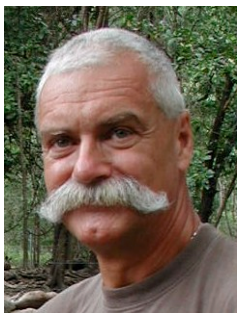
Layer by layer adsorption of Trypsin onto Polyelectrolyte -microcapsules (PEMC)



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Introduction

Recently, micro- and nano- sized polyelectrolyte capsules have been fabricated applying the layer-by-layer (L-b-L) adsorption technique (Decher 1997) on charged colloidal particles with subsequent decomposition and removal of the core (Neu et al 2001, Voigt et al. 1999, Donath et al. 1998). The size of the capsules can be varied from 0.1 to tens of microns and it is defined by the size of the template. The thickness of the capsule wall depends on the number of assembled polyelectrolyte layers and it can be adjusted in the nanometer range (Bäumler et al. 2005). A variety of colloidal particles, melamine resin latexes (MRL) Donath et al. 1998, Voigt et al. 1999, biological cells (Neu et al. 2001, Georgieva et al. 2004, Bäumler et al. 2005), organic (Antipov et al. 2001) and inorganic crystals (Caruso e al. 1999) have already been used as templates for the capsule preparation.
The properties of these polyelectrolyte microcapsules (PEMC) can be designed and are very different depending on the fabrication technique and the polyelectrolytes used. The membranes of the PEMC can change their permeability as a function of salt concentration or pH of the solution (Georgieva et al. 2004, Bäumler et al. 2005).
Recent experiments deal with enhancement of biocompatibility and thus with the wider use of these PEMC as injectable microcontainers for drug delivery or to serve as artificial RBC when loaded with hemoglobin (Bäumler et al. 2005). Since the oxygen delivery in RBCs is not dependent on the hemoglobin alone another aim is to immobilize enzymes within the PEMC membrane. These enzymes are needed for reduction, oxidation and pH stabilization within the PEMC. When immobilized by the L-b-L technique enzymes are observed to lose activity or change their pH dependent properties (Caruso et al. 2000). This work concerns the immobilization of trypsin and their enzymatic activity in the membrane of PEMC, when the PEMC consisting of five layers are coated with additional layers of trypsin and an other polyelectrolyte. Trypsin serves as a model enzyme for these experiments as it is easy to obtain and straightforward to monitor.

Material and methods

RBC stabilized with glutaraldehyde served as templates for the PEMC. The stepwise adsorption of oppositely charged polymers was performed using a filtration technique[3]. The polyelectrolyte (PE) assembly was performed either with poly(styrensulfonate, sodium salt) (PSS), (M_W 70000, Aldrich) or alginate (M_W 176, Aldrich) as negative charged polyelectrolyte followed by poly(allylamine hydrochloride) (PAH), (M_W 70000, Aldrich) as positive polyelectrolyte. For the preparation of the microcapsules, RBC with five layers $(PSS/PAH)_2PSS$ were suspended in a solution of 140 mM NaCl and 1.2% NaOCl. Within 20 min of incubation at 20^0C the cellular template was dissolved obtaining hollow polyelectrolyte capsules (Bäumler et al. 1988).
The additional coating procedures on the PEMC were performed for both PE couples trypsin/PSS and trypsin/alginate (trypsine from bovine pancreas M_W 24000, Sigma). The first layer of trypsin was applied adding trypsin solution in TRIS buffer (10mg/ml, pH 7.4) to the pellet. After an incubation time of fifteen minutes at 4°C the suspension was centrifuged (1500 x G 5min) and the supernatant removed. The PEMC were washed three times with distilled water to remove excess enzymes. To adsorb the additional polyelectrolyte layer the PEMC were resuspended in the PE solution with a concentration of 1mg/ml. After fifteen minutes of incubation time at 4°C the PEMC

were centrifuged and washed with water three times at 4°C. This complete process was repeated until the required amount of layers was built. To all used buffers calcium chloride was added in a concentration of 0.02M to protect the enzyme from autolyses.
Light and fluorescent microscopy of PEMC were performed using a confocal laser scanning microscope (CLSM) (LSM 510, Carl Zeiss GmbH, Jena, Germany).
The concentration of PEMC was determined using a Neubauer chamber.
The fluorescent intensity of single PEMC coated with different numbers of FITC-trypsin layers was determined by flowcytometry (FACS-Canto and the FACS-DIVA software, Beckton Dickinson, USA). For each probe 25,000-30,000 cells were measured to calculate the average amount of fluorescent intensity.
The protein concentration of water-solved proteins was determined according to Lowry et al. in modification by Onishi and Barr (Lowry 1951, Onishi et al. 1978) .Measurements were performed in quartz cuvettes (Binninger Analytik, Germany) using a spectral photometer (Hitachi K2800, Tokyo, Japan). After cell count the protein amount per PEMC could be calculated.
To determine tryptic activity the chromogene trypsin substrate Na-Benzoyl-D,L-arginine 4-nytroanelyde hydrchloride (DL-BAPA; M_W=434.9Da) was used. From substrate trypsin cuts N-acetyl-Ele-Glu-Pro-Asp-p-Nitroanelyde (pNa;M_W=36.46Da) and the absorption maximum can be determined photometrically.

Results and discussion

FACS measurements were performed to show whether PE layers were just replacing each other or building up. PEMC of five layers were coated with FITC trypsin and PSS or alginate, respectively. The fluorescent intensity of the PEMC was shown in a histogram for each layer. These histograms showed 3-4 peaks each. As described elsewhere (Bäumler et al. 2005), additional peaks of higher intensity are interpreted as doubles or triplets of the PEMC population under observation (fig 1).

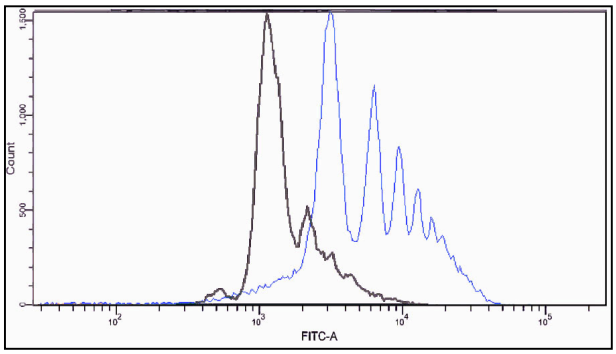

Fig. 1: Histrograms of PEMC, coated alternatively with FITC trypsin and alginate. Black line: PEMC with one layer of FITCtrypsin. Blue line: PEMC with three layers of FITC trypsin.

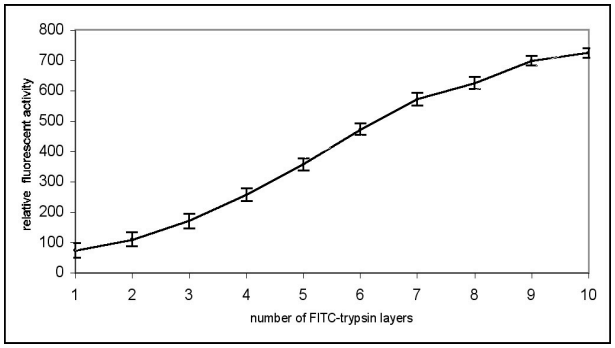

Fig 2: Relative fluorescent intensity of PEMC coated with FITC trypsin and PSS. Layer numbers indicate number of FITC trypsin layers. Mean values with standard deviation
n = 24,000-30,000.

With an increasing amount of FITC trypsin layers the fluorescent intensity constantly grows. The sigmoidal growth of the fluorescent intensity for the couple trypsin and PSS (fig. 2) can be explained with effective charge multiplication. Observations from the first three layers show the difference in charge to be less than for the following layers, which is also shown and discussed in other experiments (Caruso et al. 2000, Schlenoff 2001, Schüler et al. 2001). Decher et al. reported findings when measuring the thickness of the membrane with relatively low charged polyelectrolytes as used in this case (Decher 1997, Peyratout 2004), this effect was called the effective multi-

plication of surface charge. This means that the polyelectrolytes bind onto the PEMC membrane charges but present more than the found charges on their own in centrifugal direction on the PEMC membrane. Thus for the next layer more charges are present The flattening of the curve at high fluorescent intensities is most likely a result of the covering of lower fluorescent layers.

Since FACS measurements give out semiquantitative results, photometric experiments were performed to determine the amount of immobilized trypsin. A steadily increasing amount of trypsin per PEMC was seen with rising layer number. In comparison with one another it is noticeable that the overall slope in the trypsin/PSS series is higher (1.92pg/PEMC) than in the trypsin/alginate series (1.03pg/PEMC) (fig. 3)

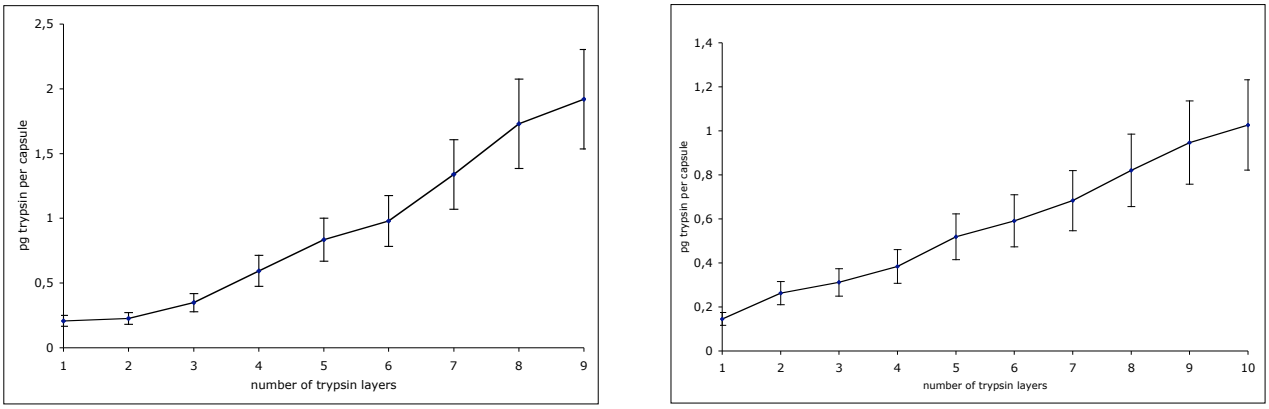

Fig. 3: Immobilized trypsin/PEMC [pg]. Coated with trypsin/PSS (left) or trypsin/alginate (right). Shown are the protein concentrations determined according to Lowry in relation to the cell count.

The higher amount of immobilized trypsin in the trypsin/PSS series is probably due to complex formation of trypsin and PSS.
Native PEMC were coated with five layers of trypsin/PSS or trypsin/alginate as described before. Activity and cell count were determined. With an increasing amount of trypsin layers the activity was seen to constantly grow. The increase in activity per PEMC is bigger for the trypsin/PSS series {0.744 (ΔE/s) / (PEMC/µl)} than for the trypsin/alginate series {0.51 (ΔE/s) / (PEMC/µl)}.
To find out whether the activity of the immobilized enzyme was different depending on the polyelectrolyte used, regardless how much enzyme was immobilized per PEMC, the activity was correlated to the enzyme concentration of coated PEMC suspensions. Within the observed range significant differences in activity when comparing the coating series trypsin/PSS and trypsin/alginate could not be found.

Conclusions

Results showed a layer buildup for both polyelectrolytes and no replacement of trypsin was found. The amount of immobilized enzyme was bigger for the coating series with trypsin/PSS compared to trypsin/alginat. This is the result of complex formation between PSS and trypsin, which leads to a higher trypsin load of the PEMC. Normalizing the enzym activity to the amount of adsorbed trypsin no significant differences between the activity of PSS-PEMC and alginate-PEMC were found.

Acknowledgements

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