O5-2	**Encapsulation of Living Cells in Polymer Microcapsules**	

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INTRODUCTION AND OBJECTIVES

Enzyme evolution is a powerful tool for improving enzyme properties such as temperature stability. Because modern techniques allow the generation of large mutant libraries consisting of up to billions of variants, the success of an optimization process depends largely on the efficiency of the high-throughput screening method used. To screen those large populations very rapidly Fluorescent Activated Cell Sorting (FACS) is the method of choice (Yang and Whiters, 2009). This method however poses several challenges, because the cells are in most cases not connected with the fluorescent substrates occurring during the enzyme assays. To achieve a link between genotype and phenotype, encapsulation of fluorescent substrates is very important. Here we report a method to encapsulate living cells, based on Layer-by-Layer (LbL) assembly of poly(allylamine)-hydrochloride (PAH) and polystyrenesulfonate (PSS) on *E.coli* loaded pre-precipitated calcium carbonate cores. We demonstrated that the generated polymer microcapsules can be used as biocompatible microreactors. Furthermore observations by Confocal Laser Scanning Microscopy (CLSM) proved that entrapped cells remained viable and are able to produce enzymes inside the polymer microcapsules. The polymer microcapsules filled with fluorescent substrates were sorted by FACS analysis properly and fast.

MATERIALS AND METHODS

Materials

Poly(allylamine)hydrochloride (PAH, average M_w ~15.000 Da) and polystyrenesulfonate (PSS, average M_w ~70.000 Da) were obtained from Sigma. All other chemicals used were of analytical grade. Pure water (ELGA Labwater) with a specific resistance of 18.2M Ω/cm was used for all preparations.

Preparation of Calcium Carbonate Microcapsules

Porous $CaCO_3$ capsules were prepared by mixing an equal volume of 0.33 M $CaCl_2$ and 0.33 M Na_2CO_3 under vigorous stirring for 30 s, followed by precipitation of the $CaCO_3$ microcapsules for 30 min. The capsules were washed three times with 0.05 M NaCl, pH 6.5 before LbL-assembly. For encapsulation of GFP expressing living *E.coli* cells, 100 µl of overnight culture (OD_{260} = 1 ~ 10^9 cells/ml) were added to a sterile reaction tube and centrifuged for 3 min at *10000* g. The cells were washed three times with TBS-buffer, finally resuspended in 10 ml of 0.33 M $CaCl_2$ solution and incubated under gently stirring for 15 min. 10 ml of 0.33 M Na_2CO_3 solution were added under vigorous stirring for 30 s, followed by precipitation of the $CaCO_3$ microcapsules for 30 min.

Preparation of the Polyelectrolyte Multilayer Microcapsules by LbL-Technique

Sterile solutions of PAH and PSS were prepared (5 mg/ml in 0.05 M NaCl, pH 6.5). The $CaCO_3$ capsules were dispersed in the PAH solution first. After absorption of the polyelectrolyte for 20 min under vigorous shaking, the microcapsules were collected by centrifugation for 5 min at *7500* g. The particles were washed three times with 0.05 M NaCl, pH 6.5 to remove excess PAH. The microcapsules were then dispersed in the PSS solution, incubated for 20 min under vigorous shaking followed by centrifugation and washing as described. The LbL procedure was repeated until the desired number of layers were applied.

Cultivation of Encapsulated Cells

Encapsulated cells with eight alternating layers of PAH/PSS were collected by centrifugation at *5000* g for 10 min and washed three times with 0.05 M NaCl, pH 6.5 followed by resuspension in 50 ml autoinduction-media in an 200 ml Erlenmeyer flask. Incubation at 37 °C for 15 h with shaking.

Zeta-Potential Measurements

The zeta-potential was measured of microcapsules after each LbL step with a zeta sizer 3000 (Malvern).

FACS-Analysis

FACS-analysis and sorting was obtained with a MoFlo Cytometer (BD Bioscience). Compartments were selected based on fluorescence activity.

Characterization of Multilayer Microcapsules by Confocal Laser Scanning Microscopy (CLSM) and Fluorescence Microscopy

CLSM images of *E.coli* loaded microcapsules were recorded using a AxioCam CCD camera connected to a LSM 510 inverted microscope (Carl Zeiss Jena) and imaging software LSM Image Browser. A drop of the microcapsule suspension was added to a cover glass and analyzed using an oil immersion objective lens (Plan-Achromat 63x/1.4 Oil DIC, Wavelength 488nm). For fluorescence microscopy images a colour digital camera (Sony) connected to a Zeiss Axiostar microscope (Filterset 44 with HBO 50/AC excitation source) was used. For optical microscopy a BA310 microscope (Motic Instruments) with an integrated digital camera and the imaging software Motic Images Plus 2.0 was used.

RESULTS AND DISCUSSION

Preparation of Polymer Microcapsules and Cell Viability: Blank and *E.coli* loaded LbL coated microcapsules were successfully prepared with a homogenous size distribution from 2 – 5 µm (for blank capsules) to 3 – 6 µm (for *E.coli* loaded capsules). The assembly of polyelectrolyte multilayers on the calcium carbonate core was monitored by measuring the zeta potential as shown in Figure 1. Uncoated calcium carbonate cores displayed a negatively charged surface, whereas after application of the first layer of PAH a positive zeta potential was observed. The alternating zeta potential confirms the assembly of polyelectrolyte multilayers on the calcium carbonate template.

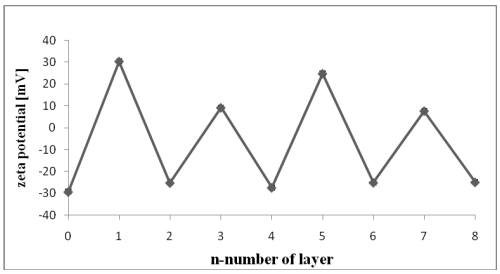

Figure 1 : Zeta potential of PAH/PSS coated calcium carbonate microcapsules

The viability of GFP producing *E.coli* cells after encapsulation was studied by cultivating the cell loaded microcapsules in an autoinduction-media overnight at 37 °C. As shown in Figure 2 a fluorescence signal inside the microcapsules could be detected indicating that the LbL pro-cess did not have an effect on the viability of encapsulated cells and protein production inside polymer microcapsules is possible.

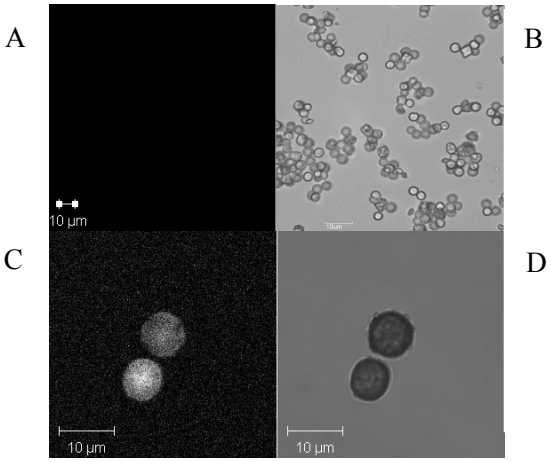

Figure 2: Microscopic images of *E.coli* loaded PAH/PSS coated calcium carbonate microcapsules. (A) Fluorescence image before expression of GFP, (B) Light microscopic image before expression of GFP, (C) CLSM fluorescence image after expression of GFP, (D) CLSM phase contrast image after expression of GFP.

FACS sorting of FITC-PAH/PSS Coated Polymer Microcapsules: For determining the ability to sort polyelectrolyte microcapsules using FACS analysis by fluorescence activity a 1 : 1 mixture of blank and FITC-PAH/PSS coated microcapsules were assayed. As shown in Figure 3, sorting of fluorescent and non-fluorescent polymer microcapsules was achieved. After the first sorting procedure over 99% of the fluorescent capsules were removed from the non-fluorescent fraction and only 20% of non-fluorescent capsules remained in the fluorescent fraction.

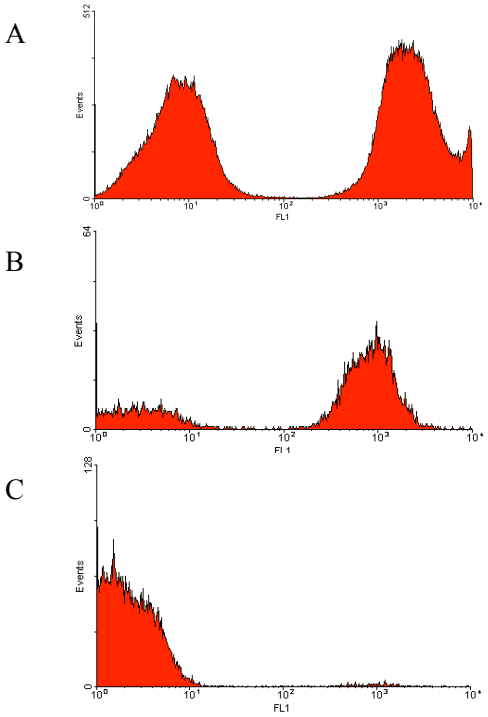

Figure 3: Fluorescence activity against capsules counted. (A) Mixture (1 : 1) of blank and FITC-PAH labled polymer microcapsules, (B) Fluorescent capsules after sorting, (C) Non-fluorescent capsules after sorting

CONCLUSION

Viable *E. coli* can be encapsulated in biocompatible PAH/PSS polymer microcapsules by LbL-Technology and produce active enzymes inside. Generated capsules had a narrow size distribution of 2 – 5 µm (blank capsules) to 3 – 6 µm (for *E.coli* loaded capsules). During cultivation, no release of encapsulated cells could be observed by CLSM. Our experiment clearly demonstrated that the polymer microcapsules filled with fluorescent substrates can be sorted by FACS analysis properly and fast. After one sorting procedure over 99% of the fluorescent capsules were removed from the non-fluorescent fraction and only 20% of non-fluorescent capsules remained in the fluorescent fraction. Currently we are working on optimizing the system for the directed evolution of biocatalysts for the transformation of renewables into fine chemicals.

REFERENCES

- Yang G.Y. et al. (2009) *Ultrahigh-Throughput FACS-Based Screening for Directed Enzyme Evolution.* Chembiochem 10(17) 2704-2715