Aus dem GKSS Forschungszentrum Geesthacht GmbH, Institute
für Chemie, Teltow

THE WETTABILITY OF BIOMATERIALS DETERMINES THE PROTEIN ADSORPTION AND THE CELLULAR RESPONSES

Dissertation

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To my husband Ivan, my daughter Borislava, and
my parents Liliana and Dimitar Tzonevi

Preface

This work was carried out at the GKSS Forschungszentrum Geesthacht GmbH, Institut für Chemie, Teltow, during the period from November 1999 to April 2003 under the guidance of Dr. Albrecht and Dr. Groth.

This thesis consists of six parts. Chapter 1 is Introduction and Chapter 2 is Literature Survey. Chapter 3 is Materials and Methods. Results and Discussion are shown in Chapter 4. Summary is given in Chapter 5 and Chapter 6 contains Perspectives.

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Abbreviations

AJ	adherent junctions
τ	surface free energy
γ	surface tension
θ	contact angle at the solid-liquid interface
β-TG	β-thromboglobuline
ABP	actin-binding protein
ADP	adenosine diphosphate
AFM	atomic force microscopy
CA	contact angle
cAMP	cyclic adenosine monophosphate
Cb	solution concentration of the protein (μg/ml)
CE	Cuprophan
C_L	limiting value of protein adsorption (adsorption “plateau”)
CLSM	confocal laser scanning microscope
Cs	adsorption amount of protein (per surface area)
DTS	dense tubular system
EC	endothelial cells
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ESEM	environmental scanning electron microscopy
FITC	fluorescein isothiocyanate
FN	Fibronectin
FNG	Fibrinogen
GP	gap junctions
HMWK	High Molecular Weight Kininogen
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule-1
K	binding constant
mAb	monoclonal antibody
MMP	matrix metalloproteinase
MTS	microtubular system
NO	nitric oxide

OCS	open canicular system
ODS	Dimethyloctadecylchlorosilane
pAb	polyclonal antibody
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffer saline
PC-PE	polycarbonate-polyether
PEI	polyether imide
PEO	polyethylene oxide
PET	Polyethyleneterephthalate
PEX	MMP-2 termed hemopexin fragment
PGI_2	Prostacyclin
PSU	Polysulfone
PF4	platelet factor 4
PTFE	poly(tetrafluoroethylene)
PVDF	polyvinylidene fluoride
RGD	arginine-glycine-aspartic acid
SDS	sodium dodecyl sulphate
TF	tissue factor
TJ	tight junctions
TNF-α	tumor necrosis factor alfa
t-PA	tissue plasminogen activator
TxA_2	Thromboxane
u-PA	urokinase type activator
VWF	von Willebrand factor
W	work of adhesion

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1. Introduction

1.1. General introduction

Polymer materials have become widely used as components of medical devices and implants, drug delivery systems, diagnostic assays, bioreactors and bioseparation processes. Most of the devices cannot avoid the blood contact in their use. When the polymer materials come in contact with blood they can cause different undesired host responses like thrombosis, inflammatory reactions, infections and others. Thus the materials must be hemocompatible in order to minimize these undesired body responses. One of the most important problems associated with the blood-contacting biomaterials is surface-induced thrombosis. The sequence of the thrombus formation has been well established. The first event, which occurs, after exposure of biomaterials to blood, is the adsorption of blood proteins. The type, the amount and the conformational state of the adsorbed proteins determine whether platelets will adhere and become activated or not. The adsorption of fibrinogen (FNG), which is present in plasma, has been shown to be closely related to surface-induced thrombosis. The protein adsorption is an interfacial phenomenon and depends strongly on the physico-chemical properties of the polymers, such as surface wettability, surface energy, surface charge density, surface roughness and others. Wettability, however, is believed to play one of the most important role for the amount of adsorbed proteins and their conformational changes during adsorption. Since the thrombus formation begins with protein adsorption, the main efforts in improving the material hemocompatibility have been directed towards controlling (mainly preventing) protein adsorption. Therefore, a modification of the material surfaces with protein-repulsive molecules has become a widely used approach for improving the hemocompatibility of the materials. The commonly used protein-repulsive molecules are proteins such as albumin, polysaccharides such as heparin and dextran, synthetic polymers such as polyethylene oxide (PEO) and phospholipid molecules such as phosphatidyl choline. Since the endothelium is the nature's most efficient anti-thrombogenic surface, growing of endothelial cells (EC) on biomaterials is another approach, which is believed to be the most ideal solution for making truly blood-compatible materials. Devices benefiting from the use of such kind of surface modifications are for instance synthetic vascular grafts**.** However the studies have shown that the EC do not adhere strongly to the currently available vascular

graft materials. Precoating of the grafts with extracellular matrix (ECM) proteins such as fibronectin (FN) and FNG has been shown to enhance EC adhesion, spreading and proliferation. The adhesive proteins bound to a solid surface provide not only a structural support for cell adhesion and spreading but they are also the critical element of the message directing from the substrate to the cell. Therefore, the correlation among surface properties, protein adsorption, and cell responses should be studied in order to increase the knowledge how the biomaterial influences the cell function and to modulate the biomaterial's surface properties in attempt to perform higher compatible materials. There is abundant evidence that the water wettable substrates facilitate the cell adhesion in contrast to poor wettable ones. That fact in general was explained with the different conformational state of adsorbed proteins. On the wettable surfaces proteins are adsorbed loosely, near to their native state in the solution and they usually keep their biological function. In contrast, the poor wettable surfaces cause unfolding of the adsorbed proteins due to the dehydratation phenomenon, which lead to the conformational changes in the protein molecule and could alter or/and change their biological function. The cells tend to organize the adsorbed and deposited proteins in fibrilar structures resembling ECM in order to spread and migrate onto the substrate. Since it was shown that FN fibrillogenesis by human fibroblasts was dependent on surface wettability, there are no data available for FN and FNG fibrillogenesis by EC as a function of surface wettability.

Many cells adhere and spread better on a mixture of several coated proteins than on single protein coating. For instance EC were found to adhere to FNG coated substrata, but for their spreading and growth the presence of FN in culture medium was required. This fact has been correlated later with the observations on the cooperative action of different adhesive proteins to form matrix-like structures, which were shown to be a prerequisite for proper cell functioning. For instance, in human fibroblasts the active FN matrix deposition was required for the retention of another adhesive proteins such as thrombospondin, collagen and FNG in fibrilar structures within the ECM.

While the cell-substrate interactions determining cell adhesion, spreading and migration, are very important for the early phase of the implant colonization with EC, the importance of cell-cell interactions may dominate at the later stages of 2D tissue formation. The adherent junctions (AJ) are one type of cell-cell contacts, which are very important in providing

integrity of the endothelium. The balance between the strength of both cell-substrate and cell-cell adhesions will lead to a well-established EC monolayer. The functionality of the seeded EC is another important feature that has to be always considered when the EC seeding is used for improving the hemocompatibility of the implants. Since prostacyclin (PGI_2) was shown to suppress early phases of thrombosis by preventing platelet adhesion, activation and aggregation, the ability of seeded EC on polymer materials to secrete prostacyclin could be used as a measure for anti-thrombotic properties of the newly established EC monolayer.

1.2. Aim of the work

The aim of the work was to study the influence of materials surface wettability on thrombogenicity of blood-contacting biomaterials. Secondly, the endothelization of the biomaterial surfaces was investigated as a promising approach for improving the blood compatibility. To reach these goals, three tasks were carried out.

The main task was to characterize the plasma protein adsorption as a function of surface wettability. For this purpose a new polymer membrane polyether imide (PEI) was introduced together with another three membranes with different wettability used in blood-contacting devices. The study was focused on the adsorption of FNG as a main protein involved in the platelet adhesion to artificial surfaces. The amount and conformational changes of adsorbed FNG was correlated with the materials surface wettability/energetics and with the rate of platelet adhesion and activation (Part I).

The second task was to develop criteria for a successful colonization of the materials with endothelial cells (EC) with respect to their wettability and protein coating (Part II). Human Umbilical Vein Endothelial Cells (HUVEC) were seeded on model surfaces with different wettability. FN and FNG were used for coating of the surfaces. Three main criteria were developed:

1. Expression of cell phenotype with regard to protein coating.
2. The ability of HUVEC to form cell adhesions (cell-substrate and cell-cell adhesions) with respect to surface wettability.
3. The matrix remodelling activity of HUVEC in dependence to surface wettability.

The third task was to study the adhesion, growth and functionality (production of prostacyclin) of seeded HUVEC on polymer membranes as a function of the polymer surface charge and the type of protein coating (Part III).

2. Literature survey

2.1. Hemocompatibility of polymers

During the past several decades, the use of polymer membranes as components of medical devices and implants increased dramatically, due to the progress in techniques such as extracorporeal procedures including cardio-pulmonary bypass, hemodialysis, bioartificial organs, as well as vascular and reconstructive surgery [Ratner 1996, Olsson 2000]. This evolution has highlighted the problems of biocompatibility of the materials, defined as "the ability of a material to perform with an appropriate host response in a specific application" [Williams 1999]. Most of the devices and implants cannot avoid the blood contact in their use [Ikada 1994]. Blood-contacting biomaterials range from hemodialysis equipment and bioartificial organs to vascular grafts and total artificial heart [Deppisch 1998, Park 2000, Clark and Gao 2002]. For blood-contact applications, biocompatibility is determined largely by specific interactions with blood and its components [Angelova and Hunkeler 1999]. When polymers come in contact with blood, they can activate diverse body defense mechanisms, which might trigger a variety of undesired responses, including thrombosis [Sefton 2000], inflammation [Marchant 1984], infection [Lamba 2000] and fibrosis [Hunter 1999]. Therefore the materials used in medical devices must possess functional characteristics to minimize these body responses in order to be biocompatible. Thrombosis on foreign surfaces in contact with blood remains a major unsolved problem in the design of extracorporeal blood-handling systems and vascular implants [Brash 1987]. Therefore much of the attention of researchers has focused on surface induced thrombosis since this is the earliest complication, and is far the most troublesome effect [Brash 2000, Sefton 2000]. The coagulation system and platelets are the main factors for thrombus formation on biomaterials. The coagulation system is composed of more then ten plasma proteins and proceeds via cascade reactions by either the intrinsic or extrinsic pathway. In physiological conditions, coagulation system prevents blood loss from damaged vessels proceeding with the formation

of thrombin that converts soluble FNG to a solid fibrin clot at the end stage [Olsson 2000]. The activation of the coagulation system on non-physiological surfaces is initiated by the intrinsic pathway [Matsuda 1989, Olsson 2000]. The initiation reaction is called contact phase activation and involves three coagulation factors (Hageman Factor, FXII; High-Molecular Weight Kininogen, HMWK; and Prekallikrein, FXI [Matsuda 1989]. The formation of this three-molecular complex on the surface is the essential requirement for the activation. Upon the activation of the three-molecular complex, coagulation factors change their conformation or are converted into active enzymes and result in generation of thrombin, which mediates conversion of FNG to fibrin (Fig.1). In parallel, platelets adhere to the adsorbed proteins, become activated, change their shape and degranulate. They release a variety of bioactive substances, which generate activation of other platelets, increase the thrombin production and lead to platelet aggregation [Grunkemeier 2000]. Large platelet aggregates under the blood flow can be detached from the surface to form thromboemboli [Sefton 2000]. Both processes are linked, most notably through thrombin a potent platelet activating agent. Thrombin is produced from prothrombin, via the prothrombinase complex, which is assembled on the surface of activated platelets [Sefton 2000] (see Fig.1).

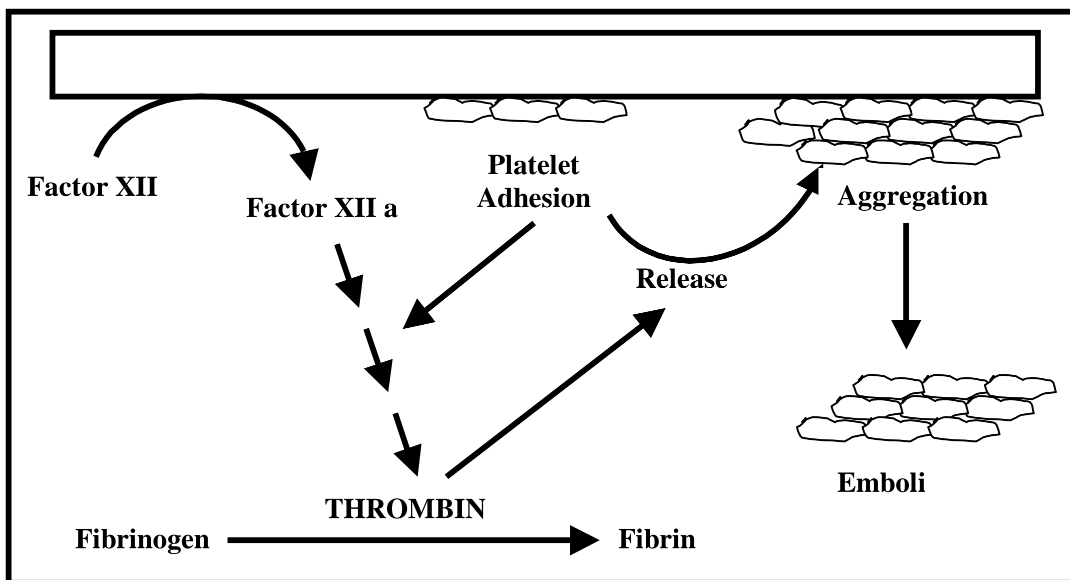


Fig.1 Simplified illustration of the major elements of biomaterial associated thrombosis. From [Sefton 2000].

2.2. Protein adsorption

2.2.1. General aspects

The first event, which is established after exposure of biomaterials to blood, is the adsorption of plasma proteins. The adsorption occurs much more rapidly than the transport of cells to foreign surfaces so that the cells interact with the adsorbed protein-material interface rather than directly with the foreign material [Bohnert 1990]. The types, the amount, and the conformational state of the adsorbed proteins determine whether platelets will adhere and activate or not [Brash 2000, Park 2000]. The generally accepted understanding is that the activation of platelets is greatly promoted on surfaces with adsorbed plasma proteins such as FNG, FN and von Willebrand factor (vWF) [Matsuda 1989, Park 2000, Brash 2000]. The adhesive proteins mediate platelet adhesion via a group of receptors in the platelet membrane (a group of receptors belonging to the big family of integrins) [Hynes 1990]. These membrane receptors recognize and bind to the adhesive site of the proteins that consists of a common amino acid sequence: arginine-glycine-aspartic acid (RGD sequence). The physico-chemical surface properties of the polymer strongly influence the protein adsorption [Ratner 1996, Williams 1999]. The distribution of functional groups on the biomaterial's surface, which governs the surface wettability and the surface charge and hence the macromolecular microstructure, is one of the key factors determining the amount and the affinity of adsorbed proteins and thus the subsequent cellular interactions [Courtney 1994]. The affinity of proteins for artificial surfaces is mainly determined by hydrophobic and electrostatic interactions [Andrade and Hlady 1986]. The protein affinity may be enhanced by the possibility of structural changes within the protein upon adsorption, which can alter their biological activity. Thus the initial and "fate-determining step" for thrombus to occur is the composition and conformational state of the adsorbed protein layer.

2.2.2. Fibrinogen adsorption - role in blood - polymer interactions

FNG is a circulating 340 kDa glycoprotein, primarily synthesized by hepatocytes and circulates as a component of blood at a concentration of approximately 9 μM with a half-life of around 100h [Herrick 1999]. It is composed of a two symmetric half molecules each consisting of one set of three different polypeptide chains termed Aα, Bβ and γ (Fig. 2). The

FNG molecule has three distinct domains: two terminal D domains (67 kDa), each linked to a single E domain (33 kDa) by a triple-stranded array of the polypeptide chains, believed to exist in the form of α helical coiled coils. The three constitutive chains and the two halves of the FNG molecule are held together by series of 29 disulphide bonds with all 58 cysteine residues of FNG participating in these interactions. FNG plays a central role in thrombosis by participating in blood coagulation and facilitating platelet adhesion and aggregation on foreign surfaces [Horbett 1993]. Surface–bound FNG has been suggested to be the major mediator of platelet adhesion to artificial surfaces since FNG is present in plasma and is adsorbed on biomaterials in much higher amounts than other plasma adhesive proteins [Mosher 1981, Brash 1987, Chinn 1991, Horbett 1998]. In addition, the dimeric structure of FNG enables platelet-platelet bridging leading to macroscopic platelet aggregation [Horbett 1993]. Thus platelet adhesion and activation to biomaterials might be affected particularly by adsorbed FNG via its direct interaction with the platelet receptor GPIIb-IIIa [Kumar 1995, Beguin and Kumar 1997, Keularts 1998, Beguin 1999].

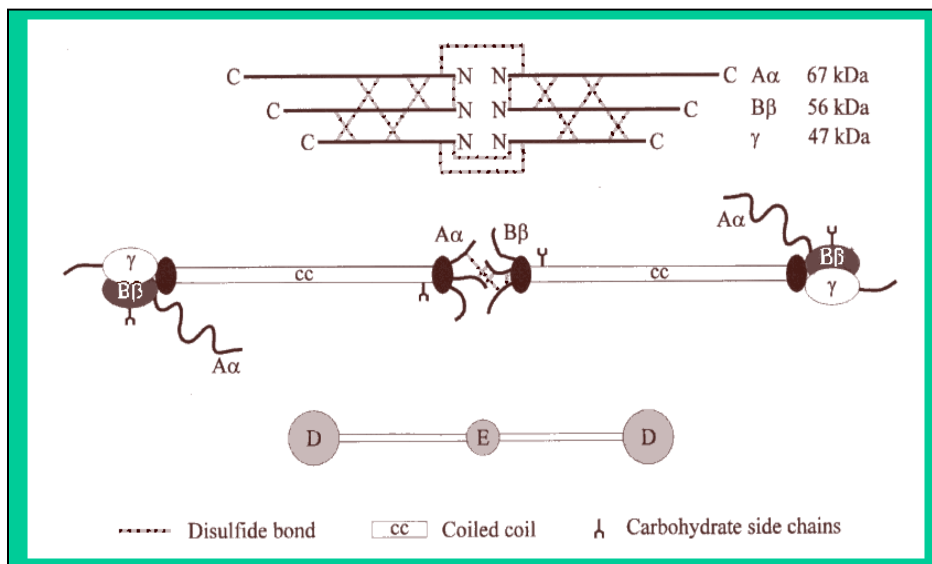


Fig. 2 Schematic representation the FNG structure**.** From [Ruggeri 1993].

There are three distinct sites in the FNG molecule that have been implicated to play a role in the binding to platelets [Grunkemeier 1996]. Two of them, the dodecapeptide (γ400-411) and the RGD sequence (Aα 572-575), which are located in the D domain of the FNG molecule, are believed to be critical for the platelet interaction with FNG [Farrell 1992].

GPIIb-IIIa ($\alpha_{IIB}\beta_3$) appears to be a prototype of an integrin receptor whose adhesive specificity and affinity is posttranslationally regulated by conformational changes by intracellular stimulus-response activation as well as extracellular ligand occupation [Kieffer 1993]. This receptor has activation-dependent and activation-independent functions. In resting circulating platelets, GPIIb-IIIa is surface exposed but does not bind soluble RGD containing adhesive proteins. However, GPIIb-IIIa in unstimulated platelets binds to adsorbed FNG, allowing platelet adhesion to FNG coated surfaces [Kieffer 1993]. This activity of GPIIb-IIIa in unstimulated platelets differs from that of GPIIb-IIIa in stimulated platelets in that it is specific for FNG with no binding to other RGD containing adhesive proteins [Kieffer and Phillips 1990, Savage and Ruggeri 1991]. The domain recognized by resting GPIIb-IIIa on surface-bound FNG corresponds to the dodecapeptide sequence of the FNG γ - chain (γ 401-411) [Kieffer and Phillips 1990]. As this domain is not recognized by resting GPIIb-IIIa on soluble FNG, it is tempting to speculate that adsorption of FNG, induces conformational changes of the molecule leading to an exposure of the dodecapeptide site, making it more easily accessible for resting GPIIb-IIIa interaction [Zammarron 1991]. On the other hand, the conformation of adsorbed FNG was found to be strictly dependent on the binding strength of the adsorption [Kiaei 1995]. That fact in turn could modulate the platelet adhesion to adsorbed FNG in dependence of the materials surface properties [Kiaei 1995, Groth 1994]. Subsequently, binding of adsorbed FNG (through the γ-dodecapeptide) to resting platelets induces conformational changes in GPIIb-IIIa receptor [Parise 1987]. This process of binding of the unstimulated platelets to adsorbed FNG via GPIIb-IIIa receptor was referred as a “substrate activation” (Fig.3) of platelets [Horbett 1994]. Furthermore, these ligand-induced changes in GPIIb-IIIa by outside-in signaling lead to the exposure of secondary high affinity binding sites for soluble FNG [Du 1991]. These are two RGD sequences in FNG α chain, one near to N-terminus (residues 95-97) and a second near the C-terminus (residues 572-574) capable to bind GPIIb-IIIa on activated platelets [Doolittle 1979]. Immuno-inhibition experiments have shown that FNG primarily uses the C-terminal RGD sequence to bind GPIIb-IIIa [Cheresh 1989]. Finally, after platelet adhesion to adsorbed FNG GPIIb-IIIa becomes activated and enabled to bind soluble FNG and thus plays also a role in platelet aggregation (Fig.3) [Kieffer 1993].

Thus plasma FNG appears to be an important factor for the thrombogenicity of the biomaterials. The interaction of the resting platelets with the adsorbed FNG most probably is the mechanism not only involved in platelet adhesion to foreign materials but also involved in platelet activation and aggregation [Kieffer 1993]. In addition the conformation-orientational state of adsorbed FNG becomes the major factor governing the platelet adhesion as well as the platelet aggregation on biomaterials [Lindon 1986, Horbett 1993, Kiaei 1995].

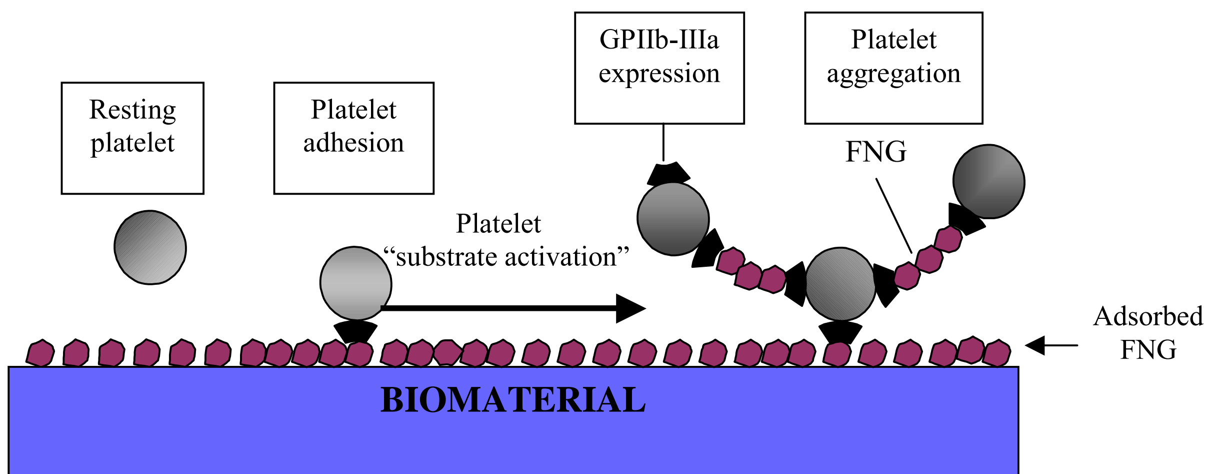


Fig. 3 Adhesion, activation and aggregation of platelets on biomaterials. Resting platelets become "substrate activated" upon binding to adsorbed FNG and express activated receptor (GPIIb-IIIa). Then activated platelets are capable to bind soluble FNG and to aggregate.

2.2.2.1. Adsorption isotherms of FNG–amount and affinity

In order to completely characterize and predict protein adsorption, a quantitative description of adsorption is required. This description is typically obtained by measuring the adsorption isotherms [Hlady 1999]. The adsorption isotherm relates the measured adsorption amount of a protein (per unit area) C_s, to the solution concentration of protein C_b (Fig.4). The slope of the linear region of an isotherm curve is proportional to the binding affinity [Wankat 1990]. The most popular adsorption model is the Langmuir isotherm (see Fig.4), probably due to its simplicity and its good correlation to experimental data. For many surfaces, FNG adsorption was shown to follow the Langmuir type [Hanson 1987, Joung 1988, Sigal 1998]. According to this model C_s increases sharply at low solution concentration of protein and levels off at higher protein concentrations approaching a limiting value C_l. It is

$$C_s = C_l \frac{KC_b}{1+KC_b} \qquad (1)$$

where C_s and C_b are the amounts of adsorbed protein and the bulk concentration respectively. K is the binding constant and C_l is a limiting value of adsorbed protein.

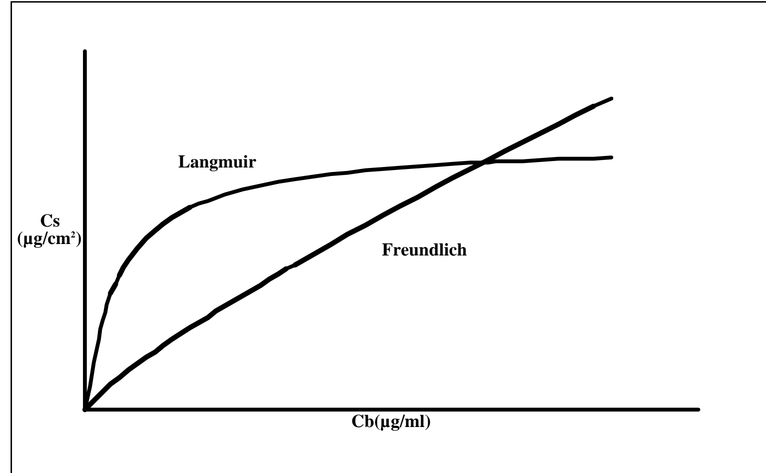


Fig. 4. Langmuir and Freundlich adsorption isotherms. The amount of adsorbed protein Cs (per surface area) is plotted against the bulk concentration C_b.

The existence of a C_l value (so called adsorption "plateau") has been interpreted as a sign that the adsorbing surface is "saturated" with protein molecules. Any further increase of the bulk concentration typically does not affect C_s. Usually the plateau value of adsorbed protein falls within the range expected for a close-packed monolayer of protein depending on the diameter and orientation assumed for the protein [Horbett 1993]. For FNG molecule with a dimension 47/5nm [Sigal 1998], the theoretical surface density of a complete monolayer of protein assuming that the long axis of the protein is perpendicular (end) or parallel (side) to the surface, was calculated to be 2.26 $\mu g/cm^2$ (end) and 0.24 $\mu g/cm^2$ (side). Also some workers have reported values for FNG "adsorption" to surfaces exposed to blood that are much higher than a monolayer amount. These reports are suggesting that a second protein layer is built, or/and the excess of FNG is not adsorbed to a surface site but rather it is bound to the surface in the form of macroscopic fibrin clots [Horbett 1993].

Hence, a well–defined plateau is not always observed in protein adsorption. Instead, adsorption may rise much more slowly at higher bulk-phase concentrations than at low concentrations (Fig. 4). In that case the Freundlich isotherm equation might be applied:

$$C_s = KC_b^{1/m} \qquad (2)$$

where m is the so-called heterogeneity parameter.

Examples for the application of the Freundlich equation were reported for FNG adsorption on polyethylene [Horbett 1993], also on polyvinyl chloride (PVC), silicon rubber, Teflon and polyurethane [Hanson 1987].

It should be noted that the mathematical isotherm equations (1) and (2) originate from gas-solid adsorption and are based on such common assumptions as that all binding sites on the solid surface are equivalent and bind only one solute molecule. Further it is assumed that the solute molecules do not react between each other when they are adsorbed, which is usually not fulfilled for the complicated protein adsorption process. For that reason, equations (1) and (2) are only empirically applied to experimental adsorption data. For instance, an equilibrium state is not achieved in many cases for protein adsorption. Some authors call the situation a *pseudo-equilibrium* at high dilution rate [Rubens 1992] or an *ill–defined equilibrium* at high protein adsorption coverage [Shaaf 1992]. This often observed effect is caused by the *irreversibility* of the protein adsorption process. Mainly responsible for that are the conformational/orientation changes in the protein molecules that occur during adsorption [Hlady 1999]. Because of this, the constant K in isotherm equations (1) and (2) can not be considered as a binding constant with a strict physical meaning, but nevertheless it can be used as a measure of protein affinity [Joung 1988], and it appears to be a good tool for a comparison of protein affinity to different substrata [Wankat 1990].

2.2.3. Physicochemical properties of the biomaterials influencing protein adsorption

2.2.3.1. Wettability

Wettability is believed to play an important role for the amount and the conformational changes of adsorbed proteins [Vroman and Adams 1969, Norde and Lyklema 1991]. The hydrophobic interactions seem to be the dominant force driving protein adsorption/unfolding

on the surface [Norde and Lyklema 1991]. The hydrophobic interactions and their importance in protein adsorption were firstly indicated by studies showing that the protein adsorption increased with the decreasing wettability of the surface [Brash and Horbett 1995] – so called "hydrophobic rule". The water structure is that which makes differences between hydrophobic and hydrophilic surfaces [Vogler 1998b]. In the case of a hydrophobic surface, water molecules are ordered in an ice-like structure at the surface and have much lower entropy than the water molecules in the bulk. The interaction between a hydrophobic surface and a protein originates mainly from an entropy gain due to water desorption from the solid surface and from the protein molecule [Norde 1986]. In contrast water molecules near to a hydrophilic surface exhibit relatively more-dense water structure in an extended 3D network of self-associating molecules. This type of water structure is less reactive and therefore it is difficult to be removed [Norde and Lyklema 1991]. To hydrophilic surfaces, the proteins are adsorbed weakly with a conformation near to their native state. As a result the protein adsorption to hydrophilic substrata is generally reversible, whereas to hydrophobic one's it is not. Denaturation of the adsorbed protein by hydrophobic–hydrophobic interactions with the substrate can also contribute to an irreversible adsorption [Chinn 1992]. As a result the biological function of a given protein could be changed and/or altered, when it is adsorbed to a hydrophobic surface (see Fig. 5). For instance Chinn et al. [Chinn 1992] have shown that the ability of adsorbed FNG to bind platelets was decreased due to post-adsorptive conformational changes in the FNG molecule by spreading and unfolding, which also resulted in a more tightly bound protein. Horbett et al. [Horbett 1998] found also post-adsorptive transitions in FNG upon adsorption to segmented polyurethanes, indicated by decreased SDS elutability leading to decreased ability to bind platelets. In another interesting study [Perez-Luna 1994] the correlation between FNG SDS elutability and binding strength of adsorption for a large number of polymer materials has been demonstrated. In several works Vogler et al. [Vogler 1995a, 1995b, 1998a] studied the activation of the coagulation cascade in a dependence of surface wettability. They showed that the wettable surfaces adsorb proteins near to their native state due to the "entrapment" or association in a strongly bound hydration layer and therefore they retain their biological function. In contrast on poorly wettable surfaces the proteins were bound by dehydration mechanism to the surface and thus they lost their biological activity. As a result there was a low activation of the

coagulation cascade leading to a fibrin formation typically for the poorly wettable surfaces and an increase of the procoagulant activity with the increase of the surface wettability.

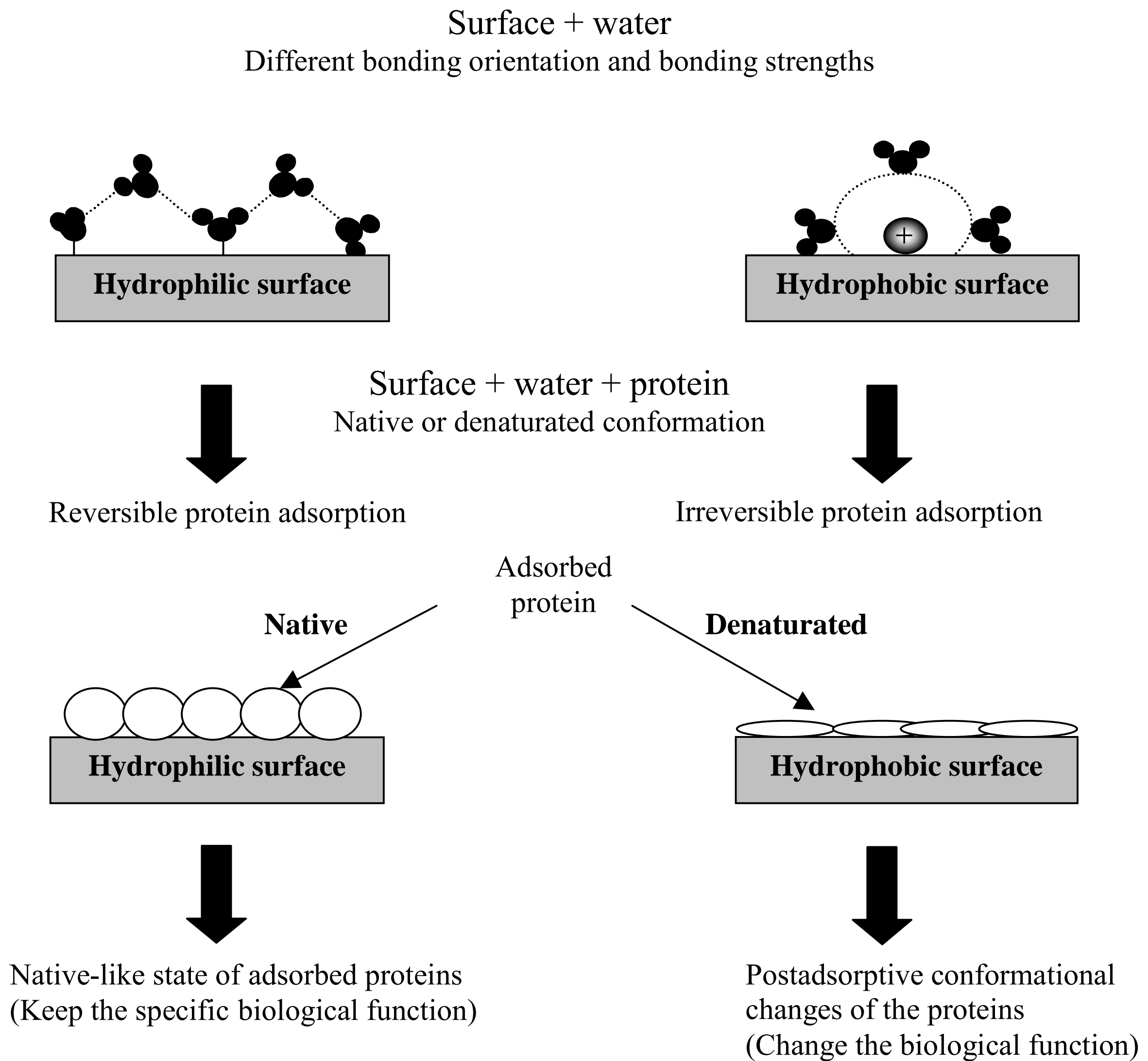


Fig.5. Schematic illustration of the events following after the blood (or plasma) contact with a hydrophilic or hydrophobic surface. Wettability affects the water structure near to the surface and therefore the protein adsorption and the cell behavior. Adapted from [Kasemo 2002].

2.2.3.2. Energetics of wetting

During placement of bulk molecules at the boundary of a material, a *surface free energy* (τ), is arising through the loss of the nearest-neighbor interactions that otherwise would be exist in the bulk phase [Vogler 1993]. In units, surface free energy per unit area is expressed in, e.g., mJ/m^2. These units are formally equivalent to a force per unit length (mN/m), which physicists call a tension (γ). The terms surface energy and surface tension could be used as synonyms [Vogler 1993]. For example, when a water-material interface is established, the interaction of the water molecules with atoms of the surface of the material along the interfacial plane lead to a unique interfacial energy or tension. For different materials, the resulting surface energy governs the different structure of water at these interfaces. According to Vogler [Vogler 1998b] water near to the material surface with water contact angle $\theta > 65$ deg – hydrophobic surfaces exhibits less dense structure, while water structure to materials with $\theta < 65$ deg (hydrophilic surfaces) is denser with extended 3D network.

A number of authors have correlated surface energy values of implanted materials to their biocompatibility [Kaelble and Moacanin 1997], Perez-Luna 1994, Vogler 1995a, 1995b, 1998a].

The surface free energy can be measured using contact angle (tensiometric) techniques and can be expressed by Young's equation:

$$\tau = \gamma_{(sv)} - \gamma_{(sl)} = \gamma_{(lv)}\cos\theta_{(sl)} \qquad (3)$$

where γ is an interfacial surface tension, τ is a surface (free) energy, the indexes $_{(sv)}$, $_{(sl)}$ and $_{(lv)}$ referred to surface-vapor, surface-liquid and liquid-vapor interfaces, and θ is the contact angle at the solid-liquid interface.

Another useful thermodynamic relationship involving τ , is the work of adhesion:

$$W = \tau + \gamma_{(lv)} \qquad (4)$$

W is the work required to remove liquid from a solid (per unit area of contact), with higher W values reflecting greater interaction of a solid with a liquid. Work of adhesion gives a complete picture of the interplay of interfacial forces that govern liquid, solid, and solute [Vogler 1993].

Further relation between protein adsorption and surface free energy of the materials could be obtained by expressing the surface free energy in its polar and dispersion components [Fowkes 1962, 1963]:

$$\gamma_i = \gamma_i^d + \gamma_i^p \quad (5)$$

where i = sv or av (index "v" is for vapour, index" a" is for alkane, index "s" is for solid).

The exposure of blood or plasma to a foreign surface produces a complex set of concurrent and sequential events, which appear to correlate with the dispersion (London–type, index d) and polar (Coulomb-type, index p) components of surface energy for the implanted material [Kaelble and Moacanin 1977].

The polar and dispersion components of surface free energy can be calculated using a set of polar and non-polar liquids. For that purpose Kamusewitz and co-workers have used solid/water/vapor and solid/water/hexadecane systems to calculate the solid surface energy and its dispersion and polar components [Kamusewitz 1997].

Kaelble and Moacanin [Kaelble and Moacanin 1977] were using 190 biological and implant surfaces demonstrated that high dispersion (i.e. low polar surfaces) provided surface energetics favoring stable plasma protein film retention. In contrast low dispersion (i.e. high polar surfaces) appears to favor weak adsorption and retention of plasma proteins, which could continuously generate and spall of emboli into the blood stream. Several groups used the approach of Kaelble and Moacanin and studied the relation between surface free energy and protein affinity for different plasma proteins to a large number of polymer membranes. Joung et al. demonstrated a correlation between dispersion part of surface free energy and protein affinity [Joung 1988]. They showed an increased strength of protein binding with increasing the dispersion component of surface free energy. Baszkin and Lyman [Baszkin and Lyman 1980] concluded in experiments with albumin, γ- globulin and FNG on hydrophobic surfaces of various degree of polarity that the ratio of the polar and dispersion components of work of adhesion (W_A^p/W_A^d) determines the degree of affinity of the protein for the adsorbent and that the maximum affinity occurs when (W_A^p/W_A^d) approaches unity. In addition Perez-Luna and co-workers [Perez-Luna 1994] also showed a correlation between γ_s^d and protein affinity to the surfaces, noting that the protein retention after SDS treatment was greater on surfaces with higher γ_s^d. Hence, the measurements of the wettability,

expressed by the contact angle in the presence of different liquids, permit to evaluate and to compare surface free energy of membranes with different physicochemical properties.

2.2.3.3. Surface charge

Surface charge is another surface characteristic, which was shown to influence protein adsorption and has been related to biocompatibility of polymers applied in medical devices [Nadarajah 1995, Werner 1998]. Using ionic and non-ionic detergents to desorb albumin and FNG from series of polymer materials, Bohnert et al. [Bohnert 1990] studied the nature of the protein/polymer bonds. Their data showed that the ionic detergent SDS removed significantly more protein than the non-ionic detergents Triton X-100 and Tween-20. Thus, they concluded that ionic interactions together with hydrophobic interactions play also a role for protein adsorption. However, the overall electrostatic interactions depend on the surface charge and protein charge, both of which are usually function of pH and solution ionic strength [Andrade and Hlady 1986]. Experimentally, proteins have been found to exhibit greater adsorption at or near the isoelectric point, perhaps because of the charge–charge repulsion among the adsorbed molecules is minimized under these conditions [Horbett 1982]. The increase of ionic strength increases protein adsorption probably due to the involvement of two mechanisms: shielding the double-layer repulsion as well as the promotion of a more globular shape of the protein [Lu 1988]. At physiological pH =7.4 most of the plasma proteins have a negative charge (FNG pI 5.5; albumin pI 4.8) and one can expect in general that positively charged surfaces will have more high impact on protein adsorption than negatively charged ones [Horbett 1982]. However the negatively charged proteins are not fully repelled from negatively–charged surfaces due to the multiple binding modes of adsorbed proteins [Horbett 1982]. It is well known that the proteins consist of polar, charged and nonpolar domains and therefore they have an opportunity to bind to different surfaces through a complex of interactions including hydrophobic-hydrophobic, electrostatic interactions and others. Thus it is possible that even a given protein has an overall negative charge it can expose to the surface positively charged or non-charged domains and hence is not repelled from the negatively charged surfaces [Andrade and Hlady 1986]. For instance COOH and OH groups are shown to have a positive effect on EC seeding most probably due to the loosely–bound proteins which could promote EC adhesion and proliferation [Curtis

1986]. High density of surface charge was shown to reduce protein adsorption by increasing repulsive effects [Angelova and Hunkeler 1999].

2.2.3.4. Topography and roughness

The surface topography and surface roughness should also be considered to play role in protein adsorption and subsequent cell response as well [Kam 2001]. The surface roughness was shown to be a rather important determinant for protein adsorption on complex solid surfaces such as block co-polymers and surfaces with flexible polyethylene oxide (PEO) molecules immobilized on them. Most probably the instability of adsorbed proteins caused by steric repulsive effects (for PEO grafted surfaces) and microdomain structure (for block co-polymers consisting of hydrophobic and hydrophilic domains) makes these surfaces to repel loosely bound proteins [Vermette 2002, Deppisch 1998]. The surface topography plays an important role in providing three-dimensionality to the materials, as would be found in the body [Dalby 2002]. It has been very well documented that many cell types react strongly to micrometric topography [Dalton 1999, Schwartz 2001], and more recently, it has been demonstrated that cells can respond to nanometric cues in vitro [Curtis 2001]. Different topographies as pits, islands or ribbons can be produced using a polymer demixing technique which can react in different ways with the ECM proteins, and hence might modulate the cell interaction with the material. On the other hand, the adsorption of proteins leads to changes in topography by forming spatial structures like fibrils, which in turn affect the cell behavior [Mondon 2003]. For instance the topography of the collagen fibers, with repeated 66nm binding, has shown to effect cell shape [Curtis and Wilkinson 1999]. Techniques based on micropatterning of biologically important proteins (e.g., laminin and FN) are of a particular interest because these proteins could provide cell guidance [Tai and Buettner 1998, Kam 2001]. For instance the adsorbed polylysin-conjugated laminin on glass was shown to form an interconnected network of narrow linear features (micrometer-scale), which was able to guide the outgrowth of hippocampal neurons along the formed network [Kam 2001]. The formation of large clusters of immobilized peptides on glass surfaces also have been shown to affect the cell-substratum adhesiveness of EC and the random motility [Kouvroukoglou 2000]. In summary, the surface roughness and the topography of the given biomaterial

achieved by the chemical surface modifications could contribute to the controlling the protein patterning, and hence to modulate the cell adhesion in accordance to the specific use.

Apparently, reviewing the mechanisms of protein adsorption, we can conclude that no single factor can explain the protein adsorption phenomena. There are always several different properties of protein and adsorbent that determine the protein-surface interactions [Matsuda 1989, Andrade 1992].

2.3. Platelets

2.3.1. General aspects

2.3.1.1. Structure

Platelets are produced by megakaryocytes in the bone marrow and have a life span of 8-10 days. They are the smallest of the human blood cells (about 2-3 microns in size) and do not have a nucleus but have mitochondria, which serve as an energy source [Ordinas 1993]. The typical shape of resting platelets is discoid (Fig. 7A), upon activation they undergo a shape change to a globular form with pseudopodia (up to 5µm long) (Fig. 7B).

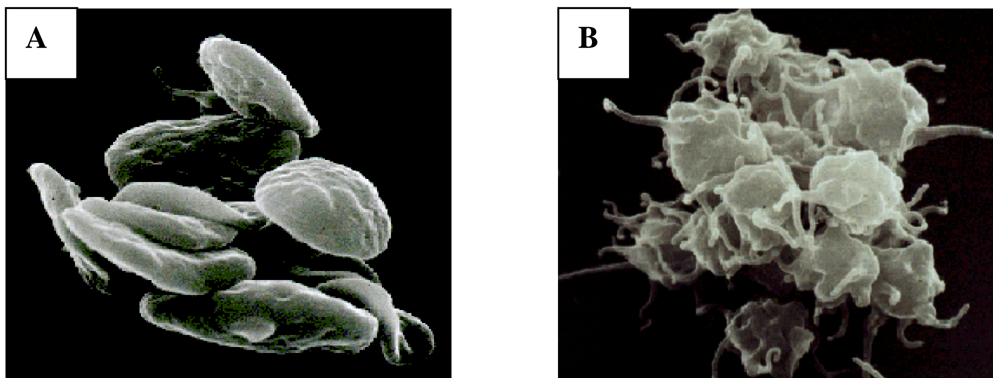


Fig. 7 Morphology oh human platelets**.** Typical smooth discoid shape of resting platelets (A) and spiny spheric shape of activated platelets (B). From Platelet web page: Anatomy of human blood platelets.

Membrane Systems

The platelet plasma membrane plays a major role in the platelet physiology (Fig.8). Series of plasma membrane invaginations form a surface-connected open canalicular system (OCS) and dense tubular system (DTS). The OCS increases the total surface area of the platelet and provides access to the interior for plasma born substances and a channel for products of the release reaction. The DTS serves as a calcium reservoir enabling platelet activation and is also the site where enzymes involved in prostaglandin synthesis are located.

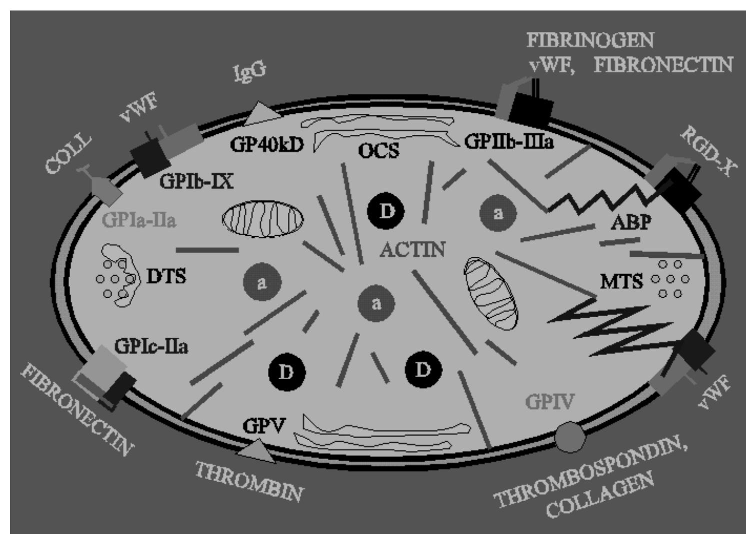


Fig.8 Platelet structure**.** From Platelet web page: Anatomy of the human blood platelets.

Platelet cytoskeleton and Microtubular System (MTS)

Actin (10-20%) and myosin (15-20%) as the major platelet proteins form a three-dimensional network through the cytoplasm of platelets. A second two-dimensional network of shorter actin fibres serves as a membrane skeleton, responsible for the discoid shape of the resting platelet, since membrane receptors are linked via an actin-binding protein (ABP) to this network. Furthermore a marginal bundle of microtubules (MTS) supports the actin membrane skeleton in keeping this discoid shape.

Organelles

Organelles are almost evenly distributed in the cytoplasm of resting platelets (Fig.8). Mitochondria serve as energy source, since resting platelets cover their energy expenditure by oxidative phosphorylation, similar to other cells. The most organelles by far are storage granules (~40/platelet). Alpha-granules contain FNG, thrombospondin, FV, vWF, beta-thromboglobuline (ß-TG), platelet factor 4 (PF4), etc. Dense bodies contain calcium, serotonin, adenine nucleotides, etc. Following activation platelets release their granula contents, contributing to diverse interactions with other platelets or other cells.

2.3.1.2. Function

The major function of platelets under normal physiological conditions is to prevent the blood loss after blood vessel injury by covering the denuded endothelium [Willoughby 2002]. The platelet plug is the final result of platelet adhesion, activation and aggregation in the response to blood vessel injury [Deitcher 2001]. The activated platelets form a temporary platelet plug (aggregation), and support the generation of fibrin by the coagulation cascade of proteins (Fig.9). The formation of a fibrin mesh stabilizes the platelet plug and forms the thrombus, which serves for closing the ruptures in the blood vessels during wound repair.

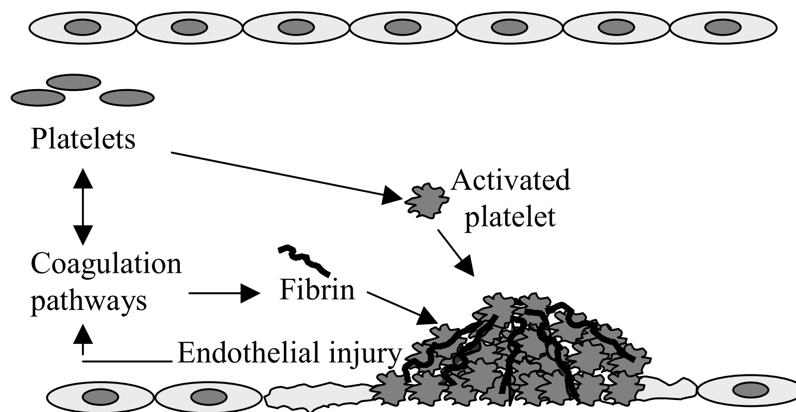


Fig. 9 Participation of platelets in clot formation following blood vessel injury. Platelet become activated and accumulates at the site of vascular endothelial injury. The formed plug (aggregation) stops the escape of blood from the circulation and supports the formation of fibrin, which stabilizes the platelet plug. From [Deitcher 2001].

However, platelet adhesion is also the first step in the development of pathological thrombi in coronary and cerebral arteries, leading to arteriosclerosis [Schrader and Berk 1990]. Thrombosis of vascular implants and extracorporeal circulatory system is likewise triggered by platelet adhesion to artificial surfaces in contact with flowing blood [Salzman and Merrill 1987]. The sequence of the events leading to formation of a hemostatic plug or platelet - fibrin thrombus may depict as follows [Casenave 1986, Hawiger 1990]. Firstly, a zone of vascular injury or protein coated artificial surface is recognized by platelets, which contact and adhere, becoming activated and changing their shape from smooth discs to spiny spheres. Secondly, platelet activation is accompanied by secretion of their granule contents, release of adhesive membrane receptors. This leads to platelet spreading on the surface and interplatelet bridging, with formation of surface bound aggregates (Fig.9). Thirdly, thrombin generation causes further platelet activation and transformation of FNG into polymeric fibrin, thus enmeshing the platelets in more resistant and stable hemostatic plugs or platelet-fibrin thrombus.

2.3.2. Activation of platelets

Platelets are activated by several physiological (thrombin, collagen, adenosine diphosphate (ADP), epinephrine, vasopresin, serotonin) and non-physiological (divalent cationophores, cyclic endoperoxides) substances [Willoughby 2002]. The platelet plasma membrane contains a large number of receptors, which specifically bind platelet-activating agonists (listed above). The interaction between a platelet-activating agonist and its receptor causes rapid mobilization of signaling molecules within the platelet, which are sufficient to initiate and complete shape change and aggregation. Because platelet functions depend primarily on adhesive interactions, it is not surprising that most of the glycoproteins on the platelet membrane surface are receptors for adhesive proteins or otherwise mediate cellular interactions [Kieffer 1993] (see Fig. 8). These include platelet membrane receptors for extracellular matrix proteins (e.g. vWF, FNG, collagen, FN, laminin), receptors involved in homotypic interactions with other platelets to form platelet plug, and receptors involved in heterotypic interactions with other cells of the vasculature to promote inflammatory response. There are currently four known families of cell membrane receptors [Hynes 1992]. These include the Ca^{2+} dependent cadherins, the Ca^{2+} independent immunoglobulin supergene

family, the Ca^{2+} dependent selectins and divalent cation dependent integrins. Members of three of these gene families are present in platelets: integrins, selectins and immunoglobulin supergene family. Platelets express also members of the leucine rich glycoprotein (LRG) gene family [Roth 1991].

2.3.2.1. LRG gene family

The GPIb-IX complex belonging to the LRG gene family is the most prominent glycoprotein of the platelet membrane and contributes to the net negative charge of the platelet surface [Clemetson 1985; Roth 1991]. The major role of the GPIb-IX complex in the platelet function is to bind to immobilized vWF on exposed vascular subendothelium and thus to initiate adhesion of platelets to the subendothelium at the site of the vessel injury [George 1984]. The cytoplasmic domain of the GPIb-IX complex has a major function in linking the plasma membrane to the short actin filaments network and thus to maintain the discoid shape of the resting platelets [Fox 1988]. Platelets constitutively express the glycoprotein GPIb-IX. The interactions between vWF and GPIb-IX promote a change in platelet morphology and induce pseudopodia generation, which together promote platelet aggregation and clot retraction at sites of vascular injury [Deitcher 2001]

2.3.2.2. Integrins

Platelets express 5 integrins which include, in order of decreasing amounts, glycoprotein IIb-IIIa ($\alpha_{IIb}\beta_3$), GP Ia-IIa ($\alpha_2\beta_1$), GP Ic-IIa ($\alpha_5\beta_1$), GP Ic'-IIa ($\alpha_6\beta_1$) and the vitronectin receptor ($\alpha_V\beta_3$) [Kieffer and Phillips 1990]. Platelets β_1 integrins, which are constitutively active receptors are essentially involved in platelet adhesion to insoluble FN ($\alpha_5\beta_1$), collagen ($\alpha_2\beta_1$) and laminin ($\alpha_6\beta_1$) present in ECM, exhibit highly restricted ligand specificity and recognize distinct recognition sequences within their respective ligands. In contrast, platelet β_3 integrins, GP IIb-IIIa ($\alpha_{IIb}\beta_3$) and the vitronectin receptor ($\alpha_V\beta_3$), are receptors for a variety of soluble adhesive proteins found in plasma (FNG, FN, vWF and thrombospondin) and recognize the tripeptide RGD sequence in these ligands. The vitronectin receptor is constitutively active, whereas GP IIb-IIIa functions both as an activation-dependent and activation-independent receptor [Kieffer and Phillips 1990].

The integrins are transmembrane heterodimers composed of two noncovalently associated transmembrane glycoprotein subunits called α and β subunits [Hynes 1990]. The binding of integrins to their ligands depends on extracellular divalent cations (Ca^{2+} or Mg^{2+}, depending on the integrin), reflecting the presence of three or four divalent-cation-binding domains in the large extracellular part of the α chain. These domains recognize the RGD sites, a sequence which is common to many extracellular ligands and which is thought to play key role in cell adhesion [Ruoslahti 1996a]. The integrins link to actin cytoskeleton via the attachment proteins talin and α-actinin.

2.3.2.3. Selectins

There are currently three known selectins: E-, L- and P-Selectins. From them only P-Selectin is present in platelets [McEver 1991]. P-Selectin becomes exposed on the surface of activated platelets upon the granule secretion and thus can be utilized to quantify the extent of platelet activation [Ritchie 2000]. P-Selectin is involved in heterotypic platelet interactions with other cells. P-Selectin functions as a receptor that mediates adherence of neutrophiles and monocytes to activated platelets [Larsen 1989, Hamburger and McEver 1990]. A possible functional role for P-Selectin in platelet-leukocytes interactions might be to localise leukocytes to the site of the vascular injury or alternatively, to localize platelets to the site of the inflammatory process. P-Selectin might also function as a recognition system for the macrophages to remove the activated platelets from the circulating blood.

2.3.2.4. Immunoglobulin supergene family

One member of the immunoglobulin super gene family, which is present in platelets, is platelet-EC adhesion molecule-1 (PECAM-1), which is also found on EC, neutrophils and monocytes [Newman 1990]. PECAM is essentially involved in homotypic cell interactions in platelet aggregation process.

During activation of platelets on foreign surfaces, the shape of activated platelets progresses in different morphological forms in a relation of their activation state [Goodman 1989]. For instance Grunkemeier et al. [Grunkemeier 2000], using different morphological categories of platelets, studied their activation on FNG adsorbed Immulon. The number of pseudopodia and the diameter of the platelets were correlated to the different rate of platelet activation.

2.4. Endothelial cells

2.4.1. General aspects – structure and function

EC are mesoderm-derived cells that constitute the inner lining (called endothelium) of blood vessels in contact with blood [Jaffe 1973]. Resting EC are heterogenous and differ in size, morphology and physiological functions depending of vessel caliber and the organ. EC are characterized by Weibel-Palade bodies. These structures serve as a depot for substances as P-Selectin and chemokines, which are synthesized and stored in response to inflammatory reactions [Mantovani and Garlanda 2001]. EC cultures represent a valuable tool not only in hemocompatibility testing, but also in the concept of designing hybrid organs [Kirkpatrick 1999]. Human umbilical vein endothelial cells (HUVEC) have remained the most widely used human EC type since they are more easily available, they are free from any pathological process and are physiologically more relevant than many established cell lines. [Marin 2001].

2.4.2. Role of endothelium

The endothelium is not only a passive barrier between the blood and the vessel wall but also a highly dynamic and reactive tissue participating in a variety of physiological processes including hemostasis, vascular tone, wound healing, inflammation and angiogenesis [Kirkpatrick 1997]. The endothelium also plays a critical role in various pathophysiological processes such as atherosclerosis, the growth of solid tumors, and metastasis [Blood and Zetter 1990, Folkman 1992, 1995, Ross 1993].

2.4.2.1. Anti-thrombogenic function of endothelium

Endothelium plays an important role in hemostasis as the ideal non-thrombogenic natural surface maintaining the balance between antithrombotic and prothrombotic factors inside a blood vessel [Pratt 1988]. The surface of the endothelium is multiphasic and highly hydrated. The smoothness of the endothelium can prevent contact activation of the platelets. Also endothelium is nature's most efficient anti-thrombotic surface, the maintenance of which depends on the production of numerous factors acting either as anticoagulants or as promoters of fibrinolysis (process of lysis of clot) [Matsuda 1989]. The anti-thrombotic factors produced by endothelium are PGI_2, nitric oxide (NO), thrombomodulin, heparan

sulphate proteoglycans, as well as tissue plasminogen activator (t-PA) and urokinase type activator (u-PA). Although under physiological conditions the anti-thrombotic activity of the EC predominates, pro-thrombotic activity can be rapidly induced by, for examples, tissue injury, proinflammatory cytokines and bacterial toxins [Nawroth 1986]. For instance, thromboxane (TxA_2) [Parente and Parretti 2003], the tissue factor (TF) [Ruf and Edgington 1994] and plasminogen activator inhibitor–1 (PAI-1) [Fujii 1992] are the most important endothelial pro-thrombotic factors.

2.4.2.2. Prostacyclin (PGI_2)

Among the important anti-thrombotic products of endothelium is PGI_2 which was shown to suppress early phases of thrombosis by preventing platelet adhesion, activation and aggregation and can even play a role in the dissolution of clots [Greisler 1990]. Together with NO, PGI_2 is also a potent vasodilator controlling the vascular tone [Orpana 1997]. PGI_2 is the main product of arachidonic acid in all arteries and veins so far tested [Vane 1983]. Arachidonic acid is a member of essential fatty acids contained in membrane phospholipids. Activation of the enzyme phospholipase A_2 releases arachidonic acid that can be further metabolized to a number of products including PGI_2 and TxA_2 [Parente and Parretti 2003] with almost opposing functions.

Under physiological conditions, circulating platelets remain inactive in part because EC secrete PGI_2 [Brass 2001]. This molecule binds and activates receptors on the surface of platelets that stimulate adenylyl cyclase, increasing the formation of cyclic adenosine monophosphate (cAMP) within the platelets. Rising cAMP levels make platelets less responsive to platelet activators. In fact, many such activators – including ADP that can be released from damaged red blood cells, work in part by inhibiting adenylyl cyclase and lowering internal levels of cAMP.

In the sites of vascular injury, the pro-thrombotic activity of the endothelium starts to predominate. In that case, the EC are damaged or removed. This exposes collagen fibrils, to which platelets adhere with the help of vWF or/and FNG. Once activated in this way, platelets secrete ADP and TxA_2. These molecules bind to receptors on circulating platelets, causing them to change shape and become activated, and recruiting them into the hemolytic plug.

Apart its role in platelet aggregation, PGI_2 is also able to down regulate the production of TF, which plays a central role in activation of blood coagulation, of thrombin generation and fibrin deposition [Edgington 1991, Crutchley 1994].

PGI_2 plays a wide spread role as a marker of various inflammatory processes [Okajima 2001]. The proinflammatory cytokine tumor necrosis factor α (TNF-α) is an important factor for PGI_2 production by cells [Okajima 2001]. For instance, in septic shock associated with Gram-negative bacteria, the production of TNF-α is increased, which leads to non controlled high levels of PGI_2 reflecting to systematic hypotension [Bone 1991]. In other cases like stress-induced acute gastric mucosal injury, the enhanced production of TNF-α by activated monocytes, in turn activates neutrophils [Konturek 2000]. The activated neutrophils release inflammatory mediators as proteases and oxygen free radicals, which damage the EC, decrease PGI_2 production and lead to increased endothelial permeability [Weksler 1987, Mizutani 2003]. PGI_2 could also be a potent anti-inflammatory factor by feedback mechanism, in which released PGI_2 inhibits monocyte production of TNF-α by interacting with cell – surface heparin-like substances [Okajima 2001].

2.4.3. Role of EC-substrate interactions

It has been very well documented that the ***in vivo*** interaction of EC with the vessel wall is mediated by ECM constituents such as FN, fibrin, FNG, vWF, vitronectin, laminin and collagens [Form 1986]. This subendothelial matrix is, in general, a thrombogenic surface that promotes platelet adhesion and activation of the coagulation system [Dejana 1993].

Under normal conditions, the presence of the endothelium represents a protection against thrombotic phenomena and plasma protein infiltration in the vascular media. Thus the capacity of EC to remain attached to the vascular surface and to migrate and proliferate to cover exposed subendothelium is an important defense mechanism against the development of vascular injury [Dejana 1993]. ECM proteins play a more complex role than only providing a substrate for cell attachment [Poot 1993, Underwood and Bennet 1993]. Cells also reorganize them and through the outside-in signaling involving specific surface receptors belonging to the integrin superfamily [Hynes 1990] providing signals for cell differentiation, growth and survival [Hay 1991].

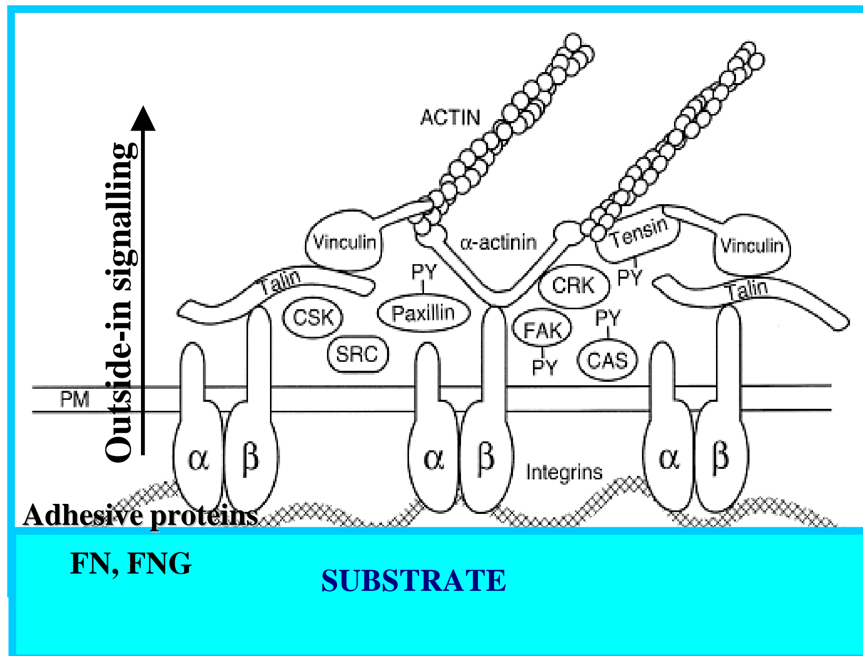


Fig. 14 Interaction between the cell and the substrate. Outside-in signaling through adhesive proteins and integrins. Adapted by [Vuori 1998].

2.4.3.1. Integrin-ECM bindings

Integrins are the main cell receptors by which the cells bind and respond to the ECM. Although some integrins are cell-type specific, most integrins are expressed in a variety of cell types, providing cells with the ability to interact with many different ECM proteins in a variety of cellular processes [Hynes 2002].

Thus, bound integrins and actin cytoskeleton cluster together giving rise to adhesion complexes named focal adhesions (elongated small regions usually a few microns in length), which are the closest contact (leave gap only ~10 - 15 nm) between the cell membrane and the substratum [Zamir and Geiger 2001]. The cell surface integrin receptors play a major role at the focal adhesions like transmembrane linkers by connecting actin stress fibers from inside of the cell to ECM proteins outside of the cell [Zamir and Geiger 2001]. Thus, the focal adhesions serve as sites to anchor actin stress fibers and to nucleate actin polymerization (Fig.15) [Hynes and Lander 1992, Garratt and Humphries 1995]. Therefore, the actin stress fibers terminating in the plasma membrane are thought to produce contractile

forces generating tension on the substrate [Geiger and Bershadsky 2002]. The cytoplasmic components of focal adhesion consists of a complex network of structural and signaling proteins [Jockusch 1995, Burridge and Chrzanowska-Wodnicka 1996] of which vinculin as a structural molecule is concentrated on the cytoplasmic side of the focal adhesions and aids in the attachment of actin filaments to the plasma membrane [Avnur 1983]. The focal adhesions contain many signaling molecules like focal adhesion kinase (FAK), ras, and src [Petit and Thiery 2000], which are involved in transmitting signals to the cytoskeleton, cytoplasm and nucleus from the ECM [Gilmore and Romer 1996, Yano 1996]. Although by definition, focal adhesions are formed by cultured cells that grow on solid surfaces [Geiger and Bershadsky 2002], structures with similar molecular properties are found ***in vivo.*** For example, adhesions formed by aortic EC with the underlying basement membrane, are closely related to focal adhesions [Kano 1996].

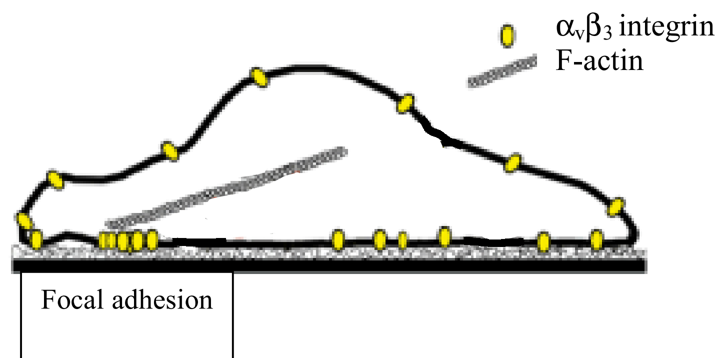


Fig.15 Organization of focal adhesions on the artificial surface.

Since the EC are anchorage dependent, the ability of a given substrate to promote and maintain the formation of stress fibers and focal adhesions is important for the performance of the material to keep the cell attached, for their growth and survival and thus could be a critical parameter for material biocompatibility [Ruoslahti and Obrink 1996b].

Focal adhesions were shown to be assembled on many artificial surfaces like glass [Schneider and Burridge 1994, Groth and Altankov 1995]; a variety of metal alloys, ultrahigh molecular weight polyethylene, hydroxyapatite, alumina, and borosilicate glass [Puleo and

Bizios 1992]; Arg-Gly-Asp-grafted polyethylene terephthalate and polytetrafluoroethylene [Massia and Hubbell 1991]; and even on silicone [Meyle 1993] by a variety of cell types.

2.4.3.2. Remodelling of ECM proteins

The ECM is the glue that holds the cells together and provides texture, strength and integrity to the tissues [Vu 2001]. However, beyond these obvious scaffolding functions, the ECM is also responsible for transmitting environmental signals to cells, which affect essentially all aspects of cell's life, including its proliferation, differentiation and death [Geiger 2001]. During all these cell-matrix signaling events, the remodelling of ECM components plays a crucial role [Matvey 1998, Sottile and Hocking 2002]. Remodelling is important for numerous different processes in the adults, including neovascularization, repair processes and others [Sottile and Hocking 2002].

The process of ECM remodelling could be relatively distinguished by two pathways: fibrillogenesis of synthesized and deposited ECM components on the one side and the breakdown and destruction of ECM on the other side [Streuli 1999, Sottile and Hocking 2002]. The balance between both pathways is controlled by ECM feedback and many normal and pathological processes like hemostasis, neovascularization, wound healing and tumor growth depend on the balance between the both pathways [Sottile and Hocking 2002].

2.4.3.2.1. Remodelling of synthesized and deposited ECM proteins

Remodelling of synthesized and secreted ECM proteins in fibrilar structures by cells was shown to facilitate cell adhesion, migration and tissue organization, as well as the external regulation of cellular functions [Loftus 1994, Geiger 2001].

FN is one the most studied ECM proteins, which can be organized in fibrilar structures by various cell types [Grinnell and Feld 1981, McDonald 1982, Christopher 1997, Pankov 2000]. FN is a high molecular mass dimeric glycoprotein (450-500kDa), which is distributed, in a soluble form in plasma and most body fluids. FN is also found in polymerized form as a part of the ECM of many connective tissues [Aguirre 1994]. FN has been longer studied as a promoter of cell attachment and migration of different cell forms including EC during embriogenesis, tumor growth, wound healing, angiogenesis and inflammation [Clark and Colvin 1985, Hynes 1990, Giancotti and Ruoslahti 1990]. FN matrices are deposited in a

temporally and spatially defined pattern utilizing both newly synthesized cellular FN and soluble FN [Mosher 1992]. Cells use these FN matrices as migratory tracks during development [Boucaut 1990]. Much more, in FN matrices is imprinted a positional information that contributes to the directional migration of mesoderm [Boucaut 1990, Winklbauer and Nagel 1991, George 1993]. Cell migration along FN matrices is also involved in wound healing [Clark and Gao 1985] and the loss of capacity to form a FN matrix is a feature of the transformed phenotype [Hynes and Destree 1978]. Thus the generation of FN matrix is an essential process in vertebrate development and response to injury, and its disruption may contribute to tumorigenesis.

FN matrix formation (fibrillogenesis) is a cell-dependent process that is triggered at specific sites of the cell surface [Pankov 2000] and depends on the unfolding of the FN molecules [Hynes 1999, Schwarzbauer and Sechler 1999]. The process of FN fibrillogenesis is driven by a co-operation between two distinct types of cell surface adhesions: the focal and fibrilar adhesions [Geiger 2001]. They cooperate in a process by which integrins and dynamic tension forces seem to unmask cryptic FN assembly sites in FN molecule that mediate FN polymerization and generate network of fibrillar ECM [Pankov 2000]. The integrins play a central role in FN fibrillogenesis [Cukierman 2001]. A number of integrins bind to FN but are not normally capable of initiating formation of FN fibrils [Zhang 1993]. The major receptor responsible for FN fibrillogenesis is $\alpha_5\beta_1$ integrin [Pankov 2000].

The process of fibrillogenesis can follow three main steps. The first phase of fibrillogenesis involves binding of FN to the surface of the cell at the focal adhesions sites, which is mediated by integrins, mainly by $\alpha_5\beta_1$, but also by $\alpha_V\beta_3$ and potentially by other integrins with lower efficiency [Geiger 2001]. A critical step in this first phase is exposure of cryptic self-association sites of FN, which is important for FN polymerization. One mechanism for exposing FN cryptic sites could be binding to integrins [Schwarzbauer and Sechler 1999] that induces conformational changes in FN molecule. A second prerequisite element for unfolding of FN (triggering FN polymerization) is generation of the static tension at the focal adhesions driven by anchoring of actin stress fibers [Wu 1995]. So the integrin molecules that connect the actin cytoskeleton to the ECM are candidates for translation the tension that is generated by the actin cytoskeleton at the focal adhesions. It was recently established that ligand-bound $\alpha_5\beta_1$ integrins are actively translocated from focal adhesions to fibrilar

adhesions [Pankov 2000]. This movement provides a potential mechanism for integrins to apply tensile forces to stretch FN and induce fibrillogenesis $\alpha_5\beta_1$ integrins move from focal adhesions along fibrilar adhesions parallel to small actin microfilaments bundles. This highly directional, escalator–like type of movement of $\alpha_5\beta_1$ becomes activated when this integrin binds FN and is associated with elongation of newly forming FN fibrils [Geiger 2001]. Thus the high static tension generated at focal adhesions is transmitted to low dynamic tension in fibrilar adhesion during fibrillogenesis.

The physical properties like deformability and elasticity of the newly formed FN matrix greatly influence the process of fibrillogenesis [Ohashi 1999]. And in turn it is very well documented the role of the substrate surface properties like wettability on the conformational/orientational changes in adsorbed FN [Iuliano 1993]. For instance, several groups [Iuliano 1993, Burmeister 1996, 1999] using model surfaces with different wettability related the EC spreading and strength of adhesion to conformation/orientation of adsorbed FN. It was confirmed that the changes in the conformation of the FN cell binding domain affect the EC adhesion and spreading since on hydrophobic surface there was a significant reduction of cell attachment most probably due to an inappropriate conformation of adsorbed FN [Iuliano 1993, Steele 1995]. Much more, the higher strength of FN adsorption on poor wettable surfaces (like silanized glass) resulting in a reduction of FN elasticity was found to be the reason for the reduced FN fibrillogenesis by human fibroblasts [Grinnell and Feld 1982, Grinnell 1987, Altankov 1996]. In contrast the moderately wettable surfaces like glass were able to reorganize adsorbed FN due to loosely bound protein [Altankov 1997]. In conclusion, the process of FN fibrillogenesis globally controls the composition and stability of the ECM and thus likely to control ECM signaling cascade that regulate many aspects of the cell behavior including cell proliferation, migration and differentiation [Sottile 1998, Hocking 2000]. Therefore the ability of cells to reorganize FN in fibrils on different materials might be used as a useful tool for the material biocompatibility.

FNG, which is abundantly available in blood, was shown to act as an adhesive protein, promoting EC adhesion, motility and growth during events associated with vessel injury repair and new vessel formation [Dejana 1990]. Moreover FNG was found to be a determining factor for EC migration and its lack alters the cell migration but not influences cell adhesion [Dejana 1990]. FNG and its derivative fibrin play an important role in

biological processes associated with normal hemostasis and with pathological development of thrombotic vascular occlusion by supporting platelet and EC adhesion during these events [Cheresh 1989]. During the initial phases of the hemostatic response after vessel injury, activated platelets adhere to the exposed sub-endothelium and aggregate with one another to form a FNG-dependent hemostatic plug [Groves 1982]. On the other hand upon the subsequent wound healing, EC in the local environment proliferate and migrate on adsorbed FNG in attempt to repair damaged vessels and to produce new ones [Dejana 1987, Nicosia and Villaschi 1999]. The adhesive phenotype of both platelets and EC is a critical factor governing such process [Cheresh 1989]. In particular, the ability of these cells to interact dynamically with FNG is certainly one of the important adhesive factors to occur [Cheresh 1989]. Many studies have shown that the adhesion of EC to FNG is also mediated by integrins, particularly by the $\alpha_V\beta_3$ integrin [Dejana 1993] which recognizes a single RGD-containing sequence near the C-terminus of the α-chain of the FNG molecule [Cheresh 1989]. It was found [Conforti 1992] that the integrin $\alpha_V\beta_3$ was localized not only basally on the EC membrane, but also apically, suggesting the role, which this integrin could play in binding of different soluble plasma proteins including FNG. For instance, in some pathological processes such as ischemia-reperfusion of the vessel wall, EC acquire a procoagulant phenotype, which is characterized by FNG accumulation on the apical cell surface [Massberg 1999]. Thus FNG accumulation directly contributes to platelet recruitment by binding through α_{IIb}/β_3 [Savage 1996] or, by its binding to intercellular adhesion molecule (ICAM)-1 that attracts leukocytes adhesion and thus participates in inflammatory processes on the surface of postischemic vessel walls [Languino 1993]. FNG was shown to form a provisional matrix, which mediates cellular functions as adhesion and spreading, proliferation, and migration of variety different cell types, including EC, fibroblasts, epithelial cells and platelets [Dejana 1987, Brown 1993, Donaldson 1989, Savage and Ruggeri 1991]. Much more, it has been shown that the EC adhesion to FNG requires FN synthesis and deposition for the proper spreading and cytoskeleton organization of EC [Dejana 1990]. When the synthesis of FN was inhibited, EC were still able to adhere to FNG but did not properly organize their cytoskeleton and adhesion structures [Dejana 1990].

In studies of tissue injuries induced by inflammation, different groups have shown that the different type of epithelial cells [Lee 1996, Guadiz 1997a] were capable to synthesize FNG

and to deposit it basolaterally and thus to be incorporated in ECM. In addition, the incorporation of FNG fibrils in ECM was found to require active FN matrix formation [Pereira 2002], while in complete absence of FN exogenously added, FNG was unable to assemble in fibrils. Furthermore the FNG assembly into ECM shows striking similarities to that of FN [Pereira 2002]. For instance, FNG like FN undergoes conformational changes upon incorporation in ECM exposing a new fibrin specific epitope, independent of thrombin or plasmin cleavage [Guadiz 1997b]. There are two fibrin-binding sites on each FN subunit that may play a role in assembly of conformationally altered FNG in FN matrix [McKeown-Longo and Mosher 1989]. Therefore, the FNG assembly into the FN matrix fibrils requires FN-FNG heterotypic association [Pereira 2002]. Thus the FNG deposition in ECM might play role in tissue repair processes by rapidly changing the topology of the ECM and thus providing a substrate for EC migration, or to participate in inflammatory reactions during wound healing [Pereira 2002].

2.4.3.2.2. ECM breakdown/destruction

The opposing process of ECM matrix fibrillogenesis is ECM destruction. The both processes are closely linked to each other and the balance between them is essential for regulation of a variety of many physiological and pathological processes as neovascularization, hemostasis, soft tissue fibrosis and tumor growth [Lochter 1998, Pepper 2001, Corbel 2002]. For instance, during angiogenesis, the EC go through several steps including the loosening of matrix and intercellular adhesion, degradation of subendothelial matrix, migration, proliferation and formation of new tubes [Pepper 1997]. Thus, they change their phenotype from adhesive to invasive.

The family of matrix metalloproteinases (MMPs) plays the main and specific role in degradation of ECM components [Mignatti and Rifkin 1996, Vu and Werb 2000]. MMPs are zinc-dependent endopeptidases known for their ability to cleave ECM molecules [Pepper 2001]. MMPs can be divided into two structurally distinct groups, namely, secreted MMPs and membrane-type MMPs. Secreted MMPs include (but are not limited to) collagenases, gelatinases (gelatinase A, or MMP-2; gelatinase B, or MMP-9), stromelysins and other MMPs. The name of each class MMPs refers to their substrate specificity. EC express mainly two types of gelatinase type metalloproteinases MMP-2 and MMP-9 [Pepper 2001], which

are involved in the degradation of ECM components during the new vessel formation. Usually, these types of MMPs are synthesized in inactive proenzymes (zymogens), which are subsequently activated by proteolytic cleavage by the membrane type 1 matrix metalloproteinase, MT1-MMP [Okada 1997, Shimada 2000]. MMP activity and ECM proteolysis can be regulated directly by integrin binding. For example, in fibroblasts, binding and clustering of FN receptors are sufficient to induce an increase in the expression of MMP-1 and MMP-3 [Werb 1989]. Signals generated through cell binding to FN also induce MMP-2 activation in HT1080 fibrosarcoma cells [Stanton 1998]. Recent studies suggest that $\alpha_V\beta_3$ integrin can bind the MMP-2 in a RGD-independent manner and thus to localize the active form of the enzyme on the surface of angiogenic blood vessels [Brooks 1996]. This enables angiogenic EC to degrade the ECM during their invasion. For instance, native collagen IV, which is one of the main constituents of basement membrane and a substrate of MMP-2 [Collier 1988], contains RGD sites that are inaccessible to $\alpha_v\beta_3$. However, after MMP-dependent proteolytic cleavage of collagen, these RGD sites are exposed and become ligated to $\alpha_v\beta_3$ [Hood and Cheresh 2002]. Thus the physiological association between MMP-2 and $\alpha_v\beta_3$ might not only facilitate ECM degradation but would enable $\alpha_v\beta_3$·- mediated EC invasion through the proteolyzed matrix by attachment to exposed RGD sites [Brooks 1996]. Negative-feedback regulation of $\alpha_v\beta_3$ MMP-2 binding is required to prevent excessive degradation of the ECM and uncontrolled tumor growth [Hood and Cheresh 2002]. For instance MMP-2 is normally expressed in stromal cells but its expression is highly elevated adjacent to metastasing carcinomas [Brooks 1996]. The suppression of integrin-MMP-2 binding is regulated by one of the fragments of MMP-2 termed hemopexin fragment (PEX), which blocks protease activation by competing with MMP-2 for binding to integrin $\alpha_V\beta_3$ [Brooks 1998]. Thus, although this type of remodelling event might be important for differentiative processes accompanying normal tissue morphogenesis when is not controlled it leads to cancer cell migration [Matvey 1998, Gullberg 2002].

Interesting other view of action of metalloproteinases was given for the process of vascular remodelling after surgical vascular graft placement. Recent studies in experimental restenosis models have shown that endothelial dysfunction correlates with higher level of collagen deposition in restenotic vessels correlating with inhibition of the enzymes known to promote

degradation of ECM [Lafont 1999]. However, on the other hand the up-regulation of MMPs expression on the artificial surfaces can lead to opposing process and possible biomaterial degradation [Gibbons and Dzau 1994]. Thus a fine control of regulation of MMP expression would be suitable for normal functionioning of artificially formed vessel wall.

2.4.4. Role of cell-cell interactions

Endothelial cell-cell junctions are essential for the initial organization of the EC monolayers and play an important role in regulating vascular permeability, leukocyte extravasation and vascular remodelling [Dejana 1995]. Endothelial cell-cell junctions are complex structures formed by transmembrane adhesive molecules linked to a network of cytoplasmic/cytoskeletal proteins. On the basis of morphological and functional characteristics at least four types of junctions have been described in EC.

2.4.4.1. Tight junctions (TJ)

These organelles, also called *zonula occludens*, form a very close contact between adjacent cells [Gumbiner 1993]. They act as a primary barrier to the diffusion of solutes through the intracellular space [Tsukita 2001]. TJ are formed by transmembrane integral protein called occludin [Furuse 1993]. On the intracellular part of the TJ, EC possess proteins such as zonula occludens -1 (ZO), zonula occludens-2 (ZO-2), cingulin and others, which may contribute to the TJ anchorage to the actin microfilaments and/or to transfer of contact-mediated intracellular signals [Anderson 1993].

2.4.4.2. Gap junctions (GP)

GJ are transmembrane hydrophilic channels (connexons) that allow direct exchange of ions and small molecules between adjacent cells [Dora 2001]. The connexons are formed by related proteins belonging to the connexin (Co) family. In EC at least three connexins have been described (Co 43, Co 40 and Co 37), which are differentially expressed in various vessels [Dora 2001]. GJ are important in EC for the establishment of homotypic (endothelial to endothelial cell) or heterotypic (EC-smooth muscle cells, EC-macrophages) communications [Polacek 1993]. The junctional coupling may provide a mechanism for

coordination of EC migration and replication during repair of injury after mechanical denudation of the endothelium and during angiogenesis [Pepper 1989].

2.4.4.3. Syndesmos or complexus adherentes

These type intercellular junctions contain the transmembrane protein desmoplakin and usually are distributed with the cadherins in the adherent junctions [Dejana 1996].

2.4.4.4. Adherent junctions (AJ)

Cell to cell AJ are cellular membrane contacts formed by cadherins as transmembrane glycoproteins that mediate the physical attachment between the cell membrane and the intracellular undercoat network of cytoplasmic proteins and actin microfilaments [Geiger and Ayalon 1992, Tsukita 1992, Grunwald 1993]. The cadherins are required for the assembly of EC into a vascular – like structure, and therefore they are critically important for the structural organization of the vascular endothelial monolayer [Vittet 1997]. Cadherins are single chain transmembrane proteins comprising a highly conserved cytoplasmic region and an extracellular domain containing Ca^{++} binding motifs. Cadherins promote homophilic, Ca^{++} dependent cell-to-cell recognition [Takeichi 1991, Dejana 1995].
EC have been found to express both specific and non-specific cadherins [Heimark 1990, Rubin 1992]. E-Cadherin (epithelial type) was found in cell membrane adherent junctions of microvascular EC [Rubin 1992]. The endothelium also expresses a specific cadherin – VE-Cadherin (vascular endothelial cadherin) [Lampugnani 1992]. The adhesive function of E-Cadherin requires its attachment to the actin cytoskeleton, the association mediated by a set of proteins collectively named catenins [Kemler 1993, Lampugniani 1995]. In adherens junctions, the intracellular domain of E-Cadherin binds directly to β-catenin that in turn, associates with α-catenin, which is thought to link the cadherin complex to the actin cytoskeleton [Tsukita 1992, Fukata and Kaibuchi 2001]. Cadherins localize at intercellular AJ only when cells come into contact [Dejana 1995]. The first step is formation of E-cadherin-β catenin-α-catenin complexes at cell junctions in preconfluent culture. The next step is the anchorage of cadherins to actin cytoskeleton, which contributes to a strong and rigid adhesion [Tsukita 1992, Kemler 1993]. The association of E-cadherin with actin microfilaments creates a lateral tension, which acts as an opposition to the forces generated

by cell contact with the substratum and thus conteracts the cell spreading, and motility [Underwood 2002].

Thus the cell shape and the cytoskeleton organization might be controlled by the strength of AJ in the establishment of vascular endothelium integrity and to contribute to cell growth or differentiation [Dejana 1995].

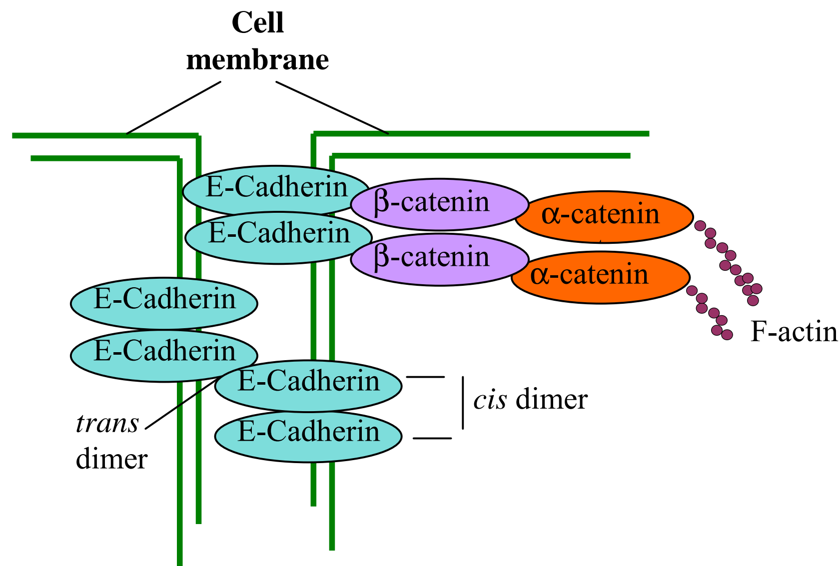


Fig. 17 Cadherin–mediated cell-cell adhesion. Cadherin calcium–dependent adhesion molecule is linked to bundles of actin filaments through β-catenin and α-catenin. Cadherins can dimerize in *cis* and *trans*, thereby forming rigid adhesions. By [Fukata and Kaibuchi 2001].

Many authors have reported the importance of AJ E-cadherin – catenin complexes for proper assembly of the AJ [Brieher 1996, Fukata and Kaibuchi 2001]. Disruption of these complexes by different agents as thrombin or inflammatory cytokines (TNF-α, INF-γ) [Lampugnani 1992] leads to increase permeability of EC monolayer and to leukocytes extravazation [Springer 1994]. Also recent data have suggested that when catenins are not bound to cadherins they can associate to other intracellular proteins and participate in signaling pathways contributing to tumorigenesis [Hülsken 1994].

In conclusion, both cell - substratum and cell - cell interactions through their specific adhesion receptors link to the cytoskeleton and play role in the establishment of the EC monolayer integrity [Nagahara and Matsuda 1996]. The formation of the EC monolayer strongly depends at the early stages on the cell - substratum interactions such as cell adhesion, spreading, migration and proliferation. At the later stage with the establishment of 2D tissue the cell – cell interactions predominate (see Fig.18).

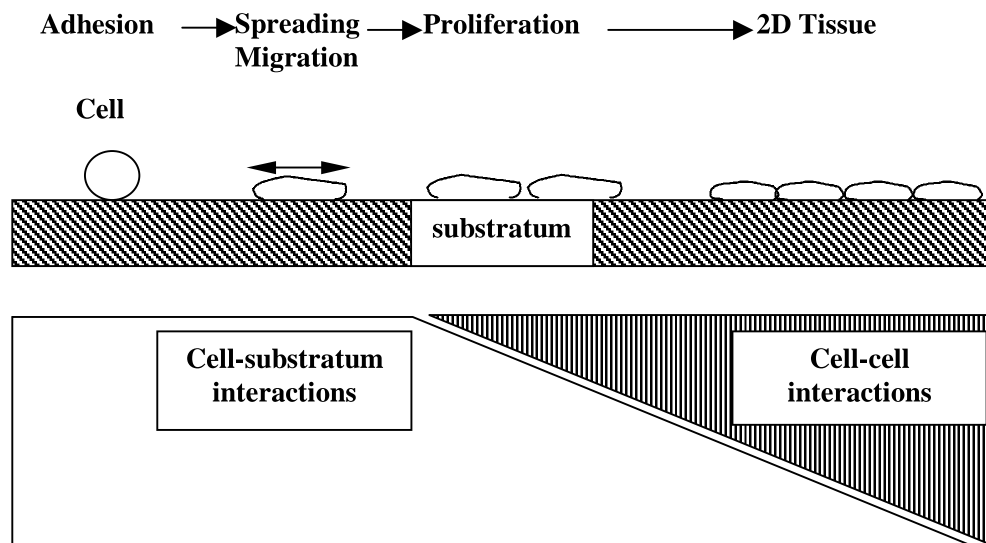


Fig.18 Dynamic process of formation of EC monolayer. The process is regulated by cell-substratum and cell-cell interactions. Their important contribution varies with time. From [Nagahara and Matsuda 1996].

2.5. Endothelization of polymer membranes.

2.5.1. General aspects

Most authors agree with the belief that the major reason for the failure of blood-contacting devices is the thrombogenicity of the internal implanted material surfaces.

Currently, various approaches aimed at molecular design of surface modifications of blood-compatible materials have been introduced with respect to the purpose and on the length of time the product will be exposed to blood [Ikada 1994]. For application requiring short-term

blood compatibility, it is important only that the device repels proteins and cells for a given time. The majority of surface modifications for short term blood compatible materials are covalent or non covalent immobilization of bioinert hydrogels onto the material surface, such as PEO [Ikada 1998, Park 2000]. Another method for a surface modification is using biologically active coatings that contain anticoagulants agents such as heparin, plasminogen activators and others [Ikada 1998]. Devices benefiting from the use of such kind of surface modifications are with longer contact with blood such as coronary stents and hemodialysis equipment.

Devices that will be permanently implanted in the body would meet benefits from being inert to both immune and coagulation system: the implanted devices will mimic the body to such a degree that they actually become invisible to the body's defense mechanisms. Artificial vascular grafts, stents, cardiac valve leaflets are examples of devices that remain in the body permanently and thus require a surface that will retain its hemocompatibility throughout its service life.

This understanding led to a shift in the focus of a research towards reconstructing the EC lining of the graft/arterial wall. Taking into account that complete prosthetic grafts endothelial lining does not occur spontaneously in humans despite a few reports [Wu 1985, Shi 1997], the concept of EC seeding before implantation has been developed to improve vascular prosthesis performance [Herring 1978, Burkel 1982]. Herring et al. were the first to suggest the ***in vitro*** lining of prosthetic implants with the host's own EC to prevent thrombus formation and to demonstrate, after implantation in dogs, that the concept was feasible [Herring 1978]. The ability to successfully seed vascular grafts with EC than became an attractive and promising approach in humans to improve long – term patency rates. The clinical benefit of this approach however, are not realized yet, as most of the biomaterials used for cardiovascular prosthesis are not designed to promote cellular adhesion in order to avoid induction of platelet activation and blood coagulation [Schneider 1993].

2.5.2. EC adhesion, spreading and proliferation on polymer membranes

Different methods exist to modify the surface of a biomaterial in order to promote EC adhesion. For instance, introduction of functional groups on polymer surfaces was shown to improve adhesion, spreading and proliferation of EC leading to formation of a confluent EC

layer. Various studies ***in vitro*** have shown that carboxyl and hydroxyl moieties are useful in this respect. [Curtis 1986]. Functional groups such as these can be inserted into the luminal surface of such materials using plasma modification, glow discharge and radiation-induced grafting, and wet-chemical technique [Pratt 1988, van Wachem 1989, Kirkpatrick 1991, Albrecht 2001]. Protein based surface modification is another technique for improving of EC adhesion [Bhat 1998]. Precoating of vascular grafts with plasma proteins such as FN [Kottke-Marchant 1996], FNG [van Wachem 1987], vitronectin [Steele 1995] or collagen [Deutsch 1997] enhanced EC adhesion. Coating graft surfaces with transglutine, a "fibrin glue" consisting of FN, FNG and vWF was also shown to enhance EC adhesion to graft surfaces [Mazzucotelli 1991]. Van Wachem et al. [van Wachem 1987] published detailed study on HUVEC adhesion and spreading on untreated poly (ethyleneterephthalate) (PET) and glow-discharge-treated PET, which usually generate COOH groups. They showed increased EC adhesion and spreading on modified PET. Furthermore, it was demonstrated that the adhesion, spreading and proliferation did increase on both surfaces after precoating these surfaces with whole blood, plasma, FN and FNG [van Wachem 1987]. However, in this study the single role of surface charge cannot be completely distinguish from surface wettability as modified PET exhibited water contact angle of 44 deg. versus 65 deg. for unmodified PET. The modification of so called "fibrin glue", consisting of FN, FNG, plasminogen, factor VIII, aprotinin and thrombin was used also by Zilla et al. to coat the internal surface of vascular prosthesis for better EC adhesion [Zilla 1989]. McAuslan and colleagues [McAuslan 1987] studied the vascular EC response to poly (hydroxyethylmethacrylate)(pHEMA) typical hydrogel, before and after surface modification by hydrolytic etching. Hydrolytic etching using sulphuric acid is capable to create negatively charged COOH groups. Poly HEMA, which without modification did not support mammalian cell adhesion, became excellent for attachment and growth of EC after the modification. Other different view gave Curtis et al. [Curtis 1986] for the influence of COOH groups on cell adhesion. He found that these groups slightly inhibit cell adhesion with the increasing of surface density of COOH groups on the surface.

2.5.3. Functionality of seeded EC monolayer (newly established endothelium)

Together with characteristics as adhesion, spreading and proliferation, the biochemical functionality of the established endothelial monolayer appears to be very important for maintaining the hemostatic balance between thrombogenic and anti-thrombogenic properties of the endothelium [Kirkpatrick 1999]. At physiological conditions the antithrombotic function of the endothelium predominates. Since PGI_2 is among the important antithrombogenic products of endothelium, the functionality of seeded EC can be assessed by measuring PGI_2 production by cells [Bhat 1998]. In several ***in vitro*** cell models it has been shown that, some of the specialized cell functions are rapidly lost when cells are removed from their natural ***in vivo*** environment [Orpana 1997]. Precoating of substrata with ECM proteins restores their original cell phenotype during their ***in vitro*** culturing. For instance, plating immediately after isolation of HUVEC on reconstructed ECM was resulted in high level of PGI_2 production [Orpana 1997]. The effect of the different protein coatings on PGI_2 production was studied also for unmodified poly (tetrafluoroethylene) (PTFE) and PTFE modified by ammonia plasma treatment [Sipehia 2001]. The extent of differences in PGI_2 production in relation to protein coating was influenced by surface chemistry of the biomaterials. For unmodified and neutral PTFE the different protein coating did not raise any significant differences in PGI_2 production. In contrast, for ammonia plasma treated PTFE, which carried charged amine groups gelatin induced significant higher amount of PGI_2 production when compare with FN and collagen.

Thus, the attempted endothelization of biomaterial surfaces for blood contacting devices must result not only in the formation of an intact EC monolayer but also in the maintenance of the EC functionality, so that, the delicate physiological balance between pro- and anti-thrombotic properties of the endothelium must be achieved.

3. Materials and Methods

3.1. Materials

3.1.1. Polymer membranes

3.1.1.1. Basic polymer membranes

Four flat membranes with different wettability were used. Fig.1 shows the structural formulas of the four membrane polymers used for membrane formation. The commercial Cuprophan® (CE) membrane was a gift from Akzo Nobel Faser AG, Membrana, Germany and polycarbonate-polyether (PC-PE) membrane was a gift from GAMBRO Dialysatoren GmbH&KG, Germany. Polysulfone (PSU) and polyetherimide (PEI) flat membranes were prepared as described in the following: PSU asymmetric flat membrane was prepared from a commercial PSU (type: ULTRASON® S, BASF, Ludwigshafen, Germany) by a conventional phase inversion process using a belt casting machine. The PSU polymer was solved in N, N-dimethylacetamide (DMAc) for 2 h at 80°C to a concentration of 15 wt.%, After cooling down to room temperature (RT°) the polymer solution was degassed and cast to a thin solution film on the woven support located to a steel belt. The steel belt and therefore the solution film were transported with a drawing speed of 0.5 m/min in a precipitation bath consisting of pure water at RTº. The casting slit width was 200 µm. After intense rinsing of the membrane with water, the membrane were tempered for 10 min at 90°C in an annealing device, dried at RTº and stored in dry state. According to the same principle, the PEI membrane was prepared from a commercial polymer (ULTEM® 1000. General Electric, New York, USA) using a 25 wt.% PEI solution in N-methylpyrrolidone (NMP) as solvent. The casting slit width was 250 µm and the drawing speed of the belt 2 m/min. In both cases flat membrane with a low porosity were obtained. The PEI membrane used later for functionalization was cast on a woven support.

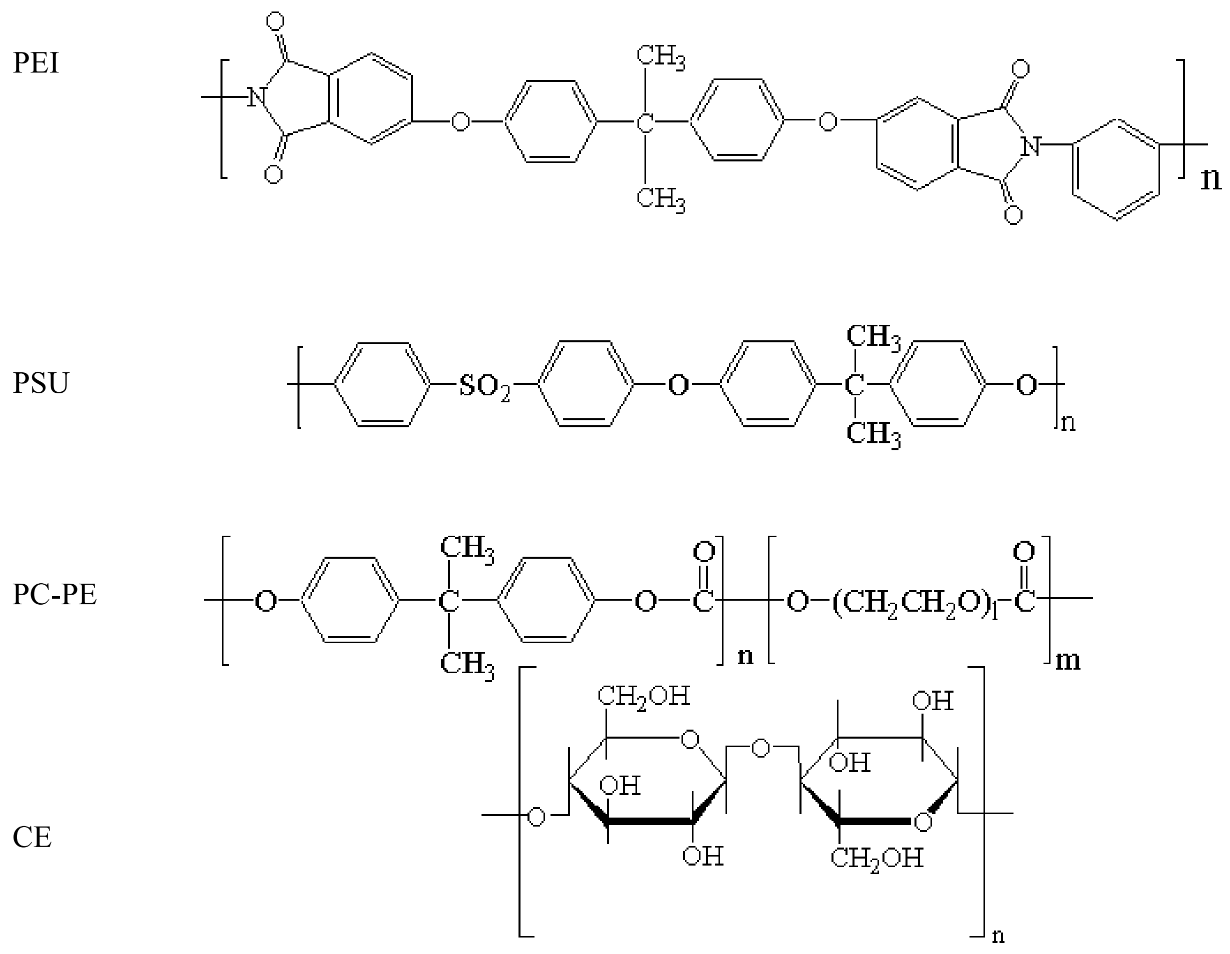


Fig.1 Chemical structure of the basic polymer membranes

3.1.1.2. Modified PEI membranes

A heterogeneous functionalization process as described above was used to modify the active layer of PEI membrane in order to introduce –COOH groups on the polymer surface. In this process the dry flat membrane was mounted onto a metallic cylinder of stainless steel (130 mm diameter) and contacted with the modifier solution under stirring at 70°C for 1 and 30 min. The modifier solution contained 2 wt-% of the sodium salt of iminodiacetic acid (IDA) solved in 1:1 mixture of 1-propanol and water. After quenching in cool water the membranes was demounted and was stored in wet state at 4°C until use.

3.1.1.3. Reference membranes

As a reference membrane for the EC study was used polyethylene terephthalate (PET) film with the thickness of 23µm and low porosity. The membrane was a generous gift from Oxyphen GmbH, Dresden, Germany. The water contact angle (CA) was found to be 77±0.99 degree.

3.1.2 Model surfaces (hydrophilic and hydrophobic glasses)

To obtain hydrophilic surfaces glass slides (Superior-Marienfeld, Germany) were cleaned in 80 % ethanol for 15 min. After extensive washing with distilled water, the glass slides were dried at 120° C for 120 min and kept in dry places until use. To obtain hydrophobic surfaces, glass slides were treated first with solution of conc. H_2SO_4 and H_2O_2 in proportion of 3:1 for 15 min. After extensive washing the slides were dried at the same conditions as above. Then the slides were treated with dimethyloctadecylchlorosilane (ODS). The slides were incubated in 2 % (v/v) of ODS (purchased from Fluka, Neu–Ulm, Germany) in n-hexane (Merck, Darmstadt, Germany) for 1 h, then rinsed with hexane and ethanol (until the slides became transparent), washed with distilled water, and air-dried.

3.1.3. Proteins

Human plasma fibronectin (FN, Roche Diagnostics GmbH, Mannheim, Germany) and fibrinogen (FNG) purified also from human plasma, fraction I, type III (Sigma, Deisenhofen, Germany) were used.

3.1.4. Fluorescent labeling of the proteins

The labeling of FN or FNG with fluorescein isothiocyanate (FITC) were carried out according to the manufacturer’s protocol of Molecular Probes, Leiden, The Netherlands. Briefly, the proteins were

dissolved in freshly prepared 0.1 M sodium bicarbonate buffer (pH 9) to give the concentration of 2mg/ml. FITC was dissolved in DMSO to give a concentration of 10mg/ml. 100µl of FITC solution in DMSO was added drop by drop to 1 ml of protein solution and incubated for 1h at RT°. Gel filtration column Sephadex G-25 (PD-10, Pharmacia) equilibrated with PBS pH 7.4 (with 5 volumes of the column) was used for separation of labeled proteins from the free dye. First fluorescent band with the conjugated protein was collected and the absorbance (OD_{280}) of the solution was measured. The protein concentration was estimated by using the Lambert - Beer low: $A = \varepsilon_{280}\, c\, d$, where A is absorbance of the sample at 280nm, c is concentration, ε_{280} is extinction coefficient and d is the thickness of the quartz cell.

When Rhodamine Red (Molecular Probes) was used the protein were dissolved in bicarbonate buffer with pH 8.3.

3.1.5. Citrate Human Plasma

Human Blood Plasma in 4% Na-Citrate used without dilution was purchased from ZBK Special Apherese GmbH Berlin, Germany.

3.1.6. Cells

3.1.6.1. Platelet preparation

Blood was collected from healthy volunteers who had not taken any medication for at least 10 days prior experiments. Sodium citrate was used as anticoagulant (3,19 g/100 ml) at a blood: citrate ratio of 9:1. Platelet-rich plasma (PRP) was prepared by centrifugation of blood at 200 x g for 10 min. The supernatant PRP was collected and the blood was centrifuged at 2000 x g for 20 min to prepare platelet-poor plasma (PPP). The platelet count in PRP was adjusted to 200,000/µl by mixing PRP and PPP.

3.1.6.2. HUVEC

Human umbilical vein endothelial cells (HUVEC) were used between passages 2 and 8 to avoid senescence of cells. They were cultured in EC growth medium (Cell Lining GmbH, Berlin, Germany) supplemented with 2% fetal calf serum (FCS), basic fibroblast growth factor (bFGF, 1ng/ml), EC growth supplement/heparin (ECGS, 0.4%), Amphotericin/Gentamicin (50ng/50µg) at 37°C and 5% CO_2. Cells from about confluent cultures were harvested with 0.05% trypsin/0.6mM ethylenediaminetetraacetic acid (EDTA) (Sigma). Trypsin was neutralized with FCS.

3.1.7. HUVEC cell lysates

To prepare extracts of total cell protein HUVEC at density of $2x10^4$ cells/cm^2 were seeded on FN coated glass or ODS petri dishes in EC growth medium for 3 days at $37^\circ C$. The EC monolayers were washed three times with ice-cold PBS followed by the addition of 200µl lysis buffer (50 mM Tris HCI, pH 7.4, 20% glycerol (v/v), 0.1 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 40 µl of aprotinin, and 40 µl of leupeptin). Cell material was scraped from the petri dishes and transferred to 2.0-ml vials, and allowed to lyse for an additional 30 min on ice. Protein concentration was determined by the Bradford method (protein assay kit, Bio-Rad).

3.2. Methods

3.2.1. Characterization of carboxylated PEI membranes

The content of the carboxylic groups on the membrane surface was measured by binding of fluorescent dye thionin acetate (THA). THA is a cationic dye that labels the carboxylic (-COOH) by salt formation. After equilibration and subsequent washing the fluorescent cation is exchanged under acidic conditions and measured in solution. For conversion of salts into carboxylic groups the samples were incubated in 0.01N HCI in water/ethanol 1:1 for 1 h. After washing with distilled water the samples (disks with 25mm in diameter) were immersed into a solution of 10mg/l THA in ethanol. The samples were shaken at RTº for 12 h. After three short washes with ethanol the samples were immersed in exactly 10 ml 0.01 N HCI in water/ethanol 1:1 and shaken for 2 h at RTº. The solution was measured spectrofluorometrically at 620 nm (594 nm excitation) and compared with a standard curve of THA.

3.2.2. Contact angle measurements

The surface properties of the membranes were characterized by contact angle (CA) measurements against distilled water using a captive bubble technique with a K10 digital tensiometer from Kruess (Hamburg, Germany). Two different CA measurements with a vapor bubble (index"v") and an n-hexadecane bubble (index"a" for alkane) were carried out. The wettability of the model surfaces (glass and ODS glass) was assessed by the sessile drop method measuring of the static water contact angle.

3.2.2.1. **Calculation of surface energy from contact angle**

The interfacial tensions of both ternary systems are related in each case to the Young equation.

$$\gamma_{(wv)} \cos\theta_{(wv)} = \gamma_{(sv)} - \gamma_{(sw)} \quad (6)$$

and

$$\gamma_{(wa)} \cos\theta_{(wa)} = \gamma_{(sa)} - \gamma_{(sw)} \quad (7)$$

where $\theta_{(wv)}$ and $\theta_{(wa)}$ are the respective contact angles for the water/vapor and water/alkane interface on the membrane (index "s" for solid). The combination of equation (6) and (7) gives

$$\gamma_{(wv)} \cos\theta_{(wv)} - \gamma_{(sv)} = \gamma_{(wa)} \cos\theta_{(wa)} - \gamma_{(sa)} \quad (8)$$

This equation contains two unknowns, γ_{sv} and γ_{sa}. In the calculations was followed the model proposed by Fowkes [Fowkes 1962, 1963], who divided the surface tension into components due to dispersive (d) and non-dispersive (p) contributions:

$$\gamma_i = \gamma_i^d + \gamma_i^p, \text{ (i=sv or av)} \quad (5)$$

Then the polymer surface free energy γ_{sv} might be expressed as sum of two terms, where γ_{sv}^d does comprise dispersion (London), orientation and induction interactions in the condensed state, and the polar part γ_{sv}^p summarizes hydrogen bonding type interactions. With the so-called Hamilton approach in combination with the harmonic mean approximation the following equations can be derived.

$$\gamma_{sv}^d = \frac{\gamma_{av}(\gamma_{wa}\cos\theta_{wa} - \gamma_{wv}\cos\theta_{wv}) - (\gamma_{av})^2}{\gamma_{wv}\cos\theta_{wv} - \gamma_{wa}\cos\theta_{wa} - 3\gamma_{av}} \quad (9)$$

$$\gamma_{sv}^p = \frac{\gamma_{wv}^p\left[\gamma_{wv}(1+\cos\theta_{wv}) - \frac{4\gamma_{sv}^d\gamma_{wv}^d}{(\gamma_{sv}^d + \gamma_{wv}^d)}\right]}{4\gamma_{wv}^p + \frac{4\gamma_{sv}^d\gamma_{wv}^d}{(\gamma_{sv}^d + \gamma_{wv}^d)} - \gamma_{wv}(1+\cos\theta_{wv})} \quad (10)$$

Using n-hexadecane as alkane with the following interface tensions to vapor and water (γ_{av} = 27.64 mN/m and γ_{wa} = 53.77 mN/m) and with the water /vapor value of γ_{wv} = 72.8 mN/m, can be calculated from the experimental contact angles θ_{wv} with equation (9) first the γ_{sv}^d values. Then with the value for the dispersive water surface tension $\gamma_{wv}^d = 21.8 \pm 0.7$ mN/m and with $\gamma_{wv}^p = \gamma_{wv} - \gamma_{wv}^d =$ 51.0 mN/m also is calculated the polar part of the solid-vapor tension γ_{sv}^p. The γ_{sv} values have been obtained from receding contact angle measurements.

3.2.3. Atomic Force Microscopy (AFM)

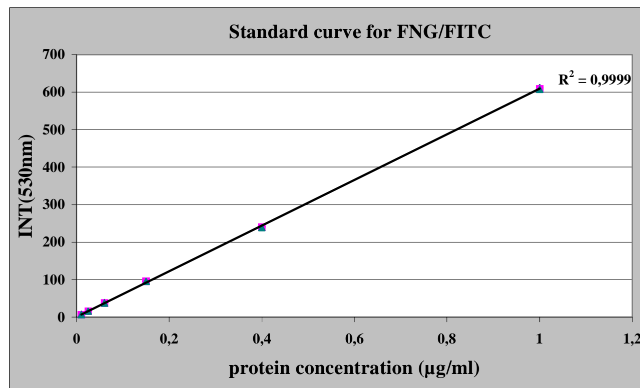
AFM images of the investigated membranes were analyzed by Atomic Force Microscope Nanoscope IIIA (Digital Instruments Inc., Santa Barbara, CA). Pointprobe siliconcantilever tip was used in contacting mode by the accompanying using Nanoscope IIIA software (version 5.12b15) the surface roughness of investigated membranes was determined. The mean value of the surface relative to the center plan Ra was calculated by the following equation $Ra = 1/LxLy\int_0^{Ly}\int_0^{Lx} [f (x, y)]\, dxdy$, where f (x, y) is the surface relative to the center plan and Lx and Ly are the dimensions of the surface. The evaluation of the roughness parameters of each membranes sample was based on three scanned areas of 40μm/40μm.

3.2.4. Desorption of plasma proteins by different eluting agents

The investigated membranes were pre-wetted with 50% ethanol for 30 min. After intensive washing in distilled water membranes were incubated with 100% Human Citrate Plasma for 1h at 37°C. After adsorption the plasma was discarded and the membranes were washed with 10mM PBS, pH7.4 four times. The desorption step was carried out using solutions of 2.5%SDS, 1%Triton-X-100, 1%Tween-20 and 0.5M NaCL (all dissolved in 10mM PBS, pH7.4) for 1h at 37°C. The supernatants were precipitated using acetone at – 20°C for 15h and centrifuged at 4000rpm for 15 min. The pellets were dissolved in 500μl of Tris 0.125M pH 7.4 and the total protein content was measured using Bradford assay (Bio-Rad).

3.2.5. Fluorescent method for protein adsorption (adsorption of FITC-labeled FNG)

The adsorption isotherms were calculated using a fluorescent-labeled protein technique for estimating the protein concentration. FITC labeled FNG in PBS (pH=7.4) at concentrations in a range between 50μg/ml – 200μg/ml was adsorbed onto the polymer membranes placed in 24 well chambers. After 1h adsorption at 37° C, the substrates were rinsed with PBS. The adsorbed protein was eluted with 1.0 ml 0.2 N NaOH for 2 hours. Each supernatant was transferred to a quartz cuvette with 1cm path length and the intensity of fluorescence was measured with a Luminescence spectrometer LS50B (Perkin Elmer Ltd, England). The excitation and emission wavelengths were set at 488/10 nm and 530/10 nm respectively. The calibration was performed at identical conditions with 0.01, 0.025, 0.06, 0.15, 0.4 and 1.0 μg/ml FNG/FITC in triplicate. The calibration fit was done by first order regression.



Validation of the method: The degrees of labeling (ratio between FITC and protein molecules) were measured before and after desorption with NaOH. There was no significant difference, which confirmed that the covalent bond between the marker and protein molecule was not disturbed by the basic conditions of the desorption step. However we have to consider the limitation of this technique since we cannot measure protein directly on the surface. Nevertheless using this technique protein adsorption in ng scale can be detected.

3.2.6. Enzyme immunoassay (EIA)

3.2.6.1. Adsorption/conformation of FNG adsorbed from plasma to basic membranes.

The investigated membranes were preadsorbed with 100% human plasma for 1 h at 37° C. The conformational state of adsorbed FNG was studied using an enzyme immunoassay. The method is based on the different binding affinity of poly - (pAb) and monoclonal (mAb) antibodies to adsorbed FNG. Polyclonal anti-human FNG (Sigma, F 2506) was used (diluted 1:2500 with 1%BSA in PBS) to quantify the total amount of FNG bound to the membranes. The mouse monoclonal anti-human FNG antibody (Clone 85D4 Sigma, F 9902), which recognizes a conformational sensitive epitope of the γ chain (302-303), was used at the same dilution to measure the accessibility of the D domain – a potential ligand for platelet binding.

The protein adsorption experiments were carried out in 24 wells test chamber where the bottom of the wells consist of the polymer membranes. For each membrane 500µl 100% citrate plasma was pipetted to four sample wells while another two wells were filled with the same amount of PBS (then used as a blank). The wells were left for adsorption for 1h at 37°C. The well chamber was covered to avoid evaporation during incubation. The test surfaces were rinsed three times with PBS then, 200 µl of mAb or pAb solution were added and incubated at 37° C for 1h. The wells were then rinsed with PBS and 200µl peroxidase conjugated rabbit anti - goat IgG, or the same amount of peroxidase conjugated rabbit anti–mouse IgG (at dilution 1:20 000) were added and further incubated for 30 min. After a

new washing procedure, the polymer flat membranes were moved into a clean test chamber to eliminate the influence of adsorbed protein to the inner walls of the Teflon upper part of the test chamber. 200 µl OPD (o-phenylene diamine) in 0.05 M phosphate citrate buffer (pH=5.01) with hydrogen peroxide (0.03%) was added and incubated for 10 min at RTº. The reaction was stopped adding 200 µl 1 M H_2SO_4. Part of the dye solution (200µl) was pipetted into 96 well polystyrene plate (Costar, Corning Incorporated, USA) and the optical density (OD) were read at 492 nm with a SPECTRA Fluor Plus, TECAN, Austria.

3.2.6.2. Adsorption/conformation of FN and FNG adsorbed from single solution to glass and ODS.

Antibodies. Four different primary antibodies were used as follows: goat polyclonal anti-human FNG (Sigma, F 2506), mouse monoclonal anti-human FNG antibody (Clone 85D4 Sigma, F 9902) specific for the conformational changes in D domain in FNG, polyclonal rabbit anti-human FN (Sigma, F 3648) and mouse monoclonal anti-human FN (Chemicon, MAB 1926), which is recognizing the RGD sequences in FN molecule. The rabbit anti-goat IgG-peroxidase conjugated (Sigma, A 5420), goat anti-rabbit IgG-peroxidae conjugated (Sigma, A 4914) and rabbit anti-mouse IgG-peroxidase conjugated (A 9044) secondary antibodies were used.

Procedure. FN or FNG (20µg/ml) was adsorbed on glass and ODS glass cover slides (1.8/1.8 cm) for 30 min at RTº. The volume of 500µl was used to cover the slides. Each slide was then individually rinsed with 10mM PBS three times. The slides then were blocked with 2% of BSA in PBS for 1 h at 37°C. The primary antibodies at a dilution of 1:1000 (for polyclonal antibodies) and 1:600 (for monoclonal antibodies) were added to the slides and incubated for 1h at 37°C. Each slide was individually rinsed with PBS three times. The secondary peroxidase conjugated antibody in a dilution of 1:10 000 (for polyclonal primary) antibodies or 1:20 000 (for monoclonal primary) antibodies was added for 1h at 37°C. The slides were rinsed three times and followed by development with 3, 3', 5, 5' – tetramethylbenzidine (TMB) substrate solution at RTº for 10min. The reaction was stopped adding 1 M HCL. Part of the dye solution (100µl) was pipetted into 96 well polystyrene plate (Costar, Corning Incorporated, USA) and the optical density (OD) was read at 450 nm with a SPECTRA Fluor Plus, TECAN, Austria.

3.2.6.3. Adsorption of FN and FNG adsorbed from single solution to modified membranes.

Antibodies. Two different primary antibodies were used as follows: goat polyclonal anti-human FNG antibody (Sigma, F 2506) and rabbit polyclonal anti-human FN (Sigma, F 3648). As secondary

antibodies were used rabbit anti-goat IgG-peroxidase conjugated (Sigma, A 5420) and goat anti-rabbit IgG-peroxidase conjugated (Sigma, A 4914).
The EIA procedure was the same as was described above. The primary antibodies were used at a dilution of 1:1000 and the secondary peroxidase conjugated antibody were used at a dilution of 1:10 000.

3.2.7. Substrate and membrane coating

When indicated, the slides and the membranes were precoated for 30 min at RT° with FITC-FNG or FITC-FN (40µg/ml), or intact FNG or FN (20µg/ml), respectively. For some experiments Rhodamine-conjugated FNG was used to coat the slides at 40 µg/ml as described above.

3.2.8. Immunofluorescence microscopy

Immunofluorescence microscopy was carried out with a Confocal Laser Scanning Microscope (CLSM, LSM510, Zeiss, Germany).

3.2.8.1. Platelets

Immunofluorescence for GPIb (which is abundantly expressed) and for P-Selectin (expressed only in activated platelets) was carried out as followed. After 1h contact of PRP with the membrane discs (d=13mm) at 37° C, samples were washed with PBS, followed by a fixation with 3% paraformaldehyde (PFA) and saturation with 1%BSA in PBS. Labeling of the platelets was performed with a mouse monoclonal antibody CD42b (anti-GP Ib) (Immunotech SA, Marseilles, France) or mouse antibody CD62P (anti-P-Selectin, Immunotech SA) at dilution 1:100, followed by 1:200 diluted polyclonal goat anti-mouse IgG antibody, Cy2™ – conjugated (Jackson Immuno Research Laboratories, USA).

3.2.8.2. HUVEC

3.2.8.2.1. Vinculin staining

HUVEC at density of $3x10^4$ cells/ml were incubated in EC growth medium on slides coated with FN (20µg/ml) or FNG (20µg/ml) for 2h in six-well tissue culture plates (Falcon, Becton Dickinson, USA). Then, cells were fixed in 3% PFA for 15 min, permeabilized with 0.5% triton X-100 (5 min), saturated with 1% BSA in PBS (30 min), pH 7.4 and incubated for 30 min at RT° with mouse anti-human vinculin (Sigma, clone h Vin-1) from Sigma at dilution of 1:100. The first antibody was visualized with goat anti-mouse IgG-Cy2™-conjugated (1:200 dilution) as the slides were incubated

at RT° for 30 min. Then samples were washed with distilled water and mounted with Mowiol on objective slides and studied with CLSM.

3.2.8.2.2. Remodelling of substratum-bound or soluble FN and FNG by HUVEC

Reorganization of substratum-bound FN and FNG was observed by incubation of HUVEC on hydrophilic or hydrophobic slides (1.8/1.8cm) precoated with FITC-FN or Rhodamine-FNG. After 4h of incubation at 37°C in 10% serum-containing medium, cells were fixed with 3% PFA, washed and mounted in Mowiol.

For evaluating of the organization of soluble FN and FNG cells were incubated for 1h on FN coated substrata and then FITC-FN or FITC-FNG (100µg/ml) was added for an additional 2h of incubation. Subsequently the samples were fixed, mounted and viewed with CLSM.

3.2.8.2.3. Distribution of integrin receptors on the ventral and dorsal cell surface

To detect integrin clustering on the ventral cell site, cells were incubated for 1h in serum-free medium (basal EC growth medium, Cell Lining) on glass and ODS slides. The slides were coated with FN (20µg/ml) for visualization of the β_1 integrin, and with FNG (20µg/ml) for β_3 integrin, respectively. Cells were then fixed, permeabilized, saturated with 1 % BSA as described above, and incubated for 30min with monoclonal anti-β_1 or monoclonal anti-β_3 antibodies (1:100), respectively, and visualized with goat anti-mouse IgG-Cy2™-conjugated as a secondary antibody (1:200 dilution). For detection of integrins on the dorsal cell surface, the cells were processed as was described above but without the permeabilization step. The samples were studied with CLSM.

3.2.8.2.4. Co-localization experiments

To detect co-localization between FNG and FN or between FNG and β_1 integrin, HUVEC were incubated on FN (20µg/ml) coated slides for 1h. Soluble FNG-Rhodamine (100µg/ml) in the presence of 10% FCS was added and the cells were further incubated for 2h. After the washing procedure the cells were fixed and saturated with 1% BSA to suppress the non-specific antibody binding. Then cells were incubated with primary monoclonal anti-FN and anti-β_1 antibodies, as specified above for 30 min, and then washed. The distribution of the labeled proteins was visualized with goat anti-mouse IgG-Cy2™-conjugated secondary antibody using CLSM.

3.2.8.2.5. E-Cadherin staining

HUVEC at density of $3x10^4$cells/ml were seeded on FN or FNG coated glass or ODS slides and incubated for 3 days at 37°C and humidified atmosphere with 5% CO_2/95% air. The samples were rinsed once with a basal EC growth medium and fixed with 3% PFA for 15 min at RT°. After three wash cycles the fixed cells were incubated with 1%BSA in PBS for 30 min. The cells rinsed three times with PBS were incubated with primary monoclonal anti-human E-Cadherin antibody (Transduction Laboratories) diluted 1:100 in PBS with 1% BSA for 30 min at 37°C. After washing three times in PBS cells were incubated with the secondary rabbit anti-mouse IgG-Cy2™-conjugated antibody diluted 1:200 in PBS with 1% BSA. After 30 min of incubation at 37°C the slides were rinsed twice in PBS and once in distilled water and mounted on objective glasses using Mowiol. The samples were analyzed by CLSM.

3.2.9. Actin staining

Actin staining using BODIPY 558/568-conjugated phalloidin (Molecular Probes, Netherlands) was applied to visualize the overall cell morphology and the organization of actin cytoskeleton of HUVEC. For that purpose approximately $3x10^4$ cells/ml were incubated in EC growth medium for 2h in six-well tissue culture plates (Falcon, Becton Dickinson, USA) containing the slides. Then, cells were fixed in 3% PFA for 15 min, permeabilized with 0.5% Triton X-100 (5 min), saturated with 1% BSA in PBS (30 min), pH 7.4 and incubated for 30 min at RT° with $4Uml^{-1}$BODIPY-conjugated phalloidin. Then samples were washed with distilled water and mounted with Mowiol on objective slides and studied with CLSM.

3.2.10. Cell attachment on glass and ODS glass

The CLSM images of HUVEC stained for actin were used to analyze the cell attachment. 10 representative images from each sample were used for cell counting. A cut drawing tool was used to outline individual cells and the cells number was calculated using KS 300 software (Zeiss, Jena, Germany). Only those cells with greater than 80% of the cell area contained within the images were analyzed.

3.2.11. Cell attachment and growth on polymer membranes

HUVEC at density $3x10^4$cells/ml were seeded on the membranes in EC growth medium. The cells were incubated for 2h (for cell attachment experiment) or 48h (for cell growth experiment) at 37°C in a humidified 5% CO2/ 95% air atmosphere. The number and the viability of the HUVEC on the

various membranes were determined by staining with 0.4% trypan blue and counting with Neubauer cell chamber.

3.2.12. Scanning Electron Microscopy (SEM)

The morphology of unmodified PEI and both modified PEI membranes were investigated by SEM. For that purpose, the membranes were fractured in liquid nitrogen and coated with gold/palladium (80/20) under vacuum. The prepared samples were studied in a JSM 6400-F field emission scanning electron microscope (Joel, Japan) at an acceleration voltage of 5 kV.

3.2.13. Western Blotting

To detect E-Cadherin, cellular protein extracts were separated by SDS-PAGE followed by Western immunoblotting. Protein samples were run in 4-12% Bis-Tris gel (Novex) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by semidry electroblotting in a buffer containing 15% methanol, 25mM Tris-HCI and 192mM glycine. Staining of the membrane with Red Ponceau was used to control the transfer. Membranes were blocked by incubation in blocking solution containing 5% nonfat dried milk in 10mM PBS, pH 7.4, and 0.1% Tween-20 (PBS-T) overnight at RT° with shaking. After four wash cycles of the membrane with PBS-T the membrane was incubated with the primary antibody (1:2500) mouse monoclonal anti-human E-Cadherin (Transduction Laboratories) diluted in PBS-T containing 0.1% BSA for 2h at RT° with shaking. Then the membranes were washed four times with PBS-T and incubated with the secondary antibody (sheep anti-mouse IgG-conjugated with horseradish peroxidase, Sigma) diluted 1:10 000 in PBS-T containing 0.1% BSA for 90 min. at RT°. After five washes with PBS-T the development was performed with an enhanced chemiluminescence detection system (ECL, Amersham, Uppsala, Sweden).

3.2.14. Immunoprecipitation

For immunoprecipitation of E-Cadherin in a cell extract, the protein was immunoprecipitated before SDS-PAGE and Western blotting analysis. The cell lysates at the protein concentration of 400µg was added to the empty spin columns and the PBS was added to give a volume of 500µl. 25µl of monoclonal anti-human E-Cadherin (Transduction Laboratories) was added and incubated overnight at 4°C with rotation. 30µl of protein G-Sepharose were added, and incubation was performed overnight at 4°C with rotation. The Sepharose was washed five times with PBS and immunoprecipitated protein was eluted by boiling the sepharose in 50 µl of Laemmli sample buffer

for 5 min at 95°C. 30µl of the supernatant were separated on a 4-12% Bis-Tris gel (Novex) and blotted onto PVDF membrane (see Western blotting) for immunoblotting analysis. The membrane was blocked with blocking solution overnight at RT° followed by incubation with 1:1000 mouse monoclonal anti-human β-catenin antibody (Zymed Laboratories Inc., South San Francisco, USA) in PBS-T containing 0.1% BSA for 2h at RT°. The membrane was washed four times and incubated with the second antibody (sheep anti-mouse IgG-peroxydase conjugated, Sigma) for 90 min. at RT°. Immunoactive protein was detected with the enhanced chemiluminescence system (ECL, Amersham, Uppsala, Sweeden).

3.2.15. Zymography

Zymography analysis for MMP-2 secreted by HUVEC was performed as HUVEC were seeded in serum free medium on FN and FNG coated glass and ODS coverslips at density of $4x10^4$cells/well. The cells were incubated till confluence and the supernatant was collected and kept at –70°C prior to use. 40 µl of the supernatant was dissolved 3:1 with non-reducing SDS sample buffer for10 min at RT°. Then samples were loaded on a 10% polyacrylamide gel containing 0.1% gelatine. After the electrophoresis, gels were renaturated in 2.5% TritonX-100 for 30 min at RT°. Substrate digestion was carried out by incubating the gel in 50mM Tris-HCL, pH 7.6, containing 5mM $CaCL_2$ and 0.2 M NaCL for 48h at 37°C. The gel was stained with 0.5% Coomassie Brilliant Blue R250 (BioRad) and the location of gelatinolytic activity was detected as clear bands in the background of a uniform blue staining. Arbitrary activity of an individual cleavage band was determined by scanning densitometry using 1D Image Analysis Software, Kodak Digital Science.

3.2.16. *In situ* Zymography on FITC-labeled Gelatine

Gelatine was dissolved in 0.25M sodium bicarbonate buffer pH 9.2 to give a final concentration of 1mg/ml, and was coupled to FITC for 1h at 4°C. HUVEC were grown on glass and ODS coverslips coated with FITC-gelatine for 4h. The samples were then fixed and analyzed by CLSM.

3.2.17. Prostacyclin assay

Secretion of PGI_2 by HUVEC was performed as cells at density of $7.5x10^5$cells/well were incubated on membrane discs (d=35mm). For basal production of PGI_2 the cells were incubated for 5 days and then the supernatant medium was collected, centrifuged (10min, 400g, 4°C) and stored at –20°C until use. For TNF-α stimulated secretion of PGI_2, HUVEC at the same density were cultivated for 24h and then were stimulated with TNF-α (10µg/ml) for 5h. The supernatant was processed as above.

PGI_2 concentration was determined using a competitive EIA for the stable hydrolysis product of PGI_2, 6-keto-prostaglandin F_{1a} (Amersham, England), a generally accepted measure for quantification of PGI_2.

3.2.18. Environmental Scanning Electron microscopy (ESEM)

The activation degree of adhered platelets on membranes seeded with EC was visualized by ESEM (30ESEM-FEG, Philips XL). For that purpose membrane discs (d=13mm) were precoated with FN or FNG and incubated with HUVEC for 3 days to reach a confluence. The samples were carefully rinsed once with PBS, pH 7.4 and incubated with PRP for 1h at 37°C. After removing the PRP the samples were rinsed with PBS and fixed with 2.5% glutaraldehyde for 30 min at RT°. The samples were rinsed 2 times in distilled water and were left in distilled water prior to the ESEM analysis.

3.2.19. Statistical analysis

All statistical computations were carried out with Graphpad Instat ®3.00 software (GraphPad Software Inc., San Diego, USA). The values were considered significantly different if the p value was < 0.05.

4. Results and Discussion

Part I. The influence of the materials surface properties on protein adsorption and platelet adhesion/activation.

In this part will be discussed the role of materials surface properties such as surface wettability for the thrombogenicity of blood contacting materials. For that purpose the role of plasma protein adsorption will be studied as the first and key determining step in blood-material interactions. The emphasis will be done on the conformational changes in adsorbed FNG as a function of the polymer surface wettability and material surface energetics. The rate of platelet adhesion and activation will be examined as a function of the degree of the conformational changes in adsorbed FNG.

4.1. Materials surface properties

4.1.1. Wettability

The observed advancing and receding water contact angles showed that PEI was the least wettable membrane followed by PSU and PC-PE. The most wettable membrane was CE with advancing contact angle of 12°. As can be seen there was no great difference in the advancing water contact angles between PEI and PSU ($p>0.05$). The block co-polymer PC-PE exhibited the highest hysteresis ~ 45° (difference between advancing and receding contact angle) most probably due to the heterogeneity arisen by microdomain surface structure.

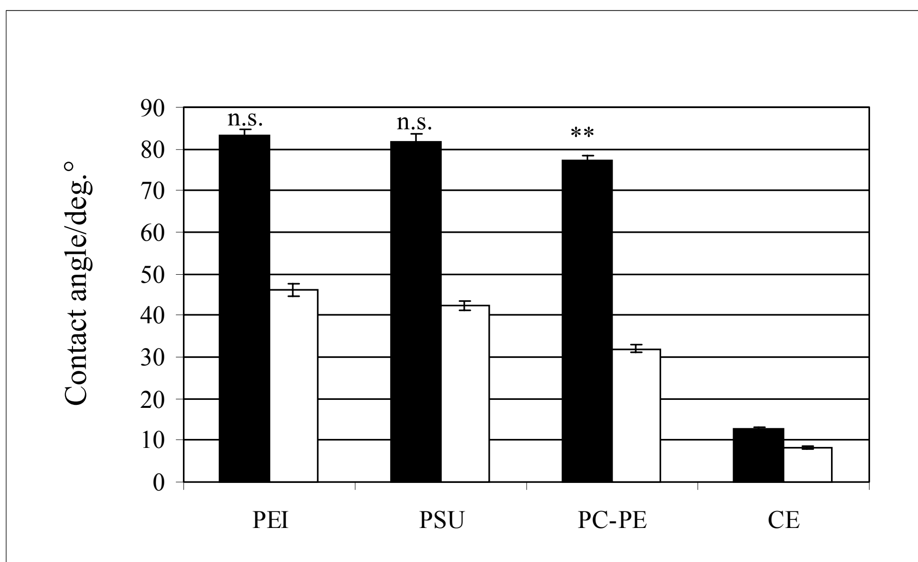


Figure 4. Advancing (black columns) and receding (white columns) water captive bubble contact angles for PEI, PSU, PC-PE and CE were measured at three different points on each membrane in quadruplicate. The error bars are two standard deviations in total height. T-test was used for the statistical analysis. n.s. – not significant, (**) - $p<0.05$.

4.1.2. Roughness (AFM measurements)

The AFM images revealed that CE, PC-PE and PEI membranes exhibited rather low average surface roughness (in nm) as follows 5.930, 12.369 and 6.414. In contrast PSU membrane showed a roughness approximately 10 times higher - 95.923 nm, which could be the explanation for the different FNG adsorption and platelet behavior on this substrate, discussed later in this part.

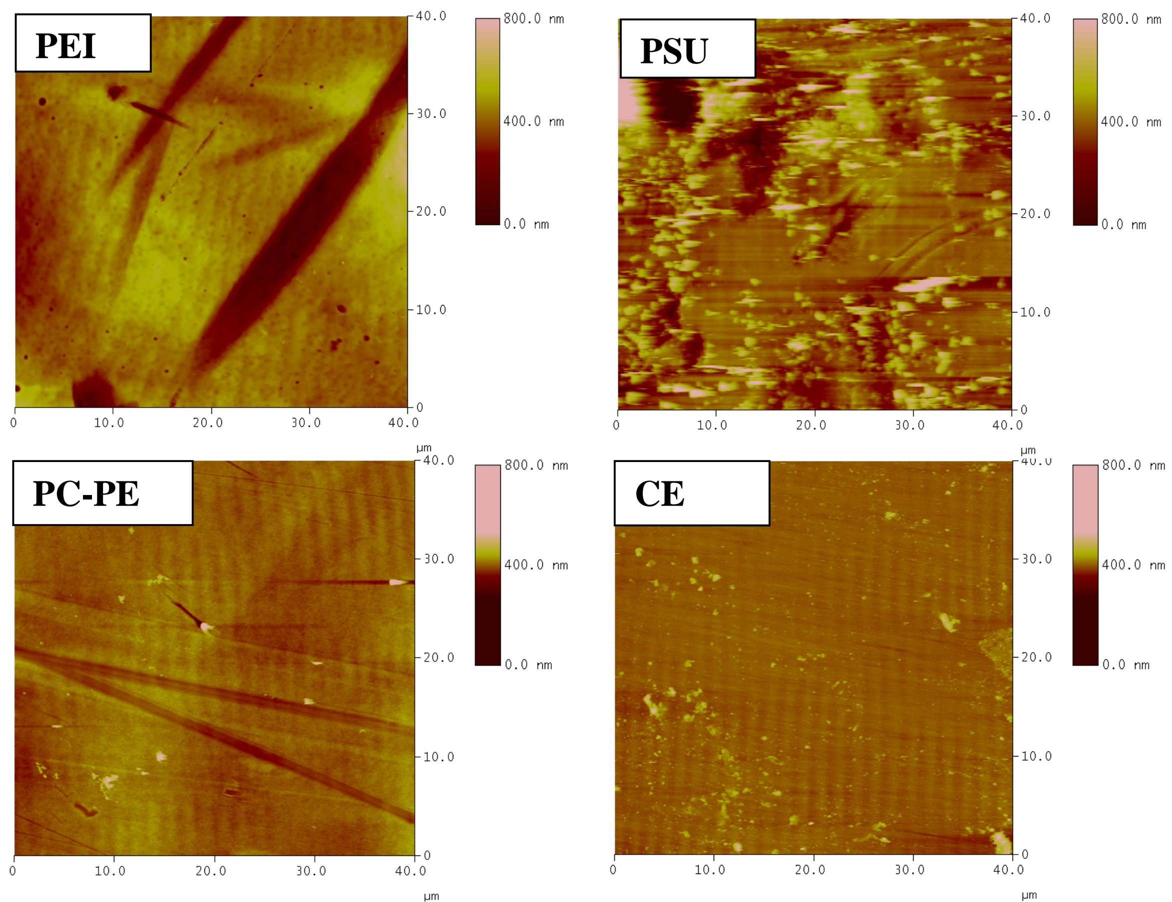


Fig. 5 Atomic force microscopic images of CE, PC-PE, PSU and PEI. The size of the images is 40µm/40µm.

4.1.3. Surface free energy

Table 1 presents the calculated γ_{sv} and its splitting up in the polar γ_{sv}^{p} and dispersion part γ_{sv}^{d}. First, a moderate increase in the total surface free energy of about 15 mN/m from PEI/PSU to CE was observed. Indicated by larger γ_{sv}^{p} - values, this is clearly caused by an increase in the number of polar groups, capable to hydrogen bond formation, which seemed to be much more available on the surface of the CE membrane. Further, the $\gamma_{sv}^{p}/\gamma_{sv}^{d}$ ratio correlates in many cases with protein adsorption and/or platelet adhesion to the polymer surface which will be discussed below. The following ratios were found: 1.08 (PEI), 1.52 (PSU), 1.92 (PC-PE) and 2.73 (CE). The $\gamma_{sv}^{p}/\gamma_{sv}^{d}$ ratio shows a higher degree of polar surface properties of PSU in comparison to PEI although the total surface free energy was very similar.

Table 1. Contact angle and surface free energy.

Polymer membrane	$\theta(vw)_r$	$\theta(hw)_r$	γ_{sv}^{d} (mNm^{-1})	γ_{sv}^{p} (mNm^{-1})	γ_{sv} (mNm^{-1})
PEI	**46.15**	**63.26**	**25.81**	**27.96**	**53.77**
PSU	**42.30**	**53.53**	**22.01**	**33.50**	**55.51**
PC-PE	**32.02**	**40.77**	**21.25**	**40.87**	**62.12**
CE	**8.21**	**10.11**	**19.74**	**53.83**	**73.57**

Water [$\theta(vw)_r$] and n-hexadecane [$\theta(hw)_r$] receding contact angles (in degrees) and the calculated surface free energy (γ_{sv}) and polar (γ_{sv}^{p}) and dispersion (γ_{sv}^{d}) part for the investigated membranes.

4.2. Protein adsorption

4.2.1. Total protein adsorption

With a desorption technique using different eluting agents the total protein adsorption from plasma to investigate membranes and the nature of protein-material interactions were studied. The results showed that the most powerful eluting agent for all membranes was SDS (anionic detergent) followed by Triton X-100 (nonionic detergent), which suggested that hydrophobic and electrostatic interactions were the main factor for protein adsorption on studied membranes. The highest protein amount eluted by SDS was obtained for PEI membrane (most hydrophobic membrane) followed by PSU, PC-PE and CE. Most interesting was the fact that the effectiveness of salt solution (0.5 M NaCI) to elute adsorbed plasma proteins was shown only on PEI membrane.

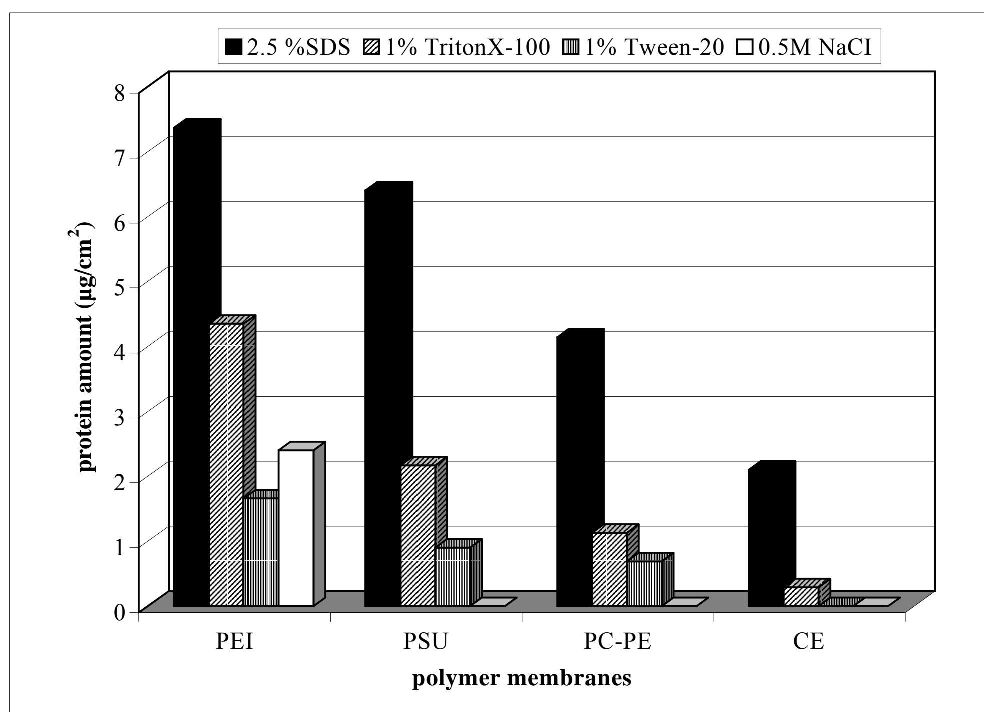


Fig. 6 Elutability of plasma proteins from polymers by using 2.5% SDS, 1% Triton X-100, 1% Tween-20 and 0.5 M NaCI. Membranes were preadsorbed with 100% Citrate Plasma for 1h at 37°C.

Nevertheless that PEI and PSU exhibited rather the same wettability (see Fig.4), the electrostatic interactions were more important for PEI than for PSU. One explanation of this phenomena could be the fact of the spontaneously generation of COOH groups on the PEI surface during the storage and adsorption procedure, which could attract the proteins by ionic interactions.

4.2.2. FNG adsorption (adsorption isotherms of FNG)

The degree of the protein adsorption and the affinity of FNG to the membranes were studied using single solutions of human FITC-labeled FNG. The adsorption isotherm data of FNG are presented in Fig.7 as plots of adsorbed protein concentration (C_s in µg/cm^2) versus the bulk protein concentration (C_b in µg/ml). The data points have been fitted by the two empirical isotherm equations (1) and (2). The resulting parameter values are given in Table 2.

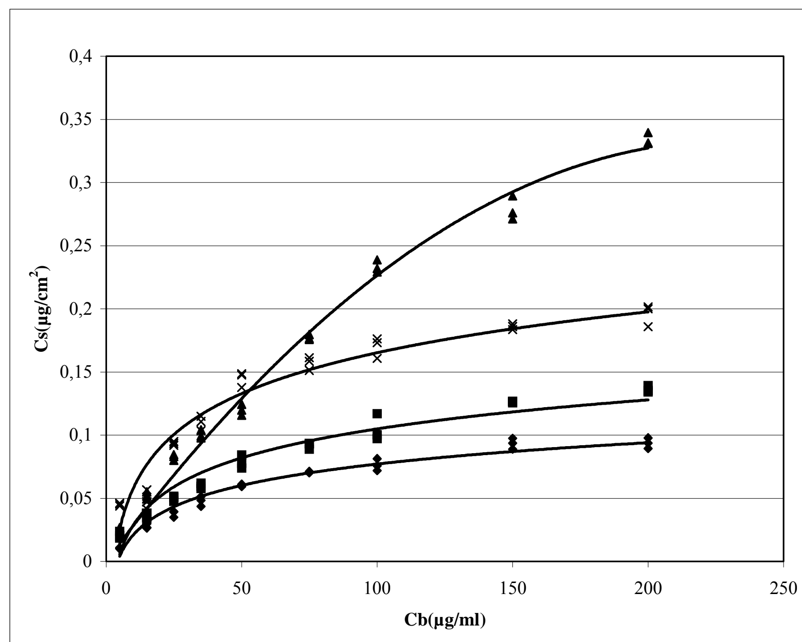


Fig. 7 Adsorption isotherms for FNG. Fluorescent labeled FNG was adsorbed for 1 h at 37°C on PEI (**x**), PSU (▲), PC-PE (■) and CE (◆). Each point was measured in triplicate.

The Langmuir equation (1) and Freundlich equation (2) fitted the experimental data with a similar accuracy. For PEI and CE the fit with the Langmuir model seemed to be accurate,

while for PSU and PC-PE the Freundlich model was fitted better. The curves in Fig. 7 are the plots of the Langmuir isotherm with the respective parameters of Table 2.
The analysis of the isotherms in Figure 7 demonstrated an increase in the adsorption with the diminishing degree of polarity of the surface, expressed by the ratio of the free surface energies (see above paragraph). This supports again the former finding that the hydrophobic interactions provide the main driving force for the adsorption of FNG and hydrogen bonds or ionic interactions play a subsequent role. The exception in our set of polymer membranes was PSU. The protein adsorption for PSU increased steadily with increasing bulk protein concentration and a plateau was not reached in contrast to the other membranes. The strength of interaction was lower compared to PEI and PC-PE (see respective K values in Table 2). However, the capacity to adsorb FNG was much higher.

Table 2. Parameters of Langmuir and Freundlich adsorption isotherms.

Equation	Parameter	PEI	PSU	PC-PE	CE
	k	**0.0202**	**0.0076**	**0.0092**	**0.0052**
Freundlich	***m***	**2.22**	**1.34**	**1.92**	**1.70**
(eq. 2)	**r^2 (n)**	**0.951**	**0.997**	**0.993**	**0.975**
	K [$cm^3/\mu g$]	**0.026**	**0.005**	**0.016**	**0.019**
Langmuir	**C_L [$\mu g/cm^2$]**	**0.24**	**0.66**	**0.18**	**0.12**
(eq. 1)	**r^2 (n)**	**0.968**	**0.993**	**0.984**	**0.988**

The adsorption parameters on four polymer membranes were calculated using the empirical adsorption equations (1) and (2). The accuracy of the fit is expressed by the correlation coefficient (r^2).

4.2.3. FNG adsorption/conformation

A clear increase in the amount of FNG adsorbed from plasma was detected by the polyclonal antibody (pAb) binding with decreasing wettability of the membranes as shown in Fig. 8. Nevertheless PEI and PSU had similar water contact angles, binding of pAb was much higher on PEI. On the other hand, the monoclonal antibody (mAb) against the conformational

sensitive epitope in the D domain showed approximately a 2-fold increase of binding for PEI and PSU in comparison to PE-PC and CE. However, PEI and PSU had almost the same binding activity. Further, we calculated the percentage of expression of the mAb binding epitope (Table 4) as a measure of the accessibility of the D domain, and thus as an indicator of conformational/orientational changes of FNG upon adsorption. As shown in Table 4 the percentage characterizing the accessibility of the D domain for PEI was significantly lower than for PSU.

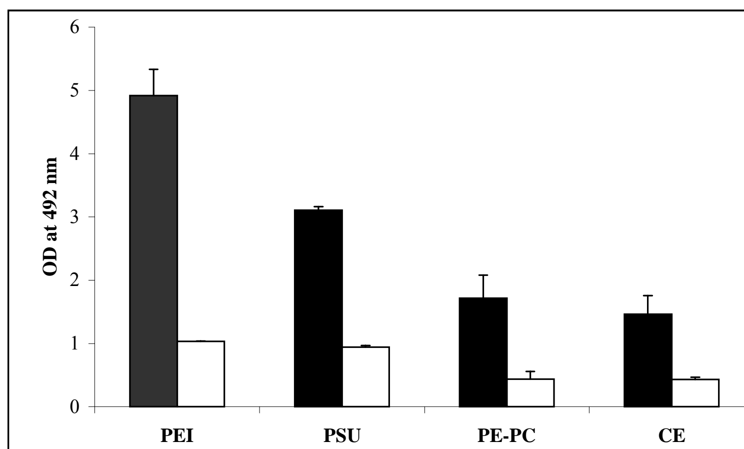


Fig.8 Polyclonal (black columns) and monoclonal (white columns) antibody binding to FNG adsorbed from 100% human plasma on PEI, PSU, PC-PE and CE. The absorbance (OD_{492}) data for polyclonal antibody for PEI and PSU were calculated with respect to dilution-4 times for PEI and 2 times for PSU. Data are means ± SD of four replicates from typical experiments out of three performed.

Table.4 Comparison of polyclonal and monoclonal antibody binding to adsorbed FNG.

membrane	pAb OD_{492}	mAb OD_{492}	Expression (in %)
PEI	4.92	1.03	20.93
PSU	3.11	0.94	30.23

FNG is adsorbed from 100% human plasma onto PEI and PSU. Percent D epitope expression (mAb signal) versus the total adsorbed FNG (pAb signal) was given as a potential measure for availability of D epitope of FNG for platelets.

4.3. Platelet adhesion/activation

4.3.1. Platelet adhesion

Platelet adhesion was visualized by CLSM using the monoclonal antibody CD42b. Significant differences in the amount of adhered platelets were observed (Fig.9 A-D). On CE (Fig.9 D) and PC-PE (Fig.9 C) membranes only single platelets with round shape were found. In contrast, on PEI and PSU (Fig.9 A-B) the amount of adherent platelets was remarkably higher. The morphology of cells on the PEI membrane showed many fully spread platelets expressing pseudopodia (Fig.10 A).

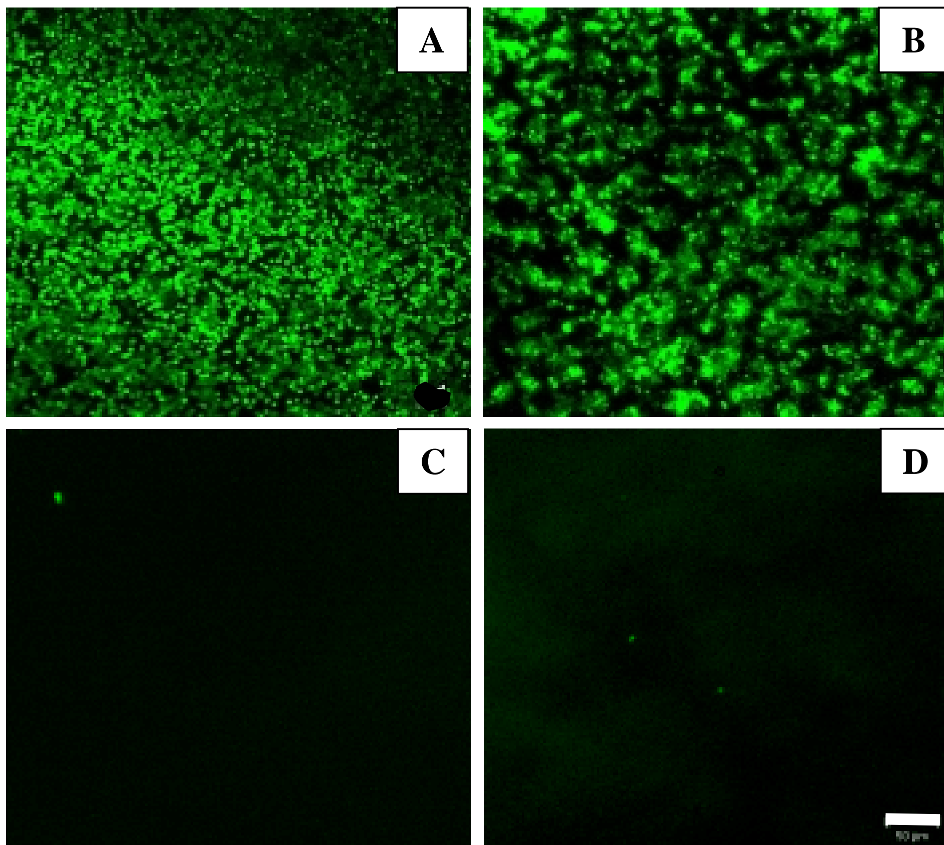


Fig.9 Detection of platelet adhesion to PEI (A), PSU (B), PC-PE (C) and CE (D) with CLSM. Adherent platelets were labeled with monoclonal antibody CD42b followed by IgG-Cy2-conjugated secondary antibody. Bar is 50µm.

Platelets on the PSU membrane however, exhibited a quite different morphology. Although a part of the cells also possessed pseudopodia, and some of them were well spread, most of the platelets had formed large aggregates (Fig.10 B).

4.3.2. Platelet activation

Platelet activation was studied by the membrane expression of P-Selectin using the CD62P antibody. The expression of P-Selectin was studied for PEI and PSU only, because of the lack of cells on CE and PC-PE. Using the same evaluation conditions for both membranes (laser intensity, amplification, etc), we found much less P-Selectin expression on PEI than on PSU (Fig.10 C-D).

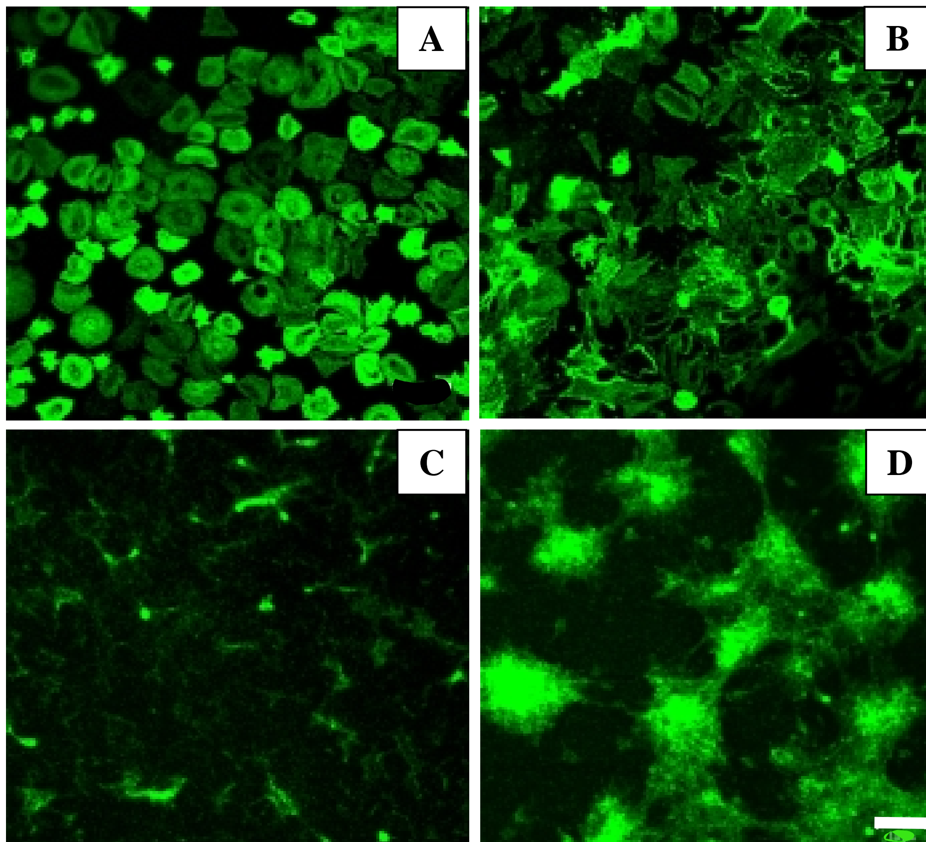


Fig.10 Detection of activated platelets on PEI and PSU with CLSM. Adherent platelets on PEI (A) and PSU (B) were labeled with monoclonal antibody (CD42b). The activation rate of adherent platelets on PEI (C) and PSU (D) was revealed by using monoclonal antibody CD62P. Bar is 10µm.

4.4. Discussion

4.4.1. Plasma protein adsorption to polymer membranes

The protein adsorption is the first and a rapid event, which occurs on all surfaces, exposed to blood, yet differences in the cellular responses to various surfaces clearly exist [Bohnert 1990]. The implied differences in the organization of the adsorbed protein layer have been attributed as well as to protein composition, amount and protein conformation after adsorption. The fundamental mechanisms underlying the interactions of proteins with artificial surfaces are therefore under active investigation. In order to investigate the nature of the polymer-protein interactions, eluting profiles of adsorbed plasma proteins from investigated membranes were studied using different eluting agents. The ionic detergent SDS is competing for hydrophobic and ionic interactions. The non-ionic detergents Triton X-100 and Tween-20 were used to break the hydrophobic interactions since a 0.5 M salt solution was used to break the ionic interactions. In general SDS, an ionic detergent was 2-3 times more effective than non-ionic detergents Triton X-100 and Tween-20. This observation highlights the contribution of both hydrophobic and ionic interactions for protein adsorption. The fact that 0.5 M NaCI solution was able to elute proteins only from PEI could be explained by the existence a population of proteins, which are bound preferentially by electrostatic interactions. This fact contributes to the understanding for the multiple states of protein adsorption, which include the presence of weakly and tightly bound proteins [Horbett 1991].

4.4.2. Surface free energy and protein affinity

FNG is a one of the major adhesive proteins governing platelet adhesion and activation [Tsai 1999]. Therefore it was interesting to study how the FNG adsorption on different wettable membranes may affect the platelet behavior. Changes in the adsorbed FNG have been studied with SDS elution [Rapoza 1990, Chinn 1991] and monoclonal antibodies that react with FNG binding domains [Horbett 1994, Grunkemeier 1996]. The FNG adsorption isotherms on different wettable membranes were studied in attempt to perform a correlation between surface wettability and the amount and the affinity of the FNG binding. The Langmuir model appeared to be a suitable model to describe the FNG adsorption on the most of the

investigated membranes. The analysis of the isotherms showed an increase in the FNG adsorption with the decrease of the wettability and free surface energy (Table 1). A higher degree of protein affinity for PEI with respect to PSU and the other membranes was also affected by the diminishing degree of the polarity of the surface, which supports again the former findings that the polar interactions play a second-rate role in protein adsorption. It can be expected that FNG will undergo unfolding on low energy surfaces to minimize the interfacial energy and maximize the protein-surface bonds. Since Perez-Luna et al. found a correlation between γ_{sv}^{d} and the resistance of the FNG elution by SDS from a variety of surfaces [Perez-Luna 1994], it was interesting to test if the FNG affinity to different wettable polymers was related to γ_{sv}^{d}. Moreover γ_{sv}^{d} might be considered, as a rough approximation, to the energy required for the replacement of water from the surface by adsorbed protein [Sigal 1998]. Hence, it can be expected that the protein affinity to the substrata will be proportional to γ_{sv}^{d}. Here, a tendency was shown that the increase of the dispersion component of surface free energy corresponded to an increasing FNG affinity. For PEI membrane the dispersion part of surface free energy almost matched the polar one. Concerning the fact that for human FNG the dispersion and polar component are similar (γ_{sv}^{d} = 4.96 (dyn/cm)$^{1/2}$, γ_{sv}^{p} = 3.67(dyn/cm)$^{1/2}$) according to Kaelble [Kaelble and Moacanin 1977], it might be one explanation for the highest affinity of FNG to PEI. Interestingly, PSU exhibited a different adsorption isotherm for FNG. The surface protein concentration increased steadily with increasing bulk protein concentration and a well–defined plateau was not observed in contrast to other membranes. The strength of interaction was lower compared to PEI (see respective K values in Table 2), but the capacity was much higher. A reason for that could be the formation of a second protein layer, or as Tsai et al. was reported that the excess of FNG might be bound to the surface in the form of macroscopic fibrin clots [Tsai 1999].

4.4.3. Platelet adhesion and activation

Platelets interact with FNG via their GP IIb/IIIa receptor in a receptor-ligand interaction. Therefore the conformation of FNG is important for adhesion and subsequent activation of platelets [Tsai 1999]. One of the methods providing an indirect evidence for the changes in

protein organization upon adsorption is the use of antibodies against specific protein epitopes. Because mAb's bind to a single protein epitope, while pAb's bind to multiple epitopes, mAb's rather than pAb's should be more sensitive to such changes [Farrell 1992, Tsai 1999]. Therefore using a combination of pAb and mAb against FNG is a useful approach to study the extent of changes in protein conformation on different substrata [Farrell 1992, Kiaei 1995, Tsai 1999]. Indeed, here was found a clear relationship between the surface wettability of the investigated membranes and the extent of conformational/orientational changes in adsorbed FNG. The results showed that the least wettable surface PEI caused higher changes in the state of adsorbed FNG with respect to the accessibility of D domain. One can relate these changes in adsorbed FNG to the binding strength of the substrata. This is in accordance to the data found for the FNG affinity (K-values in Table 2) of the membranes. Since protein affinity was the highest for PEI a great protein unfolding on this membrane might be expected. The findings here support the hypothesis of Tanaka et al. that less wettable surfaces cause unfolding by tightly binding of the adsorbed FNG and then promote platelet adhesion, because of the exposure of the binding sites for platelets [Tanaka 2000]. In contrast when the adsorbed protein is close to the native state, it does not support platelet adhesion and aggregation [Tanaka 2000]. This is also in agreement with our results for platelet adhesion and activation. We observed that on the more wettable membranes PC-PE and CE only single platelets adhered (without changes in their morphology), which is probably due to the loosely bound FNG near to the native state. Especially for PC-PE, the higher heterogeneity of the substrata (see the hysteresis in contact angle measurements in Fig. 2) could also be considered for the weak protein adsorption and thus as a repelling factor for platelets. In contrast on the less wettable membranes PEI and PSU a lot of platelets adhered in correlation with the observed larger amount of adsorbed FNG.

One of the most important findings was the observed difference in the platelet morphology between PSU and PEI. Nevertheless that the both membranes do not differ significantly in their wettability they showed strictly different properties with respect to the platelet morphology and P-Selectin expession. A high degree of platelet aggregation corresponding to the much stronger P-Selectin expression was detected on PSU membrane. In contrast, on PEI the platelets were well spread, and neither aggregation nor a strong P-Selectin activation

was found. One possible interpretation of this observation could be the different conformational/orientational state of FNG after adsorption. Platelets induce thrombosis by several modes of action: secretion of bulk phase agonists, acceleration of thrombin production and via FNG mediated platelet-platelet aggregation [Grunkemeier 2000]. The latter can be greatly influenced by the conformational state of surface-bound FNG. It is well known that soluble FNG plays a role as a molecular bridge for the aggregation of platelets [O'Toole 1994]. During this process the FNG receptors have to be re-localized to facilitate this interaction. Estry et al. [Estry 1991] observed that the receptor translocation was induced only by substrate-bound FNG. Apparently, a different state of adsorbed FNG will affect receptor translocation and subsequently the degree of platelet aggregation [Horbett 1994]. Therefore, one could suppose that conformational changes in the D domain of adsorbed FNG on PEI, leading to low domain accessibility, may hinder the translocation of the FNG receptor and would suppress platelet aggregation. Similar observations on the inhibition of receptor translocation in fibroblasts on hydrophobic surfaces were published recently concerning the β_1 and α_v integrins [Altankov 1997, Groth 1999].

In contrast, the conformation of adsorbed FNG on PSU apparently facilitated platelet aggregation. In addition, should be considered the role of higher surface capacity of PSU. It is rather well studied that the surface topography could enhance the cell adhesion by inducing a spatial reorganization of adsorbed proteins [Curtis and Wilkinson 1999, Mondon 2003]. In this respect, the surface roughness of PSU, which was shown to be approximately 10 fold higher than on other membranes, and the observed higher capacity of PSU, should also contribute to the possible larger extent of fibrin formation on this membrane. As a result a higher platelet adhesion and spreading could be expected. Also, the role of other adhesive proteins in the promotion of platelet aggregation could not be excluded. For instance, it was reported that fibrin clots enhanced platelet procoagulant activity by binding vWF from plasma [Kumar 1995, Beguin and Kumar1997, Beguin 1999]. Therefore vWF has to be considered as a strong promoter of platelet adhesion and activation and a key protein accelerating P-Selectin expression [Nygren and Broberg 1998, Broberg and Nygren 2001].

Part II. Interaction of HUVEC with model surfaces. The influence of surface wettability on protein adsorption and cell behavior

The endothelization of the polymer materials is a promising approach for improving the hemocompatibility of the blood-contacting materials. In Part I was shown that the surface wettability has influenced greatly the platelets behaviour like as adhesion and activation through the different conformational state of adsorbed FNG. Therefore here a model system consisting of hydrophilic glass and hydrophobic ODS glass is introduced in order to study the EC behavior as a function of surface wettability and the amount/conformation of adsorbed adhesive proteins. The ability of EC to adhere, to form cell-substrate and cell-cell contacts will be discussed in the light of material biocompatibility. A special emphasis on ECM remodelling by EC will be done as an important factor for proper cell functioning.

4.5. Adsorption/conformation of FN and FNG adsorbed on glass and ODS glass.

The wettability of the model surfaces was assessed by the sessile drop method measuring the static water contact angle on three different slides for each material. The CA for hydrophilic glass was found to be 24±2.04 degree, while the CA for ODS glass was 86±3.88 degree.

The FN and FNG adsorption to glass and ODS glass was studied using a set of polyclonal and monoclonal antibodies. The polyclonal antibody was used to quantify the total amount of the adsorbed protein and the monoclonal antibody was directed against the conformational sensitive cell binding epitope in the protein molecule and therefore was informative for the conformational changes in adsorbed proteins, which could alter the biological function of adsorbed proteins. The total amount of both adsorbed FN and FNG was higher on ODS glass than on glass. The percentage of expression of monoclonal antibodies revealed that on ODS glass the accessibility of cell binding domains was considerably decreased. ODS glass caused a decrease in the accessibility in the cell binding domains with 16% for adsorbed FN and with 14% for adsorbed FNG when compared to glass.

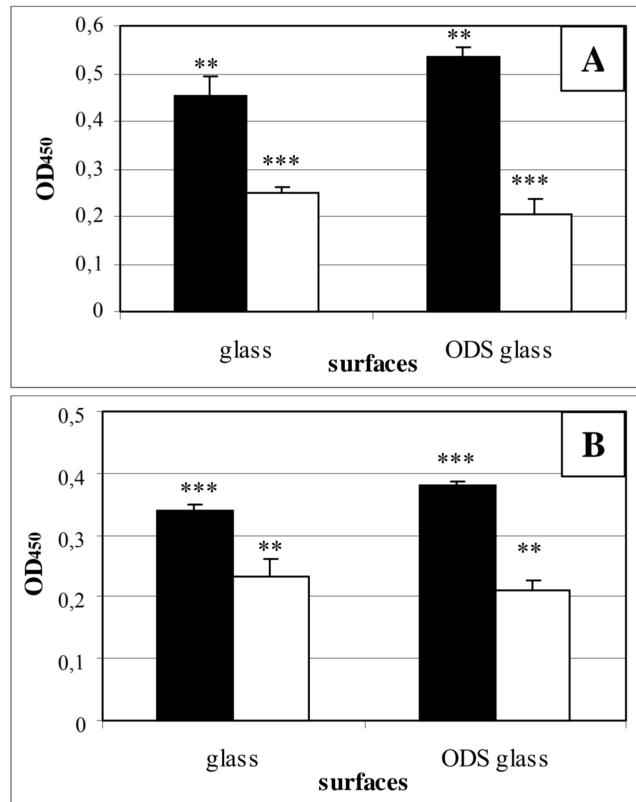


Fig. 11 Polyclonal (black columns) and monoclonal (white columns) antibody binding to FN (A) and FNG (B) adsorbed from 20µg/ml protein solution to glass and ODS glass. Data are means ± SD of five replicates from typical experiments out of two performed. The statistic was performed by unpaired t test. (**) - p<0.01, (***) - p<0.001.

Table.5 Comparison of polyclonal and monoclonal antibody binding to adsorbed FN and FNG.

FN				**FNG**			
Surface	**pAb OD_{450}**	**mAb OD_{450}**	**Expression (in %)**	**Surface**	**pAb OD_{450}**	**mAb OD_{450}**	**Expression (in %)**
glass	**0.45**	**0.25**	**54**	**glass**	**0.34**	**0.23**	**68**
ODS glass	**0.54**	**0.20**	**38**	**ODS glass**	**0.38**	**0.21**	**54**

FN and FNG (20µg/ml) are adsorbed to glass and ODS glass. Percent cell-binding epitope expression (mAb signal) versus the total adsorbed protein (pAb signal) was given as a potential measure for accessibility of this epitope.

4.6. Cell-substrate interactions

4.6.1. Actin cytoskeleton organization

Significant differences in the cell morphology of HUVEC were found depending on the wettability of substrata and the type of protein coating (Fig.12). HUVEC were allowed to adhere on FN and FNG coated hydrophilic and hydrophobic substrata for 2 h. The cells attached to the FN coated hydrophilic glass were well spread (Fig.12 A) containing prominent linear arrays of actin bundles. On hydrophobic ODS surface (Fig.12 B) the cells were less spread and exhibited predominantly circumferential organized actin filaments. Obviously, on both FN coated substrata, cells remained their adhesive phenotype. On FNG substrata cells exhibited a quite different morphology (Fig. 12 C-D). The number of adherent cells was visibly higher on glass (Fig. 12 C) than on ODS (Fig. 12 D), and the cells also spread better on glass (Fig. 12 C), although many of them possessed an irregular shape indicating an enhanced motility on this substrate. The peripheral organization of actin filaments and the formation of distinct leading and trailing cell edges were another sign for a motile cell phenotype

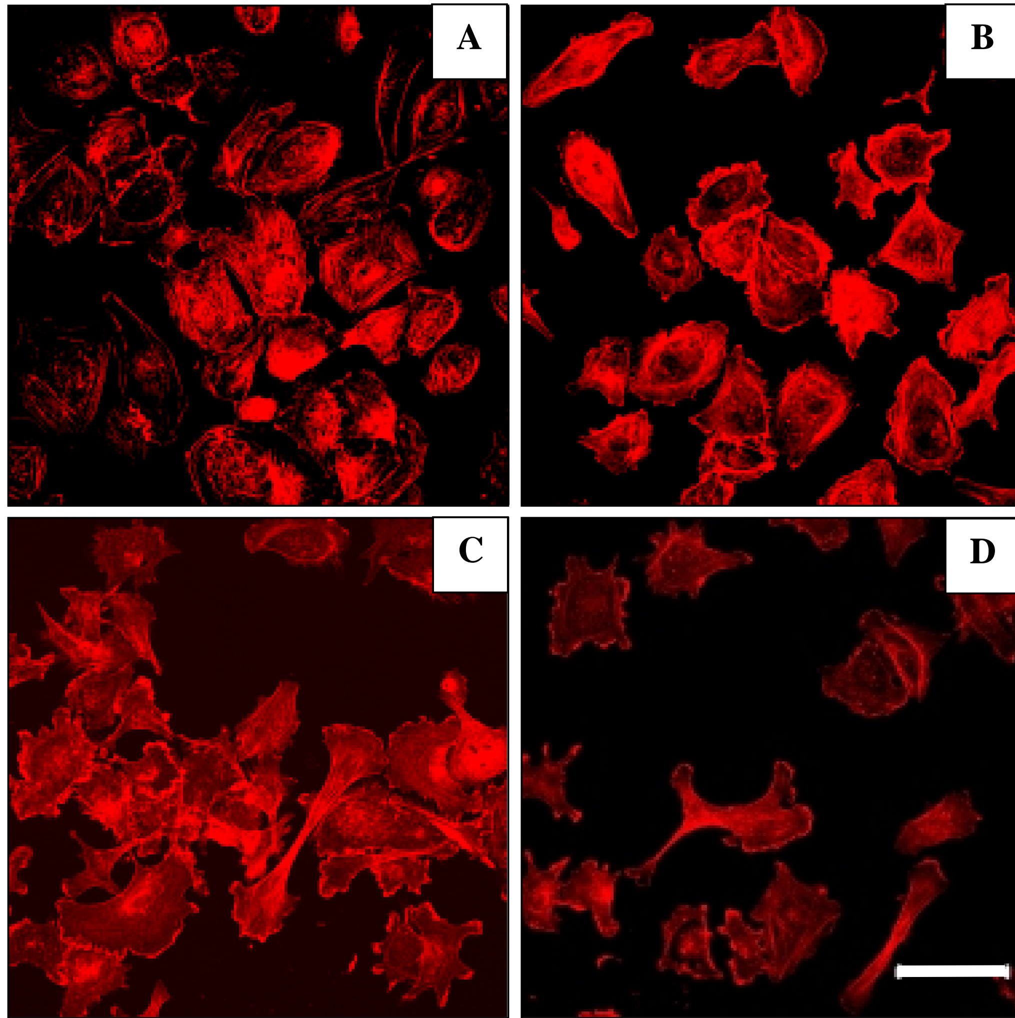


Fig. 12 Overall cell morphology of HUVEC. Glass (A and C) and ODS (B and D) slides were coated with 20µg/ml FN (A and B) or with 20µg/ml FNG (C and D). The cells were allowed to spread on the coated slides in EC growth medium for 2 h, then fixed, permeabilized, saturated and stained for F-actin using BODIPY-phalloidin. Bar is 100µm.

Confocal images of HUVEC shown in Fig. 12 were analyzed by KS 300 software (Zeiss, Jena, Germany) to determine the initial cell attachment. HUVEC attached preferentially better on FN or FNG coated hydrophilic substrata than on the same hydrophobic ones. In general the number of HUVEC attached on FN coated hydrophilic as well hydrophobic substrata was higher than cell number of attached cells on corresponding FNG coated substrata.

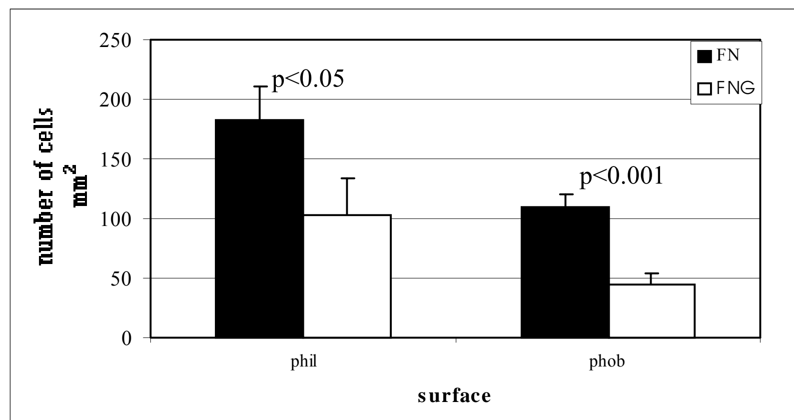


Fig.13 Cell attachment of HUVEC on FN or FNG coated hydrophilic and hydrophobic surfaces. Data are means ± SD of five replicates from typical experiments out of three performed. T-test was used for the statistical analysis.

4.6.2. Focal adhesion formation (vinculin staining)

The process of the initial cell attachment and spreading of HUVEC were analyzed by formation of focal adhesion complexes using vinculin staining. HUVEC attached to FN coated hydrophilic and hydrophobic substrata showed well defined focal adhesions (Fig. 14 A-B). In contrast, on FNG coated substrata the organization of focal adhesion contacts was considerably diminished (Fig. 14 C-D). On FNG coated hydrophilic substrata a few not well visible focal contacts can be observed (arrows in Fig. 14 C) together with single focal complexes at the cell periphery. On FNG coated hydrophobic glass (Fig. 14 D) only cortical organization of vinculin staining can be detected. However, together with the irregular cell shape on these substrata it is obvious that HUVEC seeded on FNG coated surfaces exhibited more motile phenotype.

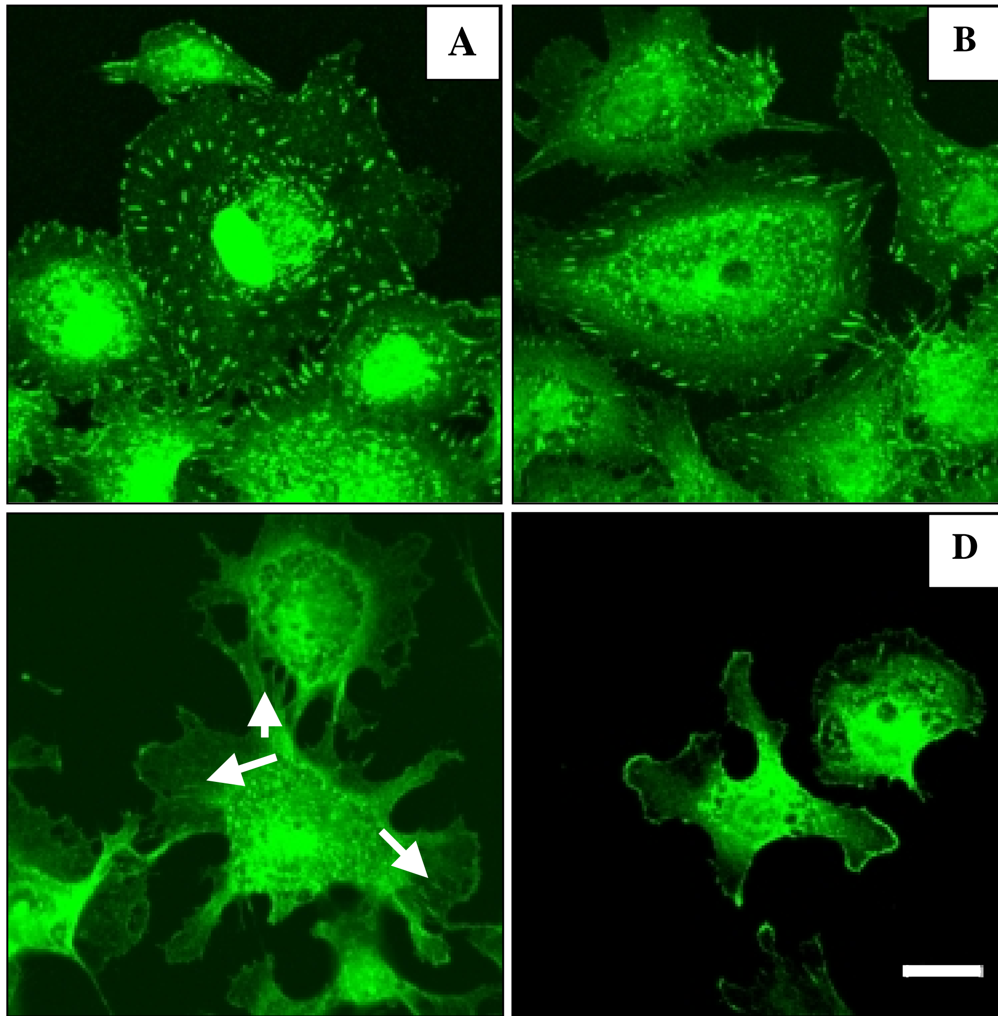


Fig.14 Formation of focal adhesions of HUVEC. HUVEC were seeded on FN coated glass (A) and ODS glass (B), and on FNG coated glass (C) and ODS glass (D) for 1h. The fixed and permeabilized cells were stained for vinculin and visualized by using confocal scanning microscope. Bar is 20μm.

4.7. Remodelling of ECM proteins by HUVEC

4.7.1. Reorganization of adsorbed FN and FNG

FITC-conjugated FN (FFN) and FNG (FFNG) were adsorbed on glass and ODS, and the substrata were incubated with HUVEC in EC medium containing 10% FCS, for 4h. This technique of a direct fluorescent labeling of protein, instead of antibody tagging techniques, was used considering the limiting antibody accessibility beneath the cells [Avnur and Geiger 1981, Grinnell 1986]. As it is shown in Fig. 15 A, significant amount of adsorbed FFN was readily removed by the cells from the hydrophilic glass and accumulated in fibril structures (arrows in Fig. 15 A) along the cell margins or beneath the cells. In marked contrast, no removal and no reorganization of FFN by HUVEC were found on ODS. On FFNG coated glass the removal was less pronounced in comparison to FFN, but well visible dark patches and thick, short streaks on the bright fluorescent background of adsorbed FFNG were observed (Fig 11 C). This fact could indicate that the reorganization of adsorbed FNG is slower then FN reorganization. Again, no removal of FFNG on ODS was detected (Fig. 15 D), although some accumulation of fluorescent FNG was observed around the cell nucleus, which can be interpreted as non-specific staining. Some internalization of the fluorescent protein however, cannot be excluded.

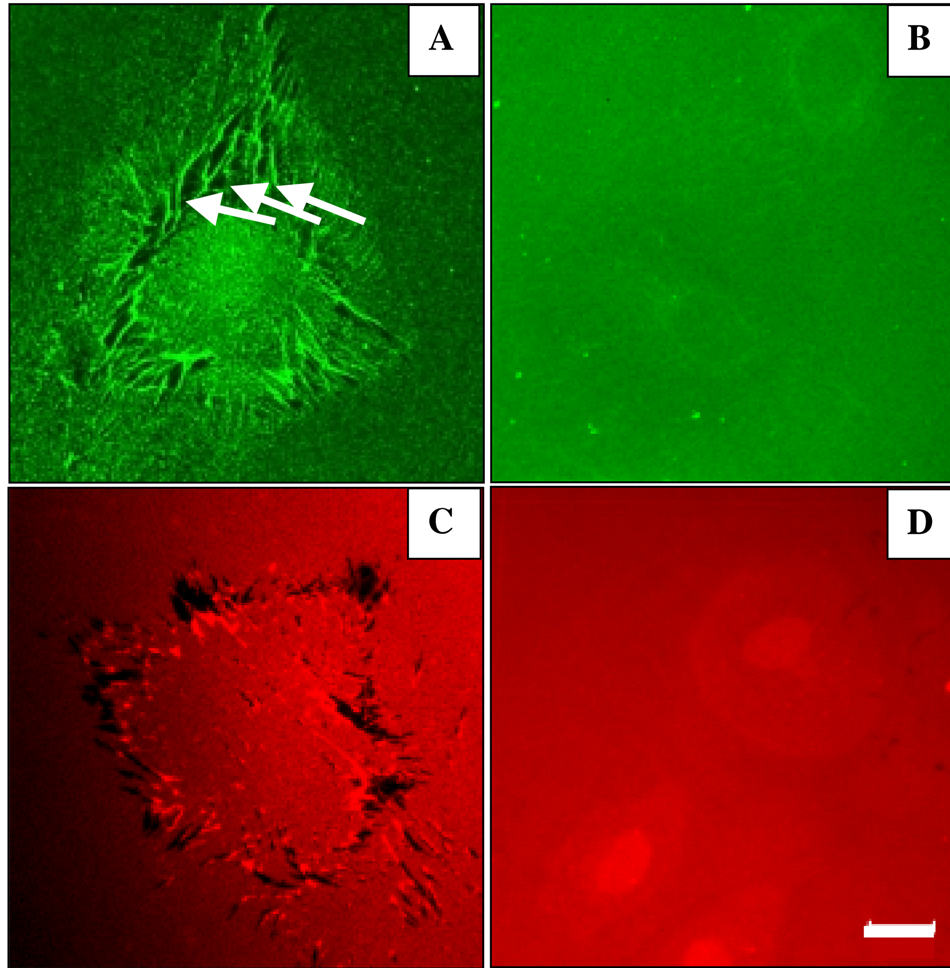


Fig. 15 Reorganization of substratum-bound FN and FNG on hydrophilic and hydrophobic substrata. Glass (A and C) and ODS (B and D) were coated with 40µg/ml FITC-FN (FFN, A and B) or with 40µg/ml Rhodamine Red-FNG (FFNG, C and D). Adsorbed FFN on glass (A) was organized in fibrilar structures at the cell periphery (arrows in A). No removal of adsorbed FFN was detected on ODS (B). FFNG was well organized on glass (C), while on ODS (D) only an accumulation of adsorbed FFNG beneath the cell center was observed. Bar is 20 µm.

To better characterize the EC interactions with the above substrata, we studied the organization of β_1 integrin, as constituent of the FN receptor ($\alpha5\beta1$), and of $\beta3$ integrin, as constituent of the FNG receptor ($\alpha_V\beta3$), on permeabilized cells. HUVEC were allowed to attach for 1 h on the respective ligands (FN or FNG), coated on hydrophilic or hydrophobic substrata, than the cells were fixed, permeabilized and stained to visualize integrins. Figure 16 A represents a typical view of EC on FN coated glass, where numerous $\beta1$-rich streaks of focal adhesions were found. Some of the streaks were spanned the cell body. On FN-coated ODS (Fig.16 B) however, single thin and slight visible streaks of $\beta1$ integrins in focal adhesions were observed. As is shown on the lower panel of Figure 16, on FNG-coated hydrophilic substrata EC organized $\beta3$ integrin in short or more elongated streaks preferentially at the focal adhesion sites (Fig. 16 C). Clustering of $\beta3$ was not observed on FNG-coated ODS (Fig. 16 D).

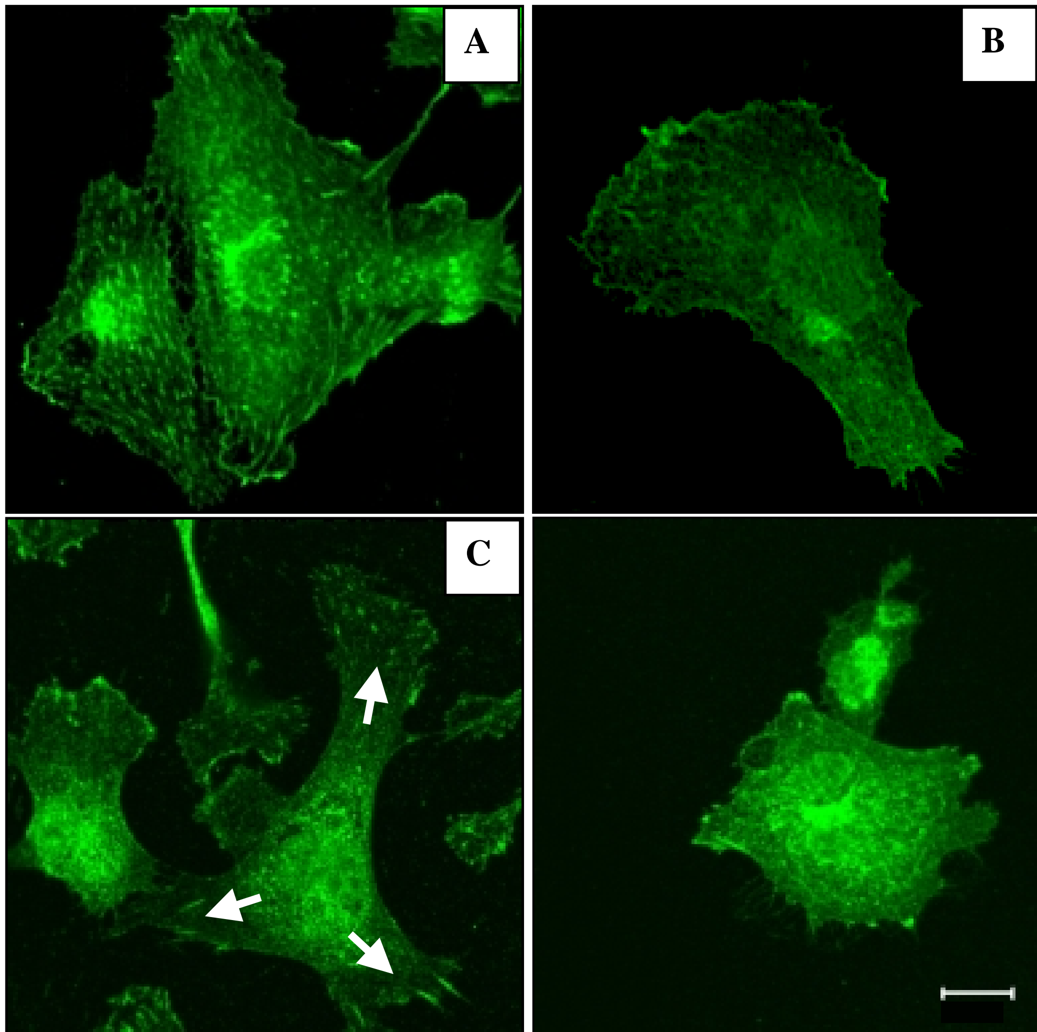


Fig. 16 Detection of integrin clustering. Glass (A and C) and ODS (B and D) slides were coated with 20µg/ml FN (A and B) or with 20µg/ml FNG (C and D). The cells were stained for β1 (A and B) or for β3 (C and D). β1 on glass (A) was localized in numerous linear streaks representing focal adhesions, while on ODS (B) only a few adhesion plaques were visible. On glass (C) β3 was localized in the form of streaks and spots or in rather elongated streaks at the cell edges (arrows in C). The clustering of β3 integrin on ODS (D) was missing. Bar is 20µm.

4.7.2. Reorganization of soluble FN and FNG

To determine whether exogenous (soluble) FN or FNG can be organized by HUVEC, the cells were plated onto FN coated glass and ODS and allowed to spread for 1 h. FFN or FFNG conjugates were added for additional 2 hours. Figure 17 A shows that FN was readily organized in fibril-like structures on glass. Confocal images showed that the FN fibrils span several cells forming a complex FN matrix. Also very thin fibrils can be observed along the whole cell body. On ODS substrate the FN fibrillogenesis was considerably reduced (Fig.17 B) and only short streaks were observed along the cell margins. In contrast to FN, soluble FNG had a tendency to become organized in fibrilar structures preferentially at the cell periphery (Fig. 17 C) on hydrophilic glass. On ODS the FNG fibril organization was less pronounced (Fig. 17 C), but still well visible FNG fibrils at the cell margins can be observed.

To study the possible role of β_1 and β_3 integrins for FN and FNG remodelling on the dorsal cell surface, HUVEC were cultured on the respective protein coated glass or ODS substrata. The cells were incubated in serum-free medium, to avoid the effect of other proteins, for 1 h and further fixed and stained (without permeabilization) for the above integrins. Fig. 18 shows the typical linear pattern of the dorsal organization of β_1 integrin on FN coated glass (Fig.18 A). On ODS the β_1 integrin organization was completely missing (Fig.18 B). In contrast on FNG coated substrata, the β_3 integrin exhibited only a punctuate staining for both hydrophilic (Fig.18 C) and hydrophobic ODS substrata (Fig.18 D).

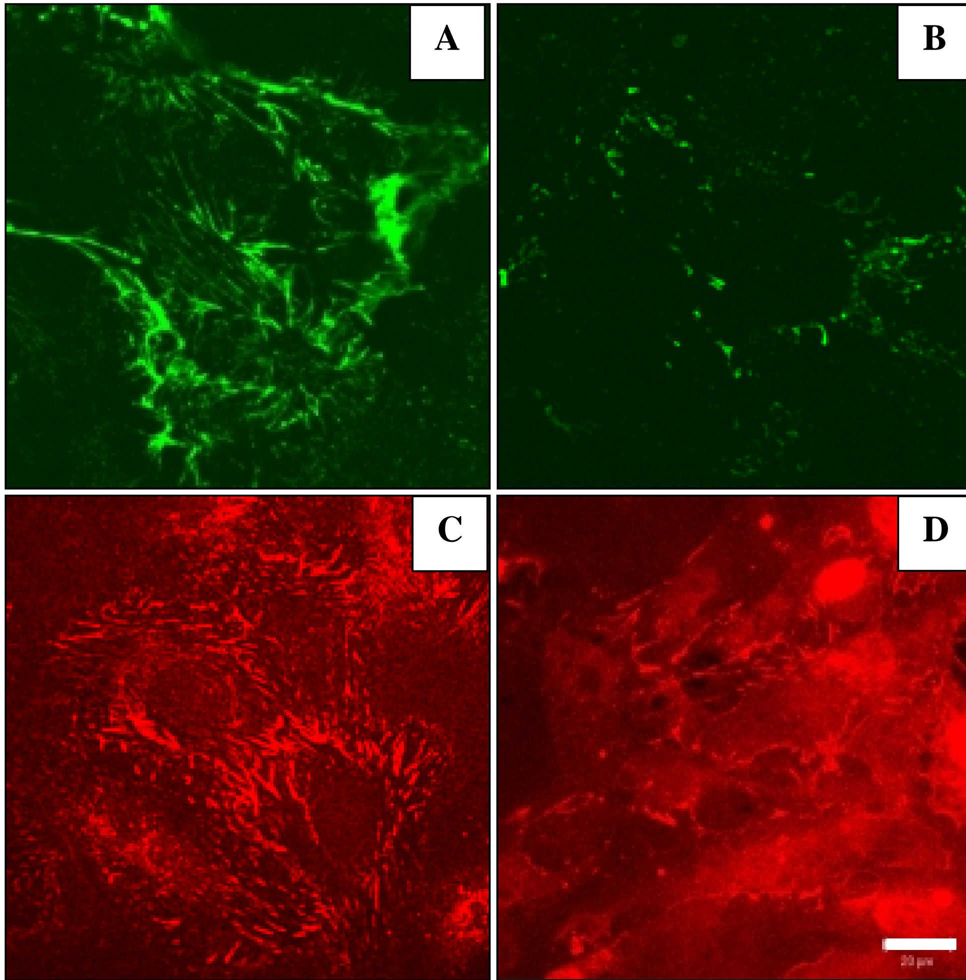


Fig. 17 Reorganization of soluble FN and FNG on hydrophilic and hydrophobic substrata. HUVEC were incubated on FN (20µg/ml) coated glass (A and C) and ODS (B and D). 100µg/ml FITC-FN (A and B) or FITC-FNG (C and D) was added for further 2h of incubation C. FN fibrils on glass (A) spanned several cells organizing a FN matrix. On ODS (B) only short FN streaks mostly at the cell margins were observed. FNG on glass (C) showed strong linear structures along the cell body (arrows in C). On ODS (D) fibrillogenesis of fluorescent FNG was considerably diminished. Bar is 20 µm.

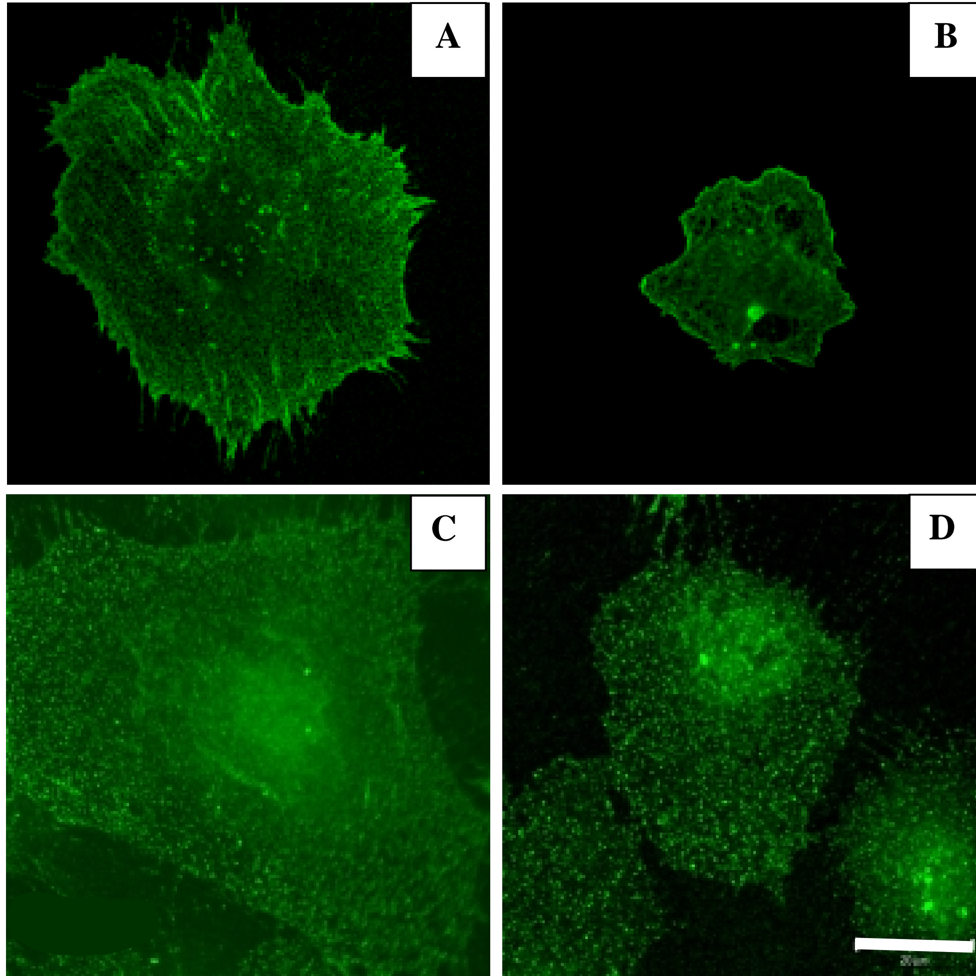


Fig. 18 Distribution of β_1 and β_3 on the dorsal cell surface. HUVEC were incubated on FN (20µg/ml) coated glass (A and C) and ODS (B and D). The cells were stained for β_1 (A and B) or β_3 (C and D). The defined linear pattern of organization of β_1 on glass (A) was greatly reduced on ODS (B). β_3 showed a punctuate staining on glass (C) with few linear streaks (arrows in C). Diffusive β_3 distribution was found on ODS (D). Bar is 20µm.

To address the question which integrins were involved in the fibrilar organization of FNG on the dorsal cell surface on hydrophilic substrata double staining experiments were conducted. Since β_3 integrin showed no linear organization on the dorsal cell site when the cells were stimulated with soluble FNG (data not shown) we tried to check the hypothesis whether β_1 is involved in FNG fibrillogenesis. For that purpose HUVEC were incubated on FN coated substrata for 1h and Rhodamine-conjugated FNG was added for the next 2 h of incubation. The cells were fixed and stained for β_1 using Cy2-conjugated secondary antibody. Since we did not find any fibrilar organization of FNG on ODS surfaces these experiments were performed with glass surfaces only. It was found that FNG fibrils co-localized with β_1 integrin. As can be seen in Fig. 19 C the FNG fibrils co-localized with the elongated β_1-rich streaks at the cell periphery (inset in Fig.19 C).

Because FNG fibrils were found to co-localize with the $\beta_1$subunit of the main FN receptor on EC, it was interesting to test whether there was some relation between FN and FNG fibrillogenesis. For that purpose HUVEC were seeded on FN coated glass and incubated for 1h. Then Rhodamine-labeled FNG was added for subsequent 2 h. Cells were fixed and stained for extracellular FN using a monoclonal anti-FN antibody visualized by secondary Cy2-conjugated antibody. After the incubation FNG fibrils were already deposited and assembled in patches (Fig.20 A). It was observed that FN matrix fibrils (Fig.20 B) co-localized with FNG fibrils at the cell periphery (Fig.20 C).

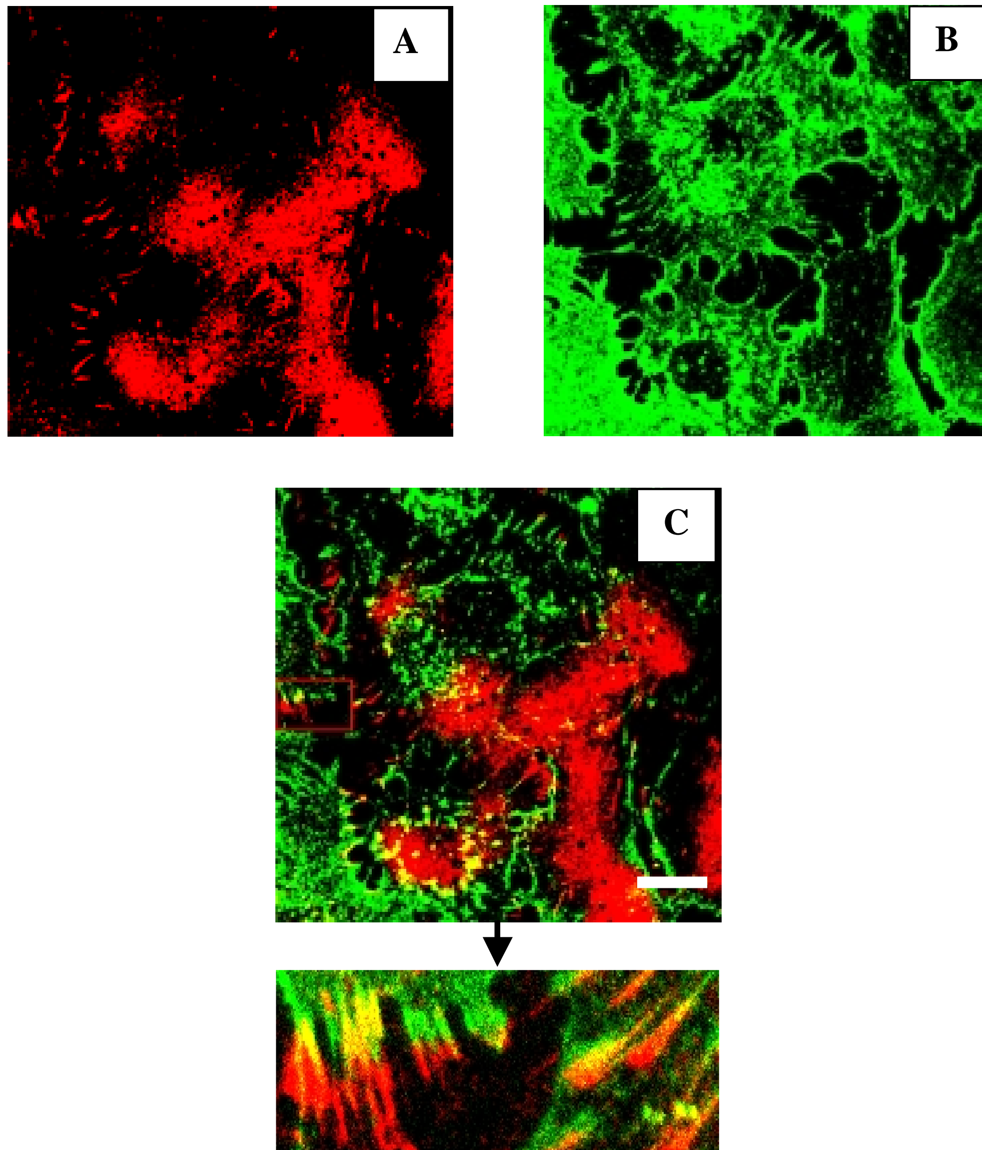


Fig. 19 Co-localization of β_1 with FNG fibrils on glass. Soluble FNG-Rhodamine Red (100µg/ml) was added to the living cells cultured on FN coated glass for 2h. Fig. 19 A visualize the fibrilar organization of FNG. The fixed cells were stained for β_1 (Fig.19 B). Fig. C (superimposed image) is the co-localization (inset in C) between FNG fibrils (red channel A) and β_1 linear streaks (green channel B). Bar is 10µm.

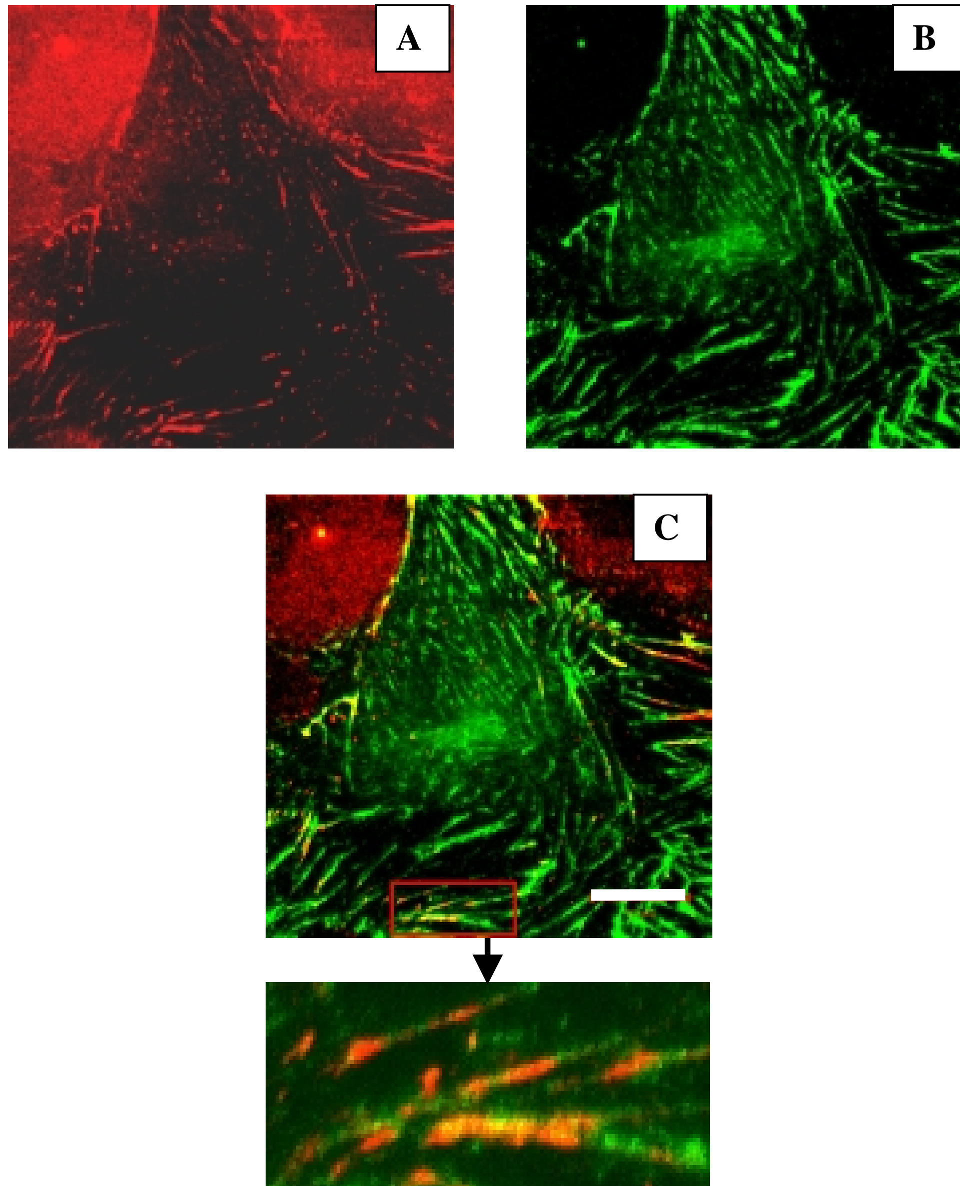


Fig. 20 Co-localization between FNG and FN fibrils. Soluble FNG-Rhodamine Red (100µg/ml) was added to the living cells on FN (20µg/ml) coated glass slides for 2h. The cells were stained for FN using anti-FN monoclonal antibody. FNG fibrils are spanned around the cell periphery (Fig. 20 A-red channel), while FN formed linear net of fibrils over the entire cell surface (Fig. 20 B-green channel). The FNG and FN fibrils co-localized mostly at the cell periphery (Fig. 20 C-superimposed image)**.** Bar is 50µm.

4.7.3. Degradation of ECM – action of matrix metalloproteinases (MMP)

Supernatants of HUVEC seeded on hydrophilic and hydrophobic substrata coated with FN or FNG showed an expression of pro-MMP-2 with molecular weight of 74 kDa. FNG coated substrata (Fig. 21, line 4, 5) showed a higher pro-MMP-2 expression than FN coated ones (Fig. 21, line 2, 3). Overall, the surface wettability did influence the differences in pro-MMP-2 expression. On both FN and FNG coated hydrophilic substrata the expression of pro-MMP-2 was higher than on hydrophobic ones. To check the direct proteolytic activity of HUVEC in dependence on surface wettability we analyzed ***in situ*** gelatinolytic activity of HUVEC. The cells were grown on fluorescent–labeled gelatine and degradation of its matrix was tested (Fig. 22) after 24 hours of incubation. However, an increase in gelatine degradation was detected on hydrophilic substrata (Fig. 22 A), whereas the degradation of gelatine by HUVEC seeded on hydrophobic substrata was diminished significantly (Fig.22 B).

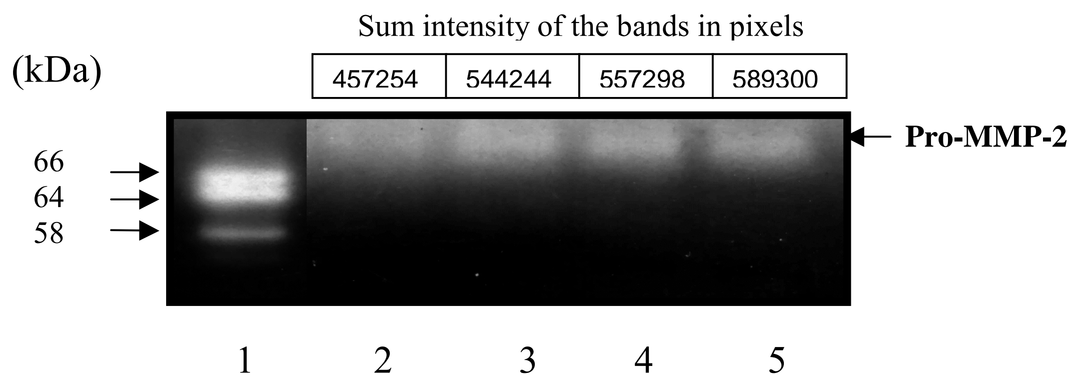


Fig. 21 Matrix metalloproteinase activity of HUVEC. Supernatants of HUVEC seeded on FN coated ODS (2) and glass (3) substrates and FNG coated ODS (4) and glass (5) substrates for 3 days were analyzed by gelatin zymography to test MMP-2 processing. The pro-MMP-2 form of 74 kDa was induced upon HUVEC seeding on different substrata. As a control (line 1) was used Gelatinase A. A representative out of three independent experiments is shown.

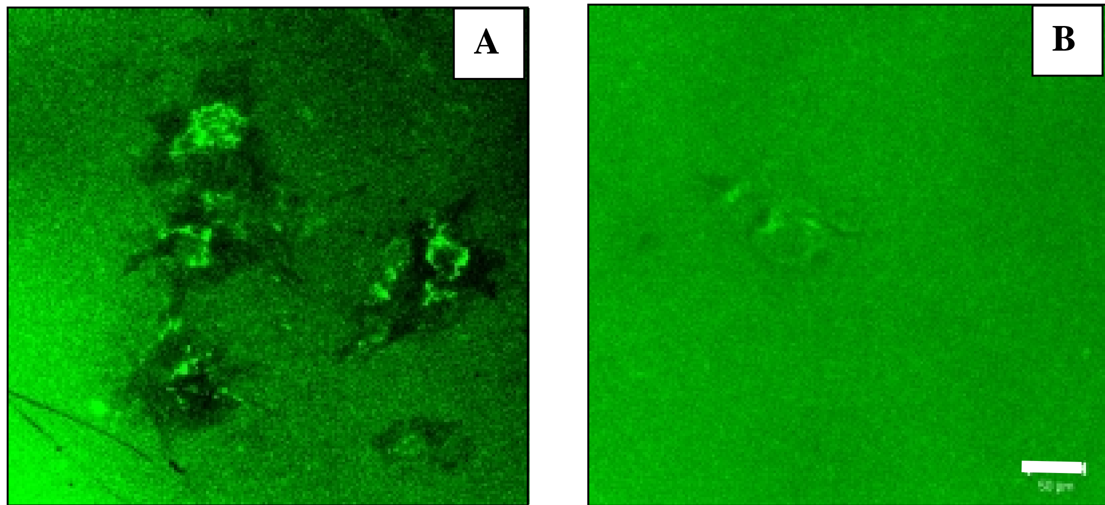


Fig. 22 Gelatinolytic activity of HUVEC. HUVEC were grown on hydrophilic (A) and hydrophobic (B) substrata coated with fluorescent–labeled gelatine. After 4h of incubation, cells were fixed and processed to be analyzed by confocal microscopy. Gelatinolytic areas were observed as black spots. Bar is 50 µm.

4.8. Cell-cell contacts

4.8.1. Adherent junctions (E-Cadherin distribution)

To determine how the wettability and the protein coating could influence the E-Cadherin distribution, the cells were incubated on FN or FNG coated normal glass and ODS hydrophobic glass for 3 days. The E-Cadherin localization was analyzed by immuonofluorescence and confocal microscopy. There was observed marked differences in E-Cadherin distribution depending on protein coating and wettability of the substrata (Fig.23). On FN coated glass (A) the E-Cadherin was localized as a bright intense immunostaining in the cell cytoplasm around the cell nucleus and was completely missing from the cell-cell contacts. In contrast, on the FN coated hydrophobic ODS glass the E-Cadherin was localized most preferentially at the cell-cell contacts and only a small amount was detected in the cell cytoplasm (B). On FNG coated glass (C) a bright intense immunofluorescent signal was detected in the cell cytoplasm, but a tendency for localization of E-Cadherin at cell-cell contacts was also observed. On FNG coated ODS glass E-Cadherin

was already localized at the cell-cell contacts (D). The immunofluorescent intensity of E-Cadherin localization at the cell-cell contacts on FNG coated ODS (D) was lower than the same on FN coated ODS (B), but however well pronounced. To correlate the marked differences in immunolocalization of E-Cadherin on FN coated hydrophilic and hydrophobic substrata, it was interesting to analyze the total amount of E-Cadherin and the corresponding β-catenin amount, which remain associated with E-Cadherin (Fig 24). Western blot analysis of HUVEC extracts showed that the level of E-Cadherin protein expression was similar on both FN coated substrata (Fig.24, Panel A). Western blot performed with monoclonal antibody specific for E-Cadherin resulted in the identification of one band for both substrata of similar intensity corresponding to the apparent molecular weight of E-Cadherin (130 kDa) (Fig. 24, Panel A). Despite otherwise comparable levels of expression of E-Cadherin on both substrata, E-Cadherin on FN coated hydrophobic substrate was found at the cell-cell junctions (Fig. 23, B) whereas E-Cadherin on FN coated hydrophilic substrate showed diffusive localization in the cell cytoplasm (Fig. 23, A).

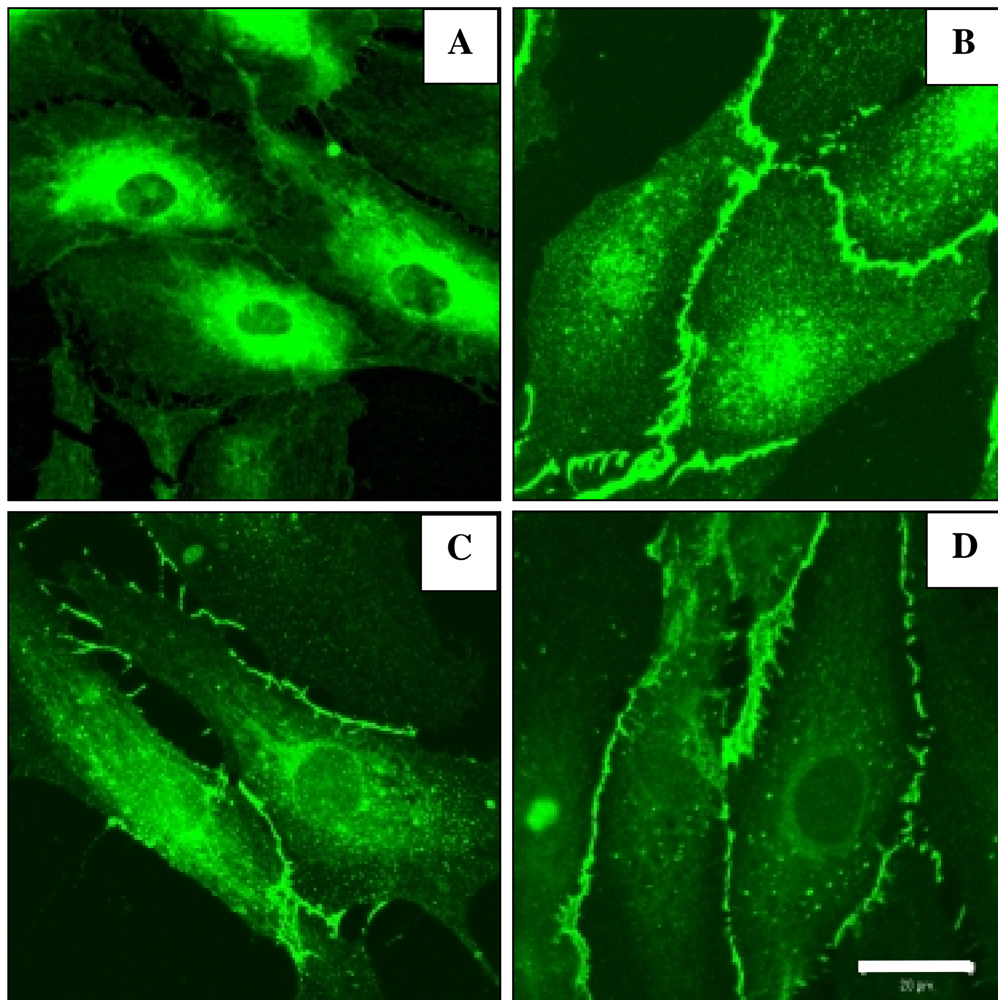


Fig. 23 Immunofluorescence for E-Cadherin. The cells were incubated on FN coated glass (A) and ODS glass (B) or FNG coated glass (C) and ODS glass (D) for 3 days. The cells were washed, fixed and stained for E-Cadherin using monoclonal anti E-Cadherin. Second Cy2-conjugated IgG antibody was used to visualize E-Cadherin distribution. Bar is 20 μm.

To study whether the different localization of E-Cadherin on FN coated glass and ODS glass could be due to a different association of β-catenin, the same numbers of HUVEC were lysated immunoprecipitated with antibody specific for E-Cadherin and sequentially were blotted with antibody against β-catenin (Fig.24 Panel B). The amount of β-catenin associated with E-Cadherin was significantly higher for FN coated ODS (Fig. 24, Panel B-b) than for FN coated glass (Fig.24, Panel B-a), nevertheless that the amount of E-Cadherin for both substrata was approximately the same (Fig. 24, Panel C). These data suggest that the localization of E-Cadherin at the cell junctions on FN coated ODS glass require an active association of β-catenin with E-Cadherin in complexes.

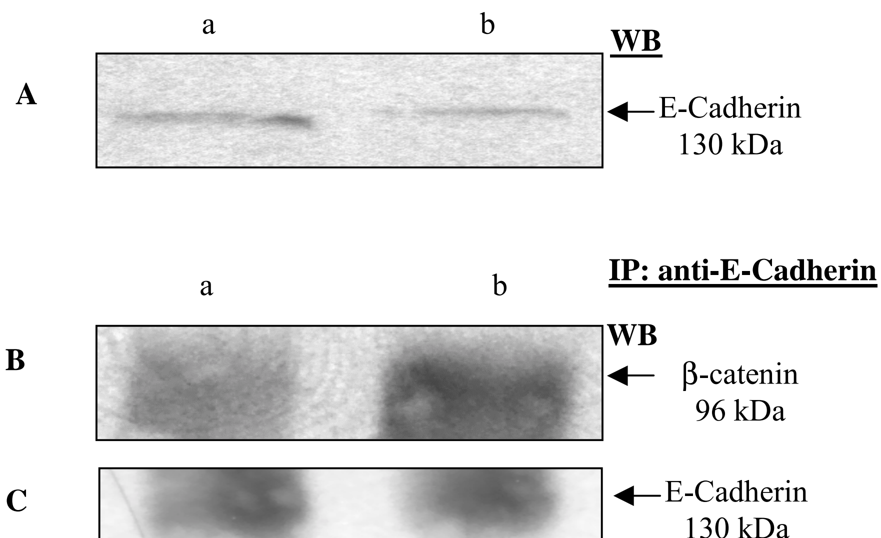


Fig. 24 *Analysis of total amount of E-Cadherin* (A). HUVEC seeded on FN coated glass (a) and ODS glass (b) were harvested after 3 days of incubation and analyzed by western blotting against anti E-Cadherin. *Analysis of associated β-catenin with E-Cadherin* (B). The cell lysates were immunoprecipitated using anti E-Cadherin /protein G Sepharose. The samples were analyzed by SDS-PAGE gel electrophoresis and immunoblotted against β-catenin. *Analysis of amount E-Cadherin in E-Cadherin-β-catenin complexes* (C). The samples from immunoprecipitation were analyzed by immunoblotting against E-Cadherin.

Table 6. Summarized results from Part II

	FN*		FNG*	
	glass	**ODS glass**	**glass**	**ODS glass**
Cytoskeleton organization	prominent linear arrays of actin bundles	Circumferential f-actin distribution	leading and trailing cell edges	leading and trailing cell edges
Focal adhesions	long streaks radiating from cell center	shorter streaks at cell periphery	few short streaks at the cell periphery	small focal complexes at the cell periphery
Adsorbed proteins reorganization	Well organized fibrilar structures	No fibrilar structures	Fibrilar structures	No fibrilar structures
Fibrillogenesis of soluble proteins	Well organized fibrilar structures	No fibrilar structures	Well organized fibrilar structures	No fibrilar structures
Integrin clustering	β_1 (+)	β_1 (-)	β_3 (+)	β_3 (-)
Integrin linear organization-dorsal	β_1 (+)	β_1 (-)	β_3 (-)	β_3 (-)
Pro-MMP-2 activity	(+++)	(+)	(++)	(+/-)
E-Cadherin deposition	(-)	(+++)	(+)	(++)

Summarized cell activity of HUVEC seeded on glass and ODS glass with different coatings.

*-type of protein coating of the surfaces. (+++) - very well presented function, (++) - well presented function, (+) - poorly presented function, (+/-) - very low presented function, (-) - missing function.

4.9. Discussion

Seeding of cardiovascular implants with EC is a desirable effect in order to improve the blood compatibility of these devices. The understanding of the substrate dependent cellular behavior is important in the surface design of blood-compatible materials [Ratner 1989, Langer 1993]. Upon seeding, anchorage-dependent cells adhere, spread, migrate and proliferate on an artificial substrate, resulting in the formation of a monolayer of tissue [Re 1994]. Tissue formation on artificial substrates is a highly regulated process that depends largely on the presence of appropriate extracellular signals [Nagahara 1996]. Two such signals, cell-substrate and cell-cell interactions have long been recognized as important regulators of cell growth both ***in vivo*** and ***in vitro*** [Ridley 1992]. On the other hand the overall cell behavior is greatly influenced by surface properties of the artificial substrate as surface wettability [van Wachem 1989, Sieminski 2000]. Since FNG and FN are the natural constituents of the wound bed at the sites of vascular injury and a new vessel formation [Dejana 1990], the coating of the material surfaces with these proteins could influence EC adhesion and spreading, especially on poor wettable substrata.

4.9.1. Cell – substrate interactions

4.9.1 1. Protein adsorption and conformation

Adsorption properties of the proteins on different wettable surfaces and subsequent cell behavior have been studied extensively [van Wachem 1985, Kottke-Marchant 1996, Webb 1998, 2000]. There is exists a hypothesis that FN is conformationally “activated” by surface adsorption by exposure of cell binding domains to the surface and that cell integrin-adsorbed ECM protein binding is stronger than soluble form of this protein [Aota 1994, Horbett 1994]. It is also true for the adsorbed FNG, since the non activated platelets binds only to adsorbed FNG, but do not bind to soluble FNG [Zammaron 1991]. On the other hand, the extent of the substrate dependent conformational changes in adsorbed proteins controls the ligand–integrin ineractions and thus strongly affects the cellular reactions [Garcia 1999]. For instance the conformational changes in adsorbed proteins on poor wettable surfaces was shown to inhibit the cell attachment and the subsequent spreading [Iuliano 1993, Tsai 1999, Koenig 2003] as well the protein remodelling by cells [Grinnell and Feld 1981, Altankov 1996]. The data

presented here underline the surface-dependent differences in FN and FNG adsorption to hydrophilic and hydrophobic surfaces. The approach of using a combination of polyclonal and monoclonal antibodies permit to correlate the total amount of adsorbed protein with the accessibility of a specific cell binding epitope, which is responsible for the biological functions of a given protein. Despite the fact that on ODS glass the total amount of adsorbed FN and FNG was higher than on glass, the accessibility of the specific cell binding domains in the adsorbed proteins was considerably decreased. The stronger protein binding and the unfolding of the proteins due to the dehydration mechanism are most probably the reasons for the decrease accessibility of the cell-binding domains [Horbett 1998, Andrade 1992]. Since the rate of the accessibility of cell binding epitope in adsorbed FN and FNG on glass was shown to favor the cell adhesion behavior (see below in the text), the decreased accessibility of the same domains on ODS glass was referred here as a „substrate inhibition" of adsorbed FN and FNG. This inhibition in accessibility of cell binding epitopes on ODS glass was in the range of 16% for adsorbed FN and with 14% for adsorbed FNG when compare to glass.

4.9.1.2. Cytoskeleton organization and focal adhesion contacts

Cell-substrate interactions are crucial for anchorage-dependent cells [Ruoslahti and Obrink 1996b]. Thus the ability of the substrate to promote the formation of focal contacts and the development of the cell cytoskeleton is important for the performance of the material [Dalby 2002]. Many authors have related the ability of different type of cells to establish and sustain the cell-substrate contacts with their biocompatibility [Massia and Hubbell 1991, Schneider and Burridge 1994, Groth and Altankov 1995]. The presented data showed pronounced differences in the overall cell morphology as well as in the ability of HUVEC to form focal adhesion contacts. The surface wettability and the type of protein coating were the two factors governing the different cell behavior. Two principle differences can be drawn from the presented data. First, the FN coating influenced the cell adhesion and spreading and led to more stationary type cells, while the FNG coating provoked motile morphology of seeded HUVEC. The lack of focal adhesions on FNG coated substrata was the confirmation for the motile phenotype of HUVEC. The results here are in an agreement with observations shown previously [Dejana 1992, Cheresh 1987], that adsorbed FNG may induce motility and growth

of EC. Second, the surface wettability influences the number of adhered cells and the integrin clustering. The number of adhered cells was higher for hydrophilic than for hydrophobic substrata. HUVEC seeded only on hydrophilic substrata showed integrin clustering. The lower efficiency of integrins to clusterize on poor wettable surfaces could be explained by the conformational changes found in the protein molecules upon adsorption, which could make them less accessible for binding to integrins.

4.9.1.3. Protein remodelling by HUVEC

However, cells not only adhere to matrix proteins, but also organize them into matrix-like structures [Hay 1991, Altankov 1996] in order to transmit signals in the cell interior for the cell functioning [Geiger 2001]. It is well known that the adsorption of matrix proteins is affected by the substratum wettability [Grinnell 1986, Iuliano 1993]. Recently it was suggested that in order to be biocompatible, materials need to adsorb FN loosely, so that it can be easily reorganized by cells into matrix-like structures [Altankov 1996]. Here was found a relation between surface wettability, protein adsorption, and FN and FNG fibrillogenesis by HUVEC. HUVEC were able to organize adsorbed and soluble FN and FNG in fibrilar structures only on hydrophilic glass, while it was blocked on hydrophobic substrata presumably because of the stronger bound FN and FNG and the lower accessibility of cell-binding domains. The main observation of the study of matrix forming activities of HUVEC was the fact that the process of FNG fibrillogenesis was found to be associated with FN matrix formation. The specific structural features of the provisional FNG matrix as a mediator of cellular functions such as adhesion and spreading, proliferation, and migration has been studied for a variety of different cell types, including EC, fibroblasts, epithelial cells and platelets [Dejana 1987, Donaldson 1989, Savage and Ruggeri 1991, Brown 1993]. Several authors have reported evidences for the relation of cell adhesion and spreading with the possible joint mechanism of interaction between FN and FNG matrix. For instance Grinnell et al. [Grinnell 1980] showed that fibroblasts did not attach to FNG substrata, but that the adhesion was supported when plasma FN was covalently cross-linked to FNG. Dejana et al. [Dejana 1990] showed that EC spreading on FNG was affected by cellular FN synthesized by the cells. The incorporation of FNG into the ECM of epithelial cells was shown to be dependent on the active assembly of a FN matrix [Donaldson 1989]. However

up to now there are no data available for the relation between surface wettability, FNG adsorption and FNG fibrillogenesis by HUVEC. The data here present a different pattern of integrin organization during the interaction with substratum - bound and soluble FNG. It is known that the adhesion of HUVEC to adsorbed FNG is mediated by $\alpha_V\beta_3$ integrin [Cheresh 1989]. Indeed, the present data also showed that β_3 integrin participated in HUVEC adhesion to FNG substrata so it was found to clusterize in the focal adhesion contacts. The absence of β_1 integrin from the focal adhesion plaques reported previously by Dejana et al. [Dejana 1990] is an indication that the FN receptor does not participate in EC adhesion to FNG. Conversely, on the dorsal cell site the FNG fibrils did not correlate with β_3 integrin linear organization. β_3 integrin was presented a punctuate distribution, in contrast to β_1 integrin, which showed a well pronounced linear pattern of organization. Integrin β_1 however, has clearly shown to be involved in FN fibril formation [Dejana 1990, Zhang 1993, Christopher 1997, Pankov 2000]. The question which araised was whether β_1 integrin could participate in both FN and FNG fibrillogenesis on the dorsal site of HUVEC and thus to link the both processes. Co-assembly of FNG and FN was shown for both epithelial cells [Guadiz 1997] and fibroblasts [Pereira 2002]. Thus, the existence of such joint fibrillogenesis was very probable for EC as well. Indeed, it was found a clear morphological evidence for the co-localization of FN and FNG fibrils on the dorsal cell surface of HUVEC. It should be noted also, that the incorporation of FNG into matrix fibrils start from the distinct place at the cell periphery, presumably the cell-cell contacts, when the FN fibrils were already spread the entire cell body. These facts could suggest the leading role of FN fibrillogenesis for FNG one. Thus the study reported here support the hypothesis [Pereira 2002] that FNG assembly into the ECM is dependent on the active polymerization of FN in matrix.

4.9.1.4. ECM protein degradation (MMP-2 production)

Matrix metalloproteinases is thought to play an important role in EC migration and matrix remodelling during different physiological and pathological processes. Here the data showed that HUVEC seeded on FN and FNG coated hydrophilic and hydrophobic surfaces produced pro- MMP-2. This is in agreement with the findings in the literature [Yan 2000] that most of the MMP-2 produced by fibroblasts and EC is in inactive form when the cells are seeded on ECM-coated substrates with a high coating concentration. For instance the authors found

about 95% pro-MMP-2 production from human capillary EC when they were plated on substrate coated with high FN concentration (2,5µg/cm^2). The concentration of protein coatings in presented experiments was even higher in the range of 5 µg/cm^2. The surface wettability influenced the production of pro-MMP-2 as on both FN and FNG coated hydrophilic substrata the proteolytic activity was higher than on hydrophobic ones (Fig. 22). The reason for that could be the lack of integrin clustering on hydrophobic substrata, which could alter the association of MMP-2 to the cell membrane and thus to increase the proteolytic activity. The conformational changes in proteins due to the stronger binding to the hydrophobic substrata could be another reason for the decreased degradability of the adsorbed proteins. In general the MMP-2 production was higher on FNG than FN coating (Fig. 22, line 4-5). The higher proteolytic activity found for FNG coated substrata, could be correlated with the enhanced cell motility on the same substrata as was already discussed earlier in this chapter (see actin cytoskeleton organization and overall cell morphology, Fig. 12). Some authors also have correlated the enhanced flexibility of the substrata to the enhanced ability of cell to migrate and to degrade the protein substrate [Tomasek 1997, Haas 1998]. HUVEC seeded on FN coated hydrophilic substrate showed slightly decreased proteolytic activity (Fig. 22, line 3), where the expression of pro-MMP-2 was considerably diminished on FN coated hydrophobic substrate (Fig. 22, line 2).
Thus the data presented here lead to the conclusion that the substrate wettability as well as the type of protein coating can be determined as factors influencing the proteolytic activity of HUVEC.

4.9.2. Cell-cell contacts

Endothelial cell-cell contracts are essential for the initial organization of the EC monolayers and play an important role in regulating vascular permeability, leukocyte extravasation and vascular remodelling [Dejana 1993]. The data presented here highlighted the role of surface wettability on the ability of HUVEC to deposit E-Cadherin at cell-cell contacts. Since the cells seeded on FN coated hydrophobic substrata formed very well cell-cell contacts, on FN coated hydrophilic glass the E- Cadherin was localized only in the cell cytoplasm and completely missing from cell-cell-contacts. Thus, the findings here support the postulated competition between cell-cell and cell-substratum adhesion [Martz 1974], so that the most

adhesive substrate (FN coated glass) was the least cell-cell cohesive substrate. The difference in localization of E-Cadherin on FNG coated substrata was not so well pronounced and in general, according to the E-Cadherin distribution HUVEC formed weaker cell-cell contacts. Cadherin-catenin complexes, which are the sign for the strength of cell-cell contacts, were found in higher quantities on FN coated ODS glass. In contrast the amount of the same complexes was considerably lower on FN coated glass. Thus the association of E-Cadherin with β-catenin strengthens the E-Cadherin binding to the actin cytoskeleton.

In conclusion, our results provide new insight into the ability of EC to interact and remodel FN and FNG in a spatially organized and coordinated manner in dependence on material surface wettability. These cellular events seem to be extremely important in the attempt to study the biocompatibility of the artificial surfaces. The interplay between cell-substratum and cell-cell adhesions and the controlling the delicate balance between them may contribute to the rational design of scaffold materials.

Part III. Endothelization of polymer membranes. The role of surface wettability and surface charge on cell adhesion, growth and functionality.

In this chapter the endothelization of polymer membranes will be discussed. PEI membrane was used as a basic material, which showed good membrane-forming properties, mechanical strength and thermal stability [Kneifel 1992]. All these features of PEI, together with its low immune response [Petillo 1994] make this material very attractive for future design of blood contacting devises. To produce active –COOH groups on the polymer surface the active layer of the basic PEI membrane was modified by a heterogeneous functionaliztion process [Albrecht 2003]. The influence of surface charge on the protein adsorption, EC attachment and growth then was studied and compared with nonmodified membranes. The functionality of seeded HUVEC will be revealed by PGI_2 production and discussed in the light of the anti-thrombotic activity of seeded EC.

4.10. Modification of PEI membrane

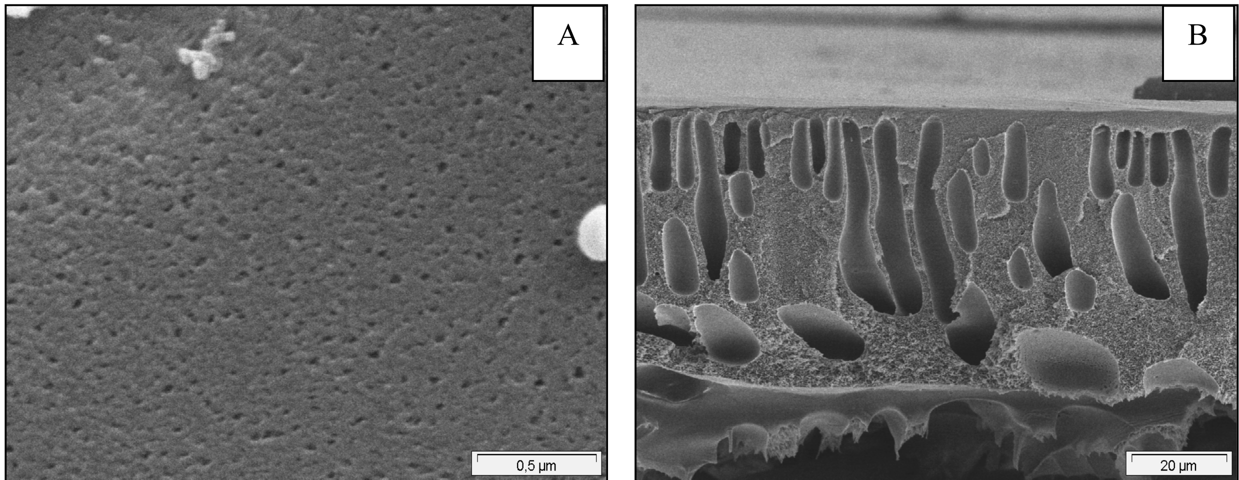


Fig. 25. SEM micrographs of unmodified PEI membrane: A–active surface layer, B–cross section.

The PEI membrane is characterized by a macrovoidal structure (Fig.25, B) typical for preparation of PEI membranes by the applied preparation procedure. The active layer of the membrane (Fig. 25 A) possesses a microporous structure with pore size in a range of 1-2nm. The functionalization of PEI membrane did not raise any significant differences in the active layer (Fig. 26, A-B). The measurements of CA also did not show any significant differences in the wettability of the modified membranes when compare with the basic PEI (see the water CA for PEI in the Chapter I).

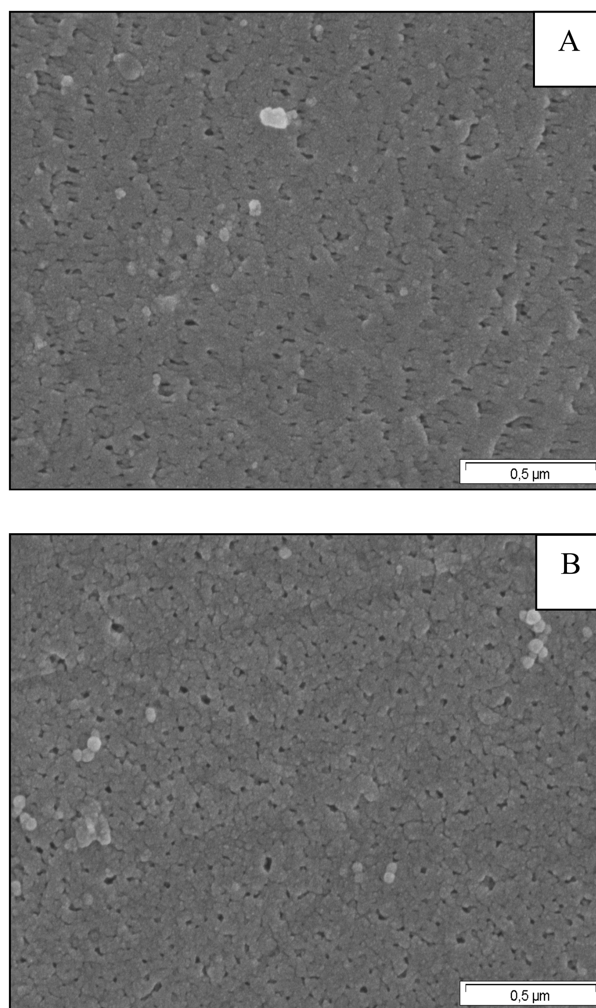


Fig. 26. SEM micrographs of modified PEI membranes. A-PEI modified with IDA for 1 min, B-PEI modified with IDA for 30 min.

To obtain the number of functional -COOH groups after modification, a thionin acetate (THA) assay was applied. For the unmodified PEI membrane (PEI-0) the THA assay gave a value of about 4.6 nmol of carboxylic groups per cm^2 of membrane area under the assumption that 1 mole of THA binds to 1 mole of carboxylic groups. Within first 10 min of the membrane modification with IDA the number of carboxylic groups raised and reached a plateau value of about 8.6 $nmol/cm^2$ indicating that about 4 nmol of carboxylic groups per cm^2 of membrane area were formed by IDA treatment. (Fig.27). For the all further experiments the modified membrane for 1 min (PEI 1) and 30 min (PEI 30) were used.

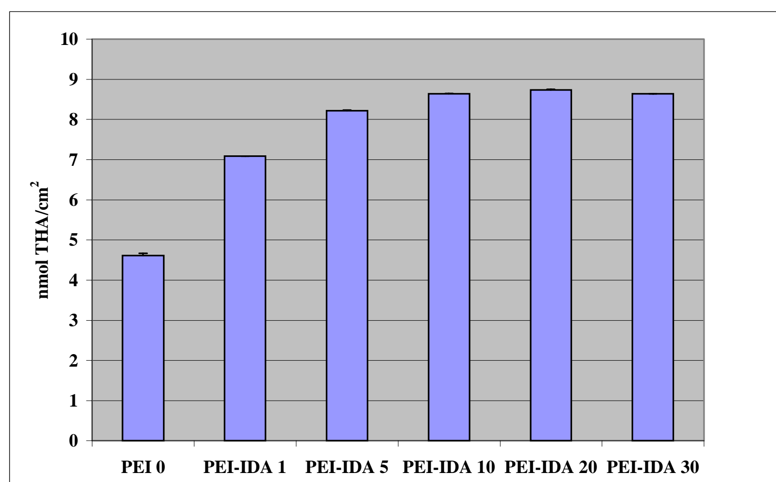


Fig. 27 Content of carboxylic groups. PEI membrane (PEI-0) was treated for 1 (PEI-IDA1), 5 (PEI-IDA5), 10 (PEI-IDA10), 20 (PEI-IDA20) and 30 (PEI-IDA30) min with sodium salt of iminoacetic acid (IDA). The content of carboxylic groups was measured using THA assay.

4.11. Protein adsorption

The protein adsorption to the membranes was performed using polyclonal antibodies against human FN and FNG. The unmodified membrane PEI was shown to adsorb the highest amount of FN and FNG. With the increasing the surface charge from PEI 1 to PEI 30 the amount of adsorbed proteins decreased. The differences in the polyclonal antibody binding to the adsorbed proteins on the both charged membranes were not significant ($p>0.05$).

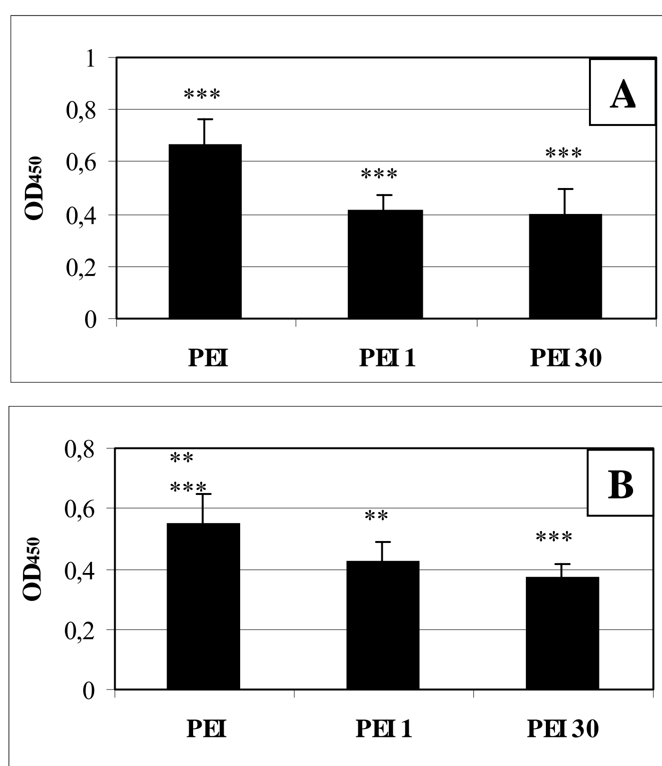


Fig. 28 Antibody binding to FN (A) and FNG (B) adsorbed from 20µg/ml protein solution to PEI, PEI 1 and PEI 30. Data are means ± SD of five replicates from typical experiments out of two performed. The statistic was performed by one way analysis using Tukey-Kramer post test. (**) - $p<0.01$, (***) - $p<0.001$.

4.12. Cell attachment

Adhesion of EC to biomaterial surface is an important prerequisite for the success of the synthetic vascular grafts [Williams 1994]. Overall, attachment of HUVEC after 2h showed that significant more cells adhered to protein coated membranes than to uncoated ones (Fig. 29). For all groups the highest adhesion showed unmodified PEI, followed by PEI modified with IDA for 1 min and the lowest HUVEC attachment showed PEI modified with IDA for 30 min in each groups of different coatings ($p<0.05$).

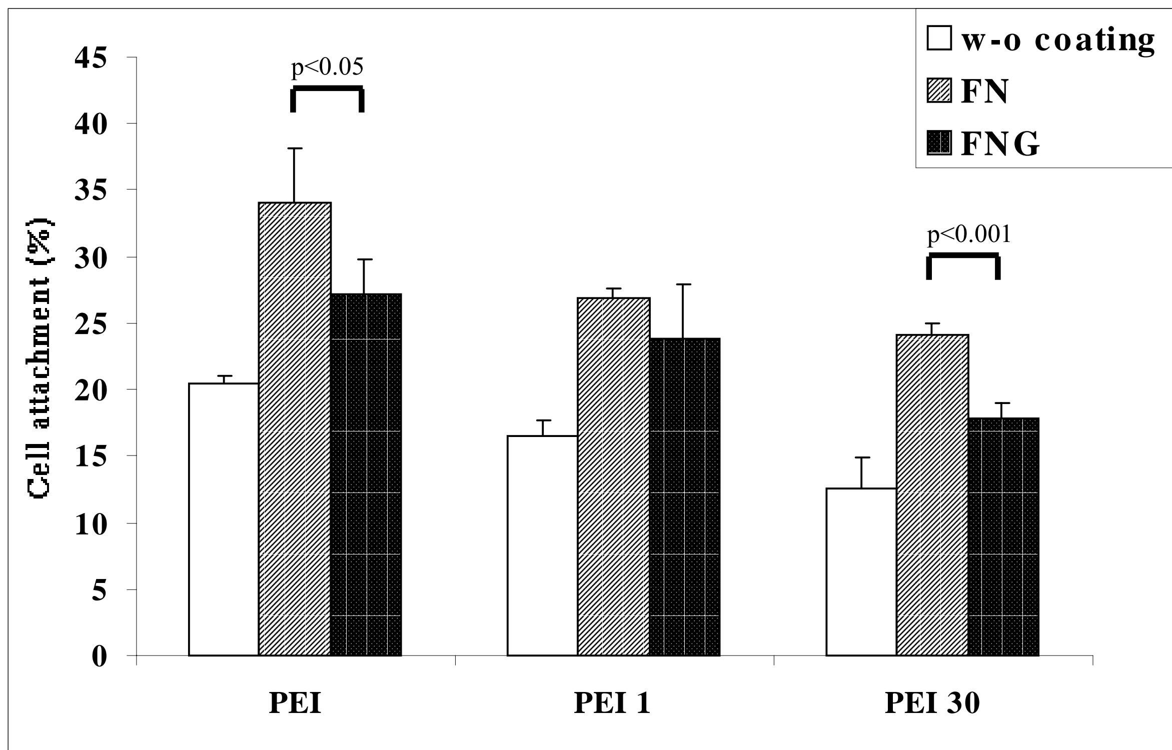


Fig.29. Attachment of HUVEC after incubation of 2 hours. HUVEC were attached to plain, FN coated and FNG coated unmodified PEI (PEI), PEI treated with IDA for 1 min (PEI 1) and PEI treated with IDA for 30 min (PEI 30). Results are the mean ±SD of two independent experiments, each performed in triplicate. For the statistical was used one way analysis with unpaired Tukey-Kramer multiple post test.

A slight, but significant ($p<0.05$) increase in the cell attachment was observed on FN coated unmodified PEI when is compared to FNG coated one (Fig 29). The difference between attached HUVEC on FN or FNG coated PEI 1 was not significant, where the cells attached to FN coated PEI 30 were higher than the same attached to FNG coated PEI 30 respectively ($p<0.001$). The viability of HUVEC attached to FN and FNG coated PEI membranes was higher than 95%. The viability of the cells attached to uncoated PEI membranes was in the range of 93-95%.

4.13. Cell proliferation

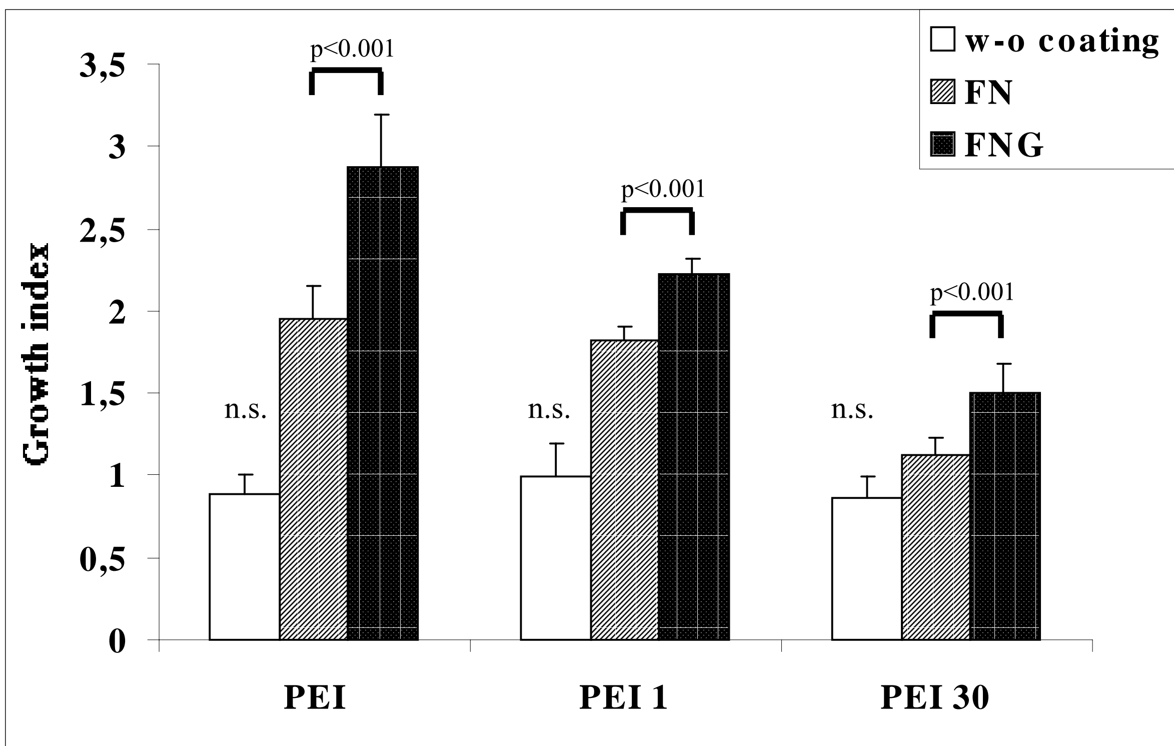


Fig. 30 Proliferation of HUVEC after 48 hours of incubation. HUVEC were incubated on plain, FN coated and FNG coated unmodified PEI (PEI), PEI treated with IDA for 1min (PEI 1) and PEI treated with IDA for 30 min (PEI 30). Results are the mean ± SD of two independent experiments each performed in triplicate. For statistics was used one way analysis with Tukey-Kramer post test.

The protein precoating of the membranes had a positive impact on the growth of HUVEC. The growth index in Figure 30 indicates the ratio between the number of HUVEC at the starting time and after 48h of incubation. The presented results showed that the uncoated membranes served as poor substrates for HUVEC growth. The results after 48h incubation showed that the number of cells on these membranes remained approximately the same when compare with the time zero ($p>0.05$) (Fig. 30). There was significant increase in the cell growth on protein coated membranes than on uncoated ones ($p<0.05$). Overall, HUVEC growth was higher on protein coated PEI, followed by PEI 1 and PEI 30. For each membrane, the FNG coating provoked enhanced cell growth when compare with FN coating ($p<0.001$). This fact could be correlated with the enhanced motility of cells seeded on FNG coated substrata as was discussed in Chapter II. The viability for FN/PEI and FNG/PEI was about 93-95 %. For all other membranes the cell viability was above 90 %.

4.14. Functionality of seeded HUVEC (prostacyclin production)

The PGI_2 production is one of the important factors for anti-thrombotic properties of the endothelium. The basal production of PGI_2 by HUVEC revealed the significant increase on the coated membranes in comparison to non-coated ones (Fig. 31 A). The type of protein coating showed a slight effect on PGI_2 production as only for PEI 30 the difference between two coatings was significant ($p<0.05$) (Fig.31 A). Overall, the PGI_2 production for FN coated PEI and PET was higher, followed by PET/FNG and PEI/FNG. The first –COOH modified PEI membrane (PEI 1) showed the same PGI_2 production on FN coating and even higher production on PEI-1/FNG when compared to the same coatings on PEI. In contrast, second –COOH modified membrane PEI 30 showed the lowest PGI_2 production for both protein coatings. After stimulation with TNF-α only HUVEC seeded on PET/FN and PEI/FN and retained a high level of PGI_2 production, as this level was highest for PET followed by PEI (Fig. 31 B). The FNG coating on the same membranes showed significant lower amount of PGI_2 ($p<0.001$). The production of PGI_2 by HUVEC seeded on the charged membranes PEI 1 and PEI 30 was very low (under 10 pg/cells x 10^5 PGI_2). Thus, the results revealed that uncharged protein coated membranes as PET/FN and PEI/FN produced significantly higher amount of PGI_2 when compare with negatively charged (-COOH bearing) membranes ($p<0.001$).

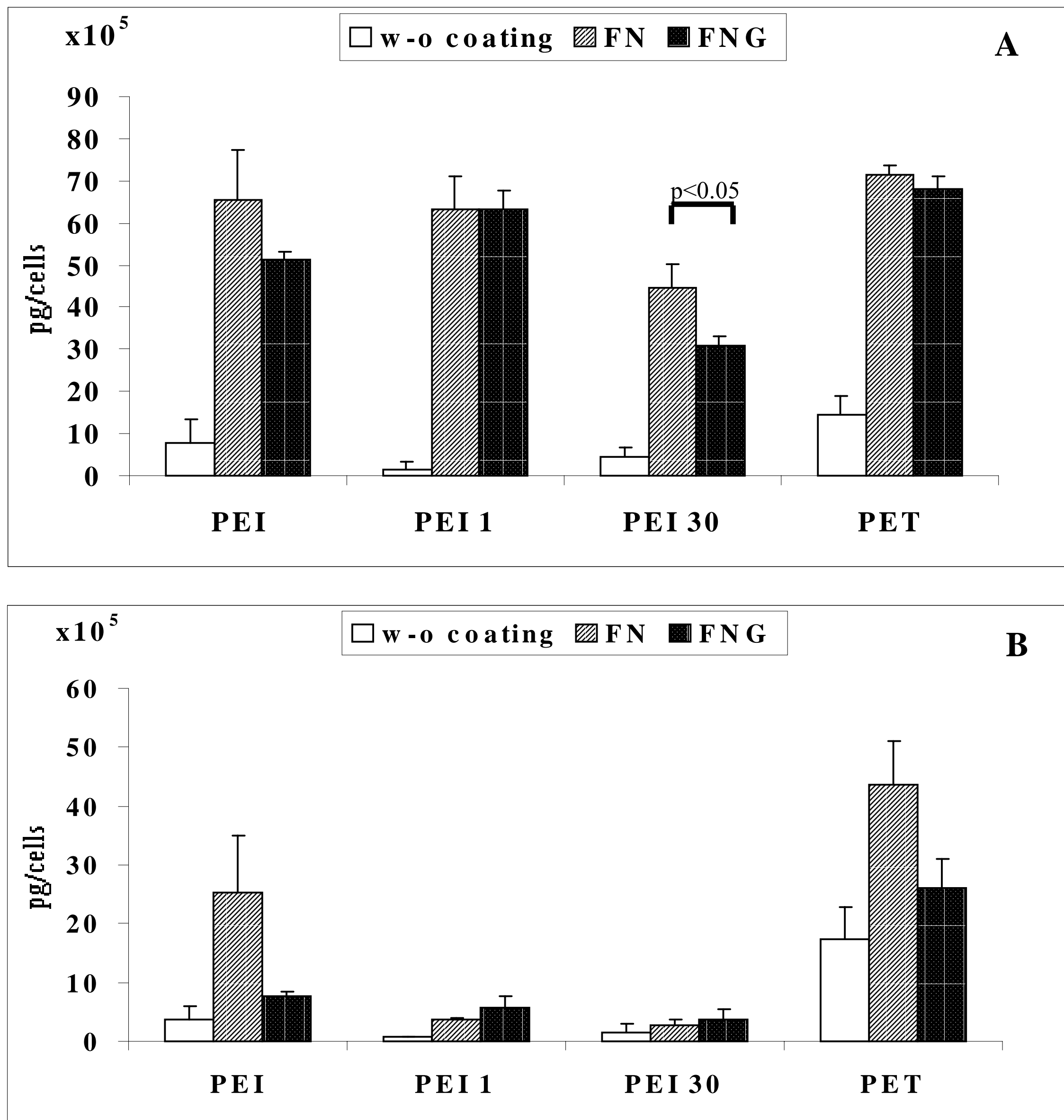


Fig. 31 PGI_2 production by HUVEC at basal and stimulated conditions. **A.** ***Basal PGI_2 production.*** HUVEC were incubated for 6 days on non-coated, FN or FNG coated unmodified PEI, PEI treated with IDA for 1 min (PEI 1), PEI treated with IDA for 30 min (PEI 30). Polyethylene terephthalate (PET) was used as controls. **B.** ***TNF-α stimulation*** The cells were incubated 24h then TNF-α (10μg/ml) was added to all samples for 5h. Results are the mean ±SD of two independent experiments each performed in triplicate. The statistic was performed by one way analysis using Tukey-Kramer post test.

Another interesting finding were that FN coating sustained higher PGI_2 production than FNG coating and thus ensure higher anti-thrombotic properties of the seeded EC (Fig.31 B).
To verify the benefit of FN coating on the platelet attachment and activation onto HUVEC endothelized substratum, we chose FN/PET, which appeared to increase HUVEC anti-thrombotic properties compared to FNG/PET used as acontrol. For that purpose the endothelized FN/PET and FNG/PET were treated with platelet-rich plasma (PRP) for 1h at 37°C. Platelet attachment and activation were visualized by ESEM. As can be seen in the Fig. 32 the type of protein coating prior to HUVEC seeding raised pronounced differences in the platelet response. On FN/PET no attached platelets were detected (Fig. 32A). Interestingly on this membrane single cells detached partly from the substrata can be seen (arrows in Fig. 32 C). In remarkable contrast, on FNG coated PET a significant amount of adhered platelets was found (Fig. 32 B). The platelets were well spread with many pseudopodia and even already formed aggregates can be observed (arrows in Fig. 32 D).

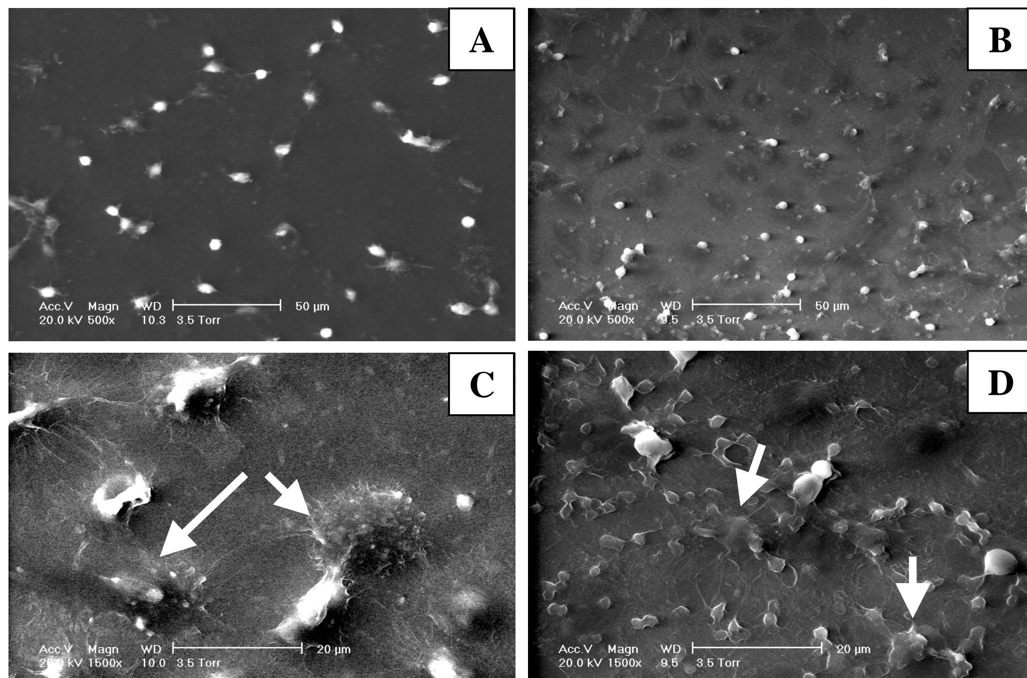


Fig. 32 ESEM analysis of platelet attachment to endothelized FN (A–B) and FNG (B-C) coated PET. HUVEC were seeded on FN or FNG coated PET for 3 days. Subsequently PRP was added for 1h at 37°C.

4.15. Discussion

EC seeding of vascular grafts is a recognized strategy to improve the patency of small–diameter synthetic vascular grafts [Wissink 2000]. Since, all existing materials used for vascular grafts are hydrophobic in order to prevent activation of coagulation cascade and platelet adhesion and activation, the protein coating was shown to be useful to promote EC adhesion [Kaehler 1989, Schneider 1993]. On the other hand it is well known that the substrate surface properties as wettability and surface charge can affect EC attachment and growth [van Wachem 1987, Pratt 1989, and Klein-Soyer 1989]. The most probable mechanism by which the surface properties affect the cellular behavior is by controlling the rate of the amount and the conformational changes in adhesive proteins upon adsorption [Steele 1995, Burmeister 1996, 1999]. And subsequently this protein deposition can regulate the expression of integrin receptors, which are key factors controlling cell adhesion and signaling [Hynes 1992, 2002]. The results of this study revealed that the protein coating had a positive effect on cell adhesion and growth as FN and FNG coated membranes showed higher adhesion and growth rate of HUVEC when compared with uncoated ones. In the study of Curtis et al. [Curtis 1986] was demonstrated that the increase of surface charge on carboxylated polymers increased the electrostatic repulsions of the surface and therefore decreased both protein adsorption and cell adhesion. The observations here were consistent with this demonstrated correlation. The adsorbed protein amount decreased with the increase of surface charge from PEI to PEI 30 and this was shown to influence the cell behavior. HUVEC were attached and grow better on plain PEI, which adsorbed the highest amount of FN and FNG. With the decreasing of the amount of adsorbed proteins on charged membranes from PEI to PEI 30, the cell attachment and cell growth were considerably decreased. An interesting observation was the fact that since FN was the best substrate for cell attachment, the FNG substrate showed higher cell growth at the later stage. The enhanced cell motility of HUVEC on FNG coated model surfaces showed above (in Results Part II) could explain the better growth on these substrata. The surface modification of PEI with loading of – COOH groups had a negative effect on cell attachment and cell growth as well. Seeding of EC on artificial substrates, however, may affect EC metabolism [Wissink 2001], which can alter the functionality of the endothelium. The PGI_2 production is an important prerequisite for the anti-thrombotic properties of the newly established endothelium [Crutchley 1994]. Its role

can be associated with down regulation of TF synthesis and inhibition of platelet adhesion and activation [Crutchley 1994]. Our results showed that FN and FNG coating of all the all polymer membranes increased PGI_2 (basal level) production. But when seeded HUVEC were stimulated with TNF–α only FN coated uncharged membranes PEI and PET sustained a relatively high PGI_2 levels. That fact was confirmed by ESEM analysis for the extent of thrombogenicity of PET membrane coated either with FN or FNG and seeded with EC. The findings that FNG coating caused platelet retention and aggregation since FN did not, supposed the role of FN substrate more than FNG as a promoter of anti-thrombotic properties of the seeded HUVEC. In conclusion, the surface charge of the membranes was shown to play also a role for the protein adsorption and subsequently on the cell behavior. The adsorbed protein amount on the charged membranes was lower which led also to the lower rate of cell adhesion and growth on these surfaces. The EC functionality together with the classical parameters of cell behavior like cell adhesion and growth must be considered for the design of the better blood compatible materials.

5. Summary

During the past several decades the use of polymer materials as components of medical devices and implants, such as hemodialysis devices, bioartificial organs as well as vascular and recombinant surgery has increased dramatically. All these devices cannot avoid the blood contact in their use. The earliest and one of the main problems in the use of blood-contacting biomaterials is the surface induced thrombosis. Since the protein adsorption on polymer surfaces is the first and "fate determining" step for thrombus to occur there is a need to study the mechanisms of protein adsorption. The protein adsorption is an interfacial phenomenon and therefore it was found to be strongly dependent on surface physico-chemical properties of the substrate such as surface wettability. FNG is present in plasma and is adsorbed on biomaterials in much higher amounts than other plasma proteins. Surface-bound FNG is related to surface thrombogenicity by participating in fibrin formation and platelet adhesion. In addition, the dimeric structure of FNG enables platelet-platelet bridging leading to macroscopic platelet aggregation.

In accordance with the first aim of the work (Chapter 1.2) namely the role of surface wettability on thrombogenicity of blood contacting devices, was found that the total amount

of adsorbed plasma proteins was in a close relation with the surface wettability of the polymer materials. The poorest wettable membrane PEI bearing the higher dispersive part of surface free energy adsorbed the highest amount of plasma proteins, followed by more wettable membranes PSU, PC-PC and CE. The main protein-material interactions driving the protein adsorption were found to be the hydrophobic and ionic interactions.

Further, the study of the FNG adsorption as a function of surface wettability revealed that the amount and the affinity of adsorbed FNG were dependent on surface wettability and surface energetics. Again the poorest wettable membrane PEI showed the highest affinity of FNG to substrata, which was correlated with the highest dispersive component of the surface free energy of the material.

The very important finding was the fact that the higher FNG affinity to the PEI membrane was related to the higher conformational changes in the platelet-binding domain in FNG. As a result, the conformational state of adsorbed FNG to PEI membrane did not make FNG absolutely resistant to platelet adhesion since many platelets were found on this substrate, but however their rate of activation was considerably low. In contrast the PSU membrane, which was adsorbed almost the same FNG amount but with less conformational changes showed a lot platelet aggregates and higher level of activation.

These data suggest that the distinct conformational changes in FNG molecule more than the total amount of adsorbed FNG should be considered as a main factor for platelet adhesion and their subsequent activation on polymer surfaces.

EC seeding on polymer materials is a promising approach to improve the blood compatibility especially for small diameter vascular grafts. Precoating of the materials with adhesive proteins present in blood such as FN and FNG has been shown to improve the cell adhesion and growth. ECM proteins play an essential role not only like a structural support for cell adhesion and spreading but also in cell signaling transmitting signals for cell growth, differentiation and survival. The ability of cells to remodel plasma proteins in matrix-like structures is an essential factor for regulating various physiological and pathological processes such as wound healing and atherosclerosis. Since FN remodelling is rather well studied by various cell types, there are no data for the influence of materials surface wettability on FNG remodelling by HUVEC.

The second aim of this work was to study and to create criteria for the successful seeding of EC on material surfaces in dependence on the material surface wettability and the type of protein coating.

The study of the FN and FNG adsorption on glass and ODS glass showed a low accessibility of the cell-binding domains in the adsorbed proteins to hydrophobic substrata when compare with hydrophilic glass.

The type of protein coating was found to be very important for the expression of different cell phenotype. Since HUVEC seeded on FN coated substrata showed typical adhesive phenotype, the FNG coating provoked a motile cell morphology, which is appear to be important for the colonization of the implants with EC.

Here is found that the ability of HUVEC to remodel adsorbed and soluble FN and FNG was strongly influenced by surface wettability since it was well pronounced only on the hydrophilic substrata.

FN fibrillogenesis was found to be a critical regulator of ECM organization and stability. An intact FN matrix was required for the deposition and fibrilar organization of FNG.

Focal adhesions and cytoskeleton organization revealed a different strength of cell adhesion in dependence on the substrate wettability. A stronger cell adhesion was found on hydrophilic substrata than on hydrophobic ones. These differences were better pronounced on FN coated surfaces.

Cell-cell interactions, which play an important role for the cell communication and EC monolayer integrity, were also found to be influenced by material surface wettability. Adherent junctions with active E-Cadherin deposition were detected preferentially on hydrophobic substrata after 3 days of cell incubation. Strictly differences in the cell deposition of E-Cadherin were found again for FN coated substrata.

The observed fact that the FN coating ensured more stationary cell morphology in contrast to the motile cell morphology on FNG coated substrata (Results and Discussion II Part), was used in the study of HUVEC adhesion and growth on polymer membranes with different charge density (increasing–COOH density on the active membrane layer).

First, the results showed a negative effect of the increasing surface charge density on the initial cell attachment and cell growth most probably due to the observed decreased

adsorption of FN and FNG on the charged membranes when compared to the unmodified PEI membrane.

HUVEC seeded on FN coated membranes showed better adhesion than those seeded on FNG coated substrata. The results here could be correlated with the observed adhesive cell phenotype on FN coated model surfaces (Results and Discussion II Part). Interestingly, the cell growth after 48h was higher for FNG coated membranes than for FN coated ones.

The study of EC functionality, revealed by the prostacyclin (PGI_2) production, showed that the protein precoating of the membranes had a strong positive effect on PGI_2 production. In general, the uncharged membranes PEI and PET revealed the higher amount of PGI_2 production when is compare with the charged membranes PEI 1 and PEI 30. Only HUVEC seeded on FN coated uncharged membranes PEI and PET sustained a higher level of PGI_2 secretion after stimulation with TNF-α and showed higher anti-thrombotic properties by not supporting platelets adhesion and aggregation.

Overall these data suggest that the process of matrix remodelling by HUVEC is an important process for the cell adhesion and spreading. A delicate balance between the strength of cell-matrix and cell-cell adhesion should be considered for the better EC colonization of the implants. The EC functionality together with the other parameters of cell behavior like ECM remodelling, cell adhesion and growth must be considered for the design of better blood compatible materials.

6. Perspectives

Studying the relation between material surface properties - protein adsorption – subsequent cell behavior could contribute to the better knowledge in biomaterials field to create more thromboresistant materials.

The functional state of adjacent endothelium should be further studied in respect to the regulation of the hemostatic balance in a favor of anti-coagulant activity.

The possible role, which the deposition and fibrilar organization of FNG could play, for the providing procoagulant and proinflammatory stimuli for newly established endothelium by binding to platelets and leukocytes, should be investigated.

Studying the regulation of the secretion of different matrix metalloproteinases, in dependence of protein coating and material surface properties, could contribute to the better understanding of the control of the endothelium functionality.

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Erklärung

Hiermit erkläre ich, daß die vorliegende Arbeit bisher an keiner anderen Hochschule eingereicht worden ist sowie selbständig und ausschließlich mit den angegebenen Mitteln angefertigt wurde.

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