Bioluminescent bioreporters encapsulated in silica gel

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Introduction

The ability of bacterial bioluminescent bioreporters to sense their surroundings can be employed to measure the bioavailability and toxicity of pollutants. Despite many potential advantages, current bioreporters do not perform well enough to comply with environmental detection standards. In cloning of bacteria it is practically impossible to completely uncouple artificial signaling chains and reporter gene expression from global cellular control mechanism (van der Meer 2006).

A construction of analytical instruments for *in situ* real time monitoring needs biorecognition elements containing bioluminescent bioreporters encapsulated in a matrix that is strong enough to endure the rigour of the environment yet resilient enough to viably maintain the fragile cells. Various techniques were developed for maintenance both viability and activity of the sensor bacteria. They include freeze drying, vacuum drying continuous cultivation and immobilization into organic and inorganic polymers. Silica based polymers possess some of the most desirable properties for immobilization of bioluminescent bioreporters; biocompatibility, transparency, controlled porosity, and chemical, thermal and dimensional stability.

Classical sol-gel procedures involving extreme conditions were modified to retain activity and viability of immobilized biomaterial. Aqueous sol-gel routes have succeeded for a full preservation of the integrity of the cell membrane during the encapsulation process (Nassif 2003). However, after gelation and during the aging process, further alcohol release from non-hydrolyzed alkoxides, cell exposure to chemical groups situated at the porous surface (e.g., silanol groups), and/or physical constraint exerted on the cells by the characteristic shrinkage of the matrix result in drop of viability of encapsulated cells. NMR and fluorescence studies have shown that cell activity decreases in a few days after encapsulation (Ferrer 2003). Analysis of the viability of encapsulated cells combining fluorescence-based determination of their metabolic activity and in situ visualization of cells by cryo-SEM has provided evidence about how deleterious for membrane integrity is encapsulation by nonaqueous route (Ferrer 2006).

Bioluminescent bioreporter *Pseudomonas fluorescens* HK44 is harboring the pUTK21 plasmid, derived from NAH7 plasmid, which codes genes for naphthalene degradation pathway divided into two operons. Both operons are positively inducible by salicylate, therefore little of their activity is present constitutively and huge increase of activity is observed after induction. *Vibrio fischeri luxCDABE* gene cassette coding bioluminescence was inserted into *nahG* gene of the salicylate operone, thus gaining the inducibility by salicylate, naphthalene (Heitzer 1992) and other aromatic hydrocarbons (Trögl 2007).

In this study we present the influence of methanol evolving during encapsulation into prepolymerized tetramethoxysilane on bioluminescence bioreporter - *Pseudomonas fluorescens* HK44 - and the microscopic observations of these silica encapsulated cells.

Material and Methods
Effect of methanol on HK44 bioluminescence.
Pseudomonas fluorescens HK44 cells were cultivated in LB medium with tetracycline (50 mg/l) to stationary phase (OD~6), centrifuged (9000 rpm, 5°C, 5 min), resuspended in YEPS medium,

centrifuged again and diluted by YEPS to concentration 10^6 cells/ml. An aliquot of 330 μl was pipetted to each test tube and after incubation (60 min, 25°C) methanol was added to reach desired v/v concentration in the range from 0 to 5%. Bioluminescence of each test tube was measured (Berthold FB-12 luminometer) after additional incubation (60 min).

Pre-polymerization of tetramethoxysilan (TMOS)
TMOS, Fluka product No. 87682, was stirred with distilled water and HCl in molar ratio TMOS/H_2O/HCl = $1:5:10^{-2}$ to form a clear solution and left to pre-polymerize for 48 hours at 4°C.

Cell encapsulation
HK44 cells were cultivated in LB medium with TC (50 mg/l) to stationary phase (OD~6), centrifuged (9000 rpm, 5°C, 5 min), resuspended in YEPS medium, centrifuged again and diluted with LB. Pre-polymerized TMOS (0.1 ml) was mixed with 0.05 M NaOH (0.1 ml) and with HK44 cell suspension in LB medium (0.4 ml, concentration of 10^8 cells ml^{-1}). The mixture was poured into Petri dishes Ø 3.5 cm to form bio-silica film thickness of 1 mm (2 % Si in the matrix). Bioluminescence of HK44 layers was monitored using Berthold FB-12 luminometer.

Microscopic observations
Immobilized cells were observed by a) optical microscope, b) fluorescence microscope and c) scaning electron microscope (SEM).
   a) A piece of biogel layer (~0.5x0.5 cm) was washed in phosphate buffer (3x) and in water (1x) before observation in differential interference contrast (DIC) with a microscope Zeiss, LSM 510 META.
   b) Samples for SEM were prepared by smashing silica biogels on a silicon wafer. The samples were left to dry freely (two days) before microscopy observation (REM DSM 982 Gemini).
   c) Samples of biogel were stained by depositing LIVE/DEAD® BacLight™ (SYTO 9:Propidum J = 1:1). The labeled biogel was examined immediately using Olympus BX60 with a filter U-MWB2.

Results and Discussion

The preparation of biorecognition elements of whole cell biosensors led us to work out modifications of silica sol-gel encapsulation, which made the encapsulation into prepolymerized tetramethoxysilane less harmful. We have encapsulated cells in the stationary growth phase instead of exponentional phase. Further, encapsulation into water diluted silica prepolymer (2% Si) and addition of nutrient rich media improved viability of silica encapsulated cells. During the time between gelation and the first immersion of the film, the cells suffer from the presence of evolved methanol and gel shrinkage. To maintain the cells alive, the films have to be immersed in water solution within few minutes after gelation. These encapsulated cells repeatedly produced bioluminescence after induction with naphthalene or salicylate for months (Trögl 2005).

For all that we observed temporal increasing of non-induced bioluminescence of *Pseudomonas fluorescens* HK44 a few days after encapsulation (Table 1). With aim to elucidate a role of methanol evolving from reactions of methoxy groups which rested in biogel, we measured the background bioluminescence of free cells in presence of methanol (Fig 1). Increasing of non-induced bioluminescence, due to small amount of methanol (0.5-2.5%) can be ascribed to membrane perturbations (Heitzer 1998). This generally negative effect however supports bioluminescence. Bioluminescence needs cyclic oxidation of long-chain aldehydes to fatty acids by luciperhase enzyme (luxAB) and reverse reduction of fatty acids to aldehydes by reductase (luxCDE) (Meighen 1999). However there is limited amount of these substrates. Perturbations cause an increase of concentrations of free fatty acids and consequently an increase of

bioluminescence. However at higher methanol concentrations the negative effect of membrane destruction predominates (Heitzer et al. 1998).

	Time from gellation (hours)	Bio-luminescence (A.U.)
Free cells	-0.5	$2*10^3$
biogel **A**	0.1	$3*10$
A	18	$1*10^5$
A	24	$2*10^5$
AW	24	$3*10^4$
A	48	$1*10^6$
AW	48	$2*10^3$
A	72	$3*10^5$
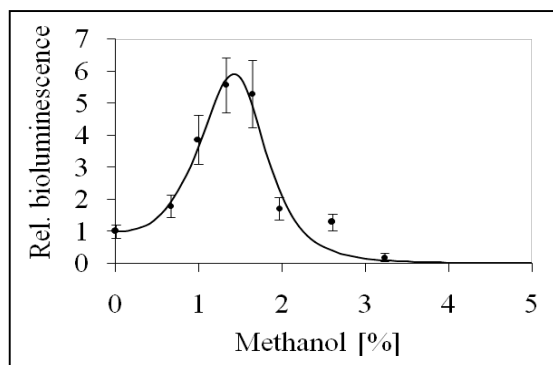
Table 1: Non-induced bioluminescence of *P. fuorescens* HK44. Biogel (silica matrix 2% Si, 10^8 cells/g) after first immersion (A) was stored between bioluminescence measurements (25°C) in phosphate buffer at 10°C. (AW) is the biogel (A) washed twice with phosphate buffer 24 hours after gelation.

Fig 1: Non-induced bioluminescence of free cells HK44 in presence of methanol.

Microscopy needs sample pretreatment such as drying, staining and metallization. This might result in destruction of cells and formation of cavities in heterogeneous biocomposite of cells inside inorganic matrix. High electron reflection from metal coated surface might create false shapes of observed biocomposite. We found out that freely dried biocomposite smashed on silicon wafer is better way of specimen preparation as compared to classical fixation with formaldehyde and drying in series of ethanol solutions. On SEM snaps the dead cells could be seen as traces and distinguished from cells that had been living in time of preparing of a SEM specimen (Fig 2 a, b). In wet biocomposite live and destroyed cells were resolvable by optical microscopy using differential interface contrast (DIC) on the surface and also inside (Fig 3). Fluorescence microscopy of specimens stained with LIVE/DEAD®, Fig 4, revealed substantially higher content of living cells dispersed in volume of soft matrix (2% Si) in contrast to more tough matrix (5% Si). In both matrices more cells were observed on edges. Large cracks in the hard matrix were evolved during specimen preparation.
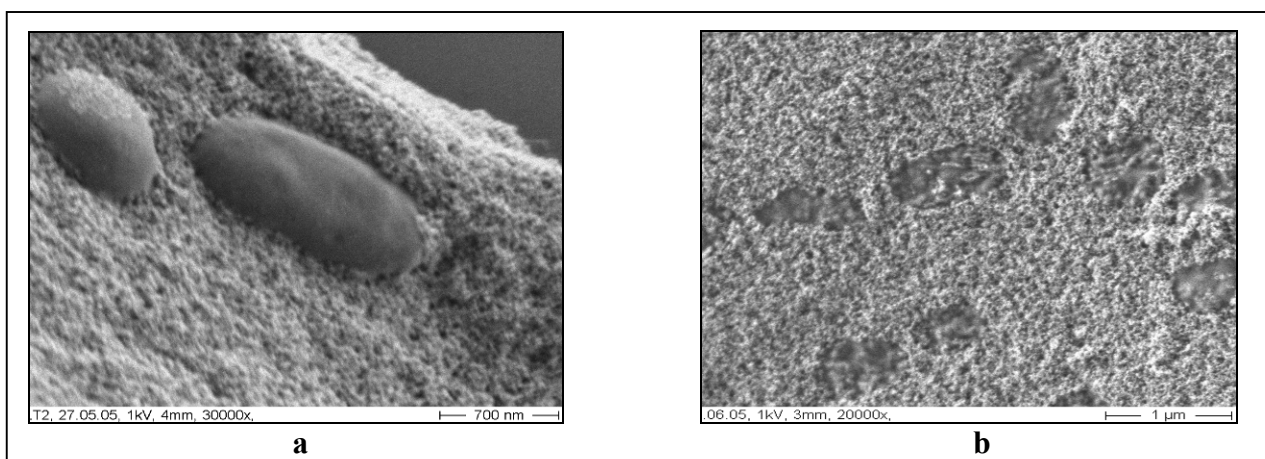

Fig 2: SEM of cells HK44 in silica gel;
a) living cells (one month after encapsulation) in the matrix with 2% Si and 10^8 cells/g
b) dead cells (19 months after encapsulation) in the matrix with with 3% Si and 10^7 cells/g.
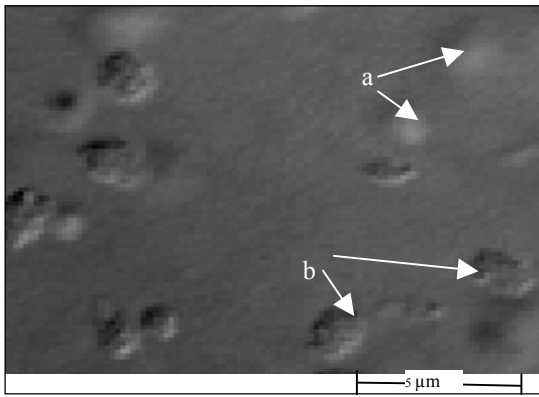
Fig 3: Optical microscopy (DIC) of cells HK44 in wet silica gel (19 months after encapsulation in the matrix with 3% Si and 10^7 cells/g; a) living cells covered with a matrix, b) destroyed cells.
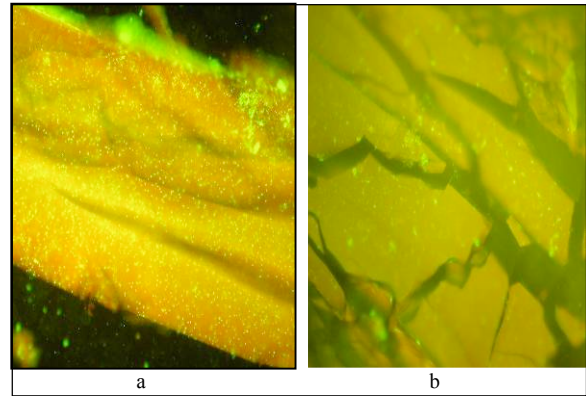

Fig 4: Fluorescence microscopy of silica gels with HK44 (stored in phosphate buffer for one month) stained with BacLight^{TM}; a) silica matrix 2% Si, 10^8 cells/g, b) silica matrix 5% Si, 10^8 cells/g.

Conclusions

In this work we demonstrated an influence of methanol on non-induced bioluminescence of cells *Pseudomonas fluorescence* HK44 encapsulated in pre-poplymerized tertramethoxysilane. In silica biogel, live and dead cell can be distinguished without or with minimum biogel pretreatment by SEM, optical microscopy with differential interfacial contrast and after staining with BacLight^{TM} using the fluorescence microscope.

References

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