a reflection unit. Information depth is associated with the wavelength and is estimated to be about 2 to 10 μm. Two hundred and fifty scans were recorded on each sample, and the spectral resolution was 4 cm^{-1}.

XPS

XPS data were collected with a Surface Science Instruments X-probe™ spectrometer (Mountain View, CA) using an Al $K_{\alpha 1,2}$ X-ray source with a 1000-μm-diameter X-ray spot size and a 35° take-off angle. A 24-eV flood gun was used to neutralize charge accumulation on the samples. Elemental composition was determined on the basis of peak areas using spectra collected at a pass energy of 150 eV. The binding environments for C, N, and O were determined using spectra collected at 25 eV pass energy. The binding energy was referenced by setting the C ls aliphatic carbon peak to 285.0 eV. The component analysis of the C ls spectrum was accomplished on an SSI ESCA 8.3 D data system by fitting Gaussian peaks. The information depth of this surface-sensitive method is 6 to 10 nm.

SIMS

SIMS spectra were obtained by a time-of-flight SIMS instrument built at the University of Mün-

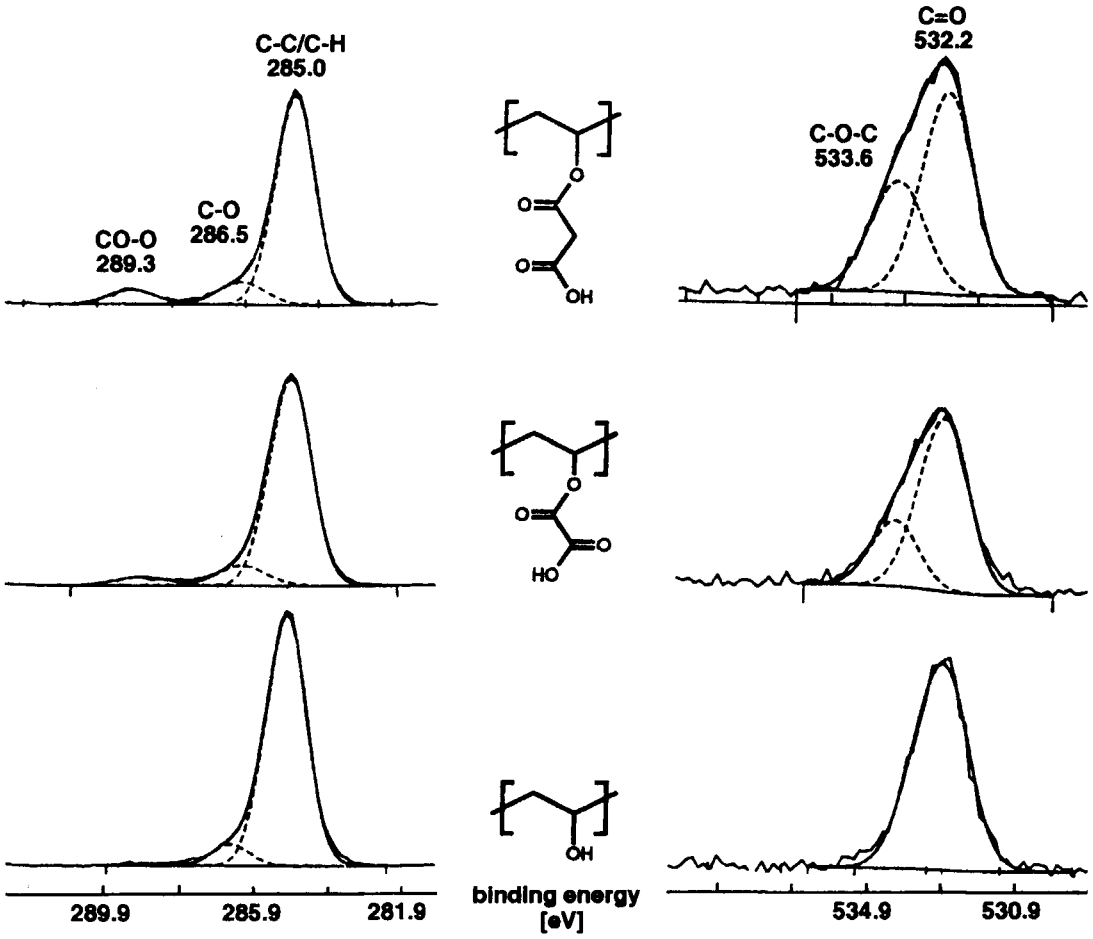
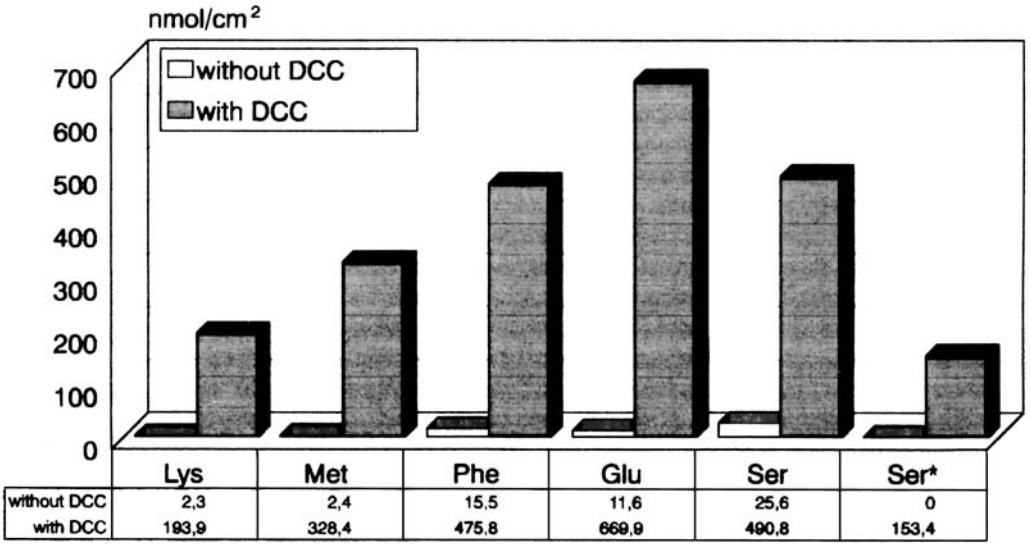


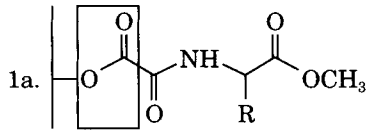
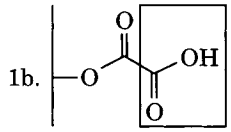
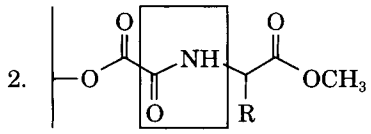
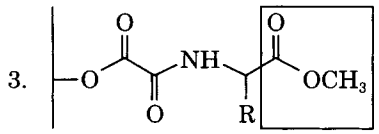
Figure 5 C ls and O ls X-ray photoelectron spectra of saponified EVA after esterification with oxalylic and malonic acid dichloride by gas-phase reaction.



	Lys	Met	Phe	Glu	Ser	Ser*
without DCC	2,3	2,4	15,5	11,6	25,6	0
with DCC	193,9	328,4	475,8	669,9	490,8	153,4

Figure 6 Amino acid concentration on oxalylic and malonic acid modified (*) EVA.

Table III Characteristic IR Absorption Bands of Immobilized Protected Amino Acids

Functional Group	IR Band (cm^{-1})	Classification
1a. 	1773 s, 1736 1197	ν(C=O), polymer ester ν(C—O), polymer ester
1b. 	1652, 1625 1232	ν(C=O), carboxylic group ν(C—O), carboxylic group
2. 	3397 1656 s 1520 1280	Amide A: ν(NH) Amide I: ν(C=O), ν(CN), $\delta(CNH)_{ip}$ Amide II: $\delta(CNH)_{ip}$, ν(CN) Amide III: $\delta(CNH)_{ip}$, δ(OCN)
3. 	1705	ν(C=O), methyl ester

ster.[6] A pulsed primary ion beam of Xe cations with an energy of 11 keV was delivered by an impact ion source. In the time-of-flight analyzer, energy focusing was achieved by a one-stage reflectron, so that a mass resolution of $m/\Delta m$ = 10,000 could be obtained. Spectra were accumulated with a primary ion dose (PID) of 10^9 PI in an area of 0.1 mm^2, resulting in a removal of only some percent of a monolayer. Charging accumulation on insulating surfaces was overcome by charge compensation with pulsed low-energy electron flooding.

Amino Acid Analysis

Modified polymer films were cut to pieces and placed in a bomb tube. After addition of 4 mL 6*M* HCl, the tube was heated to 110°C for 24 h. The extracts were evaporated to dryness and washed four times with distilled water, whereas the water was removed in vacuum each time. The amino acids and their concentrations were determined by ion exchange chromatography with an amino acid analyzer LC 6000 (Biotronik).

RESULTS AND DISCUSSION

EVA and blends of PP/E and EVA were modified in the upper surface region, as described in Scheme 1. Reactive functional groups were generated by saponifying the ester groups of the EVA compound. Esterification with oxalylic or malonic dichlorides by gas-phase reaction yielded the required monoesters and allowed further immobilization of amino acids, while reaction with α,ω-dicarboxylic acid dichlorides in solution led to diester formation.

Saponification of Vinyl Acetate Groups

Figure 1 compares the IR-ATR spectra of an unmodified and a saponified EVA film. The IR spectrum of the saponified polymer shows the total decrease of the C=O double-bond vibration at 1736 cm^{-1} and the C—O stretching vibrations at 1250 and 1019 cm^{-1}. The increasing absorption at 3300 cm^{-1} is associated with the O—H stretching vibration of the formed hydroxyl group. Hence a complete saponification in the upper 5 to 10 μm of the polymer surface can be assumed.

The elemental compositions in the upper 6 to 10 nm of the surface are determined from the peak areas of the survey XPS spectra. The XPS data of unmodified and saponified polymer blends are shown in Table II in comparison to their theoretical data, calculated for a homogeneous distribution of polymer segments. As expected, the oxygen content of the polymer surface decreases after saponification

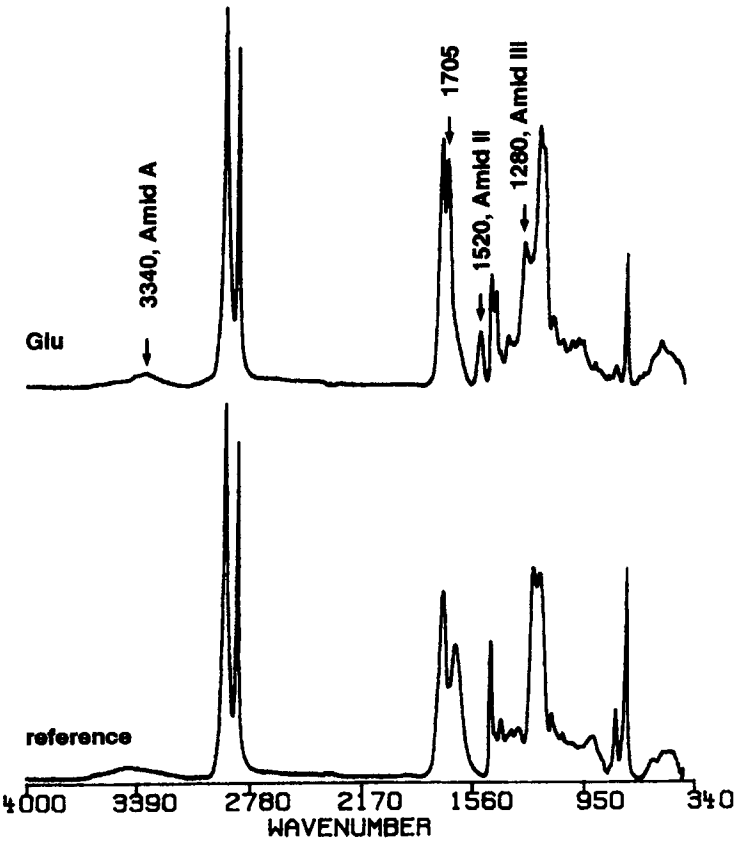


Figure 7 IR spectra of EVA after esterification with oxalylic acid and immobilized glutamic acid methyl ester in comparison to a reference sample without coupling agent DCC.

from 10.0% to 6.7%, compared to a calculated decrease from 9.2 to 5.3% (Table II). C ls and O ls core-level spectra of unmodified and saponified EVA are presented in Figure 2. The C ls spectrum of the saponified EVA (2) shows a total decrease of the ester carbon atom peak at 289.3 eV binding energy. In addition, the related O ls core-level spectrum shows a decreasing peak for the C—O—C single-bonded ester oxygen atom. Hence, total saponification in the upper 10 nm is corroborated by the XPS data.

Esterification with α,ω-Dicarboxylic Acid Dichlorides

In Solution

Figure 3 shows the superimposed IR spectra of the blend 50/50 after saponification and esterification with various α,ω-dicarboxylic acid dichlorides in THF. In the case of oxalylic acid, in the area of carbonyl absorption bands only the C=O stretching vibration band at 1730 cm^{-1} is detected. In addition, the absorption band of the ν(C—O) stretching vibration of the ester group is found at 1190 cm^{-1}, whereas the absorption of the carboxylic acid carbonyl group is missing. This gives evidence for inter- or intramolecular diester formation (e.g., ring formation between vicinal hydroxyl groups).

In the Gas Phase

Figure 4 shows the IR spectra of EVA after saponification and esterification with oxalylic and malonic acid by gas-phase reaction. The IR spectra show a decreasing ν(OH) absorption band and an accumulation of different C=O double-bond stretching vibrations in the area between 1625 and 1773 cm^{-1}. In the case of oxalylic acid, it is possible to distinguish between the C=O double-bond vibrations of the free carboxylic group at 1652 and 1625 cm^{-1} and of the polymer bonded ester group at 1773 and 1736 cm^{-1}. This gives evidence for free carboxylic groups in the polymer surface region, which allow further immobilization of amino acids. In the case of esterification with malonic acid, one carbonyl double-bond absorption is found at 1735 cm^{-1}. In this case,

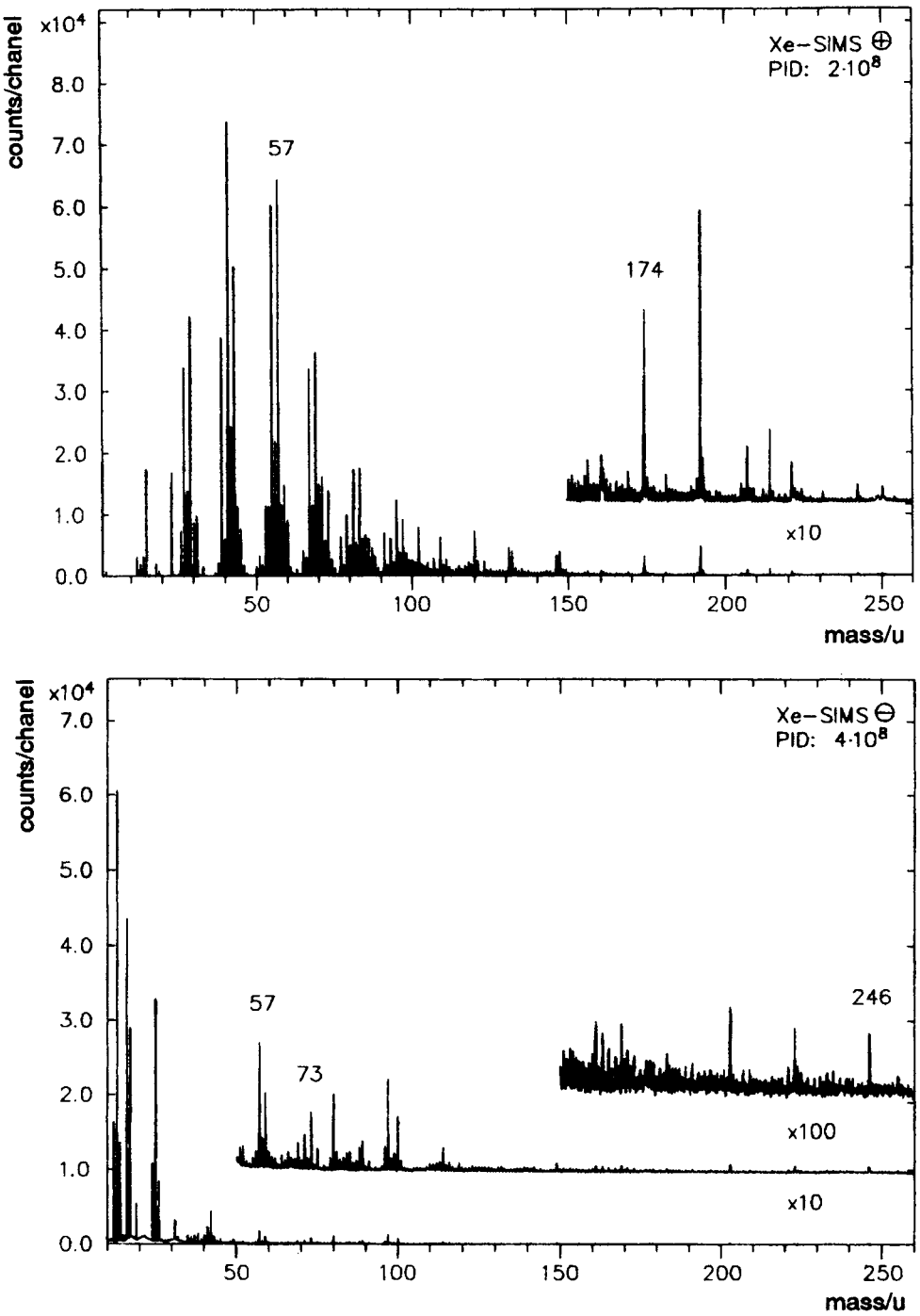
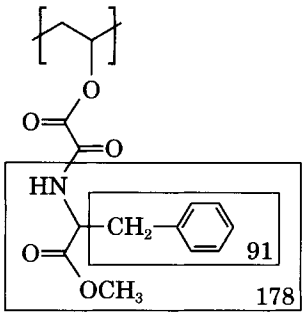
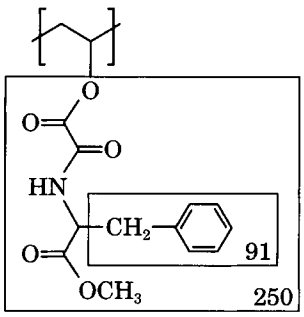
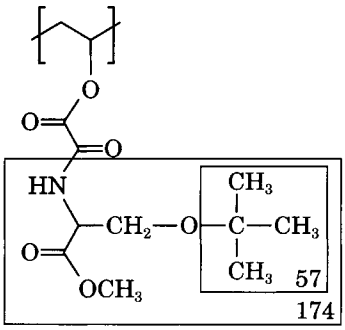
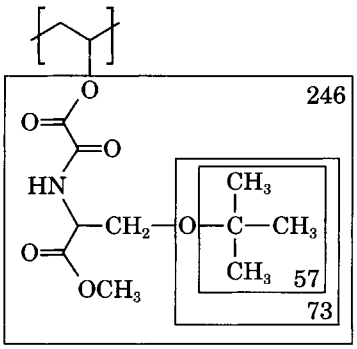
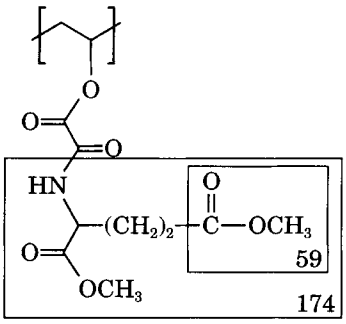
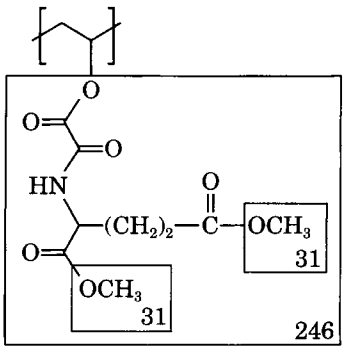
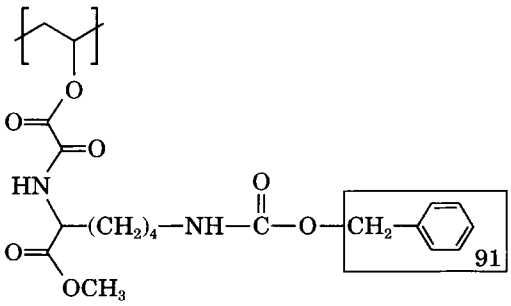
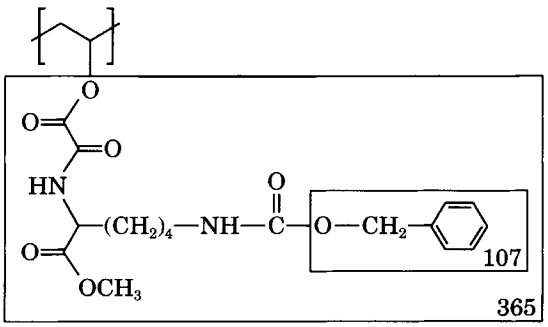


Figure 8 (a) Positive secondary ion mass spectrum of serine methyl ester immobilized on EVA, 11 keV Ar SIMS, 1×10^9 ions on 0.1 mm^2. (b) Negative secondary ion mass spectrum of serine methyl ester immobilized on EVA, 11 keV Ar SIMS, 1×10^9 ions on 0.1 mm^2.

no discrimination between C=O double-bond stretching vibration of the carboxylic group and the ester group is possible.

The carbon and oxygen core-level spectra also give evidence for covalently bonded dicarboxylic acids (Fig. 5). The ester carbon atom peak at a binding energy of 289.3 eV in the C ls spectra and the C—O—C single-bonded ester oxygen atom peak at 533.6 eV are characteristic of a successful esterification.[7,8] The nearly identical kinetic energy of

Table IV Characteristic Cationic and Anionic Fragments of Amino Acid Modified PP/EVA Blends

Amino Acid	Cationic Fragments	Anionic Fragments
Phe		
Ser		
Glu		
Lys		

Phe: phenyl alanine, Ser: serine, Glu: glutamic acid, Lys: lysine.

ester carbon electrons and carboxylic carbon electrons prohibits their resolution in the C ls spectra. The fitted peak areas of the oxygen components were expected to be equal. However, unesterified hydroxyl groups enlarge the peak area at 532.2 eV binding energy.

Immobilization of Amino Acids

Figure 6 compares the concentrations of several immobilized amino acids determined by total hydrolysis and amino acid analysis. Concentrations up to 670 nmol/cm^2 were detected in comparison to concentrations below 15 nmol/cm^2 of the reference samples. In the case of immobilization of serine, Figure 6 shows the advantage of oxalylic acid over malonic acid (*) as a spacer.

Formation of the amide bonds between spacer and amino acid leads to strong characteristic absorption bands in the IR-ATR spectra, which gives evidence for a covalent immobilization. Table III shows the detected absorptions and their assignment to the following functional groups of spacers and immobilized amino acids:

1.a. Ester group of the dicarboxylic acid
1.b. Free carboxylic acid group in the case of oxalylic acid
2. Amide bond
3. Methyl ester protecting group of the immobilized amino acid.

Figure 7 shows the IR spectrum of an oxalylic acid modified EVA after reaction with γ-methyl glutamic acid methyl ester in the presence of the coupling agent DCC in comparison to the IR spectrum of a reference sample prepared without a coupling agent. Because of the partial double-bond character, the vibrational modes tend to involve all surrounding atoms of the peptide bond. The NH group (amide A) is detected at 3340 cm^{-1}. At 1670 cm^{-1}, the strongest band in the spectrum of an amide, the amide I absorption appears. This band is superimposed by the decreasing ν(C=O) stretching vibration of the free carboxylic group of the spacer. Another strong vibration, the amide II band, is detected at 1520 cm^{-1}. As expected, the amide III band is present at 1280 cm^{-1}. After reaction of spacer modified EVA with amino acid methyl esters, an additional band in the area of the ν(C=O) vibrations appears at 1705 cm^{-1}. This signal is assigned to the methyl ester protecting group. The associated ν(C—O) vibration mode is superimposed by the analogous band of the polymer ester group.

In the secondary ion mass spectra fragments, up to 300 u are of special interest in characterizing the immobilized side chains. The lack of high-mass fragments in the case of polymer films is due to chain interactions, which reduce the emission of oligomeric fragments. Positive-ion SIMS spectra of amino acid immobilized EVA foils [Fig. 8(a)] show characteristic fragments which originate from the protected amino acid (amino acid methyl ester-H)$^+$ and the alkyl cations of the immobilized side chain and protecting groups. SIMS is also a suitable method to distinguish between different alkyl groups. The observed fragments were assigned to structural features, which are listed in Table IV. The positive static SIMS spectrum of a modified EVA film with immobilized serine is shown in Figure 8(a). The peak at 174 u arises from the protected amino acid, and the fragment at 57 u originates from the *t*-butyl protecting group.

Negative-ion SIMS spectra of modified EVA [Fig. 8(b)] show a characteristic fragment, which can be assigned to a complete elimination of the immobilized side chain. This ion comprises the spacer molecule and the protected amino acid. In contrast to the spectrum of the reference sample, there is no peak at 89 u corresponding to the oxalylic acid mono anion. This gives further evidence for the covalently bound amino acid. In Figure 8(b), the negative-ion SIMS of modified EVA with immobilized serine is shown. The peak at 246 u is assigned to the cleaved side-chain anion, and the peak at 73 u originates from the *t*-butanolate anion. The SIMS spectra give evidence for a successful covalent immobilization of amino acids in the uppermost monolayer.

CONCLUSIONS

The surfaces of EVA and PP/EVA blends after saponification were successfully modified with different spacers and amino acids. It was shown that the combined application of many analytical methods leads to a more differentiated understanding of the nature of the outermost few angstroms of films of PP/EVA blends. IR-ATR, XPS, and SIMS are suitable analytical methods to examine the different modification steps. Only detailed knowledge of the chemical composition of the surface allows evaluation of its biological significance.

We thank Hüls AG (Marl, Germany) for the polymer blends and Dr. R. Kaufmann (DWI Aachen, Germany) for the XPS measurements and helpful discussions.

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Received July 14, 1994
Accepted March 15, 1995