Z. Phys. Chem. **226** (2012) 749–759 / **DOI** 10.1524/zpch.2012.0271

Temperature Controlled Activity of Lipase B from *Candida Antarctica* after Immobilization within p-NIPAM Microgel Particles

By Kornelia Gawlitza[1,*], Changzhu Wu[2], Radostina Georgieva[3,4], Marion Ansorge-Schumacher[2], and Regine von Klitzing[1]

[1] Technische Universität Berlin, Stranski-Laboratory for Physical and Theoretical Chemistry, Institute of Chemistry, 10623 Berlin, Germany
[2] Technische Universität Berlin, Department of Enzyme Technology, Institute of Chemistry, 10623 Berlin, Germany
[3] Charité–Universitätsmedizin Berlin, Institute of Transfusion Medicine, Center for Tumor Medicine, 10117 Berlin, Germany
[4] Trakia University Stara Zagora, Medical Faculty, Department of Medical Physics, Biophysics and Radiology, 6000 Stara Zagora, Bulgaria

Dedicated to Matthias Ballauff on the occasion of his 60th birthday

(Received May 11, 2012; accepted in revised form July 9, 2012)
(Published online August 13, 2012)

Lipase B / p-NIPAM / Immobilization / Catalytic Activity / Thermoresponsibility

The immobilization of lipase B from *Candida antarctica* (CalB) within micronsized poly-*N*-Isopropylacrylamide (p-NIPAM) microgel particles with a crosslinker content of 5% is reported. The immobilization of the enzyme was reached by an exchange from polar to organic solvents. After determining the embedded amount of CalB within the polymer network, an enhanced specific activity in *n*-hexane was obtained. Due to the thermoresponsibility of the polymer particles, the activity reaction was done at 25 °C and 50 °C. The results presented show that the reversible collapse of the microgel leads to a decreased activity with increasing temperature. Hence, p-NIPAM microgels display a good opportunity to tailor the activity of CalB. An interesting side effect is that CalB presents a suitable probe to estimate the mesh size of the polymer network, since it penetrates in the unlabeled form but not after labeling with FITC.

1. Introduction

Hydrogels made of p-NIPAM are one of the most studied water swellable microgel systems which have an internal gel-like structure. They are synthesized by surfactant free emulsion polymerization forming so called “smart” hydrogels which are able to react reversible to external stimuli like temperature [1–4], pH [5–7] and ionic

* Corresponding author. E-mail: kornelia.gawlitza@tu-berlin.de

strength [8,9] depending on their chemical composition. Due to linear segments of the monomer *N*-Isopropylacrylamide (NIPAM) which shows a lower critical solution temperature (LCST) the produced polymer network undergoes a volume phase transition at around 32 °C [10]. This reversible process makes them useful for applications like drug delivery [11,12] or as biosensors [13] and enzyme supports [14, 15].

In the last decades, enzymes have gained a huge interest in biocatalysis. Due to the high regio-, stereo- and chemoselectivity [16,17], they are important in the synthesis of pharmaceuticals. It is well known that enzymes are not very stable against changes in the environment like high temperatures, different pH values or organic solvents. Hence, many methods were developed to improve enzyme stability, among them immobilization. One approach of immobilization is the embedding into a polymer matrix. Due to the low contact to the carrier, the residual mobility and flexibility of the biocatalyst is still high [18].

In literature, enzymes have been immobilized into silica [19], reversed micelles [20] and polysaccharides [21]. Polymer matrices made of p-NIPAM have been also widely studied for the immobilization of enzymes. The first adsorption of proteins onto these microgel particles was studied by Kawaguchi *et al.* in the early 1990s [22,23]. A further study demonstrates that β-D-glucosidase can be immobilized within core-shell particles where the core consists of polystyrene and the shell of p-NIPAM [24]. Although an enhanced activity is reached, the small size of the polymer particles does not allow to detect the localization of the enzyme molecules. The described studies and activity measurements were done in water. Due to the fact that substrates and products are often soluble in organic solvents while enzymes are soluble in water, there is a huge interest in designing a system where all components are in their suitable environment. In a previous study, the enzyme CalB was immobilized within agarose microgel particles by exchanging a polar solvent (water) against an organic one (isopropanol or *n*-hexane) [25]. To benefit from the low polydispersity and the high surface-to-volume ratio, CalB was immobilized within p-NIPAM microgel particles with a crosslinker content of 0.25% [26]. The possibility to disperse these microgels in organic solvents and to reach a supply of the substrate to the enzyme leads to an enhanced activity in organic solvents for these two studies. Due to the thermoresponsive behavior of p-NIPAM microgels, the supply of the substrate and therefore the activity of the immobilized system can be controlled by changing the temperature.

In the present paper, the enzyme CalB was immobilized within large p-NIPAM microgel particles with 5% crosslinker. Therefore, the solvent was changed from water to isopropanol or *n*-hexane. After immobilization the location of CalB in the polymer network was investigated by confocal laser scanning microscopy (CLSM). The activity of the immobilized enzyme was determined below and above the volume phase transition temperature (VPTT) using gas chromatography. The results show that the supply of the substrate can be controlled by changing the temperature due to the reversible collapse of the p-NIPAM particles. This leads to a decreased specific activity with increasing temperature. As side effect, it is demonstrated that CalB is a good probe to give an estimation of the largest mesh size of the polymer network.

2. Experimental

2.1 Materials

N,*N*′-methylenebis(acrylamide) (MBA) (≥99.5%) and potassium peroxodisulfate (KPS) (≥99%) were purchased from Fluka (Munich, Germany). *N*-Isopropylacrylamide (97%) (NIPAM), octanoic acid (≥99%), 1-octanol (≥99%), *bovine serum* albumin standard (BSA, 2 mg/mL) and Bradford reagent were from Sigma-Aldrich (Munich, Germany) and Fluorescein-5-isothiocyanat (FITC) was from Merck (Darmstadt, Germany). The enzyme lipase B from *Candida antarctica* (CalB) was generously donated by Novozymes A/S (Bagsvaerd, Denmark) and dialyzed for the labeling procedure. NIPAM was purified by recrystallization in *n*-hexane. Other chemicals were used as received. Water was taken from a three-stage Millipore Milli-Q Plus 185 purification system.

2.2 Preparation techniques

2.2.1 Synthesis of micronsized p-NIPAM microgel particles

Micronsized p-NIPAM microgel particles with a MBA content of 5% as crosslinker were synthesized by surfactant free emulsion polymerization using a temperature ramp according to Meng *et al.* [27]. The larger size of the particles is necessary to localize them after immobilization by CLSM. The synthesis was done dissolving 2.26 g (0.02 mol) of the monomer NIPAM and 0.15 g (0.001 mol) of MBA in 200 mL water. Afterwards, the solution was degassed for 1 h at 45 °C using N_2 while stirring at 450 rpm. To initiate the polymerization a solution of 1 mL KPS (0.08 M) in water was added to the mixture. The temperature was increased stepwise to 65 °C at a rate of 1 °C per 2 min. To finish the polymerization the solution was stirred overnight at 65 °C under N_2 atmosphere. The received microgel particles were purified by filtering over glass wool and dialysis for 2 weeks with daily water exchange. Finally, the polymer solution was freeze dried at −85 °C under 1×10^{-3} bar for 48 h.

2.2.2 Immobilization of CalB within p-NIPAM microgel particles

For the immobilization process, several samples of 5 mg p-NIPAM microgel particles were dissolved in 1.4 mL buffer (0.1 M potassium phosphate buffer, pH 7) and mixed with 0.1 mL of CalB ($c = 4.32$ mg/mL) at room temperature. These solutions were stirred overnight and centrifuged for 10 min at 9000 g. The received residues were redispersed in 1.5 mL isopropanol and washed three times by centrifugation and redispersion. After the last centrifugation step, one part of the samples was dissolved in buffer to determine the immobilized amount using Bradford assay. To investigate the catalytic activity of the immobilized system the other part of the samples was transferred into the water immiscible solvent, *n*-hexane.

To determine the location of the immobilized CalB within the polymer particles, the enzyme was labeled with FITC according to the literature [28] and freeze-dried afterwards. Two samples consisting of 5 mg p-NIPAM microgel particles, 1.1 mg FITC-CalB and 1 mL buffer (0.1 M potassium phosphate buffer, pH 7) were prepared. After stirring overnight, the samples were centrifuged for 10 min at 9000 g. The residue of

one sample was redispersed in buffer while isopropanol was added to the other sample followed by one further washing step.

2.3 Characterization methods

2.3.1 Light scattering

To characterize the size of the synthesized polymer particles, Dynamic Light Scattering (DLS) was used. Correlation functions were measured at a constant scattering angle of 45° using an ALV goniometer setup with a Nd:YAG laser as the light source ($\lambda = 532$ nm). An ALV-5000/E multiple-τ digital correlator was used to generate the correlation functions. The analysis was done by inverse Laplace transformation (CONTIN [29]). The molecular weight of the p-NIPAM microgel particles was determined by means of Static Light Scattering (SLS). Therefore, concentrations of the polymer ranging from 1×10^{-6} g/g to 9×10^{-6} g/g were measured at scattering angles from 15° to 35° with 2° steps in between using an ALV/CGS-3 compact goniometer system equipped with an ALV/LSE-5004 correlator. The temperature was controlled to be 25 °C using a Huber Compatible Control thermostat. A He-Ne laser ($\lambda = 632.8$ nm) was used as the light source.

2.3.2 Confocal laser scanning microscopy

After immobilization the location of labeled CalB within the polymer particles was investigated by CLSM. Therefore, the samples were redispersed either in buffer or in isopropanol. To prepare the measurement, roughly 20 μL of the immobilized sample was placed between two cover slips. Using a Zeiss LSM 510Meta confocal scanning unit (Zeiss MicroImaging GmbH, Jena, Germany) in combination with an inverted microscope Axiovert 200 M equipped with a 100 × oil immersion objective (numerical aperture 1.3) the samples were investigated. The 488 nm line of the argon laser for excitation and a 505 nm long-pass emission filter was used to prepare the fluorescence images. The analysis of the obtained images was done with the LSM 510 software.

2.3.3 Determination of the immobilized amount of CalB

To achieve the immobilized amount of CalB, the total protein concentration of the prepared samples was determined using Bradford reagent. The procedure was done according to the manufacturer's instruction and assuming that CalB was the only protein present in the commercial preparation (supporting information). All required UV-VIS spectra were measured with the PerkinElmer Lambda 25 UV-VIS-spectrometer.

2.3.4 Determination of the catalytic activity

The catalytic activity of the immobilized system was determined using an esterification reaction of 1-octanol and octanoic acid in *n*-hexane leading to octyloctanoate as product. For the reaction, 50 μL (216 μg) droplets of the aqueous solution of unimmobilized CalB or 5 mg of p-NIPAM particles loaded with ≈ 14.5 μg CalB were mixed with 1 mL of the substrate solution. After shaking these dispersions for 15 min at 25 °C, the upper part of the solution of CalB or the supernatant of immobilized CalB was investigated

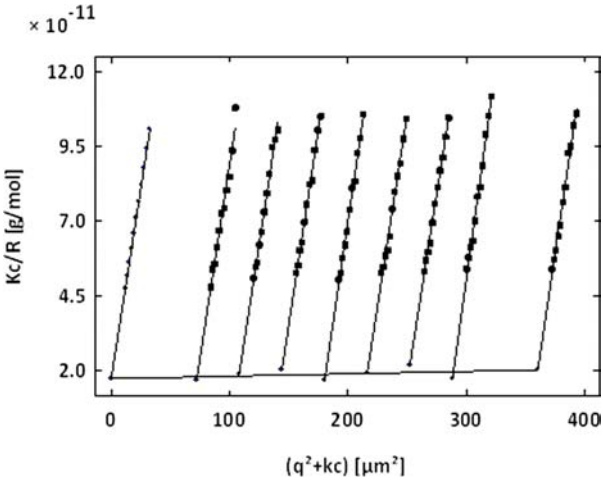

Fig. 1. Zimm plot of large p-NIPAM microgel particles with 5% MBA.

with a gas chromatograph. Every 5 min 150 µL of solution were withdrawn and analyzed for ester concentration *via* gas chromatography (Shimadzu 2010; BPX5 column from SGE: length 25 m, ID 0.22 mm; film thickness: 0.25 m; detector: FID at 300 °C; injector: 275.0 °C, injection volume of 1 µL, split model; temperature program: start temperature 80.0 °C, hold for 0.5 min, temperature rise 20 °C/min from 80 °C to 170 °C and 5 °C/min rise from 170 °C to end temperature 200 °C) [25]. The peak area of the product at a typical retention time of 10 min is used to calculate the concentration. The described studies for the catalytic activity were performed in triplicate.

3. Results and discussion

In order to synthesize micronsized p-NIPAM microgel particles with a crosslinker content of 5% MBA, surfactant free emulsion polymerization applying a temperature ramp was used. To characterize the received polymer particles, the molecular weight was determined by SLS. Applying extrapolations to the received Zimm-plot (Fig. 1) a molecular weight M_W of 5.87×10^{10} g/mol, a second virial coefficient A_2 of 1.6×10^{-10} mol dm^3/g^2 and a radius of gyration R_g of 670 nm were achieved. A residual water amount of 10% [30] and a refractive index increment dn/dc of 0.167 cm^{-3}/g [31] were used for calculation.

Furthermore, the size and the swelling behavior of the received polymer particles were investigated by DLS. Figure 2a shows the swelling curves for the p-NIPAM microgel particles dissolved in water and isopropanol. The measured correlation functions were evaluated by cumulant analysis. A correlation function for the microgel particles at 26 °C dissolved in water is shown in Fig. 2b. The cumulant analysis of this example leads to a hydrodynamic radius (R_H) of 727 nm and a polydispersity index (PDI) of 0.134 which indicates the low polydispersity of the particles. The microgel particles show a VPTT of around 31 °C which is in good agreement with the results for p-NIPAM

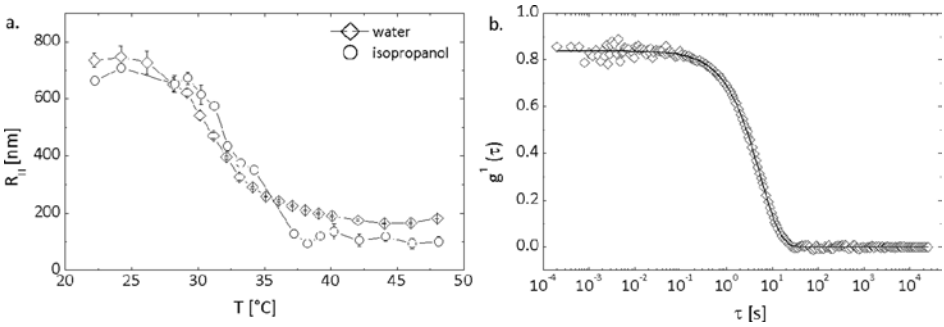

Fig. 2. Swelling curves for p-NIPAM with 5% MBA dissolved in water and isopropanol (a.) and a correlation function with cumulant fit for p-NIPAM dissolved in water (b.).

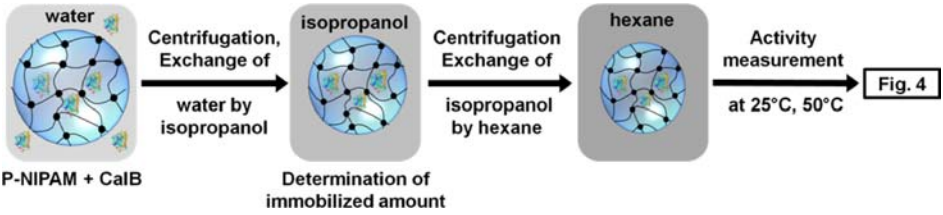

Fig. 3. Schematic process of the immobilization procedure at 25 °C. The gradient of the crosslinker in the polymer structure is simplified in the sketch for the sake of clarity.

in literature [10]. The increase in temperature to 45 °C leads to a decrease in R_H of 560 nm. Since later a solvent transfer to isopropanol will be described, the swelling behavior was also studied in isopropanol. The cumulant analysis give a R_H of around 680 nm at 26 °C and of R_H of 560 nm at 45 °C. The VPTT for this system is roughly at 33 °C. Obviously, these p-NIPAM microgel particles show the same deswelling ratio in isopropanol and water. The similar VPTT in both environments reflects that isopropanol is also a good solvent for large microgels with a crosslinker content of 5%. However, the synthesized p-NIPAM microgel particles show a suitable size for investigations of the enzyme distribution after immobilization using CLSM.

To get information on the process of embedding, it is necessary to determine the immobilized amount of CalB. Therefore, the unimmobilized enzyme is mixed with the p-NIPAM particles in buffer. Afterwards, the solvent exchange leads to an immobilization of the enzyme molecules. A sketch for the immobilization process and the following steps is shown in Fig. 3. To determine the embedded amount of enzyme, the sample is investigated using Bradford assay (see supplementary information). The received amount of CalB is 3 μg/mg p-NIPAM. Using the determined molecular weight of 5.87×10^{10} g/mol for p-NIPAM and 33273 Da [32] for CalB, an immobilized amount of 5400 CalB molecules per p-NIPAM microgel particle is determined. This value is in good agreement with the embedded amount found for p-NIPAM with 0.25% MBA [26]. This leads to the conclusion that the crosslinker content does not influence the loading efficiency.

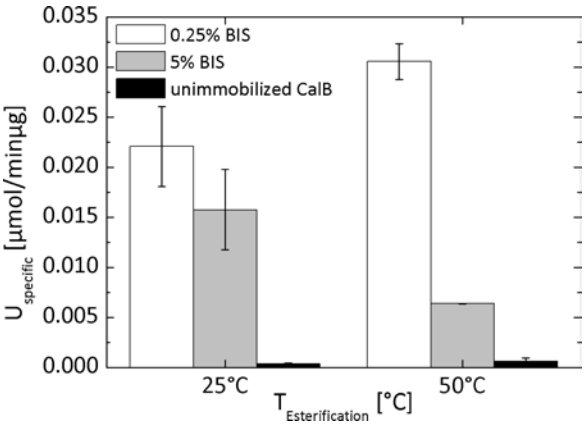

Fig. 4. Specific activity in *n*-hexane of unimmobilized and immobilized CalB investigated at 25 °C and 50 °C after immobilization within p-NIPAM with 0.25% and 5% MBA.

For the application in biocatalysis, it is important to reach a high activity after immobilization. For measurements of the catalytic activity in presence of CalB and *n*-hexane, 1-octanol and octanoic acid form octyloctanoate as product which can be determined *via* gas chromatography. For comparison the same reaction was performed with unimmobilized CalB. Due to the fact that CalB is dissolved in a buffer solution, it is still surrounded by hydration water even after transfer to *n*-hexane. In order to get information about the substrate penetration into the microgel below and above the VPTT, the activity was determined at 25 °C and 50 °C. Figure 4 shows the specific activity at both investigated temperatures of the unimmobilized enzyme and CalB after immobilization within p-NIPAM particles with 0.25% and 5% MBA. The data for the system with 0.25% MBA were taken from a previous study [26]. Figure 4 presents that an enhanced activity can be reached for both p-NIPAM systems compared to unimmobilized CalB. In general, the immobilized CalB is more homogeneous distributed in *n*-hexane reaching an enhanced activity. The unimmobilized enzyme is dissolved in buffer which leads to a phase separation and therefore to a macroscopic interphase after adding *n*-hexane to the reaction. The immobilized system creates a larger total internal interface at which the esterification reaction normally takes place. Additionally, the immobilized system possesses a higher density of CalB molecules in the polymer particles compared to unimmobilized CalB in solution. If the substrate diffuses into the microgel particles the collision frequency between enzyme and substrate increases and more product is generated. The usage of p-NIPAM with 5% MBA leads to a slight decrease in activity at 25 °C compared to p-NIPAM with 0.25% MBA. Due to the higher density of the polymer network with increasing crosslinker content, the diffusion of the substrate into the polymer particles is decreased. Beside, the substrate is not able to diffuse easily through the denser network which can lead to a decreased collision frequency. Nevertheless, the system shows still a much higher specific activity than unimmobilized CalB. Furthermore, the esterification reaction was done below and above the VPTT to investigate the influence of the collapse of the microgel particles on the supply of the substrate. As shown in the previous study, the collapse of p-NIPAM with 0.25% MBA has no signifi-

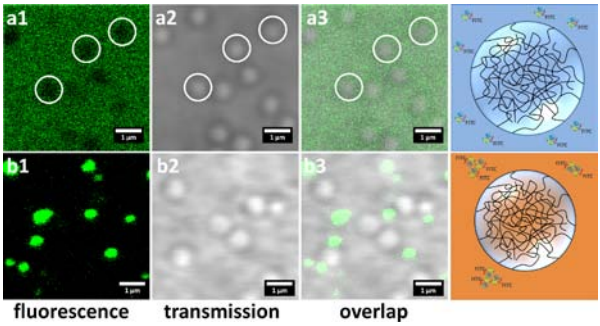

Fig. 5. CLSM-images of the residue after incubation with CalB redispersed in buffer (a) and isopropanol (b) in fluorescence mode, transmission mode and as a super imposed image of both.

cant influence on the activity. Using a higher crosslinked p-NIPAM system (5% MBA) the specific activity decreases clearly by increasing the reaction temperature from 25 °C to 50 °C (Fig. 4). The shrinkage of the microgel particles by increasing temperature leads to much smaller meshes. Therefore, one reason for the decreased activity could be that the supply of the substrate is partly disabled and the formation of octyloctanoate is decreased. Additionally, the collapsed polymer network leads to a reduced flexibility of the substrate and the enzyme within the microgel particles which could also lead to a change of the conformation of the active center leading to a disadvantage for substrate binding.

In order to observe the location of lipase B, the enzyme was labeled with FITC before the immobilization procedure. After mixing the components, the samples were centrifuged and redispersed in buffer on one hand and in isopropanol on the other hand. In a previous study it has been proven by CLSM that the enzyme is located within the p-NIPAM microgel particles with 0.25% MBA after immobilization. The explanation for this immobilization can be given by the high affinity to aqueous environment. Dissolving the sample in buffer leads to a diffusion of CalB through the microgel particles. Due to the partial hydrophobicity of the polymer matrix and the aqueous environment, the enzyme has no motivation to stay within the microgel network. By exchanging the solvent against isopropanol a residual amount of water remains inside of the polymer particles. Hence, a kind of an “aqueous cage” is presented in the microgel network. Due to the lower solubility of CalB in isopropanol compared to water, the enzyme molecules stay within the microgel particles. Additionally, the exchange of the solvent leads to a decrease in radius which was shown by DLS (Fig. 2a). Therefore, a more dense structure of the p-NIPAM microgel particles can be found in isopropanol and the enzyme is kept within this structure. Figure 5 shows the images made by CLSM for p-NIPAM with 5% MBA in buffer (a) and isopropanol (b).

Image a1 and b1 give the fluorescence of the sample and image a2 and b2 display the transmission. An overlap of these two images is reflected in image a3 and b3 and proves whether the fluorescence areas match with the postition of the polymer particles. In buffer (upper series), obviously the fluorescent enzyme is distributed over the whole scan range with the exception of some spherical shaped areas (white circles). An overlap of the transmission and fluorescence image (a3) proves that these dark areas

fit to the position of the microgel particles. This leads to the conclusion that there is no immobilization of CalB in buffer which is in good agreement with the results of p-NIPAM with 0.25% MBA. The lower series of Fig. 5 presents the CLSM images after redispersion in isopropanol. Against one's expectation, the microgel particles show no fluorescence. Obviously, some fluorescent aggregates are formed in the solution which are totally independent from the position of microgel particles. The mass of these aggregates seems to be so high that the immobilized system is not purified from them after centrifugation during the washing step with isopropanol. The images show that CalB is not immobilized within p-NIPAM microgel particles with 5% MBA after labeling with FITC. Due to the fact that CalB is not soluble in isopropanol, aggregates of enzyme molecules are formed after redispersion in isopropanol. Unlabeled CalB shows an enhanced activity in the immobilized system indicating an embedding of the enzyme within the polymer network. The adsorption only at the surface of the microgel particles can be excluded because the enzyme should also be adsorbed at the surface in the case, where it cannot penetrate the microgel. As it can be seen in Fig. 5 there is no enhanced fluorescence on the particles' surface. Hence, the labeled enzyme that is not able to penetrate into the particles is not adsorbed on their surface. These results confirm former observations [26]. To summarize, without FITC-labeling CalB is small enough to enter into the swollen microgel particles. After labeling the FITC-CalB seems to be too large for penetrating the polymer network. Since CalB does not show any strong interaction with p-NIPAM itself [26] this result allows an estimation of the mesh size of the polymer network. The size of CalB is supposed to be $3\,\text{nm} \times 4\,\text{nm} \times 5\,\text{nm}$ [32]. As determined by UV-VIS spectroscopy the average number of bounded FITC molecules per CalB molecule is 1.5. Due to the fact that no labeled enzyme is immobilized within the polymer particles, even CalB with one FITC molecule attached is not able to diffuse inside of the p-NIPAM network. The largest molecular axis of FITC is 0.7 nm. That means the largest axis of FITC-CalB can be up to $(5 + 1 \times 0.7)\,\text{nm} = 5.7\,\text{nm}$. From literature it is known that p-NIPAM microgels consist of an inhomogeneous network with larger meshes in the outer part which are probed with the described technique [33]. That means that the largest mesh sizes in the outer part of the swollen gel (5% MBA) are between 3 nm (shortest axis of CalB) and $\approx 6\,\text{nm}$ (longest axis of FITC-CalB). According to the temperature sensitive catalytic activity the shrunken gel (5% MBA) has to have a largest mesh size below 1 nm (longest axis of the substrate) or the mesh sizes are at least close to this value and lead to a reduced mobility of the substrate. In contrast to this result the largest mesh size of the swollen gel with 0.25% MBA is well above $\approx 6\,\text{nm}$ and the shrunken gel has a largest mesh size below $\approx 6\,\text{nm}$ but above 1 nm.

4. Conclusions

The aim of the presented work was to investigate the influence of the crosslinker content of p-NIPAM microgel particles after immobilization of CalB by solvent exchange. Therefore, the immobilization of CalB within p-NIPAM microgel particles with 5% MBA was compared to the system with 0.25% MBA [26]. The immobilized amount of the enzyme is independant of the MBA content and was determined to be around 5400 CalB molecules per p-NIPAM microgel particle. The increase of the network density

with increasing crosslinker content leads to a slight decrease in activity at 25 °C. By increasing the reaction temperature, the specific activity decreases clearly. p-NIPAM microgel particles collapse at temperatures above the VPTT leading to much smaller meshes. Therefore, the supply of the substrate is partly suppressed. The dependance of the activity on the temperature is a great property to reach systems with temperature controlled activity. Going to higher crosslinker contents could lead to a system where a change in temperature leads to either an active or a nonactive biocatalyst. Additonally, CalB can be used as a probe for the largest mesh sizes of such polymer particles. This is a great finding due to the fact that the mesh sizes of such microgel particles cannot be determined by other methods like small angle neutron scattering (SANS). Using this method only the network fluctuations of the polymer can be investigated which is not a realistic parameter for the mesh size.

Acknowledgement

The authors acknowledge Helmuth Möhwald and Shuo Bai for helpful discussions and collaboration regarding the solvent exchange. The authors thank Nora Konnertz for determination of the FITC concentration after labeling. This work was supported by the DFG *via* the Cluster of Excellence "Unifying Concepts in Catalysis". The authors also want to acknowledge Novozymes A/S for donating CalB.

References

1. H. Senff and W. Richtering, J. Chem. Phys. **111** (1999) 1705.
2. K. Kratz, T. Hellweg, and W. Eimer, Polymer **42** (2001) 6631.
3. I. Berndt and W. Richtering Macromolecules **36** (2003) 8780.
4. M. Stieger, J. S. Pedersen, P. Lindner, and W. Richtering, Langmuir **20** (2004) 7283.
5. A. Fernández-Nieves, A. Fernández-Barbero, B. Vincent, and F. de las Nieves, Macromolecules **33** (2000) 2114.
6. K. Kratz, T. Hellweg, and W. Eimer, Colloids Surf. A **170** (2000) 137.
7. T. Hoare and R. Pelton, Macromolecules **37** (2004) 2544.
8. M. Shibayama, F. Ikkai, S. Inamoto, S. Nomura, and C. C. Han, J. Chem. Phys. **105** (1996) 4358.
9. M. Karg, I. Pastoriza-Santos, B. Rodriguez-González, R. von Klitzing, S. Wellert, and T. Hellweg, Langmuir **24** (2008) 6300.
10. K. Kratz, A. Lapp, W. Eimer, and T. Hellweg, Colloids Surf. A **197** (2002) 55.
11. G.-H. Hsiuea, S. Hui Hsub, C.-C. Yangc, S.-H. Leed, and I.-K. Yang, Biomaterials **23** (2002) 457.
12. G. M. Eichenbaum, P. F. Kiser, A. V. Dobrynin, S. A. Simon, and D. Needham, Macromolecules **32** (1999) 4867.
13. J. R. Retama, M. S.-P. López, J. H. Pérez, G. F. Cabanillas, E. López-Cabarcos, and B. López-Ruiz, Biosens. Bioelectron. **20** (2005) 2268.
14. T. G. Park and A. S. Hoffman, J. Biomed. Mater. Res. **24** (1990) 21.
15. S. Nayak and L. A. Lyon, Angew. Chem. **117** (2005) 7862.
16. R. N. Patel, Biomol. Eng. **17** (2001) 167.
17. R. N. Patel, Adv. Synth. Catal. **343** (2001) 527.
18. M. B. Ansorge-Schumacher, in: *Handbook of Heterogeneous Catalysis Vol. 1*, S.-W. Ertl, H. Knötzinger (Eds.), Wiley VCH, Weinheim (2008).
19. J. Lei, J. Fan, C. Yu, L. Zhang, S. Jiang, B. Tu, and D. Zhao, Micropor. Mesopor. Mater. **73** (2004) 121.

20. H. Chen, L.-H. Liu, L.-S. Wang, C.-B. Ching, H.-W. Yu, and Y.-Y. Yang, Adv. Funct. Mater. **18** (2008) 95.
21. P. Grunwald, K. Hansen, and W. Gunßer, Solid State Ion. **101** (1997) 863.
22. H. Kawaguchi, K. Fujimoto, and Y. Mizuhara, Colloid Polym. Sci. **270** (1992) 53.
23. K. Fujimoto, Y. Mizuhara, N. Tamura, and H. Kawaguchi, J. Intell. Mater. Syst. Struct. **4** (1993) 184.
24. N. Welsch, A. Wittemann, and M. Ballauff, J. Phys. Chem. B **113** (2009) 16039.
25. S. Bai, C. Wu, K. Gawlitza, R. von Klitzing, M. B. Ansorge-Schumacher, and D. Wang, Langmuir **26** (2010) 12980.
26. K. Gawlitza, C. Wu, R. Georgieva, D. Wang, M. B. Ansorge-Schumacher, and R. von Klitzing, Phys. Chem. Chem. Phys. **14** (2012) 9594.
27. Z. Meng, M. H. Smith, and L. A. Lyon, Colloid Polym. Sci. **287** (2009) 277.
28. R. D. Nargessi and D. S. Smith, Methods Enzymol. **122** (1986) 67.
29. S. W. Provencher, Comput. Phys. Commun. **27** (1982) 213.
30. K. Kratz, Ph.D. thesis, University of Bielefeld (1999).
31. J. Gao and Z. Hu, Langmuir **18** (2002) 1360.
32. J. Uppenberg, M. T. Hansen, S. Patkar, and T. A. Jones, Structure **2** (1994) 293.
33. M. Stieger, W. Richtering, J. S. Pedersen, and P. Lindner, J. Chem. Phys. **120** (2004) 6197.