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NOVEL ENZYME ASSAYS USING THE ATTENUATED TOTAL REFLEXION SPECTROSCOPY (ATRS)

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INTRODUCTION: Simple and fast enzyme assays are attractive for medical diagnostics, *e.g.* in haemostaseology, when the pro-thrombin time can be monitored at the patient's home. Within the project MIOSA (miniaturized integrated optical sensor application), novel enzyme assays using attenuated total reflexion spectroscopy (ATRS) as measuring technique on a chip are being developed. To exclude optical interference with haemoglobin from blood, near-infrared chromophore-labelled peptides as substrates for thrombin and other proteases needed to be synthesized. Here we present preliminary results such as the characterization of the measuring platform and the synthesis of near-infrared-(NIR)-labelled substrates for proteases.

METHODS: ATRS measures the absorption of light within a thin sensing layer in the order of a few nanometers in z-direction of the propagating light at the Ta_2O_5-waveguide on the optical chip. The NIR-dye is covalently linked to the substrates immobilized on the chip surface. On addition of the enzyme, the dye is first cleft from the immobilized peptide and then diffuses out of the sensing layer. The enzyme activity correlates with the increasing intensity of light, which is transmitted through the waveguide by total internal reflexion.

RESULTS: The sensitivity of the ATRS photometer prototype, developed by Metanor AG, Regensdorf and in cooperation with CCS, was tested and in all three cases the sensitivity of the optical chip used was found to be satisfactory:

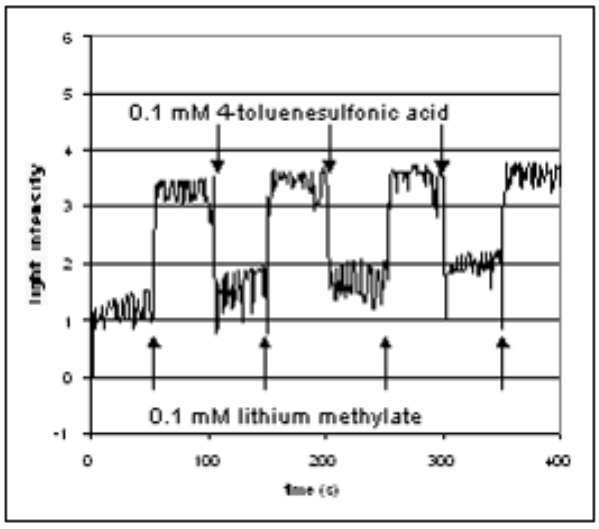

Fig. 1: The covalent immobilization of the NIR-dye on the Ta_2O_5-waveguide was tested by exposing the immobilized and pH-sensitive dye to two different methanol buffers (4-toluenesulfonic acid & lithium methylate) changing the detected light intensity of the ATRS-signal.

1) A lipophilic derivative of the same pH-indicator chromophore as used as label during synthesis of potential protease substrates was measured as indicator dye in its protonated form within methanol solutions. The detection limit, given by the signal to noise ratio, lay at about 10^{-5} mol/l indicator at a propagation path length on the waveguide of 6 mm.
2) With regard to possible immobilization of chromophore-labelled substrates within a plasticised PVC membrane, the sensitivity of the set-up within a membrane disposed on the chip surface that contained the same indicator dye was investigated.
3) The chips were functionalised with amino groups, such that the Ta_2O_5-surface was completely covered (XPS). In the next steps, a linker was first chemically bound to the functionalised chip surface and secondly the dye was linked to the linker. The covalent immobilisation of the pH-sensitive-dye was tested by exposing the chip to different methanol buffer solutions leading to different absorption spectra of the indicator dye and different light intensities of the ATRS-signal.

Preliminary experiments to test the influence of immobilization on the substrate were performed in solution. A common substrate for trypsin was derivatized at the amino-terminus by caprinic acid (Caprinyl-Phe-Gly-Arg-pNA), showing remaining cleavability in kinetic measurements.
Chromogenic substrates for trypsin-like enzymes with the peptide sequence R_1-R_2-Arginin-L-D (R_1, R_2: amino acids; L: spacer [amino acid or phenylenediamin]; D: NIR-dye) were synthesized. Since modelling experiments had suggested a linker between substrate and NIR-dye to be necessary, substrates with and without different linkers (L_1 & L_2) were synthesized:
- BOC-Ser(Bn)-Gly-Arg-D
- BOC-Ser(Bz)-Gly-Arg-L_1-D, L_1=Phenylendiamine
- BOC-Ser(Bz)-Gly-Arg-L_2-D, L_2=Phenylalanin

Cleavability studies with these substrates are under investigation.

CONCLUSIONS:

Substrates labelled with a NIR-chromophore likely to be cleft by trypsin-like proteases as suggested by modelling studies, could be synthesized.
ATRS on optical waveguides with immobilized substrates was shown to be sufficiently sensitive to realize miniaturized measuring devices.

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