IMMOBILIZATION OF OXIDASES AND HRP FOR BIOSENSOR APPLICATIONS

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Enzyme based analytical methods are well–known and broadly applied in analyses of bioprocess samples due to their simplicity. Glucose, ethanol and many other enzymatic kits with soluble enzymes are commercially available. Enzyme immobilization, in form of test strips for example, is frequently used strategy for prolonging the kits' shelf life and for making the assays more easy to use, and these products are also available on the market. Drawback of both soluble enzyme tests and test strips is that it is impossible to perform continuous measurements and that they are expensive as the enzyme is wasted during the procedure.

A flow through packed bed enzymatic column with enzymes immobilized on a suitable solid support allows for the same enzyme load to be reused in repeated measurements. We have developed a system for continuous or frequent analyte measurements based on a modified Trinder's method (Vojinović et al. 2004) with enzyme immobilized by glutaraldehyde crosslinking onto alkylamine controlled pore glass (CPG) beads. Two enzymes, an oxidase and horseradish peroxidase are immobilized separately to CPG beads. After immobilization beads are mixed and filled into a micro column (20 μl working volume). The column is connected to a FIA system for the analyses (Fig. 1). Carrier solution contains colorimetric reagents: PSA and 4–AAP (Vojinović et al. 2004). Sample is injected to the system and transported to the column. When oxidase substrate comes in contact with immobilized oxidase it is oxidazed by O_2 dissolved in the carrier solution and an equivalent of H_2O_2 is produced. Peroxidase catalyses reduction of H_2O_2 and co–oxidation of PSA/4–AAP system and strongly coloured quinone–imine dye is produced. Colour intensity is proportional to the initial analyte concentration in the injected sample and is continuously measured by a flow through spectrophotometer at 490 nm. Columns sensitive to glucose, ethanol, lactate and galactose are being tested. Recently, micro packed–bed bioreactors containing horseradish peroxidase (HRP) and *Hansenula polymorpha* alcohol oxidase (AO) immobilized on alkylamine CPG were assembled for monitoring and quantification of hydrogen peroxide and ethanol, using a flow injection analysis (FIA) system (Azevedo, et al., 2004b; Azevedo, et al. 2004c). Glucose oxidase (GO)/horseradish peroxidase (HRP) coupled column has been fully characterized and validated and is reported in this work.

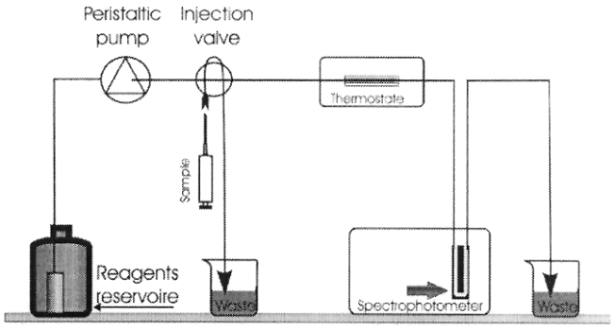

Figure 1. FIA system

MATERIALS AND METHODS

Alkylamine CPG beads were obtained from CPG (USA). GO and HRP enzymes were purchased from Biozyme (UK). Gluteraldehyde, and 4–AAP were from Sigma–Aldrich (Germany), while PSA was from Fluka (Germany).

Enzymes were immobilized by gluteraldehyde cross linking: alkylamine CPG beads were activated in a reaction with 3% gluteraldehyde (1h, room temperature, agitated), washed with distilled water and submersed in a 6 mg/l enzyme solution, and left to react over night at room temperature with agitation, (Leirão et al. 2003). Beads with immobilised enzymes were washed and 20 mg of beads with immobilised GO was mixed with same quantity of HRP immobilised beads. The mixture was filled to a glass column of 1.2 mm diameter and about 8 mm active enzymatic layer length. Activity of free and immobilised enzymes was measured by method described in (Vojinović et al. 2004).

Protein content for HRP in solution was determined spectrophotometrically at 404 nm using the molar absorptivity value 102000 $mol^{-1}dm^3cm^{-1}$ (Nicell et al. 1997). Quantity of immobilized enzyme per mass of

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CPG support was determined by dissolving the CPG beads with immobilized enzyme in a concentrated NaOH solution and then according to the standard Lowry method for the protein determination. In order to make the comparison between the two methods possible a conversion factor has been determined by comparing values obtained by the two methods for the HRP concentration when in solution.

Carrier solution contained 0.4 mM 4–AAP, 25 mM PSA in a 100 mM phosphate buffer solution at pH 7 as described in (Vojinović et al. 2004).

Column operational stability was tested by performing a continuous substrate conversion and by injecting repeatedly a standard solution over prolonged periods of time. Linear range and sensitivity were determined by making calibration curves for various flow rates and injection volumes.

The column was validated by comparing results of glucose measurement by a standard HPLC method and results of the enzymatic column method for two *E. coli cultivations*, *S. cerevisiae* cultivation, and an animal cell culture experiment.

RESULTS AND DISCUSSION

Characterisation of the CPG beads after immobilization comprised measurements of total immobilized protein content by Lowry method and immobilized enzyme activity. Results are expressed as residual activity. HRP residual activity was 7.0% and residual activity of GO was 6.3%. Loss of activity is probably due to conformational disturbances of the protein upon immobilization. Nevertheless residual activity is high enough for the biosensor applications.

Glucose enzymatic column proved to be very stable, with a prolonged shelf life (it was kept more than a year on 4°C and there was no significant activity loss). Operational stability was shown to be very good by a continuous glucose conversion experiment due to *in situ* stabilization strategy (Azevedo et al. 2004a) as shown on Fig. 2. During 12 hours of 330 mg/l glucose conversion, which is equivalent to 21600 injections of 20 µl samples there was only a 30% activity loss. Repeated injections of standard glucose solution (10 µl injections of 500 mg/l glucose solution) during 24 hours gave stable signal with relative standard deviation of 1%.

GO/HRP column validation for the *E. coli* and *S. cerevisiae* cultivations and for the animal cell culture experiment is presented on the Fig. 3 and Fig. 4.

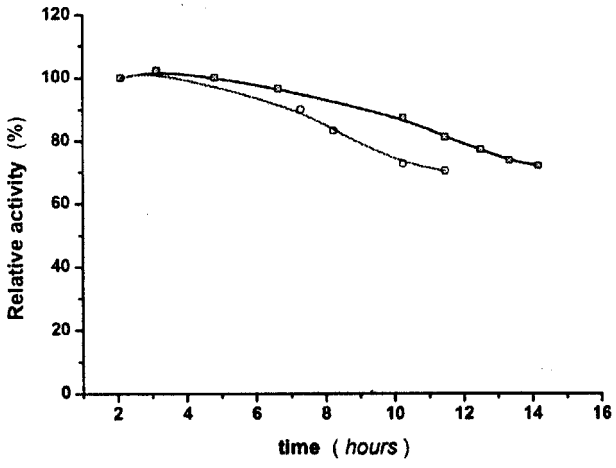

Figure 2. Column operational staility (□ mixed byenzimatic GO/HRP column, o GO column with 0.4 g/l HRP in carrier)

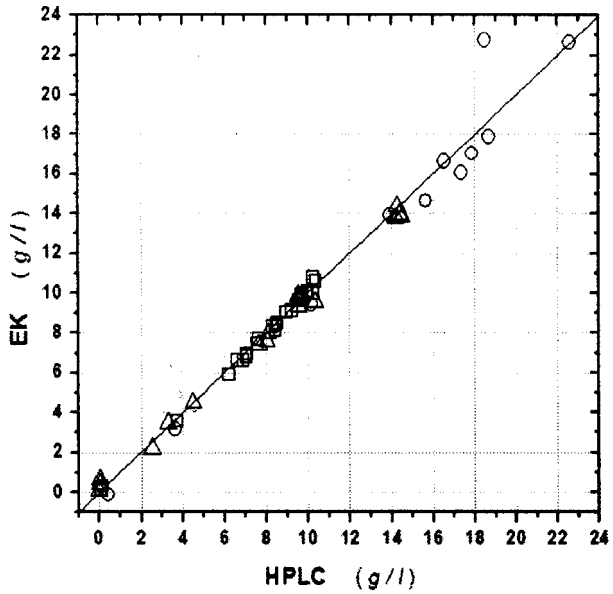

Figure 3. Enzimatic column vs. HPLC glucose measurement. o S. Serevisiae cultivation, Δ E. coli cultivation (20 g/l medium), □ E. Coli cultivation (10 g/l medium). Correlation: $[Gluc]_{EK} = 0.10\ (\pm 0.07) + 0.971\ (\pm 0.008) * [Gluc]_{HPLC}$, $R^2 = 0.997$, *number of samples:* $N = 59$.

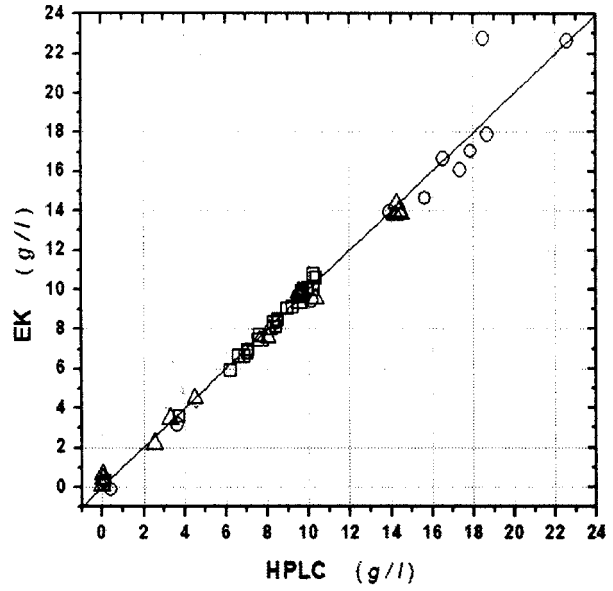

Figure 4. Enzymatic column vs. HPLC glucose measurement in animal cell media. Corelation: $[Gluc]_{EK} = 0.21\ (\pm 0.05) + 0.93\ (\pm 0.02) * [Gluc]_{HPLC}$, $R^2 = 0.996$, *number of samples:* $N = 17$.

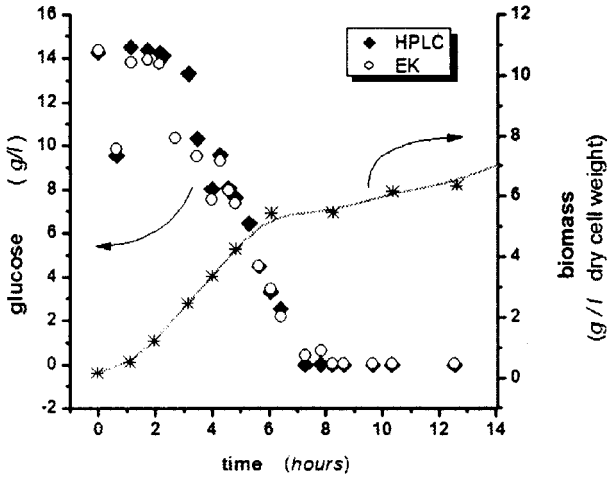

Figure 5. E. coli cell growth (glucose by HPLC (♦) and enzymatic column (o), biomass (⋆)

Relative error between the FIA and HPLC results for *E. coli* and *S. cerevisiae* were lower than 3% for all the samples with more than 2 g/l glucose. Relative error between the two methods for the animal cell culture samples was below 5% for all the samples.

The method has been tested for the quasi *on line* monitoring of *S. cerevisiae* cultivation and good results have been achieved (Fig. 5).

CONCLUSION

It is shown that the described bienzymatic GO/HRP column can be successfully used for rapid glucose concentration measurements over prolonged periods of time. It has a very good reproducibility, good linear range, high sensitivity, and there is no interference noted in the animal cell culture medium measurements.

LITERATURE

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