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Immobilization of a fibronectin fragment at the surface of a polyetherurethane film

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Vinylacetate has been grafted on to the surface of a poly(etherurethane) (pu) film *via* plasma polymerization. After hydrolysis of the acetate groups, the fibronection fragment Gly–Arg–Gly–Asp–Ser (GRGDS) has been covalently bound to the polymer surface by benzoquinone coupling to provide a cell adhesive surface appropriate for cell seeding.

1. Introduction

Arteriosclerosis is one of the most frequent of human diseases. The replacement of the sclerotic part of a blood vessel by a prosthetic device frequently results in thrombus formation and the obstruction of the synthetic vessel. To avoid these complications, caused by complex interactions between the blood and the polymer used in the artificial artery, different research groups have attempted to colonize the polymer surface with endothelial cells, thereby presenting a more natural and hopefully non-thrombogenic surface to the blood. First experiments with the pre-seeding of polymer surfaces were started by Herring *et al.* [1]. Union Carbide tried endothelial seeding on polypropylene microfilaments [2]. Sentissi *et al.* [3] coated poly(tetrafluoroethylene)-vessels with fibronectin and collagen. Two weeks after seeding these surfaces with endothelial cells, the growth of a confluent cell sheet could be observed. The application of this adhesion dependent model for long-term uses has not yet been reported.

Under conditions of flow, Poot *et al.* [4] succeeded in reducing the platelets' adhesion after endothelialization of polypropylene. Similar observations were made by Stanley *et al.* [5] and Sharefkin [6] in animal experiments. In commercially available implants no spontaneous re-endothelialization has been observed [7].

A model of a modified surface for endothelial cell seeding is described in the present article [8, 9] (Fig. 1). In this, an artificial 'extracellular matrix' (II) is covalently bound to the functional groups of the carrier polymer (I). This matrix consists of the fibronectin fragment Gly–Arg–Gly–Asp–Ser (GRGDS) whose good cell adhesion properties are known [10–15]. The experimental realization of this concept – a covalent bonding of GRGDS *via* benzoquinone as coupling agent (III) – is described.

2. Materials and methods

2.1. Plasma polymerization

The grafting polymerization of vinylacetate was carried out in a microwave plasma unit [9, 16]. The flow was adjusted by means of the MKS Multi Gas Controller (Model 147.A V 1.1; MKS) and the pressure was regulated by a unit PR 2000 type 252 A (MKS-Instrumente, München, FRG). In Table I the parameters and constants of the procedure are summarized. By the induced plasma polymerization (Table II, Sample V) the plasma was stopped after 30 s and the monomer continued to flow. In Sample VI (Table II) the polymer film was pre-treated with argon plasma for 10 s under the same conditions (Table I). The polymer substrate used in these experiments was the poly(etherurethane) Mitrathane®, in film form.

2.2. Peptide synthesis

GRGDS was synthesized using the solid-phase procedure [17] after Geiger–König [18] and the TBTU method [19]. The peptide synthesizer ACT-2 of

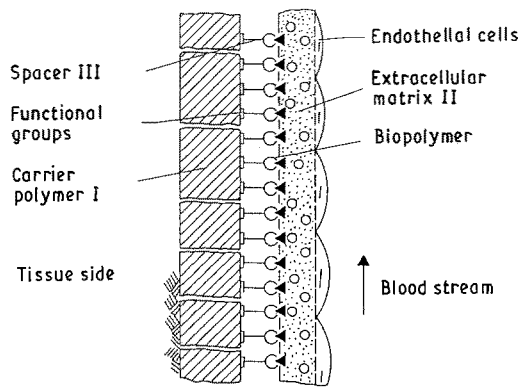

Figure 1 Composition of a modified polymer surface for endothelial cell seeding [8, 9].

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TABLE I Parameters and constants of the plasma polymerization procedure

Feeding interval	45 mm
Coating time	120 s
Gas	Vinyl acetate
Initial pressure	2 Pa
Monomer partial pressure	50 Pa
Power	240 W, 420 W
Flow rate	50 ml min^{-1}, 75 ml min^{-1}

TABLE II Glycine occupation per cm^2, dependence of the Mitrathane® surface on the parameters of the plasma polymerization procedure

Sample	Flow rate (ml min^{-1})	Power (W)	Glycine (mmol cm^{-2})
I	50	240	62.4
II	50	420	157.6
III	75	240	81.2
IV	75	420	147.1
V[a]	50	240	76.5
VI[a]	50	240	76.5

[a] V: induced plasma polymerization.
VI: argon plasma pre-treated.

Advanced Chem. Tech. (Louisville, USA) was used. Fmoc-derivates of the amino acids serine (S), asparagine acid (D), arginine (R) and glycine (G) were obtained from Bachem Biochemica GmbH, Heidelberg, FRG. After liquid–liquid chromatography (RP-column, HD-Sil-18-60-100, BESTA, Heidelberg, FRG; gradient H_2O/0.1% TFA, 0–100%) the product GRGDS showed 90–95% purity. This was determined by high performance liquid chromatography (HPLC) chromatography (LC-41 of Bruker-Frantzen, Bremen, FRG; RP-C_{18} silica-gel column, 4 mm × 8 mm × 250 mm, Nucleosil $10C_{18}5C_{10}$, Machery Nagel, Düren, FRG).

2.3. Immobilization reaction

To immobilize the glycine or the oligopeptide at the polymer surface, the ester groups of the vinyl acetate layer were hydrolysed under reflux (1 h) by 0.05% aqueous CH_3ONa solution. The material obtained was then washed with acetic acid (1%) and distilled water.

50 mmol benzoquinone was activated by addition to a solution of 20% ethanol and 80% Na_2HPO_4/NaH_2PO_4 buffer (pH 7; 0.1 M). After stirring for 15 min a piece (8.5 cm^2) of the Mitrathane® film, modified as above, was added and incubated for 2 h at room temperature. It was subsequently washed with ethanol and water until the solution was clear.

The oligopeptide was immobilized upon dissolving 200 mg GRGDS in 20 ml of a 0.1 M buffer. Three different buffer solutions, a citrate solution (pH 3), an acetate solution (pH 4–7) or a $KHCO_3$ solution (pH 8–9), were applied. The activated film was then added and left in the solution at 4 °C for 36 h. It was washed subsequently three times with 200 ml acetate buffer (0.1 M; pH 4), $KHCO_3$ buffer (0.1 M; pH 8.5) and distilled water. Each washing circle lasted 15 min. The treated film was dried in a vacuum oven for 24 h.

2.4. Analytical procedures

Fourier-transformation-infrared-spectra (FTIR) were measured in Attenuated Total Reflection (ATR) with a Nicolet 60 SXR spectrophotometer (Offenbach, FRG). A germanium crystal as a reflection unit provides a depth of sample penetration of approximately 0.3 μm.

Electron spectroscopy for chemical analysis (ESCA) was performed with an SSI 206/X/sample spectrophotometer from Surface Science Instruments (SSI, Mountain View, USA).

The scanning electron microscopy (SEM) investigations were carried out with a SEM 515 electron microscope (Philips, Eindhoven, Netherlands).

For amino acid analysis a Biotronic LC 6000 E (Maintal, FRG) was used.

3. Results and discussion

The Mitrathane® surface must be functionalized for the covalent binding of structures similar to the basement membrane. This was achieved by vinyl acetate grafting upon plasma polymerization. After hydrolysis of the acetate groups at the polymer surface hydroxy groups are obtained. Then the covalent binding of proteins at the polyetherurethane surface may be achieved.

3.1. Plasma polymerization

The grafted poly(vinylacetate) layer was characterized by means of FTIR–ATR and X-ray photoelectron spectroscopy (XPS) spectra. For further characterization glycine was bound at the Mitrathane® surface by a method later described. The glycine content per modified surface area was determined by means of amino acid analysis after hydrolysis. In Table II the dependence of the glycine content of the surface on the parameters of the plasma polymerization procedure is shown. Consequently the glycine content is highly influenced by the power of the microwave generator. A power increase from 240 (I, III) to 420 W (II, IV) results in a doubling of the glycine content. On the other hand, this parameter is only negligibly influenced by the flow rate of vinyl acetate which was increased from 50 (I, II) to 75 ml min^{-1} (III, IV). The glycine content of samples I and III and of II and IV do not differ significantly.

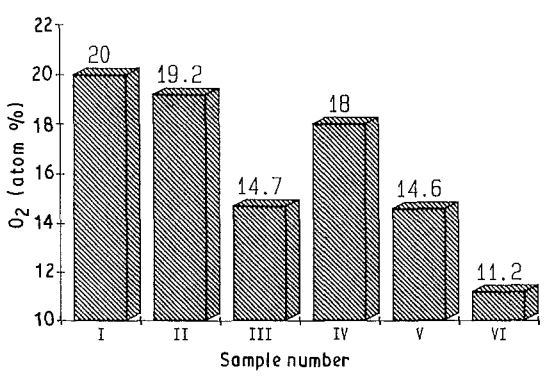


Figure 2 Oxygen content of the plasma modified Mitrathane® surface dependent on the parameters of the plasma polymerization procedure (cf. Table II).

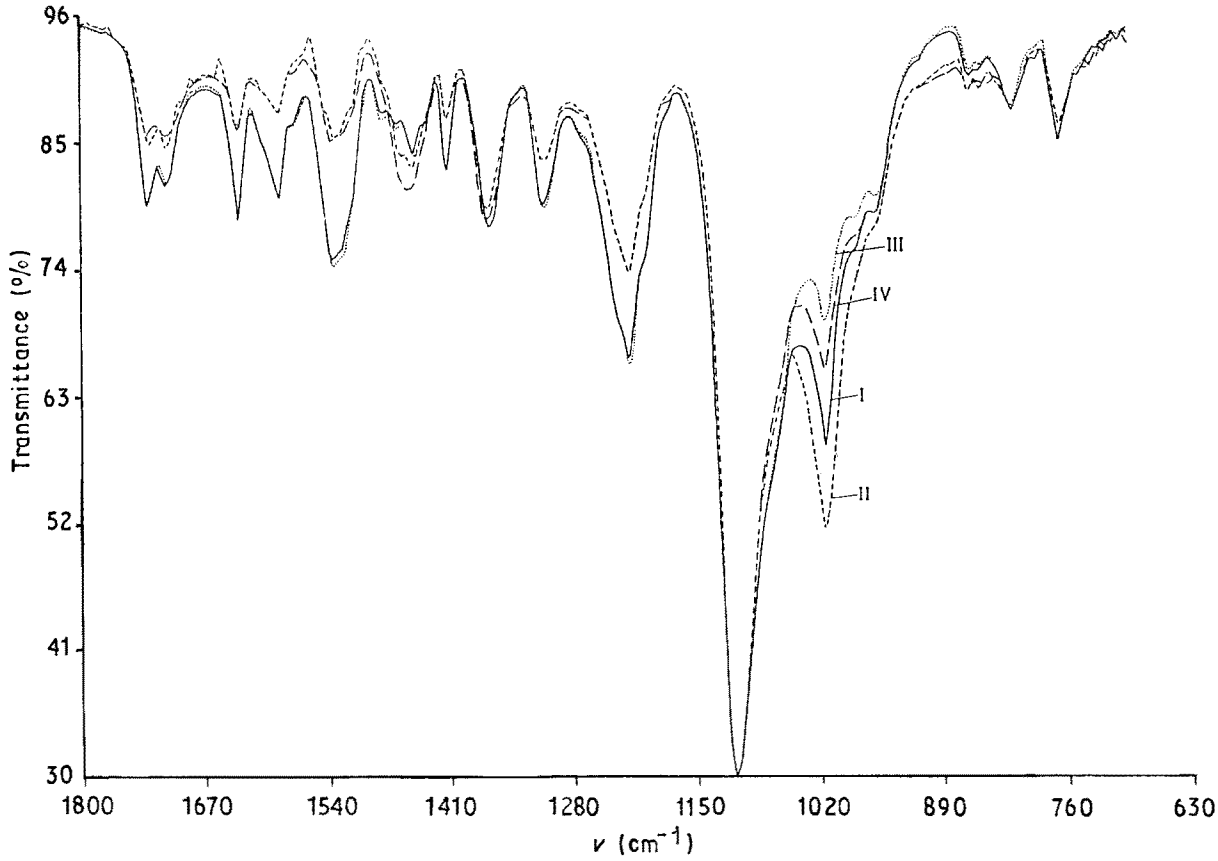


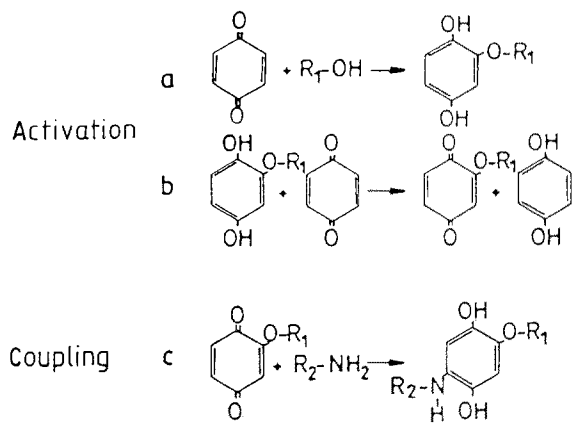
Figure 3 Superimposed FTIR–ATR spectra of Samples I–IV in the range of 1800 to 799 cm^{-1}.

Induced plasma polymerization (V) and an argon pre-treatment (VI) has no effect on the yield of covalently bonded glycine. These data correlate to the oxygen content obtained by means of XPS measurements of the plasma modified polymer surface (Fig. 2).

Samples II and IV, coated at 420 W, exert a significantly higher oxygen content (19.2 and 18.0%) than samples III, V and VI, coated at lower power. Only Sample I seems to be an exception. Probably surface contaminations are responsible for the high portion of oxygen (20%). Apart from that, the good correspondence between the glycine and oxygen content of the samples II–VI support the results. The different coating yield is also reflected by the FTIR–ATR spectra (Fig. 3). Compared to Samples I and III coated at 240 W, in the FTIR–ATR spectra of Samples II and IV, coated at 420 W, a decrease of the carbonyl absorption at 1730 cm^{-1} is observed. This can be explained by the fragmentation of the acetate groups (ester pyrolysis) in plasma conditions. Though, an inactivation of the grafted surface running parallel to this decrease was not noticed. Samples with a particularly low carbonyl content showed the highest glycine content after the coupling process (Fig. 2).

3.2. Immobilization of glycine and fibronectin fragment

To immobilize peptides at the hydrolysed polyetherurethane surface, benzoquinone was used as a coupling reagent. This coupling method allows the binding of peptides and proteins to polymer carriers under mild conditions. The reaction can be carried out in aqueous medium at room temperature over an extremely wide pH range. In Equation 1 the mechanism of the reaction according to Brandt *et al.* [20] is shown:



Glycine and the pentapeptid GRGDS were immobilized. The latter has especially good cell adhesion properties [14, 15] because it is the adhesive sequence of the fibronectin molecule [11]. The pH dependence on the coupling reaction is presented in Figs 4 and 5.

The highest yields of coupled glycine were reached at pH 8 and 3, whereas coupled GRGDS showed maxima at pH 6 and 3. This result corresponds to the reaction course taking place using basic catalysis as well as acid catalysis, because the 1,4–addition to benzoquinone is a vinylogue carbonyl reaction. The shift of the high coupling rate from pH 8 to 6 of the GRGDS immobilization compared to the glycine immobilization could be explained by the strong basic

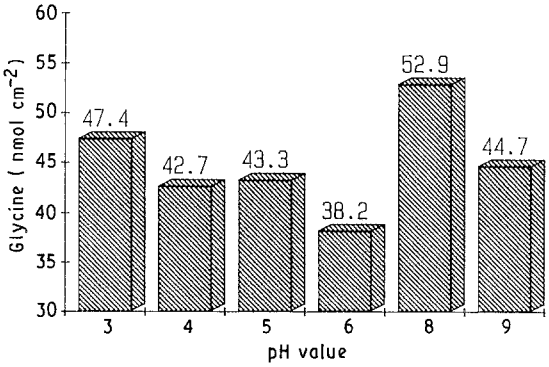


Figure 4 pH dependence on the immobilization reaction of glycine at quinone activated Mitrathane® surfaces.

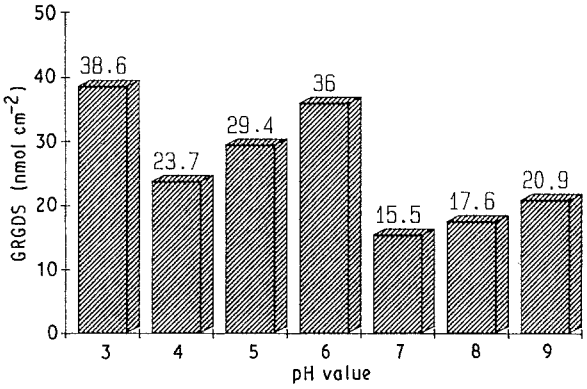


Figure 5 pH dependence on the immobilization reaction of GRGDS at quinone activated Mitrathane® surfaces.

guanido function of arginine. Generally a coupling is possible at each pH value. Figs 6 and 7 illustrate the concentration dependence on the immobilization reaction.

In both cases the concentration of the reaction solution shows a substantial influence on the deposition of glycine and *penta*-peptide at the polymer surface. Beginning at a glycine concentration of 5 mg ml^{-1} or a GRGDS concentration of 20 mg ml^{-1} the film is coated in a significant manner. A further increase of the GRGDS concentration in the solution was not investigated.

In conclusion, the results of these investigations demonstrate that the immobilization of adhesive proteins is possible by functionalizing a polyether-urethane surface during plasma polymerization. The application of benzoquinone as a spacer allows a bonding of these adhesive molecules over a wide pH range. Finally the peptide GRGDS, which has excellent cell adhesion properties, is fixed at the polymer

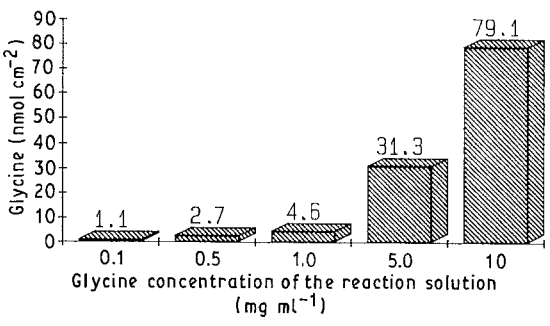


Figure 6 Concentration dependence on the glycine immobilization.

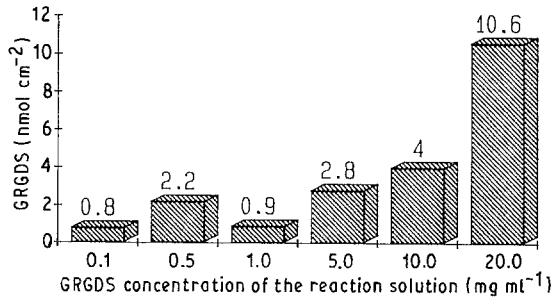


Figure 7 Concentration dependence on the GRGDS immobilization.

surface [12–14]. Thus a biologically active polymer surface is obtained, which is anticipated to facilitate cell attachment.

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