THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

COLLAGEN AND THROMBIN, TWO OF THE CRUCIAL COMPONENTS OF THROMBUS FORMATION

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Abstract

Haemostasis is the process that maintains the integrity of a closed, high-pressure circulatory system after vascular damage. When the vessel wall is breached or the endothelium is disrupted, collagen and tissue factor become exposed to the flowing blood, thereby initiating formation of a thrombus. Exposed collagen triggers the accumulation and activation of platelets, which rapidly adhere to collagen and various glycoprotein, activated and aggregate, a process that amplified by the local generation of thrombin. Whereas exposed tissue factor initiates the generation of thrombin. These events occur concomitantly, and under normal conditions, regulatory mechanisms contain thrombus formation temporally and spatially. When pathologic processes overwhelm the regulatory mechanisms of haemostasis, excessive quantities of thrombin are formed, initiating thrombosis.

This thesis is focus on the two crucial components, the collagen and the thrombin that have a major role in thrombus formation.

Collagen is one of the most thrombotic surfaces on which thrombus is growing when endothelium is damaged. Whether the collagen surface and platelet adhesion-aggregation or thrombin is the dominant process, it is shear dependent. *In vitro* thrombosis models are used to study this process at static and different shear conditions. In order to optimize the collagen surface for these experiments, **the first aim was to find the optimal condition to prepare repeatable collagen surfaces for VWF and platelet binding**.

The effects of pH, salt and ligand concentration and binding time were tested when human collagen type I and III matrices were prepared by adsorption. Surface-bound collagen and collagen-bound VWF measured by specific antibodies. Platelet adhesion was tested under

flow conditions at a shear rate of 1800 s^{-1} for 2 min. Matrices and platelets were visualized by atomic force and scanning electron microscope.

The extent of human collagens type-I and III binding to the surface was 10 and 4 times greater and binding was maximal under 8–16 hours, when coated from physiological buffer solution versus acid solution. Collagen fibrils were more developed and platelet adhesion was higher, with more organized and denser aggregates. VWF binding was parallel to the surface bound collagen in both collagen types.

In conclusion: Collagen coating of surfaces for VWF binding and platelet adhesion studies is very variable from acid solution. Our experiments provide evidences that neutralizing the acid and adding NaCl in physiological concentration, thereby facilitating formation of collagen fibril molecules in solution, results in efficient coating of human type-I and type III collagens, which then bind normal VWF equally well.

Thrombin is a coagulation protein that has many effects in the coagulation cascade, converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation and anticoagulant related reactions. Therefore thrombin is usually considered as the suitable target for anti-coagulants and antithrombotics to inhibit blood coagulation. I studied thrombin formation and examined the antithrombotic effect of a new group of thrombin inhibitors. These new groups are the aptamers. Aptamers are single-stranded DNA oligonucleotides, have been identified with high binding affinity for specific targets, including thrombin. Bock et al. selected aptamers with a consensus sequence of 15 nucleotides (C15-mer) that dramatically inhibited the ability of thrombin to clot fibrinogen. As has been shown in our earlier studies, the 4-thio-deoxyuridylate (s4dU)-containing

oligonucleotides have high affinity for a number of proteins, due to the reduced hydrophilic character of the modified oligonucleotide. **The second aim was to examine the effects of 4-thio-deoxyuridylate-containing analogs of thrombin-binding aptamers on different hemostatic/thrombotic activities of thrombin and compared them with the effect of C15-mer.**

Three different analogs (UC15-mer, aUC15-mer, vUC15-mer) of the original thrombin-inhibiting sequence, in which some of the thymidylate residues were replaced by 4-thio-deoxyuridylates, were synthesized. The inhibitory effect of modified aptamers was tested on thrombin-catalyzed fibrin clot formation and fibrinopeptide A release from fibrinogen, thrombin-induced platelet aggregation/secretion, and the formation of thrombus on coverslips coated with human collagen type III, thrombin-treated fibrinogen or subendothelial matrix of human microvascular endothelial cell.

As compared with the C15-mer, the analog with the sequence GG(s4dU)TGG(s4dU)G(s4dU)GGT(s4dU)GG (UC15-mer) showed a 2-fold increased inhibition of thrombin catalyzed fibrin clot formation, fibrinopeptide A release, platelet aggregation and secretion in human plasma and thrombus formation on thrombin-treated fibrinogen surfaces under flow conditions. Concerning the inhibition of thrombin induced fibrin formation from purified fibrinogen and activation of washed platelets, UC15-mer was 3-fold and twelve-fold more effective than C15-mer, respectively.

In conclusion: the replacement of four thymidylate residues in C15-mer by 4-thio-deoxyuridylate resulted in a new thrombin aptamer with increased anticoagulant and antithrombotic properties.

Nomenclature

AFM	atomic force microscopy
ATP	Adenosine Triphosphate
AS	average size
ELISA	enzyme-linked immunosorbent assay
GPIb	glycoprotein Ib (CD42b)
GPIIb/IIIa	glycoprotein IIb/IIIa (CD41/CD61, αIIβb3)
GPIX	glycoprotein IX (CD42a)
GPV	glycoprotein V (CD42d)
GPRP	fibrin polymerization inhibitor peptide Gly-Pro-Arg-Pro
GPVI	glycoprotein VI
HMEC	human dermal microvascular endothelial cells
HRP	horseradish peroxidase
K_d	dissociation constant
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAR	protease activated receptors
PBS	phosphate buffered saline
PPP	platelet poor plasma
PRP	platelet rich plasma
SC	surface coverage
SEM	scanning electron microscopy
TRAP	thrombin receptor activator peptide

VWD	von Willebrand Disease
VWF	von Willebrand Factor
VWF:Ag	VWF antigen
VWF:CB	VWF collagen binding capacity
VWF:RCo	VWF Ristocetin cofactor activity
WPS	washed platelet suspension

Introduction

Haemostasis is the process that maintains the integrity of a closed, high-pressure circulatory system after vascular damage. When the vessel wall is breached or the endothelium is disrupted, collagen and tissue factor become exposed to the flowing blood, thereby initiating formation of a thrombus. Exposed collagen triggers the accumulation and activation of platelets, which rapidly adhere to collagen and various glycoprotein, activated and aggregate, a process that amplified by the local generation of thrombin. Whereas exposed tissue factor initiates the generation of thrombin (Fig. 1.). These events occur concomitantly, and under normal conditions, regulatory mechanisms contain thrombus formation temporally and spatially.

When pathologic processes overwhelm the regulatory mechanisms of haemostasis, excessive quantities of thrombin are formed, initiating thrombosis.

Thrombosis is a critical event in the arterial diseases associated with myocardial infarction and stroke, and venous thromboembolic disorders that account for considerable morbidity and mortality [1]. This is why it's important to understand the mechanism of thrombus formation and its regulation by antithrombotic agents.

During the period of my PhD study I got knowledge on the field of haemostasis and thrombosis and focused my research on two major components involved in thrombus formation: the collagen, and the thrombin (Fig. 1.). Collagen is one of the most thrombotic surfaces on which thrombus is growing when endothelium is damaged. Whether the collagen surface and platelet adhesion-aggregation or thrombin is the dominant process, it is shear dependent. *In vitro* thrombosis models are used to study this process at static and

different shear conditions. In order to optimize the collagen surface for these experiments, I studied collagen coating conditions and their effect on VWF and platelet binding. Thrombin has a central role in thrombus formation and thrombin inhibitors are major regulators of thrombus formation at venous sheer conditions. I studied thrombin formation and examined the antithrombotic effect of a new group of thrombin inhibitors. These new groups are the aptamers.

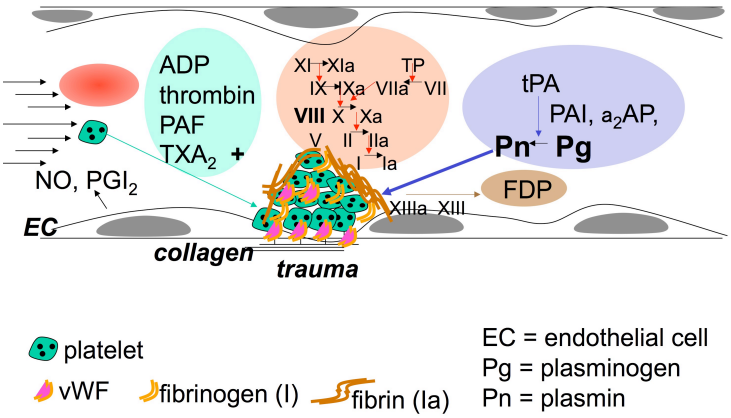

Chart 1. Formation of the primer haemostatic plug and the activation of the coagulation cascade [2]. The first step is the arrest of blood platelets on collagen of the injured vessel at arterial or higher shear rate. The interaction between high affinity VWF-binding sites and collagen then VWF-platelet GPIb results in a marked loss of velocity of the platelets. The second step is the binding of platelet collagen receptors to collagen sequences. This results in full platelet activation, firm attachment, and □IIb□3 activation. In addition, release of ADP and TXA2, in parallel, tissue factor (TF) triggers thrombin formation locally, which also contributes to platelet activation. Tissue factor activates the extrinsic pathway of the coagulation cascade leading to clot formation. (Presented by the permission of Harsfalvi)

Collagen is the most abundant protein in the human body, where it is a vital component of the extracellular matrix and connective tissue, including blood vessels. There are 29

collagens types, of which seven (types I–III, V, XI, XXIV, and XXVII) are fibrillar, and are able to assemble as stable triple helices, which then form a more complex three-dimensional fibrous superstructure. Another structure is non-fibrillar collagen, such as types IV and VI, which form two and three dimensional networks, supporting the interstitial tissue of the body. At the simplest level, the collagen polypeptide sequence is composed of G*xx*' triplets containing glycine followed by variable residues that often include proline at position *x* and hydroxyproline at position *x*'. The three polypeptide [3]chains that assemble to form a tropocollagen triple helix can be identical gene products, as for collagen III (three 1 chains) or may differ, as for collagen I (two 1 chains and one 2 chain). The tropocollagen triple helices assemble to form a supermolecular fiber (for review, see [3,4]. The two most abundant collagens of the blood vessel wall are types I and III, both present in the subendothelial intima. Collagen type I may be enriched in atherosclerotic plaque especially in its fibrous cap, but collagen III is also abundant. Several other collagens, including fibrillar type V, have been located within the vessel wall. Most information concerning receptor-collagen interaction is based on studies that used collagens type I and III of different origin. Skin and placental material is high in the mentioned collagens [5] and most frequently is the source for preparation of them.

Collagen and von Willebrand factor interaction plays a crucial role in thrombosis and haemostasis. Collagen activates platelets through collagen receptors glycoprotein GP VI and integrin $\alpha_2\beta_1$ and induces platelet plug formation and occlusion at sites of vessel damage under venous shear condition by recruiting platelets to exposed collagen. At arterial shear rates of flow, VWF and its platelet surface receptor GPIb-IX-V play a critical role in initiation of thrombus formation. The role of VWF and GPIb-IX-V is negligible in

venous shear rates of flow. Under arterial shear, VWF in circulation binds to collagen via its A3 domain, and then platelets are tethered to the complex of collagen and VWF through GPIb-IX-V binding to VWF [6]. This is followed by platelet activation mediated by the binding of collagen to GPVI, leading to inside-out stimulation of integrin $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ and platelet thrombus formation [7].

VWF is primarily synthesized in vascular endothelial cells and get released to the circulation and subendothelium, where it closely associates with the major extracellular matrix collagen [8]. VWF is a multimeric glycoprotein composed of various numbers of monomers. Each of these monomers is composed of several homologous domains that interact with other molecules. VWF interacts with the platelet GPIb-IX-V complex, heparin, cell sulfatides and non fibrillar collagen type VI through its A1 domain [9-12] and with vascular fibrillar collagen fibers (types I and III) through its A3 domain [13]. Recently Bonnefoy et al and Morales et al suggest that both A1 and A3 domains mediate the binding of VWF to collagen, with A3 being the dominant binding domain, and it appears that each domain recognizes different sequences of collagen [14,15]. Under normal conditions, VWF does not interact with platelets. However, under pathological conditions, for instance in arteries at the sites of atherosclerotic plaques, VWF binds to surface-exposed collagen fibers through its A3 domain, subsequently undergoes a conformational change allowing it to bind to platelet receptor GPIb, and thus mediates platelet recruitment to the subendothelium [16].

The interaction of collagen with von Willebrand factor requires unique structural properties in both proteins. Optimal haemostatic function requires multimerization of up to 50 VWF monomers in circulating plasma; higher-order multimers bind collagen more tightly than

smaller assemblies of VWF [17]. Lisman et al identified the sequence RGQOGVMGF (O is hydroxproline) as the minimal VWF-binding sequence in collagen type III that support platelet adhesion under static and flow conditions. In human collagen type I, a heterotrimer comprising 2 1 and 1 2 chains, a closely related sequence occurs in the 1 chain, differing by a single amino acid (RGQAGVMGF). So that VWF may bind collagen I an identical manner [18].

The quantitative or qualitative abnormality of VWF is Von Willebrand's disease (VWD). It is the most common inherited bleeding disorder with a prevalence of 0.1%-1% [19]. Three major categories of VWD are distinguished [20]. Types 1 and 3 refer to partial and severe quantitative deficiency of VWF respectively, whereas type 2 refers to qualitative abnormalities. Type 2 VWD is further divided into four subtypes (A, B, M and N). Type 2A refers to variants with decreased platelet-dependent function associated with the loss of high molecular weight (HMW) multimers of VWF. Type 2B variants show an increased affinity for platelet glycoprotein (GP) Ib resulting in a loss of HMW multimers in plasma. Type 2M refers to qualitatively abnormal variants with decreased platelet-dependent function not associated with the loss of HMW multimers. Type 2N variants show a markedly decreased affinity for FVIII.

In most laboratories, the basic screening tests used in order to evaluate a new patient suspected with having VWD are: VWF antigen (VWF:Ag) level, factor VIII coagulant (FVIII-C) activity,. and VWF function, which has classically been assessed using ristocetin cofactor (VWF:RCo) assay and ristocetin induced platelet agglutination. More recent attention has focused on another functional VWF assay the VWF collagen-binding (VWF:CB) assay, as a possible replacement for the VWF:RCo assay or as supplementary

test of VWF adhesive "activity"[21]. The VWF:CB assay is like an ELISA, measures the amount of VWF bound to immobilized collagen. Primarily HMW multimers are detected with the method [22]. However, its international acceptance has not been fully achieved. Probably due to 'apparent poor performance' in some laboratories (potentially due to use of inappropriate collagen preparations or non-optimized assay conditions), therefore it is essential to find optimized conditions for collagen coating to ELISA plate.

Furthermore collagen is also a frequently used matrix in platelet adhesion assays. Platelet adhesion assay is used to study platelet physiology and one of the tests of platelet function in the clinical practice [23]. This assay closely reflect the physiological milieu of platelets, including flow, red blood cells, and other blood components, regulating the interaction between subendothelial proteins and plasma proteins with platelet receptors in the adhesion and aggregation process. Measurement of platelet adhesion in whole blood samples under flow condition may be achieved by few technologies, for example in a collagen coated capillary, a parallel plate chamber, and cone and plate device [24]. The PFA100, platelet function analyzer is one of the best known, commercially available device. In this study I used the cone and plate apparatus which induces a laminar flow with uniform shear stress over the entire plate surface covered by the rotating cone [25].

There is no harmonization between labs, neither for VWF:CB assay, nor for the adhesion. The collagen surface itself is very uneven, see Supplementary Table 1 and Supplementary Table 2 for collagen coating condition use for VWF:CB assay and for platelet adhesion respectively. Therefore I aimed to test various collagen coating conditions for VWF:CB and platelet adhesion to find the best condition for coating collagen as presented in part of this work.

Thrombin is a coagulation protein that has many effects in the coagulation cascade, converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions. Thrombin recognizes various macro molecular substrates to function as procoagulant and anticoagulant, it contains catalytic site and two anion-binding exosites, one of which binds fibrinogen [26,27]. Therefore thrombin is usually considered as the suitable target for anti-coagulants and antithrombotics to inhibit blood coagulation [28].

In part of my PhD work I studied a new class of thrombin inhibitors, the thrombin aptamer. 20 years ago Ellington et al found oligonucleotide molecules that bind specific ligands [29,30]. Those molecules named 'aptamer' literally mean "to fit" (aptus) in latin. RNA and single-stranded DNA aptamers have been generated against a range of clinically relevant targets including viral proteins growth factors, transcription factors, and coagulation proteins. Such aptamers can be isolated from high complexity, randomized libraries that bind to essentially any targets including proteins, peptides, nucleic acids, polysaccharides, small organic molecules (amino acids, nucleotides, and other metabolites), virus particles, whole cells, and even tissues [31]. They are selected through iterative *in vitro* selection techniques known as SELEX (for “systematic evolution of ligands by exponential enrichment”). SELEX process begins with a random sequence library obtained from combinatorial synthesis of DNA. Each member in a library is a linear oligomer of a unique sequence containing random region flanked by the fixed sequences for amplification process. In the aptamer selection process, the oligonucleotide library is incubated with a target of interest and buffer of choice at a given temperature. The bound oligonucleotides

were then separated from the unbound oligonucleotides by filtration or affinity process and amplified. Several rounds of stringency selection and amplification cycles were carried out to find the high affinity molecules. Once the saturation of binding affinity to the target molecule is achieved, the enriched library is cloned and analyzed the sequences of individual oligonucleotide [32]. The SELEX procedure is quite simple and makes possible obtaining new ligands in just one or two months.

Binding affinities of aptamers for the targeted proteins tend to be very high with typical dissociation constants (K_d's) ranging from low picomolar (1×10^{-12} M) to low nanomolar (1×10^{-9} M), similar to the affinities measured for the most avid monoclonal antibody/antigen interactions. Furthermore, the intricate nature of the protein–aptamer interaction results in the production of aptamers highly specific for the target protein, like epitopes for enzyme active sites [31,33].

Aptamers have promising advantages compared to antibodies: they can be produced easily and inexpensively. They have low immunogenicity and able to fold complex tertiary structures with high specificity to their targets [34]. It is simple to chemically modify them and integrate into different analytical schemes. Aptamers can retain their binding and inhibitory behavior after immobilization on a carrier material or after delivery into animals and can be labeled with various functional groups. These properties of aptamers have led to their application in many areas of biomedical sciences such as purification processes, target validation, drug development, diagnostics, MRI-based cell tracking and even for therapy. In addition, aptamers have short half-life, minutes to hours, due to nuclease degradation, and can be rapidly cleaned from the serum by the kidneys [35]. Moreover, the effect of

aptamers can be antagonized via its inhibition using anti-sense sequences or using other ligands [36].

Selecting the oligonucleotides *in vitro* enables us to use nucleotides containing chemically modified parts. Modifications are introduced into 2' position of all nucleotides, positions 5' of pyrimidines, 7' and 8' of purines, etc. An alternative position that has been targeted for modification is the 4'-oxygen atom in the nucleotide ribose ring.

There are two approaches to obtain modified aptamers. In the first approach modified oligonucleotides are used directly during selection. The main restriction in this case is that modification should not significantly influence the ability of the nucleotide to serve as a substrate for RNA or DNA polymerase. In the second approach already obtained aptamers are modified. In this case, the variety of available modifications grows significantly (because there are no restrictions for their compatibility with enzymology of RNA and DNA syntheses), but many modifications may result in lowering the aptamer affinity to their targets. Owing to this, it is necessary to search for modifications not affecting the interaction of the aptamer with the target. Modifications are introduced to attain the following:

1. Optimize their pharmacokinetic, pharmacodynamic profiles and to promote their safety (e.g., render them non-immunogenic).

2. Provide more contacts between aptamers and their targets, which should stimulate obtaining of aptamers with higher affinity and specificity towards their targets [37].

3. Make them nuclease-resistant, which is of great importance when applying aptamers for diagnosis and therapy [38].

4. Introduction of fluorescent groups into an aptamer which is used for analysis of its binding to the protein target [39].

Aptamers have been designed for several blood-clotting factors, such as von Willebrand factor, thrombin, factor VII, and factor IXa [26,28,40-42]. The goal of a majority of these aptamers is to prevent coagulation during acute cardiovascular events. In this scenario, the short half-life of aptamers is ideal therapeutically compared to antibodies or small-molecule inhibitors. Several applications are in different stages of developments and also with pre-clinical and clinical trials. For example, a thrombin-specific DNA aptamer, NU172 (Nuvelo/Archemix) has been evaluated in Phase I clinical trials for intravenous administration during acute cardiovascular surgery and a Phase II trial is being planned (see Archemix web site: www.archemix.com).

My study focuses on DNA aptamer found by Bock et al [26] against human protease thrombin. Among their selected clones with SELEX process, they found the most conserved sequence as GGTTGG in almost all the clones. In addition much delayed thrombin catalyzed conversion of fibrinogen to fibrin (from 25 s to 169 s) was noticed with 15-mer having the sequences 5'-GGTTGGTGTGGTTGG-3' (C15-mer). The structure of this sequence and thrombin complex was solved and explaining that the aptamer is sandwiched between positively charged regions of two symmetry-related thrombin molecules making ionic and hydrophobic interactions [43]. Here I worked with three modified analogs of the original DNA thrombin aptamer (s4Du) [26], in which some of the thymidylate residues were replaced by 4-thio-deoxyuridylate (s4Du). The (s4Du)

containing oligonucleotide has a high affinity for a number of proteins, due to the reduced hydrophilic character of the modified oligonucleotide [44,45]. There are many modification of thrombin aptamers, but (s4Du) modified analogs hasn't been studied before, *see Supplementary Table 2.* We tested the inhibitory effect of the modified analogs on thrombin-catalyzed fibrin clot formation and fibrinopeptide A release from fibrinogen, thrombin induced platelet aggregation/secretion, and the formation of thrombus on coverslips coated with human collagen type III, thrombin-treated fibrinogen or subendothelial matrix of human microvascular endothelial cells. As compared to C15-mer, one of the modified analogues showed increased inhibition on different haemostatic/thrombotic activity of thrombin resulted in a new thrombin aptamer with increased anticoagulant and antithrombotic properties. Up to date there were few published modifications of C15-mer in which additional group was added in order to receive higher inhibition, extended lifetime and resistance to nucleases. The (s4Du) containing oligonucleotide is a new group -which hasn't been tested before- with promising characters.

Objectives

Our aim was to study thrombus formation at near physiological condition and its possible regulation. We focused on two crucial components of the process, the collagen and the thrombin.

Many different collagen surfaces as matrices are used to study the thrombus formation process, platelet binding to collagen and thrombin formation on the surface of activated platelets. However there is no standardization or consensus how to produce collagen surface matrices for research. The triple helical domain conformation structure of collagen is needed for recognition of collagen by it ligands (VWF, platelet and various glycoprotein), when the vessel wall is breached and collagen become expose to the flowing blood.

- The first aim was to analyze various conditions presented in the literature that influence on collagen surfaces: the 96 well plate surface, the pH of the solution used to dilute collagen, the coating time used to prepare collagen surfaces, the ionic strength of collagen solution and the collagen concentration in order to find the optimal condition.
- In addition to evaluate platelet adhesion under flow condition to different collagen surfaces to reach repeatable adhesion.
- AFM and SEM, to better understand our results, also were planed to apply, for visualize matrices and platelets.

The thrombin, which plays a pivotal role in hemostasis (converting fibrinogen into fibrin; activated factor V, VIII and XI, triggers platelet activation) and exerts anticoagulant effect (activating protein C and fibrinolysis inhibitor).

- Therefore the second aim is to study a new class of thrombin inhibitors, the "thrombin binding aptamer", which have shown promising results already, but in our study we planned to examine the effect of modification (4-thio-deoxyuridylate) containing analogs of the thrombin binding aptamer on different hemostatic/thrombotic activities of thrombin; clotting activity amidolytic activity, platelet activation and thrombus formation under shear condition and compared them with the effect of consensus aptamer in order to achieve superior thrombin aptamer inhibitor.

Materials and Methods

Materials

Pepsin-digested collagens from human placenta, Sigma (Collagen type III: Catalogue # C4407, Collagen type I: Catalogue # C7774; St Louis, MO, USA); polyclonal, rabbit anti-human collagen type-III with a 10% cross-reactivity against type I (Catalogue# AB747; Chemicon Temecula, California, USA); purified VWF, Haemate P (CSL Behring, Marburg, Germany); horseradish-peroxidase (HRP)-conjugated rabbit polyclonal anti-human VWF, goat anti-rabbit IgG-HRP, Dackocytomation (Glostrup, Denmark); 96-well plates, Nunc MaxiSorp, (polystyrene, catalogue#: 44-2404-21; Wiesbaden, Germany), Propilen E.C. (polypropylene; Pecs, Hungary), Greiner (polyvinyl chloride; medium and high binding capacity, Catalogue# 655080 and 655081 respectively; Nürtingen, Germany).

Human thrombin, hirudin, hirudin fragment 54-65 (hir54-65), purified fibrinogen, trypsin, fibrin polymerization inhibitor peptide Gly-Pro-Arg-Pro (GPRP), thrombin receptor-activator peptide for PAR-1 (SFLLRNP, TRAP-1), human collagen type III, diaminobenzidine (DAB) were purchased from Sigma-Aldrich (St.Louis, U.S). Chromogenic thrombin substrate S-2238 (H-D-Phe-Pip-Arg-pNA) was from Chromogenix (Milano, Italy). Reptilase time reagent (batroxobin) was purchased from Diagnostica Stago (Asnieres, France), Chrono-Lume kit was from Chrono-log Co. (Havertown, U.S). Aptamer 5'-GGTTGGTGTGGTTGG-3' (C15-mer) purified by ion-exchange chromatography was purchased from VBC Biotech Services (Vienna, Austria). BCA^{TM} Protein Assay Kit was from Pierce (Rockford, U.S). Collagen Horm was the product of

Nycomed, Norway. GraFit (Erithacus Software Limited, Horley, UK) was used for the calculations of the IC_{50} values.

Preparation of Collagen Matrices and Measuring Binding Efficacy

Human collagen type-III or I was dissolved in 0.05 M acetic acid (pH 2.8) to obtain 2 mg/mL stock solutions. The stock solutions were treated differently: (1) further diluted with 0.05 M acetic acid; (2) diluted with phosphate buffered saline (PBS); (3) diluted with 0.05 M acetic acid and neutralized locally by adding Na_2HPO_4; (4) dialyzed against PBS for 2 days at 4°C. The solutions were added into the wells (100 µL/well) at final concentration of 20, 10, 5, 2.5 µg/mL, and were incubated overnight at 4°C.

To study the effect of pH, collagens were coated from 6 different solutions: sodium acetate 0.02 M, pH 4.0 (1); sodium phosphate 0.02 M, pH 6.4 (2), pH 7.4 (3) and pH 8.0 (4); sodium carbonate 0.02 M, pH 9.0 (5) and acetic acid 0.05 M, pH 2.8 (6); with varying concentration of NaCl. Collagen coated wells were blocked with 3% (w/v) casein in PBS for 30 minutes at room temperature. Each step followed by washing with PBS contained 0.1% Tween-20 (PBS-T). The wells were then incubated with anti-human collagen type-III (100 µL/well diluted to 1:1000 in PBS-T) or with purified VWF (0.1 U/100 µL/well) for one hour at room temperature. The bound collagen and VWF were detected with anti-rabbit IgG-HRP or rabbit anti-human VWF-HRP (100 µL/well diluted to 1:3000 or 1:2000 in PBS-T) and the incubation continued for one hour. Then peroxidase substrate was given and the reaction was stopped with 2 M H_2SO_4, the optical density (OD) was measured by Infinite 200M reader (Tecan TradingAG, Männedorf, Switzerland), at optimal colour development of the substrate reaction, when the collagen was coated from PBS.

Atomic Force Microscope (AFM)

Drops of 10 L solutions were put onto glass coverslips and kept in a humid box for 48 h at 4°C. The final concentrations of the collagens were 200 g/mL in acid and 20 g/mL in PBS solutions. Imaging was performed with a custom-made stand-alone-type AFM (Department of Biophysics and Cell Biology, University of Debrecen, Hungary; University of Twente, Enschede, The Netherlands; Zeiss Axiovert microscope, Carl Zeiss, Jena, Germany) in tapping mode with samples under water. Cantilevers with Si_3N_4 pyramidal tips (Park Scientific Instruments, FWMS-06AU, Sunnyvale, CA) and with diameters between 10 and 30 nm were used with an average spring constant of approximately 0.06 N/m. 10×10 μm (x,y) surfaces were scanned. Images of 512×512 pixels were collected and processed with software including plane fitting and x-y flattening. Images were analyzed and processed by SPIP software (Image Metrology A/S, Lyngby, Denmark).

Blood Collection and Handling

Blood samples were obtained from healthy volunteers who had not taken anti-platelet medication for at least 2 weeks. 9 volumes of blood was anticoagulated with 1 volume of 105 mM sodium citrate for platelet aggregation studies, clotting time assay, or by 10 U mL^{-1} low molecular weight heparin (Fraxiparine, Glaxo Wellcome Production S.A.S. France) for platelet adhesion studies. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were separated by centrifugation at 190 g (15 min, 30-37°C), and 2,000 g (10 min), respectively. For the preparation of washed platelet suspension (WPS) PRP was obtained from citrated blood containing 0.18 μM prostaglandin E_1. Platelets were pelleted at 1,300 g

(15 min) and were resuspended in solution A (140 mM NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 1 mg mL^{-1} glucose, 10 mM HEPES, pH 7.4). This cycle was repeated twice and finally the platelets were resuspended in solution A containing 2 mM $CaCl_2$. Platelet count in PRP and WPS was adjusted to 290 G/L by the addition of PPP and buffer, respectively. PRP and WPS were kept at 37°C during experiments.

All donors claimed to have abstained from taking aspirin, or other drugs known to affect platelet function, in the preceding 10 days. Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

Platelet adhesion

Human collagen, diluted to 100 μg/mL in PBS or 0.05 M acetic acid, was layered over thermanox coverslips (13mm in diameter, Deckgläser) and incubated in a humid box overnight at 4°C. The coverslips were blocked with 3% casein in PBS for 30 minutes followed by washing with PBS. The coated coverslips were mounted in the wells of an Impact-R, *in vitro* flow chamber (Matis Medical, Brussels, Belgium) and anticoagulated blood was circulated at a shear rate of 1800 s^{-1} for 2 min. Then the coverslips were washed with water, stained by May-Grünwald and analyzed by light microscope connected to a camera of the Impact-R image analysis system. The surface coverage (SC; [%]) and average size (AS; [μm^2]) of the objects on the well surface were determined.

Scanning Electron Microscope (SEM)

Morphology of the platelets, which had adhered after shear stress onto collagen matrix was studied by SEM. The coverslips of the platelet adhesion experiments were washed with water and then fixed with 2.5% phosphate buffered glutaraldehyde (pH 7.2), washed

in the same buffer, and post-fixed by 2% osmium tetroxide, then by 2% tannic acid-guanidine hydrochloride. After dehydration in graded alcohol solution the alcohol was exchanged to Freon 112 (Du Pont Company, Wilmington, Delaware). The coverslips were air-dried, gold coated, and examined by a Jeol 840 scanning electron microscope (Jeol USA Inc, Peabodt. Mass) at the Faculty of Life Science (Bar Ilan University, Ramat Gan, Israel).

Calculations

The OD-s of different measurements were normalized by calculating the relative OD in each series. Relative OD was calculated by dividing the OD-s obtained for each measurement point with the OD obtained on wells coated with collagen in PBS, at 20 μg/mL concentration. The Y-axes of the presented graphs show these relative OD values. The K_d of the PBS-diluted collagen curve was calculated and used as the X value for all further calculation of Y values from each of the curves that presented the relative OD-collagen concentration response. Microsoft Excel 2004 and Grafit Version5 (Erithacus Software Limited, Horley Surrey, U.K.) were used for data analysis.

Aptamer Synthesis and Purification

Newly designed aptamers were synthesized in the laboratory of János Aradi, by standard phosphoramidite chemistry, using an automated oligonucleotide synthesizer (Pharmacia Gene-Assembler Plus). The phosphoramidites, including the 4-thio-deoxyuridyne phosphoramidite, were purchased from Glen Research (Sterling, Virginia). Crude products were purified by ion-exchange chromatography and gave a single band by denaturing PAGE [46]. The concentrations of aptamers in aqueous solutions were

determined by the measurement of their phosphorous content [47]. Aqueous solutions were diluted to 0.1 mM, aliquoted and stored at -20ºC.

4-thio-deoxyuridylate (X) was used to replace the T at various positions (Table 1). First we replaced all the T-s (positions 3, 4, 7, 9, 12, 13), which resulted in [d(GGXXGGXGXGGXXGG)] (aUC15-mer). Replacing the vicinal T-s only (position 3, 4, 12, 13) resulted in [d(GGXXGGTGTGGXXGG)] (vUC15-mer). Replacing all the T-s except the T4 and T12 in the vicinal T-s (position 3, 7, 9, 13) resulted in [d(GGXTGGXGXGGTXGG)] (UC15-mer).

Table 1: The sequences and the names of the consensus aptamer and its 4-thio-deoxyuirdylate modified analogs.

5' → 3' Position of the nucleotide base in the aptamer															
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Short name**
G	G	T	T	G	G	T	G	T	G	G	T	T	G	G	C15
G	G	X	T	G	G	X	G	X	G	G	T	X	G	G	UC15
G	G	X	X	G	G	T	G	T	G	G	X	X	G	G	vUC15
G	G	X	X	G	G	X	G	X	G	G	X	X	G	G	aUC15

The C15-mer was modified via replacing the T at various positions with 4-thio-deoxyuirdylate, represented as X.

Thrombin Clotting Activity Measurement

0.1 mL fibrinogen solution (2 mg mL^{-1}) or pooled normal human plasma was incubated at 37°C with 0.1 mL Owren's buffer containing various concentrations of aptamers for 1 min prior to the addition of 0.1 mL thrombin (final concentration 0.6 NIH U mL^{-1}). Alternatively, the aptamers were first pre-incubated with thrombin for 1 min and the reaction was started by the addition of fibrinogen or plasma. Clotting time was measured by KC-1 coagulometer (Amelung, Lemgo, Germany). To test the specificity of the aptamers toward thrombin, in certain experiments thrombin was replaced by 5 batroxobin unit (BU) mL^{-1} Reptilase.

Fibrinopeptide A (FpA) Measurement by LC-MS

Thrombin was added to plasma aliquots preincubated without or with increasing concentrations of aptamers as described above. A synthetic internal standard peptide used for quantification was also included in the incubation mixture. In certain samples thrombin was preincubated with aptamer and the reaction was triggered by the addition of plasma. All reactions were stopped when plasma sample without aptamer was clotted (at 30 sec) by the addition of three volumes of 96% ethanol. Samples were centrifuged (13,600 g , 4ºC, 15 min) and 90 µL supernatant was dried in Speedvac. Dried samples were reconstituted in 90 µL 10% acetonitrile, 0.1% formic acid.

FpA was quantified by LC-MS (API 2000, Applied Biosystems). 10 µL sample was injected on a 2.1 x 50mm C18 reverse phase column and eluted by 15-45% gradient of acetonitrile:water containing 0.1% formic acid. The elution of FpA (retention time: 3.8 min) and internal standard (retention time: 4.7 min) was monitored by MS detecting their double-protonated masses (m/z=769.3 and m/z=870, respectively). Quantification was performed by comparing the peak area ratio of FpA:internal standard to a calibration curve.

Amidolytic Assay of Thrombin Activity

The hydrolysis of chromogenic substrate S-2238 by thrombin was measured at 405 nm, 37°C. 0.1 mL thrombin (0.2 U mL^{-1}) was added to 0.1 mL buffer (20 mM Tris-HCl, 50 mM NaCl, pH 8.3) containing various concentrations of aptamers. The reaction was started by the addition of 0.1 mL substrate (2 mM). The OD of released p-nitroaniline was followed in a microplate reader. In the experiments investigating the effect of UC15-mer on the inhibition of thrombin by hirudin, thrombin activity was measured in the presence of

2.5 nM hirudin and various concentrations of UC15-mer.

Platelet Activation

Platelet activation studies were performed in the presence of 1.25 mM GPRP at $290*10^9$ L^{-1} platelet count, in a lumiaggregometer (Chrono-log, PA, USA). Thrombin (0.5 U mL^{-1}) or collagen Horm (5 μg mL^{-1}) was used as platelet agonist. In the first series of experiments aptamers were pre-incubated with PRP for 1 min prior to the addition of thrombin. In the second series of experiments thrombin pre-incubated with aptamers was added to PRP. The same experiments were also carried out with WPS. The extent of platelet aggregation was quantified by measuring the slopes of aggregation curves. Platelet secretion was monitored simultaneously by measuring the released ATP using luciferin-luciferase reaction. ATP release was calculated by comparing the peak luminescence record from samples with that of ATP standard (4 μM).

Thrombus Formation at Shear Condition

The effect of aptamers on thrombus formation was studied at a shear rate of 650 s^{-1} using a home made [22] or Impact-R (DiaMed AG, Cressier sur Morat, Switzerland) cone and plate adhesion device. Glass coverslips (18*18 mm) were coated with human collagen type III (100 μg mL^{-1} in PBS), or extracellular matrix of HMEC-1 [23]. HMEC-1 was provided by Drs. E.W. Ades and T.J. Lawley (Center for Disease Control and Prevention and Emory University School of Medicine, Atlanta, GA). Wells of Impact-R were coated with 100 μg mL^{-1} fibrinogen at 4°C overnight, then treated with 1 U mL^{-1} thrombin for ten minutes before the adhesion experiment (thrombin treated fibrinogen). Intensive washing with PBS was followed by 5 min circulation of heparinized blood containing various

concentrations of C15-mer, UC15-mer or hir54-65. After perfusion the surfaces were washed 3-times with PBS, stained with May-Grünwald-Giemsa and thrombus formation was evaluated by light microscopy. Alternatively, the total amount of protein in the surface associated thrombi was eluted in the working reagent of BCA protein assay kit and quantified according to manufacture instruction kit.

Results

Coating Conditions Matter to Collagen Matrix Formation Regarding Von Willebrand Factor and Platelet Binding

Collagen surfaces, prepared to study VWF and platelet binding, differed not only in the collagen types but also in the coating conditions. We prepared Human type-I and type-III collagen matrices by passive adsorption technique in 96-well plates or on microscope coverslips. We tested the effect of various coating times and salt concentration and pH of the collagen solutions used to prepare the matrices. We measured the amount of the collagen adsorbed during the coating by specific antibody against it, and parallel measured VWF binding capacity of the coated collagen matrix.

Coating Conditions

Using 96-well plates from different materials, namely polystyrene (Nunc), polypropylene (Propilen E.C.) and polyvinyl chloride with medium- and high binding capacities (Greiner), we demonstrated the maximum binding of the collagen diluted in PBS was comparable in cases of all different plate materials except high binding capacity plates. For that the B_{max} was 1.5-fold higher calculated from one-site curve fittings of the collagen concentration response OD-s (data not shown). In another series of measurements coating at constant collagen concentration (100 μL of 10 μg/mL), different dilutions of purified VWF were pipetted into the wells to characterize VWF binding efficacy of the collagen matrix. K_d calculated from concentration response curves increased 1.91-fold in the case of high binding capacity plates. Therefore, the high binding capacity plates were used for further experiments. We coated collagen from PBS solutions for 2 and 4 hours at 37°C, or

one or two overnights at 4°C, in a well-sealed humid box, in order to see what is the optimal coating time. The duration of coating time did not influence the amount of collagen coated as detected by antibody against collagen. On the other hand, the variation of OD-s within each plate was high at 2 and 4 hours coating and low at one or two overnights, when 20 wells were coated with 100 μL of 5 μg/mL collagen for each time period. The variation coefficient was 58%, 25%, 12% and 11%, respectively. As a result of this we used overnight coating for the subsequent experiments. In order to test how the collagen matrix was affected by the ionic strength of the coating solution, we applied increasing concentrations of NaCl. The type-I and type-III collagens were diluted from 2 mg/mL acid stock solution to 5 μg/mL in phosphate buffer (pH 7.4). As physiological buffers contain phosphate either in 0.01- or 0.02M concentrations, we used both concentrations in the different series of experiments. As shown in Fig.1, the OD increased, showing saturation in response to increasing salt concentration, both in the case of the type-I collagen (Fig.1A) or the type-III (Fig.1B). When we used 0.01 M phosphate buffer the K_d-s [M] were 0.0743±0.0187 and 0.1114±0.0804, in the case of 0.02 M phosphate buffer the K_d-s [M] were 0.0455±0.0186 and 0.0784±0.0205, respectively, although the constants are not reliable if a curve not a typical rectangular hyperbola. 2way ANOVA showed significant differences between data series at the first 3 concentration of the salt for the type-III collagen only. For the subsequent experiments we used PBS containing 0.2 M phosphate and 0.15 M NaCl unless otherwise stated. We made serial dilutions of collagen in solutions presenting 6 different pH values, as listed in the method, in order to see the effect of pH on the matrix preparation. Because collagen solutions used for matrix preparation in VWF and platelet binding studies are very different and many of them did

not contain any salt, we carried out these experiments in the absence and in the presence of 0.15 M NaCl at each pH. Series of dilutions at different pH and the detection of collagen or VWF binding resulted in four series of data sets (relative OD-s) for collagen type-I and four for type-III. Analyzing the relative OD-s in response to collagen concentrations by one-site ligand binding curve fittings we calculated Y value from each of the curves using the K_d of the PBS-diluted collagen curve as the X value. The Y is a relative OD value and the higher the Y is, the more the collagen is bound to the surface. Table 2A (collagen type-I) and in Table 2B (collagen type-III) show the highest Y values when collagens were diluted in phosphate buffer pH 7.4. The addition of 0.15 M NaCl to the solutions resulted in higher Y values. It is noteworthy that Y values were very low for acid solution (see Table 2).

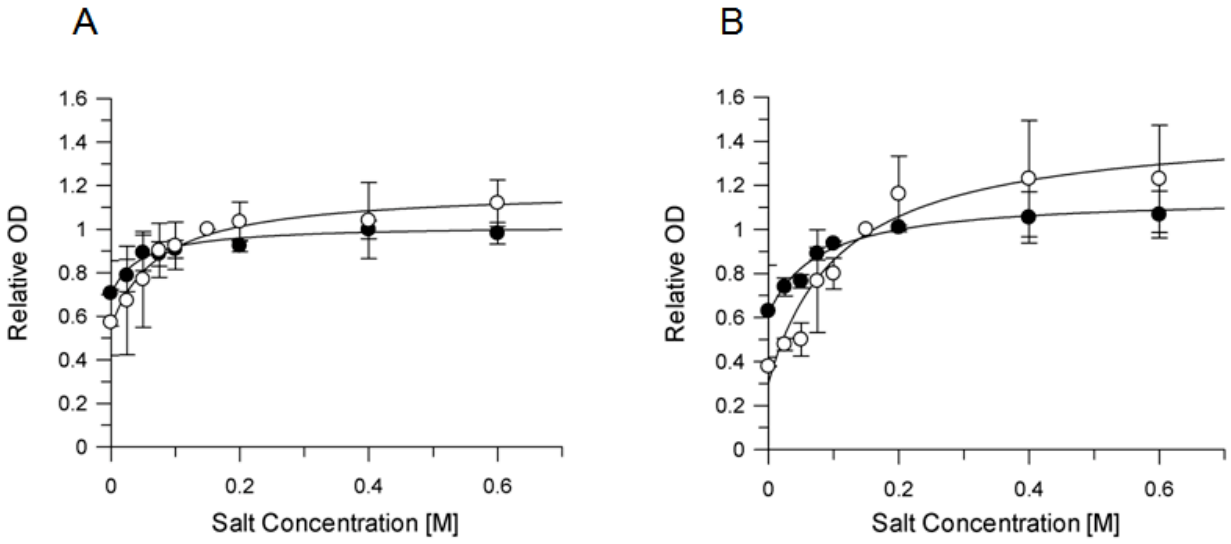

Fig.1. Effect of salt concentration on collagen coating. Human collagen type-I (A) and type-III (B) diluted in phosphate buffer 20 mM (□) and 10 mM (o) with increasing concentration of NaCl for coating to ELISA plate. VWF binding to the coated collagens were detected as describe in the methods. Relative OD was calculated within plates by dividing the OD-s with OD at 0.15 M NaCl concentration. Values represent mean ± standard deviation of three independent experiments.

Table 2. Effect of salt concentration on collagen coating.

A. Human collagen type-I

Ionic strength	0.02M PB		0.02M PB + 0.150M NaCl	
pH of coating solution	[1]Y±SE	[2]Y±SE	[1]Y±SE	[2]Y±SE
acetic acid pH~3	0.170±0.047	0.108±0.027	0.277±0.084	0.243±0.028
Sodium acetate pH:4	0.271±0.097	0.249±0.036	0.434±0.081	0.358±0.084
Phosphate pH:6.4	0.257±0.065	0.170±0.064	0.375±0.030	0.326±0.061
Phosphate pH:7.4	0.358±0.041	0.391±0.028	0.634±0.074	0.510±0.030
Phosphate pH:8.0	0.300±0.047	0.169±0.053	0.600±0.098	0.454±0.025
Carbonate pH:9.2	0.213±0.025	0.152±0.062	0.358±0.095	0.463±0.027

[1]detection by collagen-specific antibody binding

[2]detection by VWF binding

B. Human collagen type-III

Ionic strength	0.02M PB		0.02M PB + 0.150M NaCl	
pH of coating solution	[1]Y±SE	[2]Y±SE	[1]Y±SE	[2]Y±SE
acetic acid pH~3	0.177±0.065	0.144±0.054	0.190±0.096	0.238±0.065
Sodium acetate pH:4	0.315±0.047	0.272±0.081	0.359±0.071	0.420±0.086
Phosphate pH:6.4	0.270±0.053	0.280±0.081	0.304±0.069	0.438±0.087
Phosphate pH:7.4	0.407±0.071	0.413±0.071	0.505±0.088	0.584±0.024
Phosphate pH:8.0	0.355±0.092	0.326±0.095	0.301±0.071	0.549±0.030
Carbonate pH:9.2	0.199±0.037	0.169±0.065	0.314±0.071	0.354±0.088

Binding Characteristics of Collagens from Acid and PBS Solutions

Many studies on VWF or platelet binding to collagen use collagen to coat surfaces dissolved and diluted in acetic acid or dialyzed against phosphate buffers. When we dialyzed the acetic acid solution of the collagen against PBS, we did not find different coating compared to collagen directly diluted in PBS. However, acetic acid diluted collagens resulted in very different, but definitely low coating efficacy.

To compare the binding of collagen from acid or from PBS solutions, we designed a series of experiments shown in Fig. 2. Human collagen type-I (A,C) and type-III (B,D) diluted to 20, 10, 5, 2.5 g/mL with PBS (○) or with acetic acid (●) were applied into the wells. Na_2HPO_4 was added to one series of acid diluted collagens to neutralize acid (△). All the wells were incubated overnight at 4°C. Next day wells were emptied and detection was proceeded with collagen-specific antibody (A,B) or through VWF binding (C,D). Binding of collagen showed a saturation curve from PBS solutions with both detection methods. The relative OD-s were low when the surfaces with collagens were coated from acid solution. However, neutralization of the acid with Na_2HPO_4 also resulted in a saturation curve. The same was true of another series of acid-diluted collagens, which after the overnight coating, were transferred into new wells where the acid solution was neutralized (▲). Furthermore, the emptied acid wells were filled with PBS (□) and left at 4°C for coating as before, but hardly any collagen-specific antibody or VWF binding could be detected. See quantitative data in Table 3.

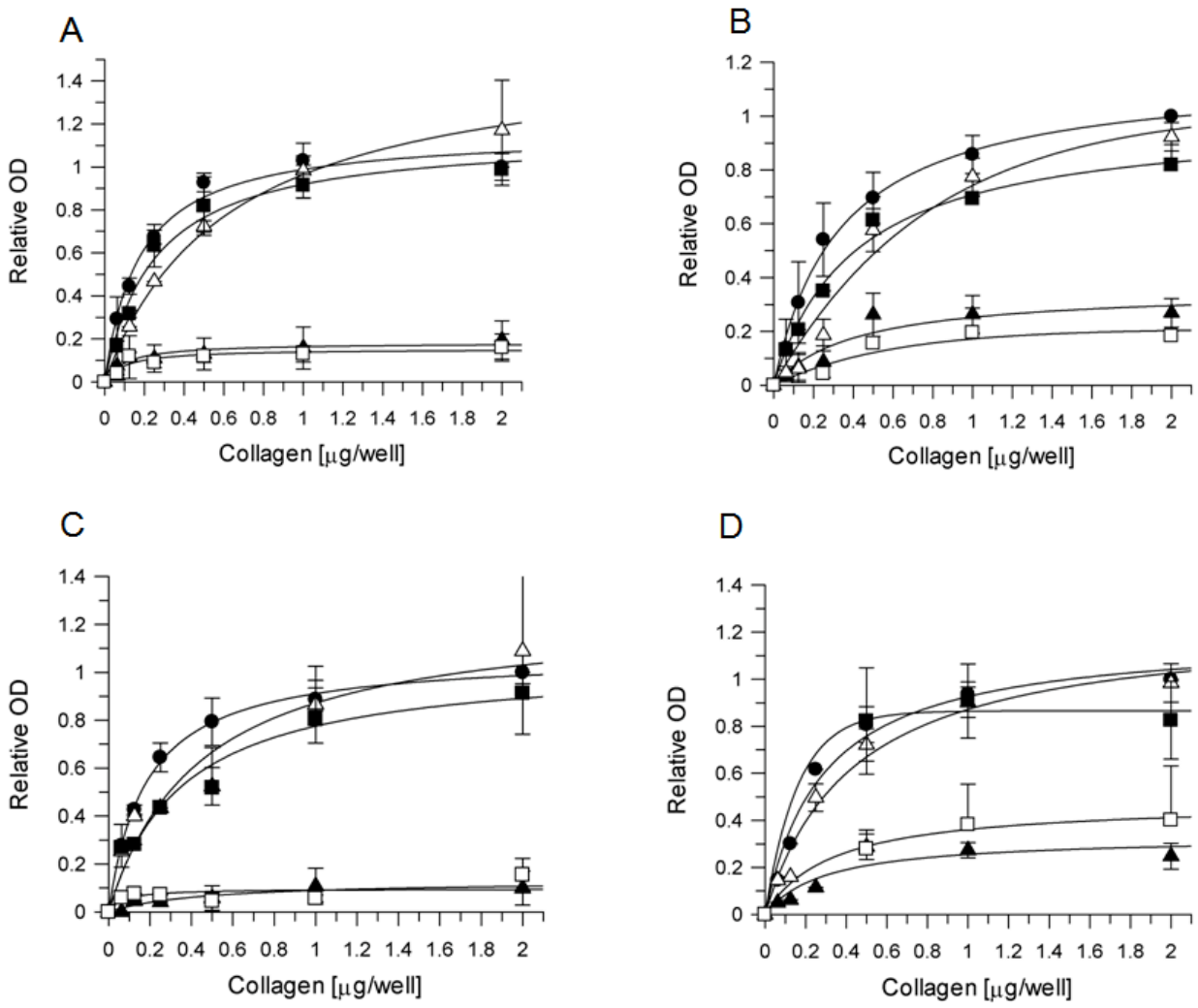

Fig.2. Binding characteristics of collagens prepared in PBS or acid solution.

Human collagen type-I (A,C) and type-III (B,D) diluted with PBS (□) or with acetic acid (□) were applied into the wells. Na_2HPO_4 was added to one series of acid diluted collagens to neutralize acid (△). All the wells were incubated overnight at 4°C. Next day wells were emptied and detection was proceeded with collagen-specific antibody (A,B) or through VWF binding (C,D). In another series of experiments some solution after the overnight coating, were transferred into new wells where the acid solution was neutralized (□). Furthermore, the emptied acid wells were filled with PBS (□) and left at 4°C for coating and the detection was proceeded as before.

Table 3. Binding characteristics of collagen prepared in PBS or acid solution for coating.

Collagen coating conditions*	Human Collagen Type I		Human Collagen Type III	
	[1]Y±SE	[2]Y±SE	[1]Y±SE	[2]Y±SE
PBS	0.594±0.017	0.572±0.023	0.591±0.025	0.570±0.019
AA	0.124±0.074	0.047±0.037	0.168±0.06	0.102±0.057
AA+Na_2HPO_4	0.432±0.086	0.420±0.017	0.410±0.145	0.513±0.077
s(AA)+PBS	0.088±0.006	0.027	0.024	0.224±0.015
tAA+Na_2HPO_4	0.486±0.054	0.371±0.092	0.409±0.065	0.596±0.224

[1]detection by collagen-specific antibody binding

[2]detection by VWF binding

* PBS and AA: human collagen type I and type III, were diluted with 0.02M phosphate buffer saline or with 0.05 M acetic acid.
* AA+Na_2HPO_4: collagen diluted in acetic acid neutralized with Na_2HPO_4.
* tAA+Na_2HPO_4: collagen diluted in acetic acid after overnight coating transferred into new wells and neutralized with Na_2HPO_4.
* s(AA)+PBS: surface of the emptied acid collagen coated wells were incubated with PBS.

In order to quantify the results, we fitted the relative OD-s with equations and calculated Y as it is written in the Materials and Methods. No significant differences were observed between collagen type I and III when collagen-specific antibody or VWF binding was measured. These results also show that coating resulted in the highest binding of collagen-specific antibody or VWF binding when collagens were in PBS, and the lowest when in acetic acid. However, when acetic acid solution was neutralized in the wells, there was no difference compared to coating from PBS.

Correlation of the Collagen Coated and the VWF Captured

When we analyzed the result obtained by measuring the collagen-specific antibody binding and the VWF binding to the coated collagen, we found high correlation with linear regression both for type-I and III collagen: r^2=0.937, y=(0.938±0.0233)x+0.0651±0.012, n=112 and r^2=0.902, y=(1.02±0.0206)x+0.0773±0.0102, n=268, respectively; where x is the relative OD of the collagen detection and y is the relative OD of the VWF detection.

Morphology of Collagens

AFM was used to test if the quantity was different only or the structure of collagens, when 5 L of collagens (200 g/mL) in acid or (20 g/mL) in PBS solutions were dropped onto glass coverslips. 10-fold higher collagen concentration was applied in acid solution, because nothing was visible at lower concentration. Images were presented on Fig. 3.

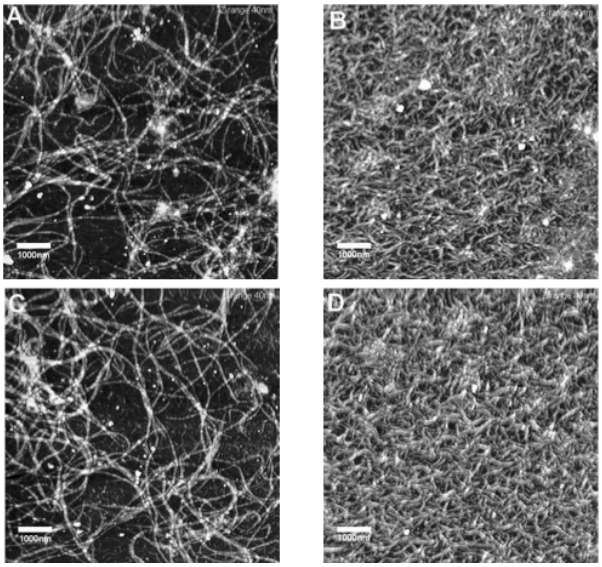

Fig.3. AFM images of human collagens bound to glass from acid or PBS solutions. Collagen type-I (A,B) and type-III (C,D) bound after 48 hours. The concentration of the collagen was 200 μg/mL in acetic acid (A,C) and 20 μg/mL in PBS solutions (B,D). 10-fold higher collagen concentration was applied in acid solution, because nothing was visible at lower concentration. Scanning was done by tapping mode in liquid (area 10×10 □m, resolution: 512×512 pixels).

We found quantitative differences between collagens bound in acid solution (A,C) or in PBS (B,D) and slight structural differences can be seen too. The structure of the collagen type-I (A,B) was similar to type-III fibrils (C,D). The average width of the collagen fibrils on the surface prepared in acid solution was 80-120 nm (A,C) and the average width of those prepared in PBS solution was 120-150 nm (B,D).

Platelet Adhesion

Collagen type I (Sigma) coated thermanox coverslips were mounted in the Impact-R wells and then exposed to anticoagulated blood under shear condition, the plasma protein VWF immediately binds to the fibrillar collagen surface (but not to the acid coated collagen) and platelets adhere to this composed surface. The total amount of platelets

adhering to the surface was determined as the percent of surface covered by individual or aggregated platelets. The coverage of surfaces coated by collagen from PBS or acid solution was 12±4% and 1±1%, respectively. The size of the platelet thrombi, characterized as the average size of the objects, was 90±31μm^2 (n=30) and 44±9μm^2 (n=14). In order to examine the structure of the platelet aggregates on the fibrilar collagen coated surface the sample was examined by SEM (Fig. 4A) in comparison to platelet deposition on a plastic surface directly coated, upon exposure to the blood, by plasma proteins (e.g. VWF and fibrinogen) representing the regular Impact-R test (Fig. 4B). The fibrilar collagen coated surface demonstrated much more "developed" aggregate with fully activated and merging platelets compared to the adhered, spread platelets with filopodia on the plastic surface. The surface coverage of the acidic collagen coated slides was under the limit of the quantitation, and therefore it was not rational to take it for SEM.

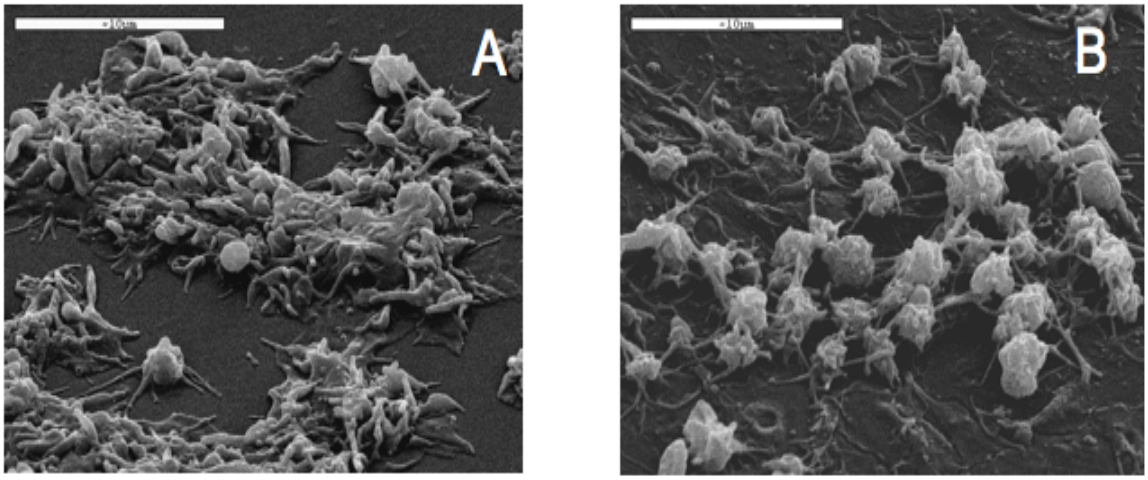

Fig.4. SEM images of platelet adhesion onto thermanox coverslips with (A) or without (B) fibrilar human collagen type I coating. The thermanox coverslips were mounted in the Impact-R wells, exposed to anticoagulated blood under a shear rate of 1800 s^{-1} for 2 min, and then the surfaces were processed for SEM as described in the method.

Inhibition of Thrombin with Synthetic Oligonucleotides

The Effect of Aptamers on the Clotting and Amidolytic Activity of Thrombin

Exosite 1 is a major fibrinogen recognition site on thrombin. To determine whether the modified aptamers binds to this region and competes with its native substrate, thrombin clotting time assay was performed with purified fibrinogen and human plasma in the presence of the aptamers. In the first series of experiments aptamers were pre-incubated with fibrinogen solution or plasma and then thrombin was added. C15-mer and UC15-mer inhibited the clotting of purified fibrinogen and plasma by thrombin (Fig . 5); aUC15-mer and vUC15-mer were ineffective.

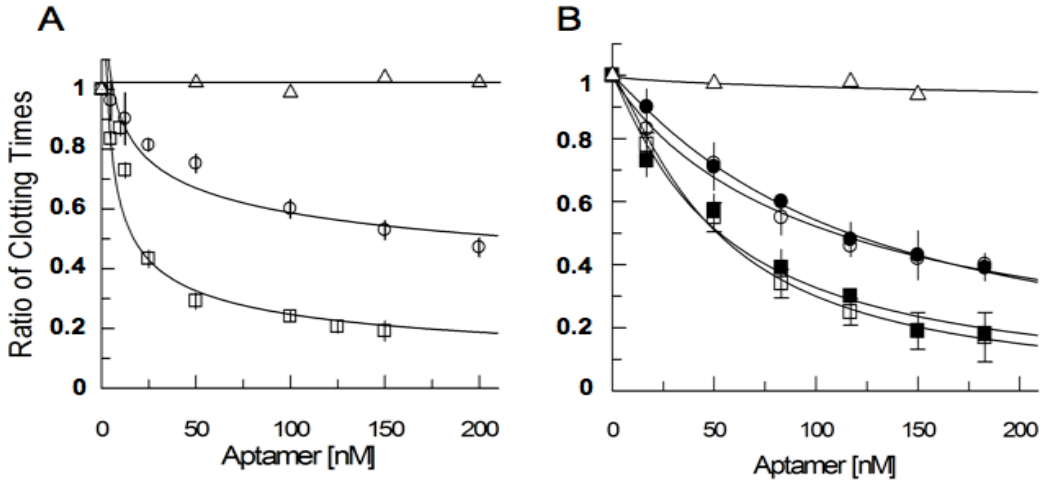

Fig.5. Effect of aptamers on thrombin induced clotting time in fibrinogen solution (A) and in plasma (B). The following aptamers were used in the experiments: C15-mer (●,O), UC15-mer (□,■), and vUC15-mer (△). In the first series of experiments, purified fibrinogen solution (A) or normal human plasma (B) was preincubated with various concentrations of aptamers (O,□). The reaction was initiated by the addition of thrombin, and clotting time was recorded. In the second series of experiments, the aptamers were preincubated with thrombin, and the reaction was started by the addition of plasma (●,■). The results are expressed as the ratio of clotting times measured in the absence and in the presence of one of the aptamers. Means ± SD of four parallel experiments, each performed in duplicate, are shown.

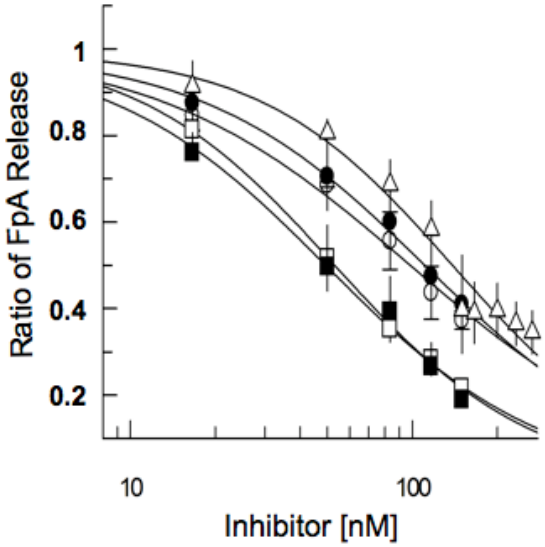

Fig.6. The effects of aptamers and hir54-65 on thrombin-induced release of fibrinopeptide A (FpA) in plasma. In the first series of experiments, various concentrations of C-15-mer (O), UC15-mer (□) or hir54-65 (△) were preincubated with normal human plasma, and the reaction was initiated by the addition of thrombin. In the seconds series of experiments, thrombin was preincubated with C-15-mer (●) or UC15-mer (■), and the reaction was started by the addition of plasma. The results are expressed as the ratio of FpA release measured in the absence and in the presence of one of the inhibitors. Means ± SD of three parallel experiments are shown.

In the case of fibrinogen solution the IC_{50} for UC15-mer was three-fold lower than for C15-mer, in the case of plasma the difference was 2.2-fold (Table 4). When the inhibition of FpA release by thrombin was measured the effectiveness of aptamers was practically identical with that obtained in clotting time experiments (Fig. 6). Both aptamers were about 2-fold more effective in fibrinogen solution than in plasma. The same difference was observed when plasma clotting (Fig. 5B) or FpA release was initiated by thrombin that was pre-incubated with aptamers.

Table 4. **IC_{50} values of C15-mer and UC15-mer**

	C15-mer	UC15-mer
	IC_{50} (nM) ± SE	IC_{50} (nM) ± SE
Clotting time		
Fibrinogen	52.9 ± 5.4	18.3 ± 2.6
Plasma	116.2 ± 2.3	52.5 ± 4.3
Fibrinopeptide A release (plasma)	110.6 ± 2.3	48.3 ± 4.6
Platelet aggregation (WPS)	26.0 ± 7.7	2.2 ± 0.34
Platelet aggregation (PRP)	274 ± 32.6	145 ± 3.9
ATP release (PRP)	232 ± 33.3	128 ± 16.6

Aptamers were preincubated with plasma or fibrinogen prior to initiation of the reactions with thrombin. The IC_{50} values of aptamers were calculated as described in the text. Results are presented as means ± SE from four different experiments, each performed in duplicate.

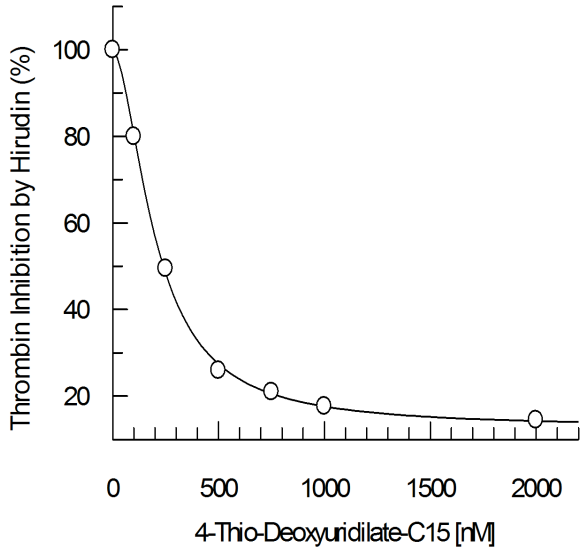

Fig.7. UC15-mer suspends the inhibition of thrombin by hirudin. Results are expressed as percentage of the inhibition exerted by hirudin in the absence of aptamer. Thrombin activity was measured with the chromogenic substrate S-2238.

These assays were repeated with Reptilase, a snake venom enzyme that forms fibrin clot by cleaving off FpA from fibrinogen Aα-chain. None of the aptamers had any effect on this reaction, confirming the thrombin-specific effect of aptamers. The amydolytic effects of thrombin on the chromogenic substrate, S-2238, was not influenced by the aptamers (not shown), indicating that the aptamers did not exert their effect on the catalytic center.

As inhibition of thrombin by hirudin requires binding to exosite 1, UC15-mer, if it is bound to the same site, should suspend the inhibitory effect of hirudin, as measured by the hydrolysis of chromogenic substrate. Indeed, thrombin was relieved from the inhibitory effect of hirudin by increasing concentration of UC15-mer suggesting that UC15-mer competes with hirudin for thrombin exosite 1 (Fig.7).

Effect of Aptamers on Thrombin Induced Platelet Activation

PRP or WPS was incubated for 1 min with increasing concentration of aptamer and platelet activation was induced by thrombin. In PRP C15-mer and UC15-mer inhibited aggregation (Fig. 8) and ATP secretion in dose-dependent manner.

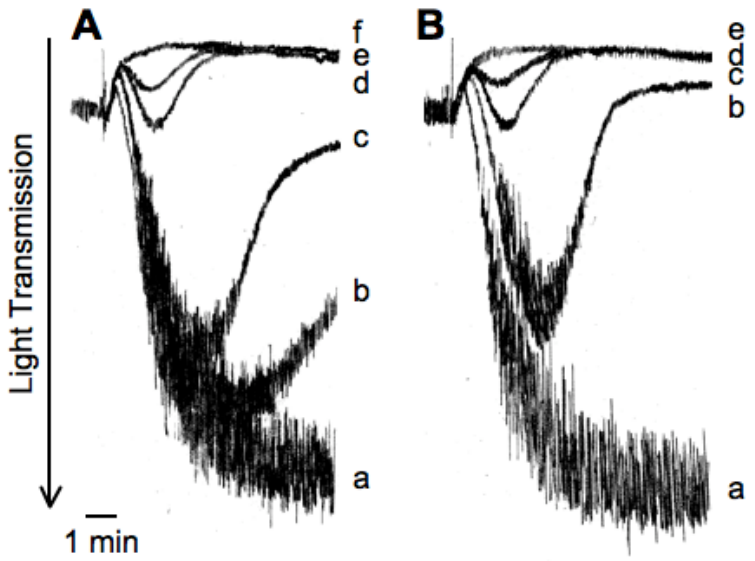

Fig.8. Effect of aptamers on platelet aggregation in human platelet-rich plasma.

Aliquots of PRP (395µl, 260*10^9 platelet/L) in the presence of 1.25 mM GPRP with either (A) C15-mer, (B) UC15-mer: (a) 0, (b) 0.1 µM, (c) 0.2 µM, (d) 0.3 µM, (e) 0.5 µM , (f) 0.7 µM . The mixture was stirred at 37°C in lumiaggregometer, and thrombin was added to final concentration of 0.5 U mL^{-1}. The results represent one of four experiments with similar results.

In both cases the IC_{50} values for UC15-mer were only half of the IC_{50} values for C15-mer (Table 4). Neither the IC_{50} values nor their relative ratio was changed when thrombin was first incubated with aptamers, and then added to PRP (not shown). In WPS the IC_{50} values drastically decreased for both aptamers and UC15-mer became 12-fold more effective than C15-mer (Table 4). It should be noticed, that shape change was not influenced even at aptamer concentrations 4-folds higher than the maximum inhibitory concentrations (Fig. 6.A, B). The lack of aptamer effect on TRAP-1 and collagen induced aggregation (not shown) demonstrates its thrombin specificity.

Effect of Aptamers on Thrombus Formation at Shear Condition

Thrombus formation in whole blood was tested on thrombogenic surfaces in cone and plate chamber. Without aptamers and hir54-65 the HMEC-1 matrix and thrombin treated fibrinogen surfaces were equally covered by large thrombi (Fig. 9Aa,Ba). When aptamers were present at high inhibitory concentration, both extracellular matrix of HMEC-1 and thrombin treated fibrinogen surfaces were covered with higher number, but smaller and less thick thrombi and many individual platelets (Fig. 9Abc,Bbc). The effect of aptamers was comparable to the effect of hir54-65 (Fig. 9Ad,Bd). There was no detectable change in thrombus formation on collagen surface in the presence of aptamers (data not shown).

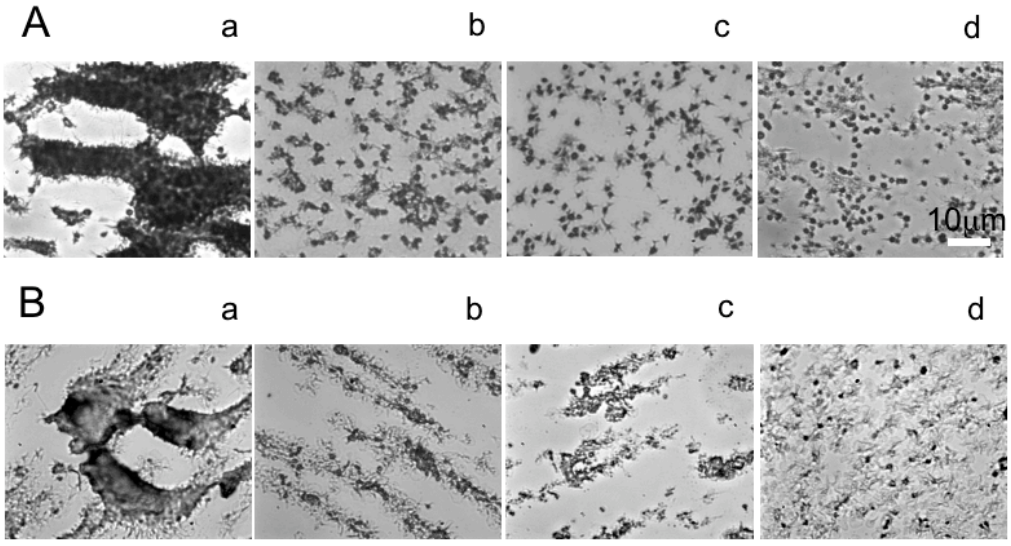

Fig.9. Effect of aptamers on thrombus formation, at shear rate of 650/s using cone and plate chamber. Coverslips were coated with (A) matrix of human dermal microvascular endothelial cells (HMEC-1) or (B) fibrin formed by in situ thrombin treatment. Blood, anticoagulated with 10 U/mL LMWH, was circulated over the matrix for 5 min at 37°C. A representative field is shown (original magnification 1000x), bar=10um, (a) control (b) preincubated with 1.5 µM C15-mer, UC15-mer, (c) 1.0 µM hirudin fragment (54-65) (d). The coverslips were stained with May-Grünwald-Giemsa.

Determination of proteins present in thrombi formed on the surface was used to evaluate the effect of aptamers on thrombus formation quantitatively. Using this technique, IC_{50} values for the inhibition of thrombus formation could be calculated (Fig. 10). UC15-mer was 2-fold more effective than C15-mer, the IC_{50} values were 210 ± 29 nM and 400 ± 38 nM, respectively. The effectiveness of hir54-65 was comparable to that of UC15-mer (IC_{50} = 148 ± 16 nM).

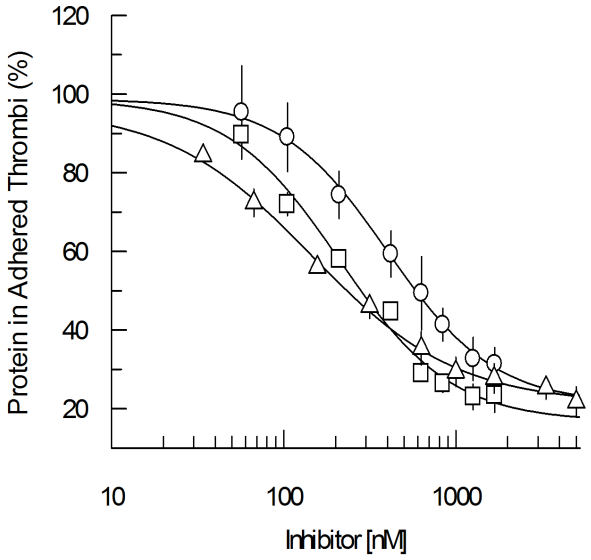

Fig.10. Aptamer concentration-dependent inhibition of thrombus formation on thrombin-treated fibrinogen under flow conditions. Experiments were carried out as described in the legend to Fig.7B, with the exception that in this case various concentrations of C-15-mer (O), UC15-mer (□) or hirudin fragment (54-65)(△) were added to citrated blood samples and the amount of total protein in thrombi adhering to the coverslips was measured according to the instruction of BSA protein kit . Results obtained in the presence of aptamers or hirudin fragment are expressed as a percentage of total protein on the coverslip in their absence. IC_{50} values were calculated as described in the text.

Discussion

Collagen matrix

Random attachment of different types of collagens leads to a large variation of matrices used by different studies for collagen VWF and platelet interaction. These matrixes mainly depend on the source and preparations of collagens used for coating and have important experimental implications, *see Supplementary Table 1*. A very recent publication of SSC committee of the ISTH, titled "Collagen surfaces to measure thrombus formation under flow: possibilities for standardization" recommends coating in both acid and collagen buffer [48].

In the present study, we used human fibrillar collagens type-I and III at varying conditions to prepare collagen matrix for VWF and platelet binding. We tested plates with different binding capacities, the pH and ionic strength of the buffers used for the dilution of collagens, the binding time and the temperature. Along with the VWF, binding of collagen-specific antibody was used to measure the matrices. This kind of systemic study has not been done before.

Testing the collagen binding to 96-well plates from different materials that are commonly used, we found that high protein binding capacity plate provided higher binding capacity as expected. In addition, it resulted in a 1.91-fold higher K_d for VWF, which means a wider measurement range. Chemical modification of collagen resulted in its covalent immobilization onto plates that functioned well to measure VWF binding to collagen [49]. The covalent binding allowed the use of low concentration of collagen

solution for coating, compared to other methods. According to our results the same collagen concentration of the neutralized collagen solution was equally effective.

In the literature, the coating time applied for binding collagen to the wells varies from a few to 72 hours, see *Supplementary Table1*. According to our measurements, the binding of collagens to the surface showed saturating kinetic even at 2 hours coating time. However, with the shortening of the time to less than 12 hours the error of the VWF binding capacity of the matrix increased. Extending the time over this period did not improve its antibody or VWF binding. Moreover, a long coating time may result in a change of the pH of the acid solutions associated with initiation of collagen binding in the wells, resulting in uncontrolled coating condition. Collagen fibril formation is an ongoing process during the standing of solutions, which may influence coating and may explain the result of previous studies [50].

We found that collagen binding reaches saturation around the physiological salt concentration, when either 0.01 or 0.02 M phosphate buffer was used. It was demonstrated that collagen fibril formation depends on salt concentration [51]. Hayashi et al. stated that the halftime of fibril formation is maximum at 0.15 M chloride ion concentration [52]. Based on these publications we explain our results, that a higher salt concentration shifts the equilibrium of collagen in different solutions to fibrils, which may bind better to surfaces. These publications demonstrated that collagen fibril formation markedly depends on phosphate concentration too, which explains our finding concerning the lower K_d at 0.02 M phosphate buffer compared to 0.01 M in this series of experiments.

As many studies used collagen dissolved in acetic acid or dialyzed it against PBS, we tested the effect of pH on the coating efficacy of collagen from different solutions. The

collagen diluted from acid stock solution to pH 7.4 was superior to those diluted to pH 4.0, 6.4, 8.0, 9.2. Studies on collagen fibril formation showed that neutralize condition is needed to obtain well-ordered fibrils [53], [54,55]. We can deduct from a series of our experiment that collagen present in acid solution is not in the right binding conformation, because it was coated well after neutralization locally. Adding salt to these solutions to reach the physiological concentration we observed increased collagen coating at all pH-s. It is shown that under *in vitro* conditions, acid-soluble collagen type-I molecules can form fibrillar intermediates and varying fibrillar assemblies over widely ranging pH, even in the absence of salt, but fibrils formed under physiological salt concentration are more regular [56]. It seems that fibril formation and coating efficacy of collagen is a linked process.

Collagens in acetic acid resulted in very variable coating efficacy. However, after a similar coating period, coating efficacy was consistently lower than that of collagen in PBS. Knowing that the triple helix itself is resistant but the triple-helical structure of the collagen can be transferred to random-coiled α-chains during isolation and preparation of collagen, and the degree of renaturation can be different as well as the rate of fibril formation controlled by electrostatic interactions, we raised the question whether the collagen from acid solution does not bind to the surface, or binds, but in a functionally disabled form. We have investigated why the acidic coating conditions lower the binding capacity of the resulting collagen surface. The acidic coating solution was exchanged with PBS at the end of the coating step to examine, whether low amount of collagen bound to the wells or a different conformation of the collagen in the acidic solution is responsible for the phenomenon. We have found, that incubating the well with PBS after removing the acidic coating solution did not result in normalized VWF binding capacity. In view of these

data, we concluded, that the low binding capacity of the acidic coated collagen surface was caused by the low amount of collagen bound to the wells and not because of its inappropriate conformation. Another approach to answer the question was the morphological investigation of the collagen with AFM. This technique revealed fibrillar structures from PBS solutions and from acid too. However, fibrils from acid solution, even under extreme conditions, were considerably fewer, well separated and thinner compared to fibrils from PBS solution. Our findings are supported by Nerlich et al. [57] who showed that binding of human collagen type-I and III from acetic acid solution to mica (sheet silicate) was reduced and there was no difference between collagen type-I and III fibrils. The diameters were in the same range and the well-separated fibrils were very long. Our size parameters of AFM fibrils are comparable to other published results too [58,59].

Paczuski et al. studied the effect of collagen source and coating conditions [50] and contrary to our results showed that human type-I collagen does not bind VWF and type-III is superior to type-I and III mixtures when acetic acid solubilized collagens were diluted with carbonate buffer (pH 9.2) for coating. This difference could be caused by the use of carbonate rather than phosphate buffer, although coating conditions for the collagens are not detailed and the influence of ionic strength was not studied in this publication. Our optimal collagen concentration for coating from PBS was consistent with the concentration shown on a representative figure in the article of Paczuski et al., and contrasted with other studies, which used higher collagen concentration. However, in most of the studies collagens were used without titration.

The extent of platelet adhesion was very low in our study when the collagen was coated from acid solution and it was similar to the results of other studies when the collagen was

coated at optimal pH and salt concentration [60]. It is known from real time analysis that fibrillar collagen structure influences the extent [61] and the mechanism of thrombus formation under flow [62], however, our end point detection technique did not allow the exploration of this kind of difference. As regards the difference in platelet adhesion to collagen coated from acid solution, our explanation is that the collagen binding was partial to the surface, as it was in the 96-well plate. The partial adhesion was feasible due to instant but limited immobilization of both plasma fibrinogen and VWF on the polystyrol surface serving as a thrombogenic substrate [63] but those few adhering platelets were activated but not aggregated. Considering the recent publication of SSC committee of the ISTH [48] our experiments demonstrate that under optimal coating conditions not only the adhesion is better or increased but also the aggregates are different in morphology.

As we have seen many different collagen surfaces or matrices are used to examine VWF and platelet binding in the scientific literature. The SSC committee of the ISTH published a review recently and recommended a procedure in the last part of the supplementary material, which based on publications and on the authors' experiences. The recommendation includes coating from acid or collagen buffer, without differentiating between them [48]. Our study provides evidences for first time that collagen in acid solution demonstrates limited surface coating resulting in limited and variable VWF binding and platelet adhesion. Neutralizing the acid and adding NaCl in physiological concentration, thereby facilitating formation of collagen fibril molecules in solution, results in efficient coating. In addition, normal VWF binds equally well to human type-I and type III collagens. Although it is known, that preparation of type I collagen with less than 5% contamination with type III is almost impossible. Thus, most of our experiments performed

with 'purified type I collagen' would include some type III collagen. As collagen-binding activity of VWF is measured routinely when differential diagnosis of VWD is needed, we advise the standardization of the methods regarding to the collagen coating condition.

Thrombin inhibiton

The published IC_{50} for C15-mer in thrombin induced fibrinogen clotting varied in the range of 25-200nM; these data obtained at different thrombin concentrations are difficult to compare, but our result (52.9nM) fits into this range [26,27,44,64-66]. The IC_{50} for fibrinogen clotting activity was 2.8-fold lower with the UC15-mer than with the C15-mer, i.e., the inhibition exerted by the new aptamer was stronger than the inhibition by C15-mer. A 2.2-fold higher inhibitory activity of UC15-mer was also demonstrated with plasma. Similar results were obtained when thrombin activity was measured by the release of FpA. The IC_{50} for both aptamers was higher in plasma than in fibrinogen solution. Specific binding of the aptamers to prothrombin [67] and, perhaps also the less specific matrix effect of other plasma proteins might be responsible for the discrepancy. The effect of plasma proteins on the inhibition by UC15-mer is somewhat more considerable than on the inhibition exerted by C15-mer.

It was shown by chromogenic substrate that UC15-mer, similarly to C15-mer [65,68,69], did not exert its effect through the catalytic site of thrombin. To prove that thrombin exosite 1 was involved in the inhibition of thrombin clotting activity by UC15-mer, we tested the interaction of UC15-mer and hirudin. Hirudin contacts exosite 1 of thrombin through its extended C-terminal tail residues and as a consequence its N-terminal domain occludes the active site [70-73] blocking both clotting and amydolitic activity. If UC15-mer was bound to exosite 1, it should relieve the inhibition of amidolytic activity.

Indeed, as measured by chromogenic substrates, the presence of UC15-mer suspended the inhibitory effect of hirudin.

The 4-thio-deoxyuridylate modification of C15-mer also resulted in a 2-fold more potent inhibition of thrombin induced platelet aggregation and secretion in PRP. The IC_{50} obtained for C15-mer in PRP was about 3-4-fold higher than that obtained by Li et al [64]. The difference might be due, at least in part, to the different method of evaluation; they quantified the extent of aggregation by planimetry of the area under the aggregation tracings, while in our study calculation of IC_{50} values was based on the slope of aggregation curves. Aggregation and secretion induced by collagen, a thrombin independent trigger, was not influenced by either of the aptamers; similar results have been published for C15-mer [64,74]. TRAP-1, a synthetic peptide that mimics the tethered ligand of thrombin receptor, activates the receptor independently of the action of thrombin. TRAP-1 was used to verify that UC15-mer affects thrombin and not its receptor. TRAP-1-induced platelet aggregation was not influenced even by the highest concentrations of the aptamers, confirming that binding of aptamers to thrombin blocks its ability to cleave PAR-1.

The inhibitory effect of both aptamers was also tested in WPS. In WPS the aptamers were far more effective than in PRP. The difference between IC_{50} values measured in PRP and WPS is considerably more than the difference in IC_{50} values for plasma and fibrinogen clotting. The reason for different effect of plasma proteins on the inhibition of thrombin-induced clotting and platelet aggregation is not clear. It could be due to the 3-fold higher concentration of plasma proteins, including prothrombin, in the experimental set-up for PRP aggregation, than for the plasma clotting. The absence of fibrinogen in WPS could

also be a factor. Finally, the concentration of free Ca^{2+} in WPS is about 20-fold higher than in citrated plasma and the effect of citrate on platelet aggregation and on its inhibition by certain platelet antagonists has been demonstrated [75,76]. The difference between the inhibitory effect of C-15-mer and UC15-mer is also much higher in WPS than in PRP; in WPS the IC_{50} for UC15-mer is one magnitude lower than that for C15-mer.

In contrast to aggregation and secretion, the thrombin-induced platelet shape change was unaffected up-to 4 μM aptamer concentration. In two studies the shape change was not specifically evaluated, but according to the Figures in the publications it was unchanged in the presence of aptamer [27,65]. In a third study the shape change was retained at lower C15-mer concentrations, but complete inhibition occurred at 1.45 μM [64]. It is important to note that the blockage of both thrombin-induced platelet aggregation pathways (PAR-1 and glycoprotein Ib) left thrombin-induced platelet shape change un-effected [77]. D-Phe-Pro-Arg chloromethyl ketone also inhibited thrombin-induced aggregation without affecting shape change [70,78]. These results suggest that either there was sufficient amount of residual free thrombin exosite 1 to induce shape change, or shape change is an exosite 2 mediated process.

We also tested the effect of aptamers on thrombus formation. When the endothelial cell layer is damaged platelets rapidly adhere to the adhesive constituents of exposed subendothelium and become activated. Platelet activation is amplified by the local generation of thrombin and fibrin formed during the ongoing thrombus formation process. To evaluate how aptamers affect thrombus formation at near physiological condition, we used HMEC-1 matrix, thrombin-treated fibrinogen and collagen in a flow chamber. Extracellular matrix of HMEC-1 is a very thrombogenic surface that does not contain the

main types of reactive collagens (types I, III and VI), but facilitates thrombin generation [79]. It has also been shown that surface covered with thrombin or thrombin-treated fibrinogen initiate thrombus formation via thrombin receptor dependent adhesion [80]. In our experiments the large thrombi formed on the matrix of HMEC-1 and thrombin treated fibrinogen was inhibited by both aptamers, but platelet adhesion did not seem to be affected. Similar results were obtained with a thrombin inhibitor peptide [72]. The suppression of FpA generation and reduction of platelet deposition by thrombin aptamer in heparinized human blood perfused over a balloon-angioplasty injured rabbit aorta, also support this finding [64]. It has also been shown that the aptamer inhibited thrombin-fibrin interaction and displaced thrombin that was already bound [81]. Like other thrombin specific inhibitors [66], aptamers did not influence platelet adhesion to collagen. The results suggest that thrombin exosite 1 is involved in the accumulation of platelets into large thrombi on HMEC-1 and thrombin-treated fibrinogen, but not on collagen surfaces. Measurement of total thrombus protein, which includes platelets and plasma derived proteins, predominantly fibrin, gives a complex overview on the effect of aptamers on thrombus formation. In this complex system the IC_{50} values for both aptamers were somewhat higher than in plasma or in PRP, but the higher efficacy of UC15-mer was observed in this system, as well.

In summary, the replacement of thymidylate at positions 3, 7, 9, and 13 of the consensus aptamer by 4-thio-deoxyuridylate resulted in more potent inhibition of thrombin-induced fibrin clotting and platelet activation, although the difference in the effectiveness of the two aptamer varied between 2 to 12-fold in different experimental systems. The new aptamer was also more effective in decreasing thrombus deposition on

thrombin-treated fibrinogen surface. The replacements introduced two major changes into the physico-chemical characteristics of the molecule. 1/ Due to the tautomer conversion of the modified nucleotide, it may carry (in enol form) reactive –SH group or groups, and this group positioned properly, may interact with –SHs of proteins. Since there is no free –SH on thrombin, this change does not explain the increased inhibitory capacity of UC15-mer. 2/ The thiono group at position 4 renders the base more hydrophobic, which may result in stronger hydrophobic aptamer-protein interactions. We have shown that the homo-oligonucleotide composed of 4-thio-deoxyuridylates is highly resistant to nucleases [45]. Since the UC15-mer contains more than 26% thiolated nucleotide, it should be more stable in biological environment than its unmodified counterparts. This feature may also be of significant advantage in the case of *in vivo* application. Further optimization of 4-thio-deoxyuridylate containing aptamers presents an opportunity for the generation of more potent thrombin inhibiting aptamers with potential use as antithrombotic agents.

In this thesis I research two major components involved in thrombus formation: the collagen, and the thrombin. I found that collagen is very thrombotic surfaces that use for research studying platelet adhesion and routinely in vWF:CB assay . Up to date there no standardization how to produce collagen surface matrices for research or routine. I and my colleagues found that collagen coating of surfaces is very variable form acid solution. Our experiments provide evidences that neutralizing the acid and adding NaCl in physiological concentration, thereby facilitating formation of collagen fibril molecules in solution, results in efficient coating of human type I and type III collagens, which then bind VWF equally well. As for thrombin, which plays a pivotal role in haemostasis but at pathological

condition is a major target for anticoagulation. Thereby I studied the antithormbotic effect of a new thrombin aptamer. We found that replacing of four thymidylate residues in C15-mer by 4-thio-deoxyuridylates resulted in a new thrombin aptamer with increased anticoagulant and antithrombotic properties.

My publications: [82,83]

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Supplementary Table 1

Literature list of conditions used for collagen coating to VWF binding (A) and for binding platelet and VWF (B).

A. Collagen Binding to VWF

Collagen type	Concentration	Solution	pH	Coating conditions and treatment	Coating time	Refer-ence
Human placenta type III, I, IV, V, bovine type I, I and III, horse type I and III	2 □g/ml	NS	NS	NS	NS	[1]
Human placenta type III	2-25 □g/ml	0.1 M citrate buffer	3	NS	2, 24, 48h	
		0.1 M acetate buffer	3			
		0.1 M citrate buffer	4			
		0.02 M PBS	7.4			
		0.015 M carbonate buffer	9.2			
Rat tail tendon	20 □g/ml	0.03 M phosphate, 0.03 M Tris.	7.3	20-30°C	NS	[2]
Bovine tendon	2 µg	0.1 M acetic acid	NS	RT	NS	[3]
Bovine tendon	100 □g/ml	0.4 M sodium phosphate buffer	7.4	18°C		
Calf skin type I and III	1000-3000 µg	0.02 M sodium phosphate and 0.13 M NaCl	7.4	NS	N	[4]
Bovine dermal	NS					
Human, bovine, chick collagen type VI,	30 µg/ml	0.05 M bicarbonate buffer	9.6	RT	24h	[5]
Calf skin type I and III.	1870 µg/ml	0.05 M acetic acid	NS	RT	1.5h	[6]
Bovine type I, calf skin type I	1800 µg/ml	0.02 M sodium citrate buffer	6	NS	1.5h	[7]
Human placenta type III and I	100 µg/ml	0.65 M sodium phosphate buffer	6.5	37°C	1h	[8]
Bovine collagen type I and type III	NS	0.05 M carbonate buffer	9.6	NS	NS	[9]
Human placenta type III	10 µg/ml	NS	NS	Dissolved in 0.05 M acetic acid and dialyzed against PBS	NS	[10]
Human collagen type I	25 µg/ml	NS	NS	Dissolved in 0.05 M acetic acid dialyzed	Overnight	[11]

Collagen type	Concentration	Solution	pH	Coating conditions and treatment	Coating time	Refer-ence
				against PBS,		
[1]Equine tendon or bovine collagen type I and III	10-50 µg/ml	HORM buffer	2.8	RT, wet box	4 days	[12]
Human placenta type III	1-5 µg/ml					
Equine collagen type I and III	50 µg/ml	HORM buffer	2.8	22°C	48h	[13]
Calf skin type III or human placenta	20 µg/ml	NS	NS	4°C	Overnight	[14]
Rate tail tendon	4000 µg/ml	NS	NS	Dissolved in 0.1% acetic acid, 0.2 M NaCl and dialyzed against 0.1 M PBS	NS	[15]
Human type III	NS	NS	7.6	Dialyzed against 0.02M Na_2HPO_4 at 4°C	NS	[16]
Human collagen type III	1000 µg/ml or 100 µg/ml	NS	NS	Dissolved in 0.05 M acetic acid, and dialyzed against 0.02 M PBS.	NS	[17,18]
Humen umbilical arteries type I and III	NS	NS	NS	Dissolved in 0.05 M acetic acid and dialyzed against 0.02 M Na_2HPO_4.	NS	[19]
Bovine tendon type I	33 □g/mL	PBS	NS	4°C	Overnight	[20]
Bovine dermal collagen I type III	3300 µg/mL	NS	NS	NS	NS	[21]
Human type III	NS	Sodium acetate buffer	4.5	NS	NS	[22]
Human type III	3 µg/mL	PBS	NS	NS	NS	[23]
Human type III	10 µg/mL	NS	NS	Dissolved in 3% acetic acid, diluted in PBS, coated at RT	1h	[24]
Human type III	4 µg/mL	NS	NS	Dissolved in acetic acid, diluted in PBS, coated	24h	[25]

Collagen type	Concentration	Solution	pH	Coating conditions and treatment	Coating time	Refer-ence
				at RT		
				4°C	48h	
Human type III	3 µg/mL	NS	7.4	4°C	Overnight	[26]
Human type III	3 µg/mL	PBS	7.4	4°C	Overnight	[27]
				RT	1h	
Human type III	100 µg /mL	NS	NS	Dissolved in 3% acetic acid and diluted in PBS	Overnight	[28]
Human type I, IV and V	20 µg/ml	NS	7.4	Dissolved in 0.1% acetic acid and diluted in buffer 0.136 M NaCl, 0.0027 M KCl, 0.0042 M NaH_2PO_4. 0.12 M $NaHCO_3$,0.0055 M glucose and 0.005 M HEPES,	Overnight	[29]
				RT	1h	
Human type III	6 µg/mL	Carbonate buffer	NS	4°C	Overnight	[30]
Human type III	20 µg/ml		NS	Dissolved in 3% acetic acid, and diluted in PBS, coated at RT	1h	[31]
Human type I	50 µg/ml	0.05 M acetic acid	NS	4°C	Overnight	[32]
Equine type I	30 µg/mL	NS	NS	4°C	48h	[33]
Human type III	100 □g/ml	PBS	NS	4°C	Overnight	[34]
Bovine type I/III	35 □g/ml	PBS	NS	NS	48h	[35]
Equine type III (Gradipore)	VWF:CBA kit	NS	NS	NS	NS	[36]
Human type III, human type I, human type VI (Technoclone)	VWF:CBA kits	NS	NS	NS	NS	
Human type III (Progen)	VWF:CBA kit	NS	NS	NS	NS	
Equine type III (Corgenix/ Reaads)	VWF:CBA kit	NS	NS	NS	NS	
Human type III	VWF:CBA kit	NS	NS	NS	NS	

Collagen type	Concentration	Solution	pH	Coating conditions and treatment	Coating time	Refer-ence
(Roche/Stago Asserachrom)						
Equine type III (Life Therapeutics)	VWF:CBA kit	NS	NS	NS	NS	[37]
Human type III (Precision Biologic)	VWF:CBA kit	NS	NS	NS	NS	[35]
Equine type I (Vital Diagnostics)	VWF:CBA kit	NS	NS	NS	NS	

[1] the author tested other types of collagens except those in table, which he found for the best CBA method: human placenta type IV, calf skin type I, bovine Achilles tendon type I, equine tendon type I, Equine type III.

B. Collagen Binding to Platelet and VWF

Collagen type	Concentration		Solution	pH	Coating conditions and treatment	Coating time	Reference
	Spray	Adsorption					
Human collagen type II, III, VI, Bovine type I		100 µg/ml	0.01 M acetate	5.0	NS	NS	[38]
Calf skin collagen type I		1000 µg/ml 50 µl/cs	0.05 M acetic acid	NS	NS	2h	[39]
Human placenta type I,III,IV and calf skin type I	30 µg/cm^2		0.05 M acetic acid	NS	Dissolved in 0.05 M acetic acid and dialyzed against PBS	NS	[40]
Bovine collagen type III	30 µg/cm^2	100 mg/ml,	NS	NS	NS	overnight	[41]
Bovine type I and human placenta type I		2.5 mg/ml, 200 µg/cs	0.5 M PBS	NS	NS	1h	[42]
		2.5 mg/ml dialyzed against PBS	0.1 M acetic acid				
Human type I	NS		NS	NS	NS	NS	[43]
Hors tendom mix I/III	1000 µg/ml, 100µl/cs		NS	NS	NS	NS	[44]
Human placenta type III, I, IV		100 µg/ml	0.065 M Sodium phosphate,	6.5	37°C	1h	[8]
Human I, III	100 µl, 1	1 mg/ml, 100	NS	NS	NS	NS	[45]

Collagen type	Concentration		Solution	pH	Coating conditions and treatment	Coating time	Reference
	Spray	Adsorption					
	mg/ml	□l/cs					
Hors tendom mix I/III or human placenta III		200 µl of 1000 µg/ml	HORM buffer	NS	NS	NS	[46]
Human collagen type III	6.5 µg/cm^2		NS	NS	NS	NS	[10]
Human placenta or calf skin		100 µg/ml	NS	NS	4°C	Overnight	[14]
Calf skin type I and III)		3200 µg/ml, 100 □l/cs	HEPES	NS	37°C	Overnight	[47]
Calf skin type I Calf skin type I Equine tendon collagen type I		2000 µg/ml or 100 µg/ml	NS	NS	RT	3h	[48]
Bovine type I		1000 µg/ml not mention if it spray or absorbtion	NS	NS	NS	NS	[49]
Horm type I		100 µg/ml	NS	NS	4°C	Overnight	[50]
Bovine tendon type I, Calf skin type I		0.1 mg/ml	NS	NS	4°C	Overnight	[51]
Human type III		0.1 mg/ml	0.05 M acetic acid	NS	4°C	Overnight	[52]
Human type I		3 mg/ml	HEPES buffer saline, 0.1 M NaOH	7	RT	150 min	[53]
Bovine type I and III		0.1 mg/ml 5 µg/ ml	0.136 M NaCl, 0.0027 M KCl, 0.0042 M NaH_2PO_4, 0.12 M $NaHCO_3$,0.00 55 M glucose and 0.005 M HEPES	7.4	4°C	Overnight	[54]

cs-coverslip, BSA-bovine serum albumin, HSA-human serum albumin, RT-room temperature, NS-not specified.

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Supplementary Table 2

Literature list of modifications used for C15-mer.

Modification	Location	Result	Reference
3 -biotin-streptavidin bioconjugates of aptamer		Protect from blood nuclease in vitro, but it did not slowdown in vivo clearance.	[1]
5'-5' polarity inversion	3'GG5'-TGGTGTGGTTGG 3'	Chair like structure, thermal stability higher then C15 but prothrombin inhibition decreased.	[2]
Guanosine carrying several substituents	at 8 (C_8)position or at the exocyclic (N_2)amino group	Dependent on substitutions group: some decrease the activity of the aptamer due to perturbation on the chairlike, and other increase the thrombin inhibitory activity, presumably due to the stabilization of a chairlike structure.	[3]
Replacement of negatively charge phosphodiester groups with neutral formacetal groups.	Same sequence as C15 but contain one or more neutral formacetal group as the replacement for phosphodiester linkages.	Mono substitution of phosphodiester groups -no critical effect. Two substitutions -the sum of two substitutions. Four - substitutions increased in vivo prothrombin time and extend in vivo half-life compared to C15. (T_{13} f T_{14}, T_4 f G_5)	[4]
Multivalent circular DNA aptamers		Increase affinity, stability and half-life (›10h). EC50 value of thrombin time 2-3 fold better than C15.	[5]
C15 flanking duplex motif with either disulfide or triethylene glycol	5'and 3' ends	Increased thrombin inhibition and improved resistance to nucleases	[6]
Addition of duplex region to C15	Using genetic algorithm to select the inhibitory sequence of the duplex region.	Extended clotting times (thrombin time).	[7]
Adding 8 mer oligonucleotides to both 5' and the 3' end of C15, and then repeated evolution in silico.	Similar structure to that of Tasset et al [27]	Higher inhibitory activity then C15.	[8]
2'-deoxy-2'-fluoroarabinonucleotide residues	On different G-quadruplexes	Enhancement in binding affinity to thrombin along with increased thermal stability and nuclease resistance	[9]
Thiophosphoryl internucleotide	Quadruplex loops	Better stability then C15 in serum against nucleases and anticoagulation properties similar to C15	[10]

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