Optical monitoring of oxygen concentration and cells viability in polymer film with entrapped cells.

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Introduction

Monitoring of fluorescence intensities of biogenic flourophores during aerobic-anaerobic (AA) transition of immobilized cells has been used for determination of influences of immobilization processes on cell viability (Podrazky O. et al., 2005). The modified instrumentation, a cuvette window coated with oxygen sensitive film, enabled us to control oxygen concentration from both side of a biofilm. Thus we could obtain information about gas permeability of both: film matrix and biofilm with immobilized cells. The method is advantageous to determination of viability of cells entrapped in insoluble and inorganic matrices.

Silica host matrices made by sol gel process have emerged as a promising platform for encapsulation cells in construction of whole-cell biosensors (Avnir D. et al., 2006). Silica carriers are relatively inexpensive to synthesize and have several desirable properties including chemical inertness, optical transparency and resistance to microbial attack. But process of encapsulation into silica by sol-gel technique has often detrimental effect on cell viability (Nassif N. et al., 2003). This negative influence of immobilization might be overcome by entrapment into mixture of silica pre-polymer with natural polymer that does not damage cell viability (Coradin T. et al., 2003).

In this work we compare films prepared from pre-polymerized tetramethoxysilane (TMOS) and mixture TMOS + alginate. During AA transition we monitored fluorescence intensity of NAD(P)H, that reflects viability of immobilized *Sacharomyces cerevisiae* DBM 60, simultaneously with fluorescence of ruthenium complex, which corresponds with oxygen concentration (Lakowicz J.R., 1999).

Materials and methods

Oxygen sensitive film

ORMOCER® containing oxygen sensitive ruthenium complex [*Tris(1,10-phenantrolin)-ruthenium(II)chloride hydrate*], purchased from Aldrich, (1%) was dropped on quartz glass (a window of the flow through cuvette) and cured under UV (10 min) to form film thickness of 0.1 mm.

Microorganisms and media

Saccharomyces cerevisiae DBM 60 obtained from Department of Biochemistry and Microbiology of Institute of Chemical technology in Prague (Czech Republic) were cultivated in liquid YPG medium (20 g/l glucose, 10 g/l peptone and 10 g/l yeast extract) at 30 °C for 2 days (stationary growth phase). After cultivation, cells were centrifuged at 2000 x *g* for 5 min, washed with physiological solution (9 g/l NaCl) and resuspended in non-fluorescent Horvath-Spangler (HS) medium (Horvath J.J. et al. 1992) without glucose to prepare cell concentration in gel 50 $g_{\text{dry cell}}$/L.

Immobilization

TMOS pre-polymerization: Tetramethoxysilane (TMOS), purchased from Fluka was mixed with distilled water and HCl in TMOS:H_2O:HCl mol ratio = $1:5:10^{-2}$ to form a clear solution and left to prepolymerize for 24 h at 4°C.

Alginate/TMOS biofilms: 3.2% (w/v) sodium alginate (Sigma-Aldrich, type IV) (0.167 ml) was mixed with 0.5 M NaOH (0.5 ml) and pre-polymerized TMOS (0.333 ml) then the cell suspension (1 ml) was added. The mixture was vortexed and poured into the cuvette and after gellification (within 5 min) the biofilm was bathed in $CaCl_2$ solution (7 g/l) for 30 min.

Fluorescence measurements
The cuvette with a film was mounted into a measurement chamber of fluorescent spectrophotometer Hitachi F-4500, connected with an aerated and tempered reservoir (Fig. 1). HS medium circulated with velocity 10 ml/min and a record of fluorescence intensities of the oxygen sensitive film (EX/EM=460 nm/580 nm) and NAD(P)H (EX/EM=340 nm/440 nm) of immobilized cells had been started. After 30 minutes, we switched the bubling in reservoir from air to nitrogen (AA transition).

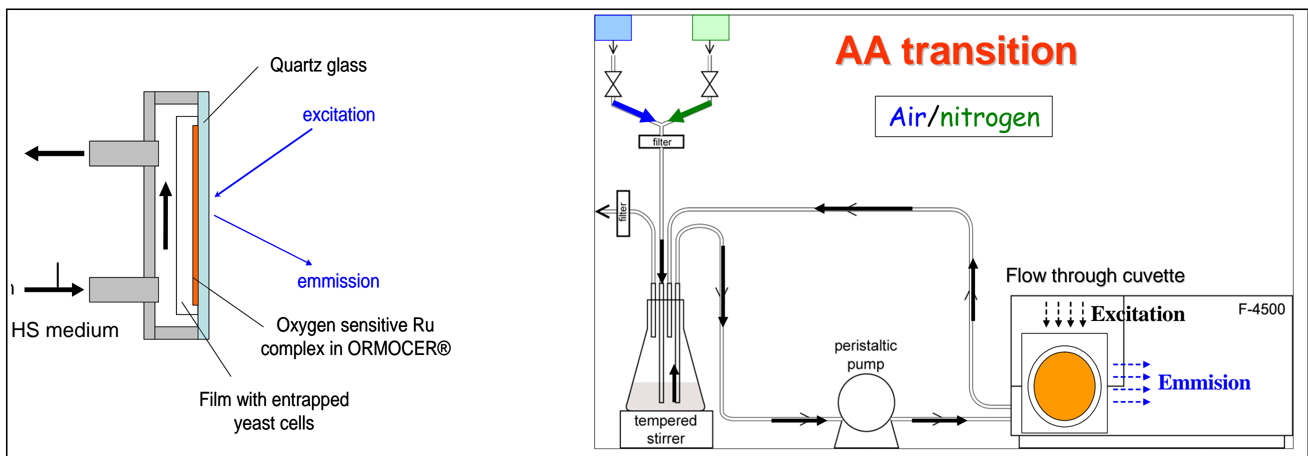

Fig.1: The flow-through cuvette and scheme of the apparatus.

Results and discussion
We used changes of fluorescence of ruthenium complex during AA transition for characterization of oxygen permeability of silica and silica/alginate films. Simultaneously monitored changes of NAD(P)H fluorescence were utilized for determination of viability of immobilized cell.
We compared AA transitions:
(a) in the cuvette with ORMOCER® and ruthenium complex
(b) in the cuvette with ORMOCER® and ruthenium complex covered with silica or silica/alginate film
(c) in the cuvette with ORMOCER® and ruthenium complex covered with silica or silica/alginate film with entrapped living cells in stationary growth phase
(d) in the cuvette with ORMOCER® and ruthenium complex covered with silica or silica/alginate film with entrapped living cells in exponential growth phase.
Changes of fluorescence intensities were characterized with ΔI and Δt (Fig. 2). ΔI is a relative change of fluorescence intensity during AA transition. ΔI_{Ru} is proportional to a change of oxygen concentration. Δt is a time between switch of bubbling from air to nitrogen and a begin of a rise of fluorescence intensity. Δt_{Ru} is a time constant of the instrument, which is increasing with decreasing of gas transport through the film. Oxygen consumption by cells immobilized in film lead to Δt_{Ru} decreasing.
The intensity of fluorescence of NAD(P)H started to rise after complete exhausting of oxygen, which was represented by maximum fluorescence of ruthenium complex $\Delta I_{Ru\ max}$ (Fig. 3) whereas $\Delta t_{NAD(P)H}$ reflects velocities of oxygen transport and cells oxygen consumption. $\Delta I_{NAD(P)H}$ is proportional to an increase of intracellular concentration of reduced form of NAD(P) during AA transition. Higher $\Delta I_{NAD(P)H}$ indicates higher cell viability.

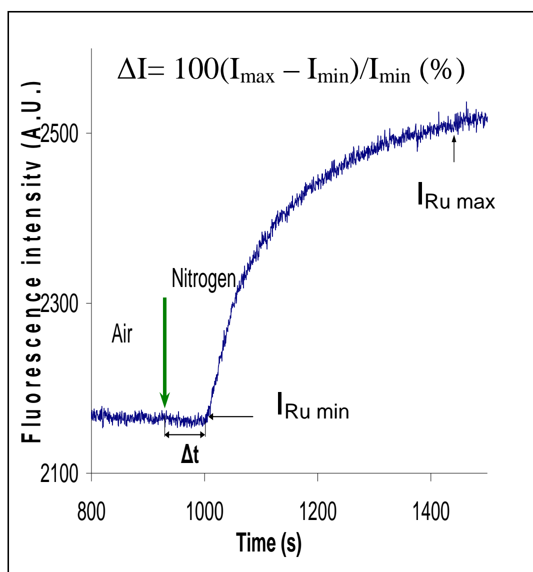

Fig. 2: Fluorescence intensity of ruthenium complex during Air-Nitrogen transition (AA).

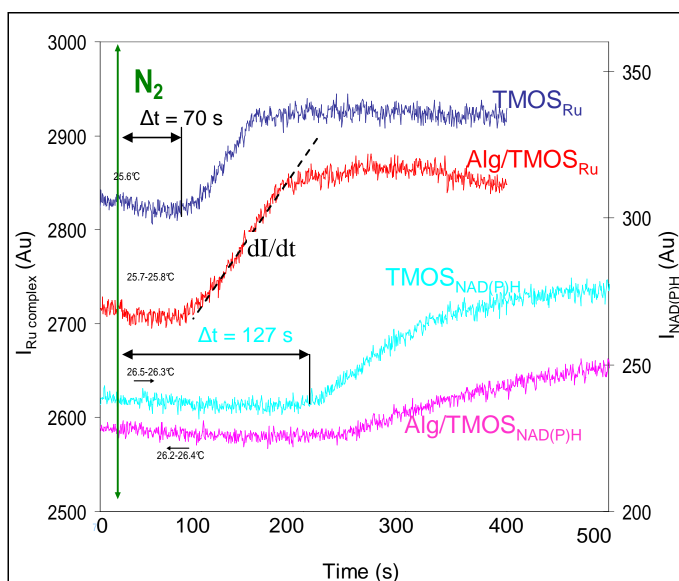

Fig. 3: Responses to Air-Nitrogen transition. ORMOCER® film with Ru complex coated with film (t=0.5 mm) with living *Sacharomyces cerevisae* DBM 60 (50g/l) immobilized in stationary growth phase.

Table 1. : Relative fluorescence intensities and time delays of ruthenium complex and intracellular NAD(P)H in silica and silica/alginate films.

AA transition	Film composition				Monitored fluorescence		
			living cells				
	TMOS	Alginate	Stat. phase	Exp. phase	ΔI (%)	Δt (s)	dI/dt
					ΔI_{Ru}	Δt_{Ru}	dI/dt_{Ru}
(a)	-	-	-	-	11	70	2.11
(b)	+	-	-	-	11.4	*76*	*1.58*
(b)	+	+	-	-	10.5	*80*	*1.19*
(c)	+	-	+	-	3.5	*70*	*1.7*
(c)	+	+	+	-	4.8	*70*	*1.84*
(d)	+	-	-	+	2	*74*	*0.8*
(d)	+	+	-	+	1.7	*80*	*0.7*
					$\Delta I_{NAD(P)H}$	$\Delta t_{NAD(P)H}$	$dI/dt_{NAD(P)H}$
(c)	+	-	+	-	14	*127*	*0.26*
(c)	+	+	+	-	8	*158*	*0.12*
(d)	+	-	-	+	11	*100*	*0.14*
(d)	+	+	-	+	12	*86*	*0.24*

Table 1. shows the influence of film composition on changes of fluorescence intensities of ruthenium complex and NAD(P)H during AA transition.

In empty cuvette, only with oxygen sensitive film **(a)**, 70 s after the switch of bubling in medium from air to nitrogen the intensity of fluorescence begin to rise. In the cuvette with a carrier film **(b)**, Δt_{Ru} increased to 76 s due to slowdown of oxygen transport through the silica film and Δt_{Ru} was 80 s in

silica/alginate film. These values indicated slower oxygen transport in silica/alginate carrier, which was confirmed also by lower first derivatives. Relative changes of fluorescence intensities, ΔI_{Ru} (corresponding to change of dissolved oxygen 0-100%) were within experimental errors, in empty cuvette **(a)** and in cuvette with carriers **(b)**.

ΔI_{Ru} was decreased by entrapment of living cells **(c, d)**, which is an evidence of constant oxygen depletion inside biofilm, especially in a place adjacent to window, regardless of flow in the cuvette with oxygen saturated medium.

Lower ΔI_{Ru} in **(d)** than in **(c)** were caused by higher oxygen consumptions of cells in exponential growth phase. This corresponded with expected higher viability of cells in exponential growth phase. ΔI_{Ru} and dI/dt_{Ru} were significantly more influenced by cells growth phase than by matrix composition.

As indicated ΔI_{Ru} of **(c, d)** that were lower as ΔI_{Ru} of **(b),** $\Delta I_{NAD(P)H}$ in a part of immobilized cells did not accord with the change of concentration of dissolved oxygen (DO) from 0 to 100 %. Solely cells close to film surface, in contact with circulating medium, were exposed to 100 % DO transition. Consequently $\Delta I_{NAD(P)H}$ values, showed in Table 1., are averaged $\Delta I_{NAD(P)H}$ through film thickness because inside the film, cells were exposed to various oxygen concentrations depending on distance from the film surface.

Nevertheless higher $dI/dt_{NAD(P)H}$ of cells in stationary growth phase in TMOS then TMOS/alginate indicated their different physiological state, which we used to ascribe to higher viability. In case of cells in growth phase, the effect of matrix was opposite (see $dI/dt_{NAD(P)H}$). Thus the results of NAD(P)H measurements impart clearly positive influence of alginate only on cells immobilized in growth phase.

Conclusions:

The application of optical measurement based on AA transition, in a determination of influences of an alginate addition into pre-polymerized TMOS revealed that alginate might decreased oxygen permeability. Biofilms containing 50 g of living cells per liter of gel, TMOS as well as alginate /TMOS, could not be completely aerated. Positive impact of alginate addition on viability was clear in biofilms with cells immobilized in exponential growth phase. Further measurements using more advanced instrumentation having temperature fluctuation ± 0.05 °C or better, and allowing measurements of films with cell concentrations two orders of magnitude lower are needed.

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