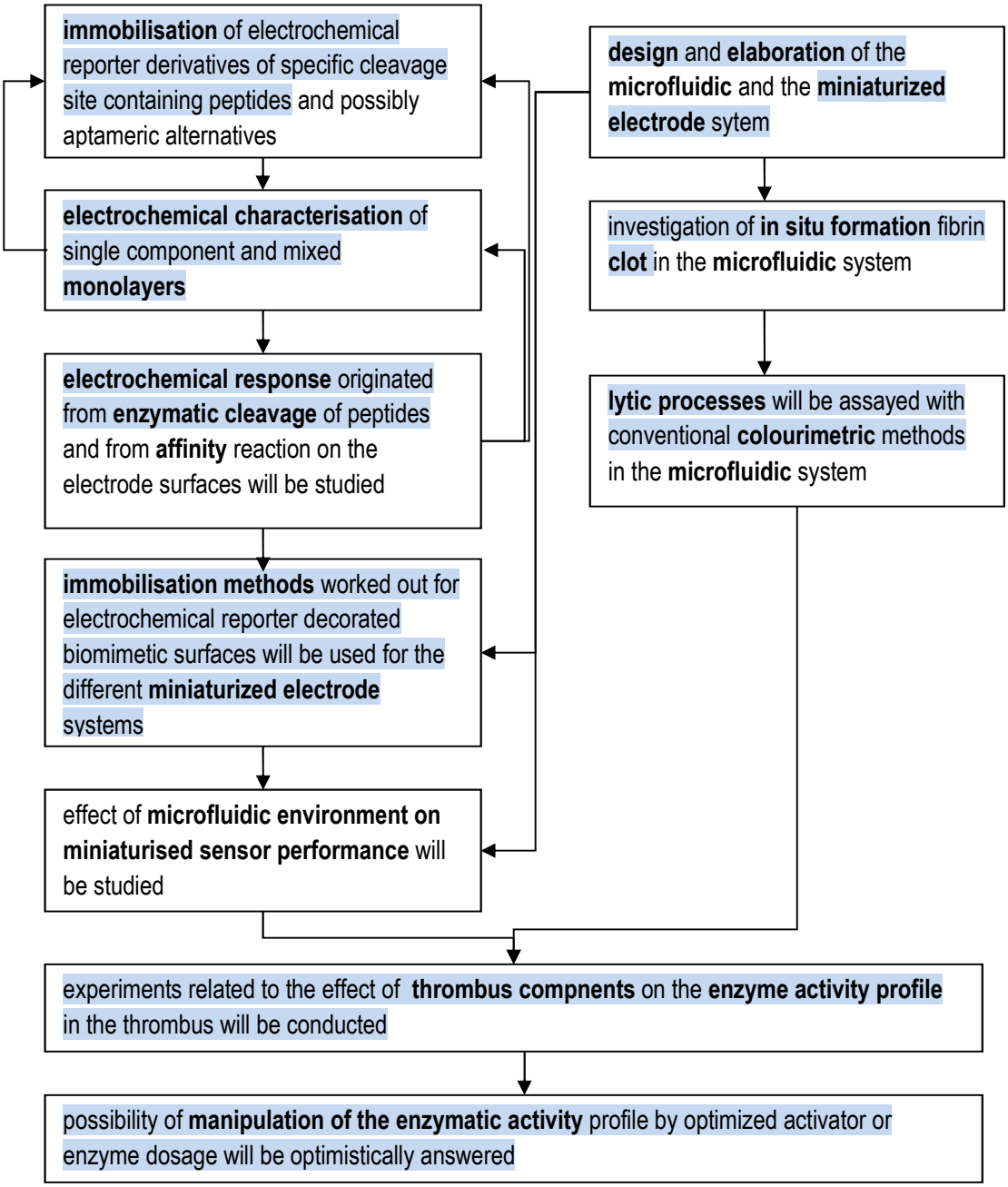
Final report on PD100753 project
„Biomimetic nanosensors for proteolytic process monitoring"

Summary

The project was aiming to develop a versatile detection tool to follow up a thrombolytic reaction front by electrochemical methods. By means of such development, a real-time investigation of thrombolytic therapy efficiency in model thrombi was proposed. The workplan was covering different stages of surface engineering, measurement technology and biochemical assay developments.
The original workplan of the proposed research was essentially completed, the alterations are summarized by the following flow-chart, where the completed tasks are highlighted in the original scheme.



Highlights

In the following, the realisation of different stages of proposed experimental route will be discussed in detail:

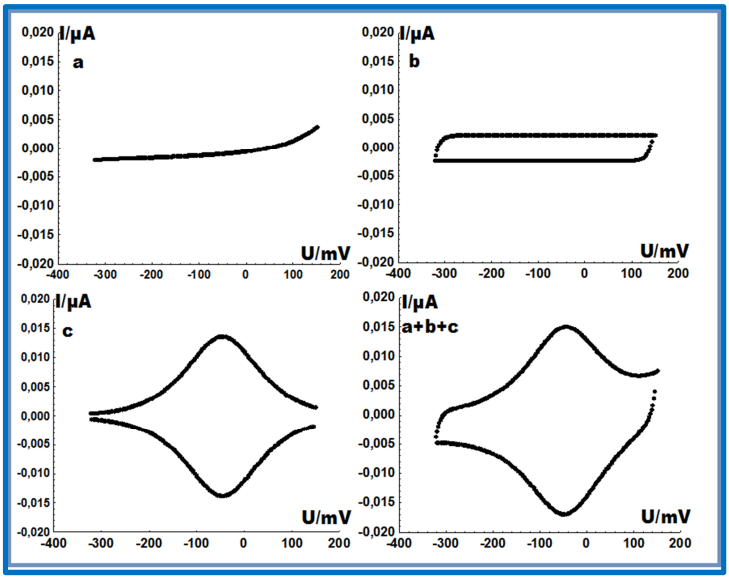
- **immobilisation of electrochemical reporter derivatives of specific cleavage site containing peptides**

- The availability of the artifical enzyme substrate, tagged with an electrochemical reporter and an anchoring group suitable for surface immobilisation, was a key factor in the realisation of the project. The purchase of the required biomolecules has raised more difficulties then it was originally expected, as it was commercially unavailable. We are greatly indebted to the generous help of Prof. Gábor Mező (Eötvös Loránd University, Research Group of Peptide Chemistry) and Prof. Antal Csámpai (Eötvös Loránd University, Department of Inorganic Chemistry) for the implementation of ferrocene-peptide conjugate molecule synthesis and for providing such a substantial amount of chemicals, so that we were able to conduct successful experiments. The structure of the peptide conjugate is based on previous publication [1], where the head group of the molecule is the electroactive ferrocene lable, and the anchoring group is a cysteine aminoacid attached to the plasmin specific sequence and the spacer peptide.

- The proposed affinity reaction providing aptamer molecule – an artificial oligonucleotid sequence with high affinity binding to the target – specific for plasmin enzyme was also commercially unavailable, and no documented previous attempts have been made to conduct selection process to identify the possible sequence with necessary binding affinity. We have contacted international suppliers offering to realise complete selection and synthesis process, but unfortunately the quote of such activity reached beyond the financial possibilities of present project, so we had to step back from this research direction.

- The immobilisation procedure of peptide conjugate on electrode surfaces was based on self-assembling process, performed with dip coating/washing procedure. The electrode surfaces used were either commercial 2 mm diameter gold electrode surface (CH Instruments) or small size microfabricated gold or platinum electrode arrays prepared at the Institute of Technical Physics and Material Science, Centre for Energy Research.

- **electrochemical characterisation of single component and mixed monolayers**



The characterisation of the self-assembled layers was performed by cyclic voltammetric measurements in biological buffer. The electrochemical response was analysed by fitting the resulted voltammetric curves by taking into account a) the Faraday process in the background, b) the double layer charging, and c) the charging of the surface confined redox film.

Figure 1.
Fitting of cyclic voltammetric measurement curves on immobilized ferrocene-peptide conjugate layer on MFA platinum electrode arrays in 0,01 M PBS, pH=7,4. Quasi-reference electrode material was platinum.

The tightly packed homogenous layer of molecules gives an electrochemical response related to the theoretically complete molecular coverage, thus providing the maximal possible surface confined substrate concentration on the electrode surface. The application of mixed layers of ferrocene-peptide conjugate and 6-mercapto-1-hexanol offered better steric availability for the enzyme-substrate cleavage reaction, lowering the limit of detection, but due to the partial electroactive substrate coverage the signal to noise ratio was worse.

- **electrochemical response originated from enzymatic cleavage of peptides**

In natural conditions, the enzymatic cleavage of fibrin is the result of plasmin enzyme activity. Plasmin (PL) is a proteolytic enzyme formed from its zymogen plasminogen (PG) by activators, such as tissue plasminogen activator (tPa) or streptokinase. The experimental assays measured can be summarized in the following figures:

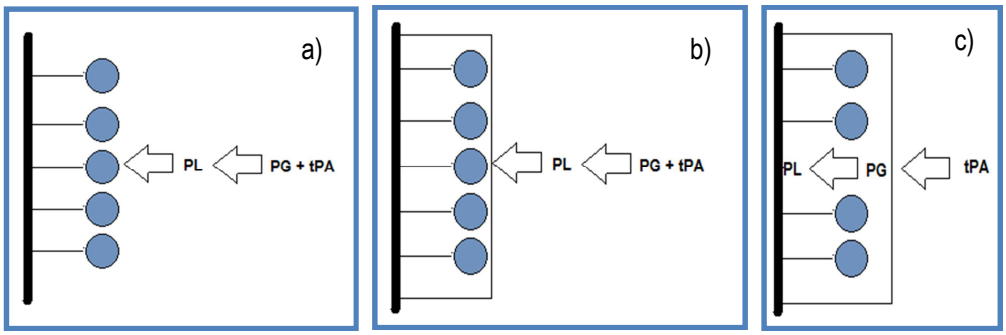


Figure 2.

Experimental schemes of enzymatic cleavage measurements. (a) Plasminogen is preactivated and plasmin is applied to ferrocene-peptide conjugate covered electrode surface (b) Plasminogen is preactivated and plasmin is applied to ferrocene-peptide conjugate in fibrin gel (c) Plasmin is generated in-situ in the fibrin gel by applying plasminogen activator to plasminogen adsorbed in fibrin.

- Electrochemical response due to enzymatic cleavage was analysed by representing the peak current change, i.e. the actual peak current value related to the starting peak current value. This dimensionless relative value compares only the changes, but is not taking into account the possible differences of substrate availability due to different surface coverage frequently occurs in real experimental conditions.

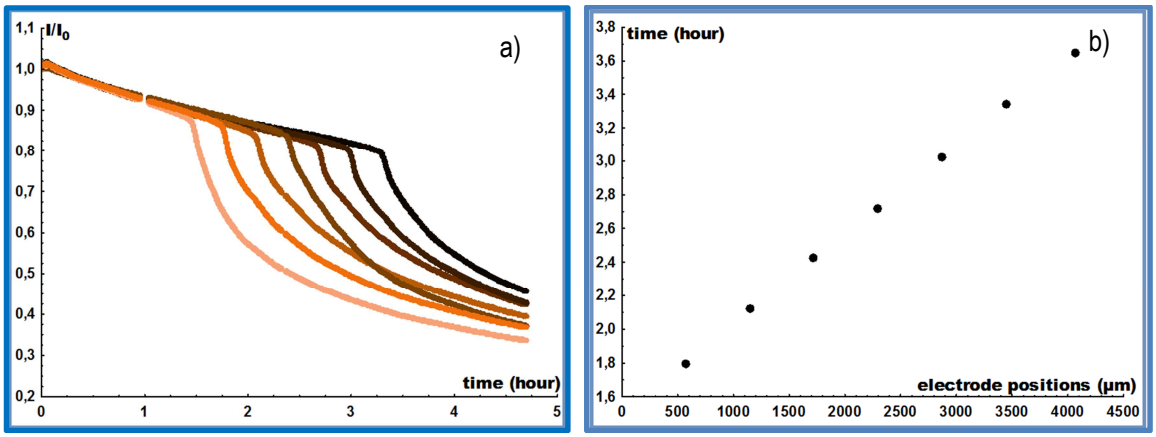


Figure 3.

Example of characteristic cleavage front progress as surface adsorbed zymogen is activated. (a) Decrease of relative peak current values, at different electrodes of the array, resulted by the activation of the zymogen in the vicinity of the electrode surfaces. (b) The inflection points of the curves are represented as the function of electrode position, showing the velocity of lytic front progress.

- To overcome this non-precise approach, the surface charge concentration was determined by applying fitting procedure described above, and calculating the integrated surface confined charge on unit surface area, giving the actual substrate concentration on the surface. By knowing exact surface charge density and substrate depletion in time, enzymatic reaction parameters can be calculated, such as K_M (Michaelis constant) to indicate the affinity of the surface bound electroactive substrate (ferrocene-peptide conjugate) to the enzyme reaction in the given solution or gel environment.

- **immobilisation methods worked out for electrochemical reporter decorated biomimetic surfaces will be used for the different miniaturized electrode systems**

One of the key steps of self-assemly immobilisation procedure is the electrode cleaning process, which was differently applied for the two types of electrodes. The polishing step of commerical „macro" electrode (1-0.05 µm alumina powder) was not a possibility for the „micro" scale array, so in the latter case only electrochemical cleaning in 0,5 M H_2SO_4 acidic solution was applied which, besides the cleaning, also gives possibility for the quality control of the process. Otherwise dipping/washing immobilisation procedure was the same as applied for „macro" electrodes.

- **design and elaboration of the miniaturized electrode sytem**

- We are very greatful for the help of Dr. Zoltán Fekete (neuroMEMS Research Group, Institute of Technical Physics and Material Science, Centre for Energy Research) for providing us the specifically project aimed 8 channel electrode arrays. Square-shape (80 µm x 80 µm) linear array of platinum recording sites are formed on a silicon microelectrode. The shaft length, width and thickness are 12 mm, 225 µm and 380 µm, respectively. The relative position of the recording sites is 575 µm. The fabrication of the microelectrode is based on standard silicon MEMS micromachining processes. In order to insulate the metallization from the bulk silicon carrier, a thin film stack of 50 nm SiO_2 / 300 nm (non-stoichiometric) SiN_x is formed by thermal growth and low pressure chemical vapour deposition (LPCVD) on a 380 µm thick (100) single-crystalline silicon wafer. Sputtered, 15 nm / 270 nm TiO_x/Pt wires and recording sites are patterned by lift-off technology. The final passivation layer of300 nm SiN_x was deposited by LPCVD again. Selective removal of SiO_2 and SiN_x from shaft contour and from the top of recording sites and bonding pads were performed by reactive ion etching. Through-wafer etch of the silicon substrate (Bosch process) is completed in deep reactive ion etcher (Oxford Plasmalab 100, Oxford Instruments Plc, UK) using a 7 micron thick photoresist mask (Megaposit SPR 220, Microchem GmbH, Germany). The released microelectrodes are mounted on custom-designed printed circuit board by wire bonding.

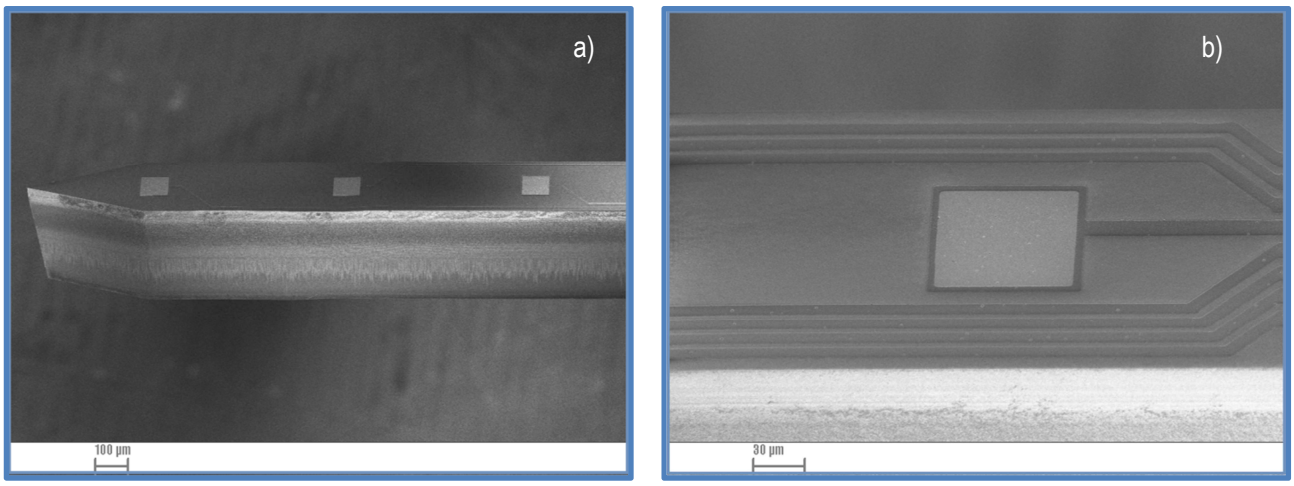


Figure 4.
Scanning electron microscopic images of the electrode array. (a) Image of array (b) Electrode wiring

- The electrode array was connected to a non-commerical 8 channel potentiostate constructed by Dr. Gábor Mészáros (Functional Interfaces Research Group, Institute of Material and Environmental Chemistry, Research Centre for Natural Sciences). The potentiostate has been fabricated to be able to follow 8 channel voltammetric signal simultaneously to very low current limits, making recording experiments on microelectrodes possible.

- In the experimental setup used in the thrombolytic model investigations we applied stereotactic frame controlled positioning of electrodes over 300 µl microplate arrays. By using the exact positioning the geometric parameters of tip penetration were well controlled.

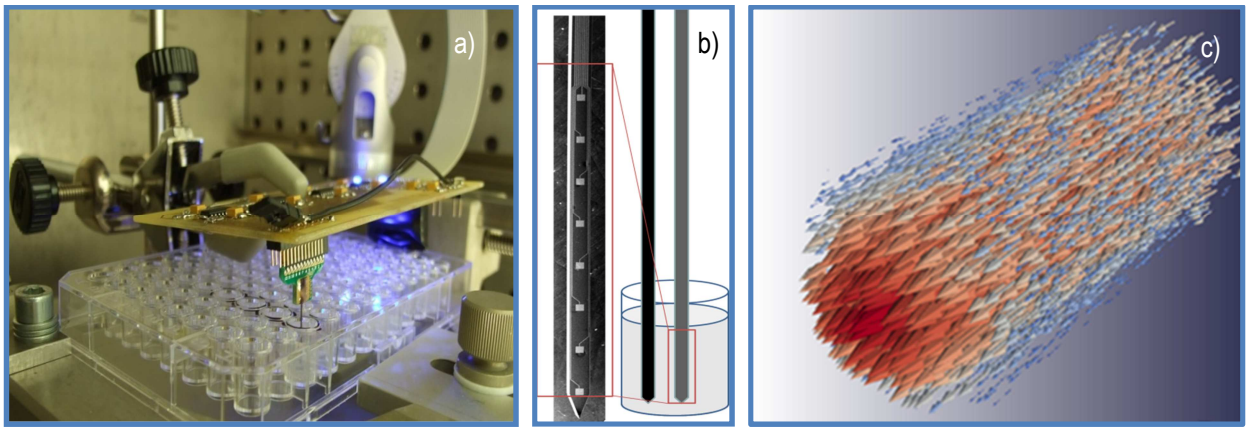


Figure 5.
Experimental setup of model thrombolytic studies and transport model. (a) Electrode positioning system set up in thermostate to control temperature dependent clotting and lysis (b) The electrode array tip and the reference electrode is immersed with fixed geometrical setup into the thrombus model gel (c) OpenFOAM® model of lytic front and active enzyme distribution.

- **investigation of in situ formation fibrin clot**

Several attempts have been made to conduct experiments on preformed throbus models by penetrating the electrode tip into fibrin gel after clotting. Unfortunately, in this case, plasmin reaction front was guided by the interfacial void formed between the electrode and the clot. This resulted a rapid transport of enzyme flow next to the electrode, not reftecting to the transport through the clot.
As the result of the above findings, the strategy of clot formation with embedded electrode structure was chosen. The fibrin clot was formed from fibrinogen monomers under the action of thrombin. The ferrocen-peptide conjugate modified electrode was placed in the microplate well, where fibrinogen as well as thrombin was added. The clotting of fibrin was followed in paralel turbidity change measurements. A technical limit of the clot variabilty was met because of the sensitivity of ferrocene-peptide conjugate for thrombin lytic activity. This non-specificity of synthetic plasmin substrate made the experimental variables to remain in smaller range then planned. We could not investigate the effect on lytic resistance of different fibrin filament structures prepared with different amount of thrombin, because we had to use the smallest possible concentration of thrombin for clot preparation to get a well detectable electric response on the electrode by the end of clotting time. For this reason optimisation of fibrin concentration / thrombin concentration / clotting time variables has been performed.

Microfluidic assays have not been elaborated due to the difficulties of batch experiments, but also due to emphasis shift considering the not special clinical relevance of capillary size thrombi dissolution. Versatile flow-assay setup construction is under progress.

- **lytic processes will be assayed with conventional colourimetric methods**

Colourimetric assays were used with commercial chromophore-tagged plasmin substrates (SPECTROZYME®) to determine the exact activity pf plasmin enzyme prepared freshly from zymogen plasminogen and tissue plasminogen activator or streptokinase. Purified plasminogen and fibrinogen was supplied by the Haemostasis Research Division, Department of Medical Biochemistry, Semmelweis University (Prof. Kraszimir Kolev). Turbidity measurements were applied in each experiments paralel to electrochemical measurements, both to follow clotting procedure, and also to follow the clearing of gel after plasmin enzyme addition.

- **experiments related to the effect of thrombus components on the enzyme activity profile in the thrombus will be conducted**

- The most straightforward question on the effect of fibrin filament thickness could not be investigated for the above mentioned non-specificity reasons, so we initiated research on the effect of blood clot components of neutrophil cell origin on the lytic resistance of the fibrin clot. Previous experiments, by using optical methods showed that components of neutrophil extracellular traps, such as DNA and histone affect the overall behavouir of fibrin clot gelation [2]. For this reason we have added DNA or histon or both components to the clotting fibrinogen and the complex gel lysis was followed by the electrode array. The time difference between electrode signal is related to the lytic resistance of the given system. A difference of reaction front velocity could be observed between the pure fibrin system and both DNA and histone containig systems, in this way the influence of thrombus components were demonstrated.

- Whole blood measurements were also conducted in limited numbers, with blood samples from lab volunteers. Pure and plasminogen enriched blood samples were clotted on the electrode array, and effect of tPA on lysis front speed was followed. The effect was observable, althoug real blood sample manifest further challenges, such as other natural proteolytic enzymes interfering with electrode confined substrates, as well as the contracting effect of cellular components of blood on the gel structure making the reaction space poorly defined.

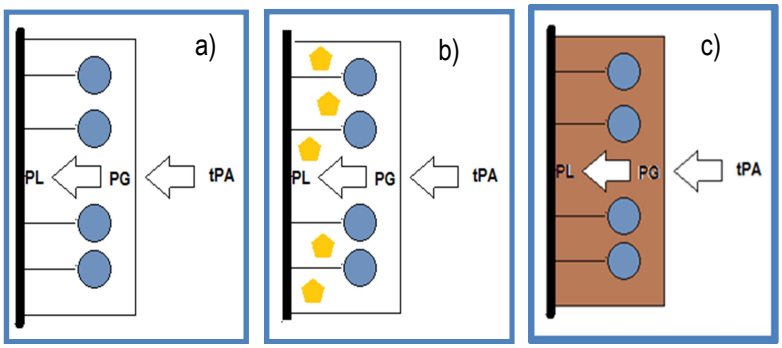


Figure 6.

Experimental schemes of enzymatic cleavage measurements influenced by other thrombus components. (a) Reference system, when plasmin is generated in-situ in the fibrin gel by applying plasminogen activator to plasminogen adsorbed in fibrin (b) Plasmin is generated in-situ in the fibrin gel by applying plasminogen activator to plasminogen adsorbed in fibrin already containing DNA or histone (c) Plasmin is generated in-situ in the blood clot by applying plasminogen activator to plasminogen adsorbed in whole blood.

- **possibility of manipulation of the enzymatic activity profile by optimized activator or enzyme dosage will be optimistically answered**

In respect of optimisation we have conducted experiments by addition of preactivated plasmin enzyme to pure fibrin gel and also by addition of tPA to zymogen containing fibrin gel. Lytic front progress difference was well observable, the preadsorbed zymogen was initialting an accelerated cleavage.

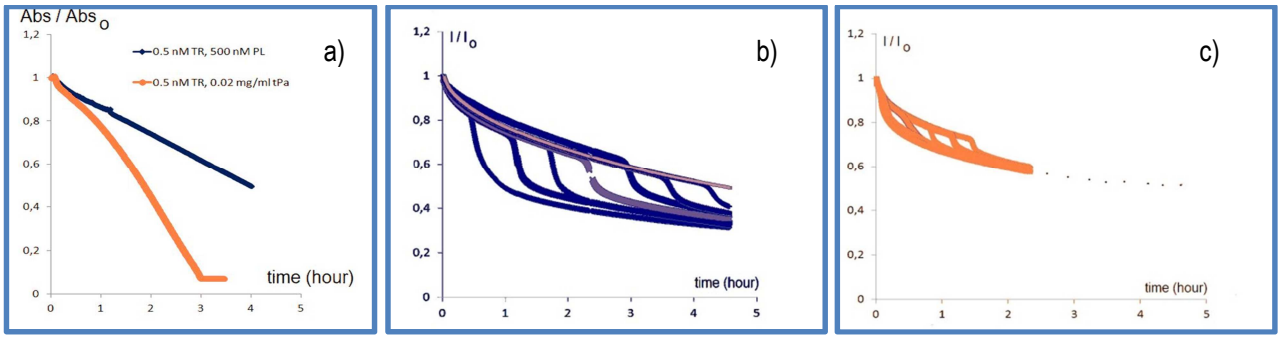


Figure 7.

Differences in lytic front progress in case of preactivated and in situ acivated plasmin. (a) Turbidity change of the system during proteolytic action in both type of enzyme addition (b) Plasminogen is preactivated and plasmin is applied to ferrocene-peptide conjugate and fibrin gel covered electrode surface (c) Plasmin is generated in-situ in the fibrin gel by applying plasminogen activator to plasminogen adsorbed to fibrin.

Epilogue

The present research report is summarizing a quite complex research subject with lot of technical dependencies, originally not well foreseen. By the end of the prolonged project all technical prerequisite have been established, and we were able to conduct quite a few interesting and promising experiments. The results of the research is under publication process, one paper on the technical realisation details and the further possibilities of the novel measurement technique, an other paper on the effect blood components of neutrophil cell origin on the dissolution reaction front progress.

Acknowledgement

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References

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