Synthesis of Peptides onto the Surface of Poly(Ethylene terephthalate) Particle Track Membranes

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ABSTRACT: In this article, we describe the synthesis of polypeptides onto the whole specific surface of poly(ethylene terephthalate) particle track membranes. In this case, initial functionalization is necessary. It had been done by an optimized surface oxidation in accordance with the method of Marchant-Brynaert, J.; Deldime, M.; Dupont, I.; Dewez, J.-L.; Schneider, Y.-J. (J Colloid Interface Sci 1995, 173, 236). Subsequent activation with carbodiimide and reaction with multifunctional amines yielded an aminated surface. The synthesis of peptides was carried out by two different means: first, by the coupling of single amino acids analogous to the method of Merrifield, and second, by coupling of presynthesized polypeptides consisting of 18 helix-forming amino acids. Analytical characterization was carried out by fluorescence spectroscopy by using the label Fluram® and confirmed by the results of X-ray photoelectron (XP) spectroscopy. Only the stepwise synthesis led to a dense surface functionalization with peptides, whereas the coupling of fragments resulted in lower yields of coupling. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 74: 1669–1674, 1999

Key words: peptides; PET; membranes

INTRODUCTION

The chemical modification of polymeric membrane surfaces is of extraordinary interest for adjusting defined surface characteristics of polymeric membranes. The use of amino acids for surface modification facilitates a biofunctionalization of the membranes. In recent years, a lot of effort was made to investigate the interactions of polymeric surfaces and biological material.[1,2] Peptides as biomimetic agents should help to control these interactions.

A wide range of applications are possible with peptide-functionalized membrane surfaces. One application is the synthesis of sequences for molecular recognition. Thus, it is possible to use those membranes as biosensors or for affinity separation. Another application can be seen in the generation of biomaterials with defined interactions between the membrane and biological material or the immobilization of enzymes with peptide spacers.[3] A completely different application could be the use of that kind of membranes for synthesis of peptide libraries (spot synthesis), which are of outstanding interest in pharmaceutical research. (The search for bioactive drugs requires the synthesis of thousands of peptide sequences. By using spot synthesis, it is possible to reduce the amount of each sequence to be synthesized to a minimum.)

The aim of our investigations is to synthesize peptides onto the surface of polyester membranes with very high yields. Particle track membranes were chosen which had been manufactured by using a heavy ion accelerator for generating latent ion tracks. Etching of these tracks leads to cylindrical pores with a uniform pore geometry and a very narrow pore size distribution. The

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functionalization is aimed at the growing of a lawnlike layer of peptide chains at the surface of both the outer membrane surface and the pore surface area. We investigated the possibilities and limits of peptide synthesis at the surface of this nonswelling polymeric material by using two different ways of synthesis: first, the stepwise synthesis with single amino acids, and second, the coupling of presynthesized peptide fragments. To assure a defined secondary structure of the growing peptide chains, only helix-forming amino acids and peptides were used for the synthesis.

EXPERIMENTAL

Materials

Poly(ethylene terephthalate) (PET) particle track membranes with pore diameters of 30 (PET 30), 50 (PET 50), 80 (PET 80), and 450 (PET 450) nm were products of Oxyphen (Dresden, Germany). Fluorenylmethyloxycarbonyl- (Fmoc) and *t*-butyloxycarbonyl- (BOC) protected amino acids and dipeptides, as well as hydroxybenzotriazole (HOBt), were purchased from Bachem (Heidelberg, Germany). Fluram® and thionine acetate were products of Fluka (Buchs, Switzerland); tetraethylenepentamine (TEPA) and Acid Orange II were products of Aldrich (Steinheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany) and used without further purification.

Hydrolysis of PET Membranes

The membranes were immersed in a solution of 10 g $KMnO_4$ in 200 ml 0.75*N* H_2SO_4 at room temperature and shaken for 2.5 h. Afterwards, the membranes were rinsed with water (two times) and then treated with 6*N* HCl (four times for 2 min). Finally, the membranes were washed with water (four times) and ethanol (two times) and dried.

Fluorescence Staining

A measure of 10 mmol/L thionine acetate was dissolved in ethanol and the carboxylated membranes were shaken in the solution for at least 12 h. The samples were removed from the solution, rinsed with ethanol (three times at 30 s), and then immersed in 10 mL of a solution of 0.01*N* HCl in a mixture of ethanol/water (1 : 1). After shaking for 1.5 h, a fluorescence spectrum of the solution was recorded (λ_{ex} = 594 nm, λ_{em} = 620 nm).

Reaction with Amines

The carboxylated membrane samples were immersed in a solution of 1.26 g diisopropylcarbodiimide and 3.06 g *N*-hydroxybenzotriazole in 200 mL dimethylformamide (DMF) and shaken at room temperature for 30 min. Subsequent washings with 50 mL DMF was followed by shaking in a 1 wt % solution of the amine in DMF for 3 h. Afterwards the membranes were washed two times with DMF, two times with ethanol, and dried.

Staining with Acid Orange II

A measure of 500 mmol/L Acid Orange II was dissolved in water (pH = 3, HCl). The samples (approx. 2 cm^2) were shaken in the solution for at least 12 h at room temperature. The solution was removed and the samples were washed three times with water (pH = 3). Removal of the dye was carried out by shaking the samples in 10 mL of water (pH = 12, NaOH) for 15 min. The absorption of the solution was recorded at 485 nm.

Coupling of Amino Acids

A measure of 2 mmol of the respective BOC-protected amino acid together with 4 mmol *N*-hydroxybenzotriazole, 2 mmol diisopropylcarbodiimide, and 4 mmol diisopropylamine were solved in 30 mL DMF. The membrane sample (approx. 25 cm^2) was immersed in the solution and shaken at room temperature for 4 h. The membranes were washed twice in DMF and ethanol and dried. Deprotecting of the amino group were carried out with 1*N* HCl in dioxane for 1 h followed by washing with dioxane and ethanol (both twice) and drying.

RESULTS AND DISCUSSION

To start the peptide synthesis, it was necessary to introduce appropriate functional groups on the membrane surface. This initial modification was done analogously to the method of Marchant-Brynaert et al.[4] Thus, after treating the membrane with potassium permanganate in sulfuric acid, we were able to realize a concentration of carboxylic groups in a range of 150–200 pmol/cm^2 open surface (see Table I). Analytical characterization was

Table I Concentration of Carboxylic Groups onto Oxidized PET Membranes with Different Pore Diameters, Determination with Fluorescence Dye Thionine Acetate

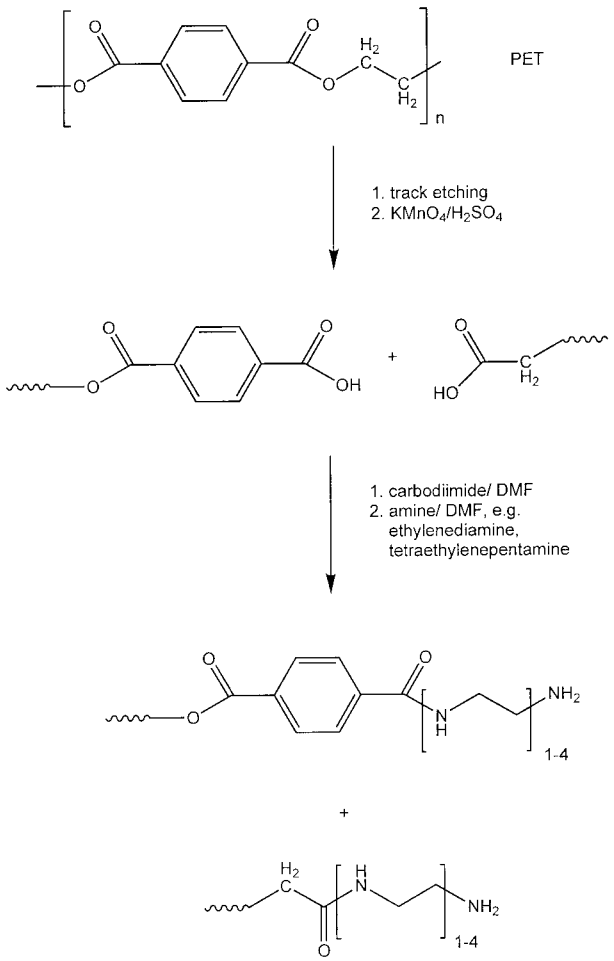
	Surface Relation[a]	c_{COOH} (pmol/cm^2)[b]	c_{COOH} (pmol/cm^2)[c]
PET 30	54	8100	150
PET 50	52	7440	143
PET 80	42	6510	155
PET 450	11	2030	185

[a] Relation of total surface area to membrane area.
[b] Concentration of membrane area.
[c] Concentration of total surface area.

done by fluorescence labeling with thionine acetate. The result of the same amount of carboxylic groups per squared centimeter for all different pore sizes (and therefore different specific surface areas) allowed the conclusion that the whole membrane surface was functionalized.

The peptide synthesis (on the membrane surface) should be done from the carboxylic to the amino end of the peptide, because this way, a great variety of coupling and protecting chemistry could be utilized. Therefore, amino groups at the membrane surface were needed. To assure a surface selective reaction, amino groups were not directly introduced (see ref. 5). Instead, the carboxylic groups were used for a further reaction. An activation with carbodiimide and subsequent reaction with diluted multifunctional amines yielded aminated membrane surfaces (Scheme 1). The reaction took place quantitatively because no carboxylic groups could be detected after amination. Analytical characterization was carried out by means of XPS (Table II) and staining the amino groups with the anionic dye Acid Orange II.[6] The XPS data indicate that the reaction with TEPA and ethylenediamine (EDA) yielded a degree of substitution of the repeating units of about 5% (repeating unit PET: $C_{10}H_8O_4$; PET–EDA: $C_{12}H_{15}O_2N_2$; a degree of substitution of 5% means: 95 ($C_{10}H_8O_4$) and 5 ($C_{12}H_{15}O_2N_2$) $\Rightarrow$ N/C = 10/1010; 0.01).

Staining of aminated membranes of different pore sizes (and therefore different specific surfaces) revealed that the functionalization took place on both the outer surface area and the pore surface area (Table III). Again this result can be deduced from the finding that all the different pore sizes contained the same concentration of amino groups regarding the whole surface area.



Scheme 1 Oxidation of track-etched PET membranes with $KMnO_4$ and sulfuric acid.

The introduced amino groups were used for the coupling of amino acids using BOC protection strategy and activation of the carboxylic group with carbodiimide–hydroxybenzotriazole (Scheme 2).[7] Compared with solid-phase synthesis onto the Merrifield resins, particle track membranes do not swell

Table II XPS Data for the Initial and Aminated Membranes (PET 30)

	C_{1s} (%)	O_{1s} (%)	N_{1s} (%)	N/C
PET[a]	71.5	28.5	<0.1	0
PET–EDA[b]	72.5	25.4	0.75	0.010
PET–TEPA[c]	72.2	25.1	1.53	0.021

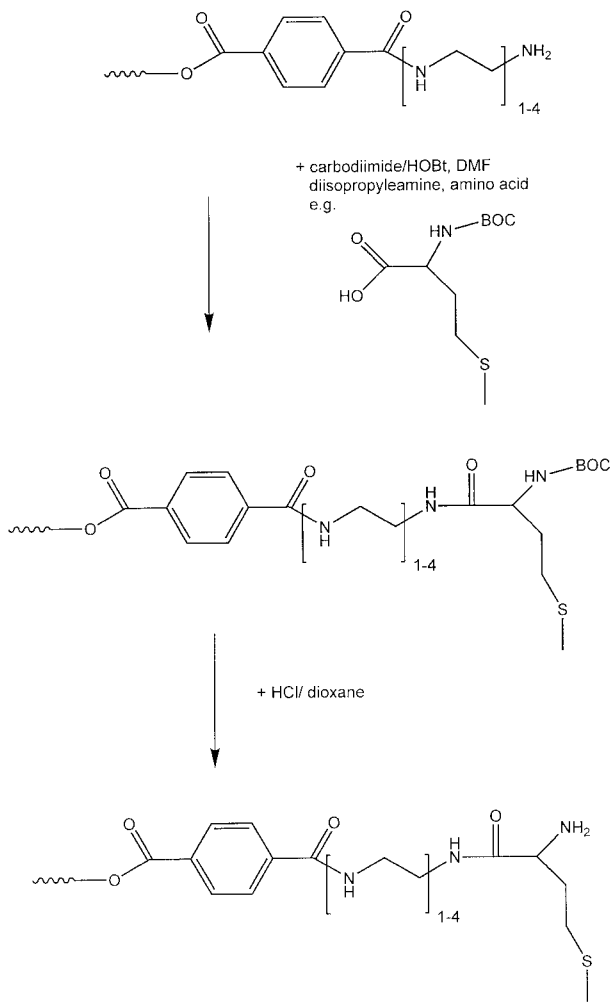
[a] Native membrane.
[b] Aminated membrane (ethylene diamine).
[c] Aminated membrane (tetraethylene pentamine).

Table III Concentration of Amino Group onto PET Membranes with Different Pore Diameters, Staining with Acid Orange II

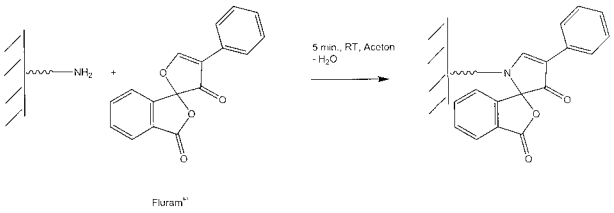
	Surface Relation[a]	$c_{NH_2'}$ (nmol/cm^2)[b]	$c_{NH_2'}$ (pmol/cm^2)[c]
PET 30	54	1.5	27
PET 30–TEPA[d]	54	34	630
PET 450–TEPA[d]	11	8.5	770

[a] Relation of total surface area to membrane area.
[b] Concentration of membrane area.
[c] Concentration of total surface area.
[d] Aminated membrane (tetraethylene pentamine).

remarkably in contact with the solvent. Therefore, a quantitative yield of the coupling of amino acids is difficult to realize. Thus, a longer reaction time, double or triple coupling, and intensive shaking were applied to realize a high yield.



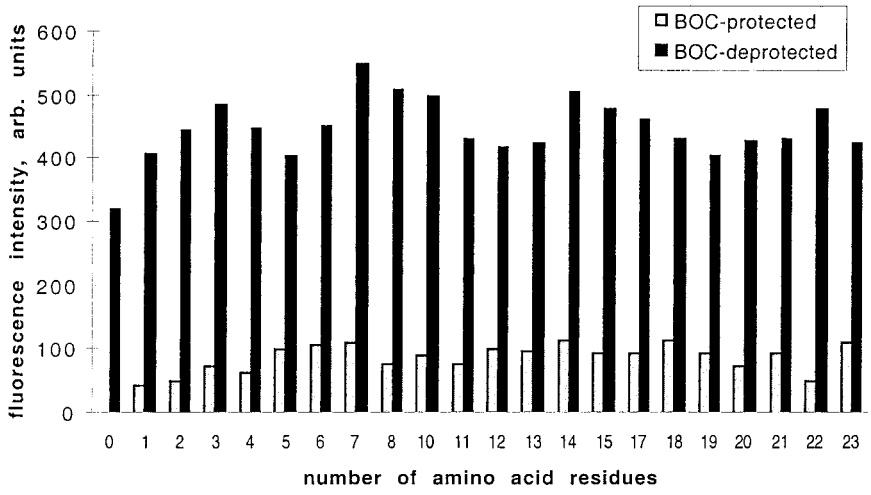
Scheme 2 Reaction of aminated membranes with amino acids (activation of the carboxylic group by carbodiimide-hydroxybenzotriazole).



Scheme 3 Reaction of fluorescence-labeled Fluram® with primary amino groups.

Analytical characterization was the major problem of investigating the reaction. To determine the yield of every step of synthesis, it is possible to use the disappearance and appearance of primary amino groups after every coupling and deprotecting step, respectively. Amino groups were quantified by fluorescence labeling. The label Fluram® covalently couples exclusively to primary amino groups (reaction, see Scheme 3 and ref. 8). Thus, by means of fluorescence intensity at the membrane surface, every step of synthesis could be quantified. Figure 1 shows the changing fluorescence intensities for the synthesis of a polypeptide consisting of 23 amino acid residues. It can clearly be seen that the fluorescence intensity changed with every step of synthesis. In accordance with the standard deviation of the measurements, the error of the method is 10%. Thus, no statement could be made about yields between 90 and 100%. However, all the coupling steps proceeded with a yield of at least >90%. Because the average intensity of the fluorescence remained the same during all the coupling steps, it is highly probable that the synthesis proceeds quantitatively. The fluorescence intensity of the aminated membrane (Fig. 1, entry 0) is remarkably lower than the intensities after all the following coupling steps. This indicates that the secondary amino groups (from TEPA) were used for coupling of amino acids too (secondary amino groups will not be detected by Fluram®).

To investigate the possibility of the coupling of dipeptides onto the membranes with the same yield as the single amino acids, a dipeptide from methionine (Met–Met) was coupled to the membrane surface. As can be seen in Figure 2, the reaction proceeds with the same yield as the coupling of a single methionine residue (Met) because the amount of sulfur corresponds to the expected value (therefore, no statement can be made about the amount of sulfur for three introduced Met



sequence (one letter code): PET-M-L-G-L-M-A-L-M-M-A-M-L-M-A-L-M-M-A-M-A-M-A-K

Figure 1 Fluorescence intensities for the synthesis of a polypeptide onto PET 30. Light columns: fluorescence after coupling of protected amino acids (and dipeptides). Dark columns: fluorescence after deprotecting the terminal amino group.

residues). The data from fluorescence labeling confirms this result.

A second strategy of synthesis was the coupling of presynthesized peptide fragments. A helix-forming peptide fragment described by Krause et al.[9] was synthesized according to the literature (but manually, without the use of a peptide synthesizer). Side-chain protection of amino groups of lysine was done with BOC. Therefore, Fmoc was used for temporary protection of the terminal amino group. These fragments were coupled by the same procedure as the amino acids. As can be seen in Figure 3, the fluorescence intensity for the deprotected samples decreases from coupling step to coupling step. This means that the reaction did not proceed quantitatively. A yield of about 80% is not sufficient at all to synthesize a pure peptide onto the membrane surface. The lower yield is due to the fact that the fully protected fragment is not quite soluble in the solvent (not >3 mmol/L compared to >100 mmol/L for amino acid coupling). Thus, only the coupling of single amino acids is a proper method to synthesize peptides onto the membrane surface.

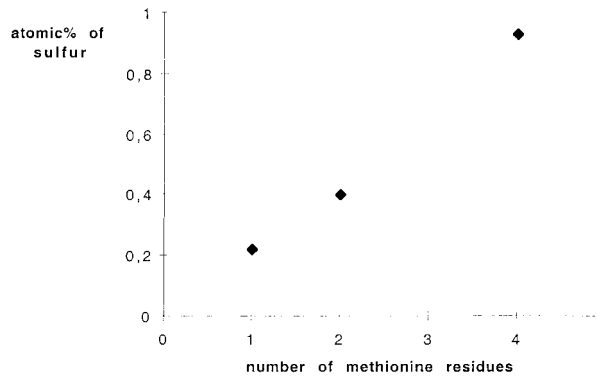
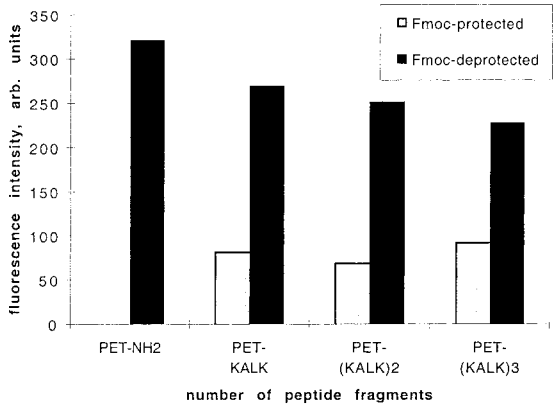


Figure 2 Dependence of sulfur content from the amount of introduced sulfur via methionine coupling.



KALK: peptide fragment; sequence: K-A-L-K-L-K-L-A-L-A-L-L-A-K-L-K-L-A

Figure 3 Fluorescence intensities for the coupling of presynthesized polypeptides (KALK) onto PET 30. Light columns: fluorescence after coupling of protected amino acids (and dipeptides). Dark columns: fluorescence after deprotecting the terminal amino group.

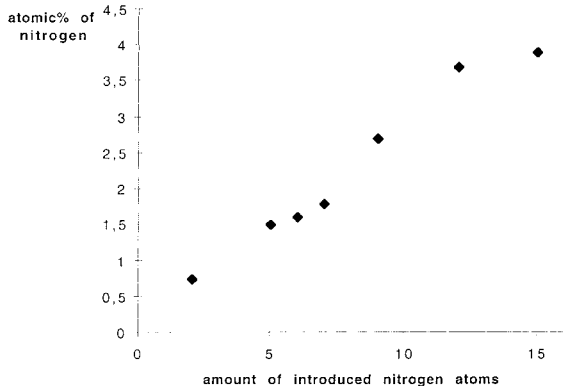


Figure 4 Dependence of nitrogen content from the amount of introduced nitrogen via amino acid coupling.

To prove the data of yields, the fluorescence method had to be verified by an independent analytical method. XPS was utilized to investigate the amount of elements on the very surface of the membranes. Therefore, oligopeptides with different chain lengths and sequences were synthesized to relate the composition of the membrane surfaces with the introduced heteroatoms. The composition of the surface of the initial membranes was in accordance with the data from the literature.[10] Figure 2 shows the amount of sulfur in relation to the coupled methionine residues (containing one atom of sulfur per residue). As can be seen in Figure 2, the content of sulphur agrees with the number of methionine residues. In Figure 4, the same graph is shown for the content of nitrogen. In this case, the initial aminated membrane already contains 2 or 5 nitrogen atoms, respectively. Thus, the measurable effect for the coupling of one amino acid (containing one nitrogen atom) is smaller than for the sulfur content. However, a clear dependence can be seen. This result means that the fluorescence method can be used for the quantification of every coupling step.

CONCLUSION

A method for introducing primary and secondary amino groups onto the whole accessible surface (including the surface of the pore walls) of poly(ethylene terephthalate) particle track membranes is described. The amino groups were used to couple both single amino acids (and dipeptides) and presynthesized peptide fragments. Both methods yielded a modification of the membrane surface with growing peptide chains. Only the coupling of single amino acids led to a quantitative reaction during all the steps of synthesis, whereas the coupling of peptides proceeded with a yield of not more than 80%. Therefore, the latter method does not yield a dense peptide layer at the membrane surface or a sequence-pure peptide. This is mainly caused by the low solubility of the fragments. In contrast, the coupling of single amino acid residues turned out to be a useful method to synthesize peptides onto the surface of particle track membranes.

The described method offers a way to generate biofunctionalized polyester membrane surfaces for various kinds of applications as biomaterials.

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