Modulation of Transaminase Activity by Encapsulation in Temperature-Sensitive Poly(*N*-acryloyl glycinamide) Hydrogels

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Smart hydrogels hold much potential for biocatalysis, not only for the immobilization of enzymes, but also for the control of enzyme activity. We investigated upper critical solution temperature-type poly *N*-acryloyl glycinamide (pNAGA) hydrogels as a smart matrix for the amine transaminase from *Bacillus megaterium* (*Bm*TA). Physical entrapment of *Bm*TA in pNAGA hydrogels results in high immobilization efficiency (>89%) and high activity (97%). The temperature-sensitiveness of pNAGA is preserved upon immobilization of *Bm*TA and shows a gradual deswelling upon temperature reduction. While enzyme activity is mainly controlled by temperature, deactivation tended to be higher for immobilized *Bm*TA (≈62–68%) than for free *Bm*TA (≈44%), suggesting a deactivating effect due to deswelling of the pNAGA gel. Although the deactivation in response to hydrogel deswelling is not yet suitable for controlling enzyme activity sufficiently, it is nevertheless a good starting point for further optimization.

Introduction

Enzymatic cascades are a valuable alternative to traditional step-wise chemical synthetic processes, since substrate specificity, stereo- and regioselectivity of enzymes are typically outstanding.[1] Especially for products with challenging stereo conformation, they can help to avoid expensive and time-consuming isolation of by-products and intermediates that is typically necessary when applying classical chemical synthesis.[2] Unfortunately, the development of efficient enzyme cascades remains a challenge: The more enzymes are combined, the more crucial it is to avoid cross-reactivity and to adapt unpaired enzyme activity to achieve high product yields and high purities.[3]

Bacillus megaterium transaminase (*Bm*TA), for example, is an enzyme which can be applied in multi-enzyme catalyzed processes.[4] *Bm*TA is very potent, e.g. for the synthesis of valuable amino alcohols, but also prone to side reactivity as it inherently shows substrate promiscuity. There are a number of technical solutions to solve the issue e.g. the sequential addition and removal of catalyst after each reaction step,[3d] or the spatio-temporal separation of the reaction steps.[5] Still, if enzyme activity could be adjusted dynamically and remotely by on/off-switching or up/down regulation of the catalyst's activity,[6] running the cascade in one pot might be possible while preventing unspecific substrate uptake leading to by-product formation as well as intermediate accumulation due to unpaired enzyme activities at the same time.

Enzymes with "switchable" activities have been known for a long time, e.g. changes in enzyme activity upon irradiation of enzymes functionalized with photo-isomerizable groups were already reported in the 1970s.[7] A more universal approach is the immobilization of enzymes in stimulus-sensitive hydrogels and/or microgels,[8] as it does not rely on extensive enzyme engineering. This approach promises the regulation of virtually every enzyme, combined with the advantages of immobilizing enzymes i.e. increased long-term stability and improved reusability.[9] Literature on lower critical solution temperature (LCST)-type hydrogels showed that a change in swelling degree leads to a change in enzyme activity.[8] Typically a reduction in enzyme activity was reported correlated with shrinking of the gels.[8a–h] Yet, the change in activity of the immobilized enzymes varied immensely depending on the enzyme-material combination used.[3e]

An alternative could be the immobilization of enzymes in upper critical solution (UCST)-type hydrogels. UCST materials are especially interesting, as they show increasing swelling degrees with higher temperatures, where the inherent enzyme

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activity, according to the Arrhenius plot,[6b] is also higher. Meaning that in contrast to LCST-type materials, here, the effect of temperature on enzyme activity (decreasing activity upon decrease of temperature[6b,10]) is expected to go hand in hand with the effect of hydrogel shrinking (decreased swelling of hydrogel upon decrease of temperature; decreased enzyme activity upon decrease of hydrogel swelling).[3e]

In contrast to the broad range of literature available on the use of LCST polymers, fewer studies can be found dealing with polymers exhibiting an UCST in water and aqueous electrolyte solutions.[11] Commonly used UCST materials are copolymers, interpenetrating polymer networks or polymer blends of poly (acrylamide) (pAAm) and poly(acrylic acid) (pAAc).[12] Also the non-ionic polymer poly(*N*-acryloyl glycinamide) (pNAGA) was reported to exhibit strong UCST properties in aqueous solutions[11a,13] and the ability to form high strength hydrogels.[13e,g,14] pNAGA was investigated for a broad range of applications, e.g. drug delivery,[14a,15] tissue engineering,[14c,d,16] photodynamic therapy,[17] capture of proteins,[18] self-healing material[14b,19] or catalysis.[20] Still, reports on enzyme immobilization in pNAGA,[21] or also other polymers exhibiting an UCST-type phase transition,[22] are still very limited. UCST phase transition was so far applied making use of soluble-insoluble transitions above/below the UCST for easier recycling of the biocatalyst,[22a–c] or enzyme activity control due to the precipitation.[22e] Studies on changes in enzyme activity due to hydrogel deswelling were to the best of our knowledge not conducted so far.

Solutions of 1 %wt pNAGA showed a phase transition at temperatures around 22–23 °C,[23] fitting perfectly to the temperature range in which many enzymes, in case of this publication *Bm*TA,[4,24] can be applied. The temperature-sensitiveness of pNAGA hydrogels was additionally reported to be preserved in buffered solutions.[13g] With pNAGA and *Bm*TA being well characterized by themselves, the combination of pNAGA with *Bm*TA is a very useful test system to investigate 1) whether pNAGA is a suitable material for *Bm*TA immobilization in general (see Figures 1a), and 2) whether temperature-related changes in swelling degree of UCST-type hydrogels affect the activity of the immobilized enzyme (see Figure 1b). This could in future be useful for modulating enzyme activity by varying temperatures on demand. If the activity could be almost completely suppressed in the shrunken state, then this would solve the problem of the substrate promiscuity of *Bm*TA.

Christiane Claaßen received her M.Sc. degree in Chemistry from the Niederrhein University of Applied Sciences (2014) and completed her Ph.D. at the University of Stuttgart (2018) focusing on hydrogels for controlled release of therapeutic proteins. She has been a Postdoctoral Fellow in the group of Prof. Dr. Dörte Rother at the Forschungszentrum Jülich since 2018. Her interdisciplinary research focusses on modulation of enzyme activity by entrapment in stimulus-sensitive hydrogels. Additionally she implements nuclear magnetic resonance spectroscopy for online analytics of biocatalytic reactions, which can be used as a basis for self-regulating enzyme cascade reactions.

Results and Discussion

The experiments in this publication were performed with poly (*N*-acryloyl glycinamide) (pNAGA) hydrogels. Uniform gels (cylindrical shape; 1 mm height and 3 mm diameter directly after cross-linking) were obtained by photo initiated free radical polymerization and physical *in situ* gel formation without chemical cross-linking (see Figure 1a). The physical cross-linking in the pNAGA gels is mainly based on hydrogen bonding and is therefore reversible upon drastic changes in the environmental conditions.[13g] In preliminary experiments, the hydrogels showed no visible change in shape and good stability at 35 °C for at least two weeks in both water and buffer solution (see Supporting Information Figure S11). In Addition, reversible temperature-dependent swelling behavior was observed in the investigated range of 20–35 °C, with an equilibration time of about 90 min when the temperature was increased/lowered 5 °C (see Supporting Information Figure S12). The temperature-dependent swelling behavior, however, showed only a gradual change upon increasing/decreasing the temperature (see Supporting Information Figure S12), in contrast to many LCST-type materials that were reported to collapse at a certain temperature.[8a,b] In the next step, these pNAGA hydrogels were used for immobilization of the transaminase *Bm*TA.

Loading of *Bm*TA to pNAGA hydrogels

Immobilization efficiency

pNAGA hydrogels were loaded with *Bm*TA either by adding the enzyme to the monomer solution before cross-linking (encapsulation, abbreviated with encaps-X) or by leaving the cross-linked, washed and fully swollen hydrogels in enzyme solution for 24 h at 35 °C to allow the protein to diffuse into the gel (diffusion, abbreviated with diff-X). The X represents the amount of *Bm*TA applied for loading e.g. 10 μg protein per mg gel dry weight loaded by encapsulation in case of encaps-10. Immobilization by encapsulation and by diffusion were compared in terms of immobilization efficiency and retained enzyme activity.

The immobilization efficiency was determined by direct measurement of the amount of *Bm*TA within the hydrogels. If the supernatant had been measured, the data only represent the protein part that is not bound, but would not specify whether the protein is truly bound in the hydrogel or e.g. absorbed to surfaces. For protein determination, the hydrogels were in a first step dissolved by adding urea and heating to 95 °C, taking advantage of the physical and thus reversible network formation within the hydrogels. Protein concentrations were measured in a second step by Bradford assay with an adapted calibration for dissolved pNAGA hydrogels. Protein

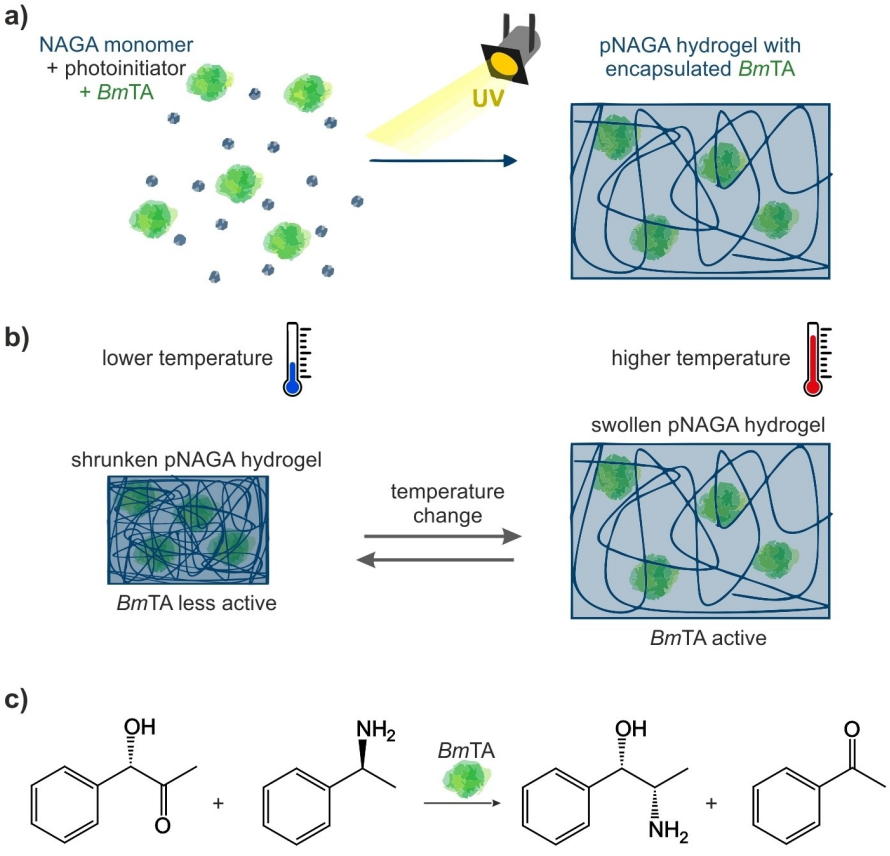

Figure 1. a) Encapsulation of *Bacillus megaterium* transaminase (*Bm*TA) in poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels. b) Expected effect of hydrogel swelling onto immobilized *Bm*TA (according to Refs. [8a–h]). c) Formation of (1*S*,2*S*)-3-(2-amino-1-hydroxypropyl)phenol from 1-hydroxy-1-(3-hydroxyphenyl) propan-2-one by *Bm*TA.[4]

determination by bicinchoninic acid (BCA) was not possible, as the amide bond in the NAGA monomer led to high background absorbances.

Figure 2 shows the immobilization efficiency of *Bm*TA in pNAGA hydrogels that were loaded by encapsulation (encaps-X) or diffusion (diff-X). Directly after immobilization, the gels loaded by encapsulation showed high immobilization efficiencies $>89\,\%$. After performing the reaction to determine enzyme activity, the protein amount in the encapsulation gels decreased to $73\,\% \pm 8\,\%$. Similar leaching of *Bm*TA occurred in control experiments where no substrate was added as well. In contrast to the gels loaded by encapsulation, the gels loaded by diffusion showed very low protein amounts corresponding to approx. 4% immobilization. However, these values have to be considered with caution as they were below the quantification limit of the applied Bradford protein assay.

From the data, it is evident that protein immobilization *via* the encapsulation approach was much more effective compared to the diffusion approach in terms of protein loading. Nevertheless, protein loss occurred during washing and reaction, most likely due to the physical encapsulation of *Bm*TA. This means that *Bm*TA was able to diffuse out of the hydrogel meshes. The very low protein contents in the diffusion gels

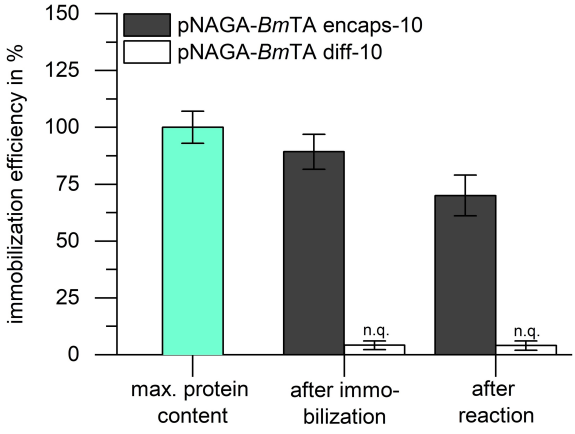

Figure 2. Immobilization efficiency (%) of *Bm*TA in poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels that were loaded by encapsulation (encaps-X) or diffusion (diff-X). The X represents the amount of *Bm*TA applied for loading which was 10 μg protein per mg gel dry weight. Immobilization was determined using an adapted Bradford assay for dissolved hydrogels ($n=3$).

were a little bit surprising as *Bm*TA was able to diffuse out of the encapsulation gels during washing (24 h, 30 °C) and reaction (20.5 h, 35 °C), but not into the diffusion gels during

loading (24 h, 35 °C), although similar reaction conditions were used. This leads to the hypothesis that the mesh size of the gels and the hydrodynamic diameter of *Bm*TA cannot be the only factor responsible for the low loading efficiency in the diffusion gels, but that other factors, such as the protein repellency of the pNAGA gels, must also be considered. Indeed, first studies have shown that pNAGA is useful as an antifouling coating with very low protein absorption, supporting the hypothesis that pNAGA hydrogels are protein repellent.[25]

Activity of immobilizates

The specific activity of immobilized *Bm*TA was first determined using a standard initial rate enzyme activity assay (Figure 3a; monitoring of product formation for 45 min). The data indicated a substantially reduced specific initial rate activity for *Bm*TA immobilized in pNAGA gels compared to free *Bm*TA. While the encapsulation gels at least showed some retained activity (3–5%), no product formation and therefore no activity was measured for gels loaded *via* the diffusion approach (pNAGA-*Bm*TA diff-10) as already expected from the low immobilization efficiency.

However, when looking at product formation in a time-course conversion experiment results were different (Figure 3b). Here, product formation between 1 h and 20.5 h reaction time was determined to be 17.8 μmol min^{-1} mg^{-1} (pNAGA-*Bm*TA encaps-10)-28.4 μmol min^{-1} mg^{-1} (pNAGA-*Bm*TA encaps-20) corresponding to product formations of 56.9–90.7% compared to free *Bm*TA. Also here, pNAGA-*Bm*TA diff-10 gels showed no measurable substrate conversion. Considering the results from the time-course conversion experiments it is most likely that initial rate activity was so low because of diffusion limitations within the pNAGA gels, meaning that the short time period in which initial rate activity is measured was most likely not suitable to characterize the activity of *Bm*TA immobilized in pNAGA.

In general, a number of factors i.e. enzyme deactivation by formation of free radicals during cross-linking,[26] an unfavorable chemical environment within the gels[26b] or diffusion limitations[9c,26b,27] can play a role explaining a activity loss upon immobilization. As the preserved activity in the time-course conversion experiments was high, strong effects due to enzyme deactivation by free radicals can be excluded. Because of the low protein immobilization and the low activity, gels loaded *via* diffusion were not investigated in further experiments. The preserved activity of *Bm*TA immobilized by encapsulation in the time-course conversion experiments was suitably high to be used in further experiments.

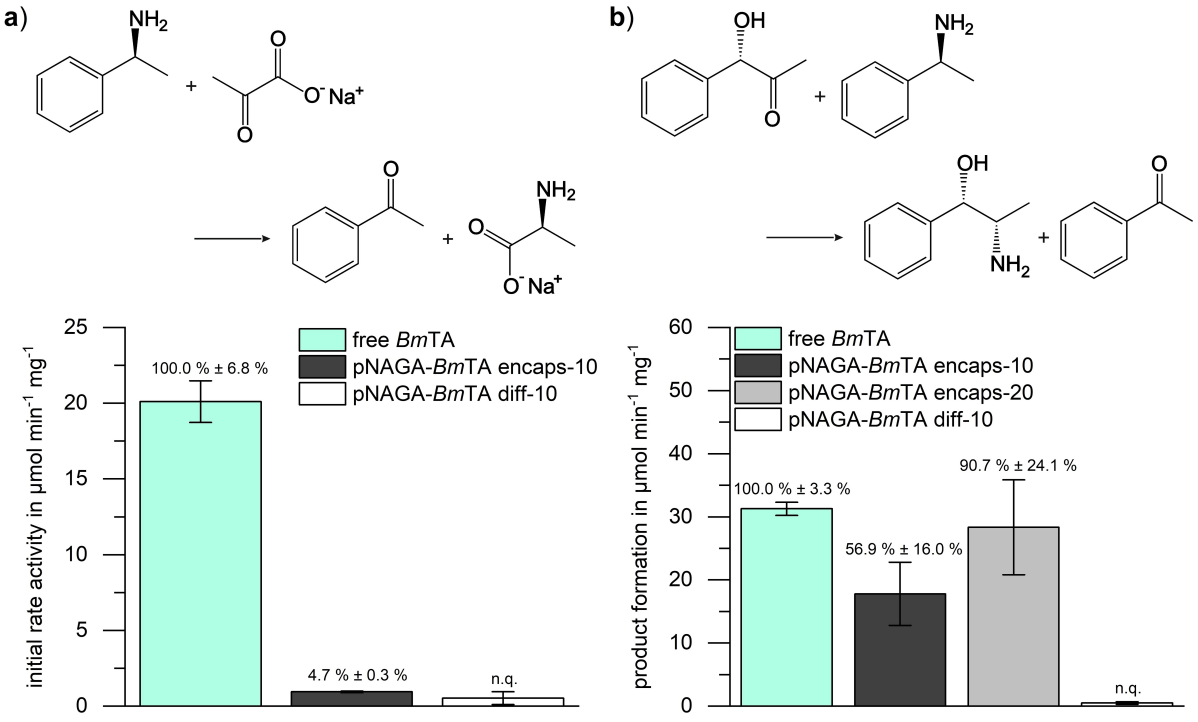

Figure 3. Activity (μmol min^{-1} mg^{-1}) of free *Bm*TA and *Bm*TA immobilized in poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels that were loaded by encapsulation (encaps-X) or diffusion (diff-X). The X represents the amount of *Bm*TA applied for loading which was 10 μg protein per mg gel dry weight. a) Initial rate activity was determined at 35 °C in HEPES buffer over the course of 45 min using a model reaction converting α-methylbenzylamine to acetophenone ($n = 3$). b) Product formation determined within a time-course conversion experiment at 35 °C in HEPES buffer with 1-hydroxy-1-(3-hydroxyphenyl)propan-2-one as substrate and (1*S*,2*S*)-3-(2-amino-1-hydroxypropyl)phenol as product (product formation from 1 h to 20.5 h reaction time) ($n = 3$).

Stability and reusability of immobilizates

Although the immobilization procedure is often associated with decreased enzyme activity compared to free enzyme, immobilization can increase long-term stability and favor reusability.[9] To investigate whether this holds true for the immobilization of *Bm*TA in pNAGA hydrogels, the stability of the immobilizates was first tested with regard to protein retention and retained activity. For this purpose, the hydrogels loaded *via* encapsulation were shaken in HEPES buffer at 30 °C for 14 days. After 7 days and 14 days the remaining protein amount within the gels (Figure 4a) and the enzyme activity (Figure 4b) were determined. The data are given as percentage of the initial values prior to incubation.

The retained protein amount (Figure 4a) decreased significantly over the course of 14 days to 52 %±7 % ($p < 0.01$). Protein loss was expected to a certain extent, as *Bm*TA was only physically entrapped within the hydrogel meshes and the experiment was conducted in the swollen hydrogel state.[28] As long as the enzyme does not show a strong affinity to the hydrogel material, physically entrapped proteins tend to diffuse out of the gel when meshes are big enough.

The enzyme activity of *Bm*TA immobilized in pNAGA was compared to free *Bm*TA that was left under the same conditions for 14 days (HEPES buffer, 30 °C, shaking). While enzyme activity of immobilized *Bm*TA was preserved to 79 %±37 % after 14 days, activity of free *Bm*TA was already almost completely lost (0.3 %) after 7 days. In spite of higher standard deviations, especially for *Bm*TA immobilized in pNAGA, the trend was still obvious: *Bm*TA was stabilized in pNAGA gels compared to free *Bm*TA in terms of retained enzyme activity.

In the next step, the reusability of immobilizates in consecutive biocatalytic reactions was investigated. To do so, encaps-10 and encaps-20 gels were used in three consecutive syntheses each for 24 h at 35 °C. Formation of the product (1*S*,2*S*)-3-(2-amino-1-hydroxypropyl)phenol (3OH-AHP) was determined *via* HPLC after 24 h reaction time. Product formation for the encapsulated *Bm*TA was compared to free *Bm*TA. After each synthesis, pNAGA-*Bm*TA gels were removed by filtration and reused; free *Bm*TA was removed from the reaction solution by ultrafiltration and re-used.

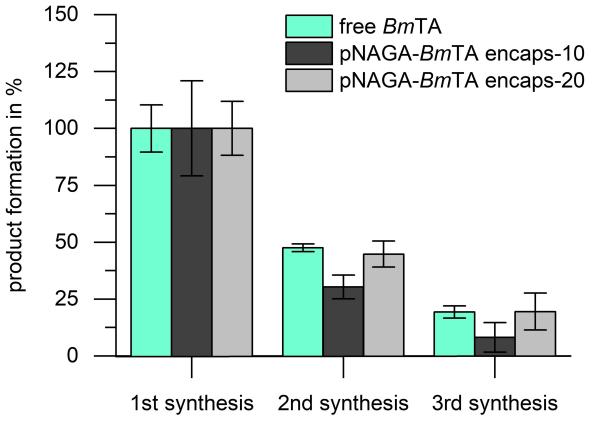

Figure 5. Formation of (1*S*,2*S*)-3-(2-amino-1-hydroxypropyl)phenol from 1-hydroxy-1-(3-hydroxyphenyl)propan-2-one in three subsequent syntheses by free *Bm*TA or *Bm*TA immobilized in poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels via encapsulation (loading with 10 μg protein/mg gel dry weight or 20 μg protein/mg gel dry weight, respectively) after a reaction time of 24 h in HEPES buffer at 35 °C. Free *Bm*TA was removed from the reaction solution by ultrafiltration and re-used; pNAGA-*Bm*TA gels were removed by filtration and reused ($n = 3$).

The data (Figure 5) are presented as percentage of product formation of the first synthesis. It is obvious that product formation decreased significantly ($p < 0.05$) over the course of the repetitive syntheses for both, *Bm*TA immobilized in pNAGA as well as free *Bm*TA. Due to the productivity decrease, more than three consecutive syntheses were not investigated. An advantage of immobilization with regard to product formation after re-using was not found. Still, deactivation was comparable for free *Bm*TA and pNAGA-*Bm*TA encaps-20. And reusing immobilized *Bm*TA is much easier compared to free *Bm*TA especially when aiming at higher reaction volumes than 1 mL.

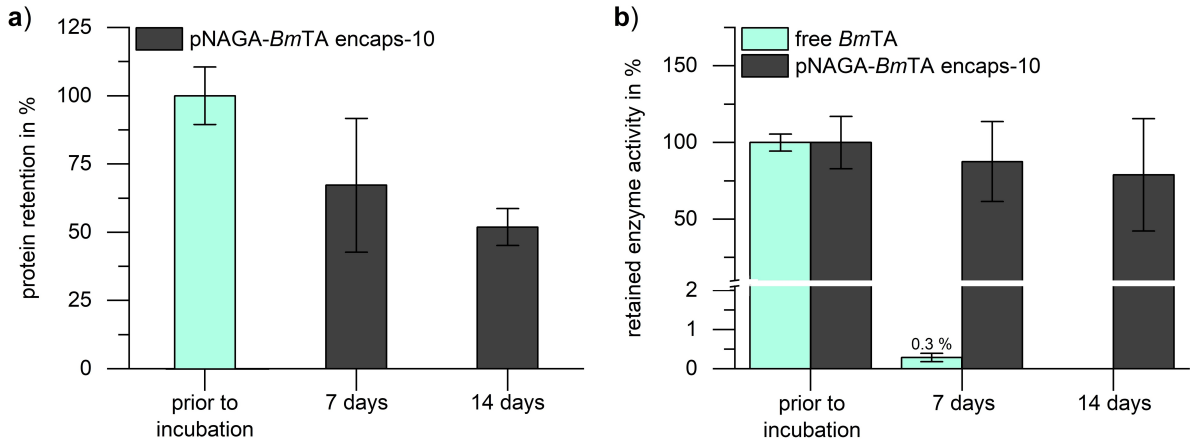

Figure 4. Retained protein amount (a) and enzyme activity (b) of *Bm*TA immobilized in poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels *via* encapsulation after 7 days and 14 days in HEPES buffer at 30 °C (loading 10 μg protein per mg gel dry weight (encaps-10)). Enzyme activity (b) of the immobilized *Bm*TA was compared to free *Bm*TA that was left in HEPES buffer for 7 days and 14 days in HEPES buffer at 30 °C ($n = 3$).

This makes pNAGA-*Bm*TA encaps-20 superior for application in repetitive biocatalytic syntheses compared to free *Bm*TA.

For future experiments a small increase in reusability for both, immobilized and free *Bm*TA, might be achieved by running all reactions in the dark: A recent study published by our group showed a 10–20% decrease of *Bm*TA activity upon blue light exposure, and also some activity loss upon laboratory light exposure.[24b]

Temperature influence

Investigation of swelling degree

In a next step, the response of the immobilizates to temperature changes was investigated in terms of swelling degree and enzyme activity. In a first step, it was tested to what extent the swelling medium (water, buffer and reaction medium with substrate) influences the degree of swelling of pNAGA gels without encapsulated enzyme (Figure 6a). Preferably, the temperature-sensitiveness of pNAGA gels should be preserved in buffered solutions, as the use of buffer is often necessary for good performance in biocatalytic reactions. Next, the effect of enzyme immobilization onto the swelling degree and the temperature-sensitiveness was assessed. Temperature-sensitiveness was tested in the range of 35 °C to 20 °C. For the investigation of the swelling degrees, pure pNAGA gels without immobilized *Bm*TA served as control (Figure 6b).

Figure 6a shows the equilibrium swelling degrees of pure pNAGA gels in ultrapure water, in 10 mM HEPES buffer and in the reaction medium necessary for the enzymatic conversions at 35 °C and 20 °C. The data show that the hydrogels shrank in the buffer solutions compared to ultrapure water, while the effect was even more pronounced in the reaction medium compared to HEPES buffer alone. A temperature-induced decrease in swelling degree upon decrease of temperature was measured independent of the solvent. However, the factor between the swelling at 35 °C and 20 °C seemed to decrease upon addition of buffer salt (HEPES buffer, factor 1.4 ± 0.1; $p > 0.05$) and buffer salt and substrates (reaction medium, factor 1.2 ± 0.2; $p < 0.05$) compared to ultrapure water (factor 1.7 ± 0.2) indicating a weaker temperature response of pNAGA gels in the salt containing solutions.

The swelling degree of hydrogels is in general determined by the ratio of intra- and intermolecular polymer-polymer and polymer-water interactions.[29] In pNAGA these interactions are mainly based on hydrogen bonding, and were therefore reported to be less sensitive to electrolytes compared to ionic polymers.[13d] Still, electrolytes can influence the swelling degree by salting-out effects, where the solubility of protein-like polymers such as polyNAGA is lowered at higher electrolyte concentrations.[13d,30] Previous studies investigating chemically cross-linked pNAGA reported only a mild shrinking in phosphate buffer,[13g] the data in this study, however, showed a pronounced shrinking upon addition of buffer salt to physically cross-linked pNAGA gels. Physically cross-linked gels are thus more sensitive against addition of salts.

The second observation, the decrease in temperature-sensitiveness upon addition of electrolytes, can be explained by a shift or partly suppression of the volume phase transition (VPT) as reported before.[13d] The weaker temperature-response in electrolyte containing solution as well as the lower swelling degrees of pNAGA in this study have to be accepted because while the system might still be applicable with even lower buffer concentrations or water as solvent, the substrates for the enzymatic reaction (partly electrolytes themselves) have to be added to the reaction to enable conversion.

The swelling degrees of pNAGA gels dependent on the temperature are shown in Figure 6b. There was no significant difference between the swelling without loading of *Bm*TA (pNAGA) and with loading of *Bm*TA (pNAGA-*Bm*TA encaps-10) ($p > 0.05$) in the investigated temperature range of 35 °C to

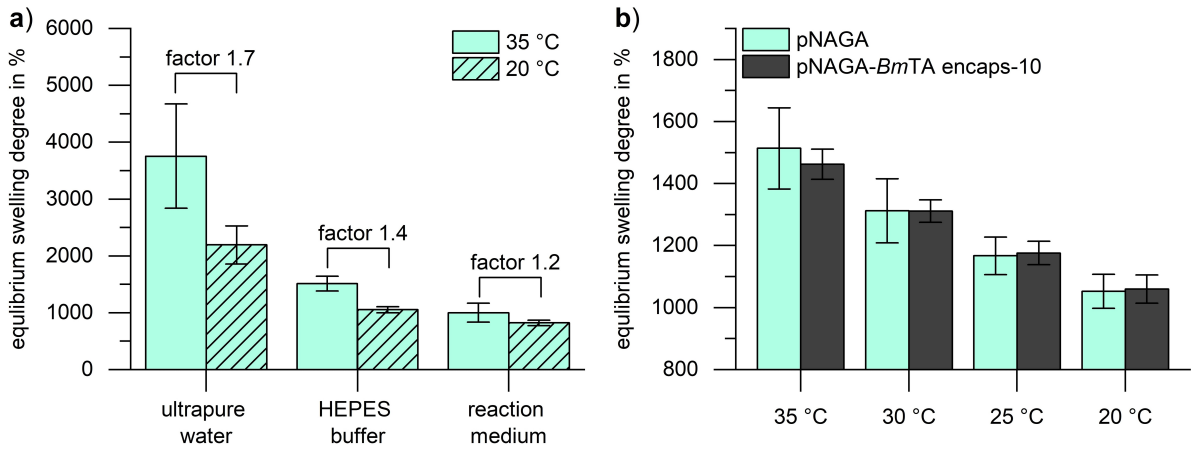

Figure 6. a) Equilibrium swelling degree of poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels without *Bm*TA at 35 °C and 20 °C in ultrapure water, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) (pH 7.5), or reaction medium (10 mM HEPES + 10 mM α-methylbenzylamine + 10 mM sodium pyruvate + 0.1 mM pyridoxal 5'phosphate, pH 7.5). The factor represents the fold-change of the equilibrium swelling degree between 35 °C and 20 °C ($n = 3$). b) Equilibrium swelling degree of pNAGA hydrogels without *Bm*TA, and *Bm*TA immobilized by encapsulation (encaps-10 = 10 μg protein per mg gel dry weight applied for loading) in 10 mM HEPES buffer at pH 7.5 upon decrease of temperature ($n = 3$).

20 °C. Meaning that the *Bm*TA immobilization did not alter the swelling degree of pNAGA nor the temperature-sensitiveness. All gels showed a gradual decrease in swelling degree upon decrease of temperature for all 5 °C steps ($p < 0.05$). The gradual change of swelling degree upon of temperature reduction was expected from literature.[13g,20,30]

Investigation enzyme activity

Since pNAGA gels showed a temperature-dependent swelling independently of whether *Bm*TA was immobilized or not, the next step was to investigate the influence of temperature change associated with change in swelling degree onto *Bm*TA activity. To investigate whether additional deactivation of *Bm*TA occurred due to hydrogel shrinking, the activity of immobilized *Bm*TA was measured at 35 °C and 20 °C, and activity values were compared to free *Bm*TA under the same conditions. In order to have a measure independent of the exact absolute activity value, a quotient of the activity at 35 °C and 20 °C (35 °C/25 °C = fold-change) was calculated:

- similar deactivation: fold-change free *Bm*TA ≈ fold-change immobilizates;
- stronger deactivation in immobilizates: fold-change free *Bm*TA < fold-change immobilizates;
- weaker deactivation in immobilizates: fold-change free *Bm*TA > fold-change immobilizates.

The conversion of 3OH-PAC to 3OH-AHP was measured and product formation was calculated between 1 h and 20.5 h reaction time at 35 °C and 20 °C for free *Bm*TA and *Bm*TA immobilized in pNAGA *via* encapsulation (Figure 7a). Product formation decreased with decreasing temperature for all formulations. The fold-change (secondary axis, orange) between 35 °C and 20 °C tended to be higher for the immobilizates compared to free *Bm*TA, yet the differences were not significant due to relatively high standard deviations.

The conversion curves of the reaction for free and immobilized *Bm*TA at 20 °C and 35 °C in Figure 7b show a more clear picture. It can be seen that in case of pNAGA-*Bm*TA encaps-20, conversions per mg enzyme were similar to free enzyme at 35 °C. However, at 20 °C, conversion of free *Bm*TA was higher compared pNAGA-*Bm*TA encaps-20 ($p < 0.05$), suggesting that temperature-induced shrining of the gel partially reduced enzyme activity.

The data give a first hint that there might be an additional deactivation of *Bm*TA immobilized in pNAGA hydrogels in time-course conversion experiments due to hydrogel shrinking upon temperature reduction; however, the effect was very small compared to the effect of temperature. With regard to the original goal of preventing the formation of by-products by remotely controlling *Bm*TA activity, the current system is not sufficient because *Bm*TA deactivation is not high enough when the temperature decreases, especially if the enzyme shows higher k_{cat} for the undesired substrate. Still, the data are a useful first step towards further development of enzyme activity control.

In order to verify the deactivating effect further studies are needed. In particular, data on the 3D-structure of the enzyme within the hydrogel in the swollen and shrunken state would be desirable, however, standard methods like CD-spectroscopy, IR-spectroscopy, TEM, fluorescence imaging are in the authors experience not applicable to characterize enzymes within the hydrogel network, or at least not without changing the hydrogels properties. In the end, even with imaging techniques it will be almost impossible to distinguish between effects on the enzyme activity resulting from:

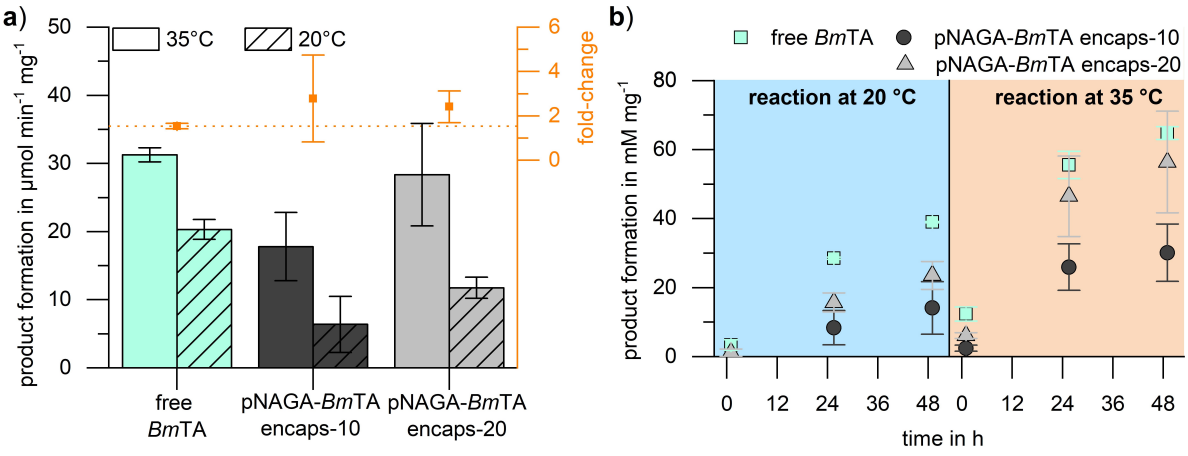

Figure 7. a) Product formation (µmol min^{-1} mg^{-1}) of free *Bm*TA and *Bm*TA immobilized in poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels that were loaded by encapsulation (encaps-X). The X represents the amount of *Bm*TA applied for loading e.g. 10 µg protein/mg gel dry weight in case of encaps-10. Product formation was determined within a time-course conversion experiment using 1-hydroxy-1-(3-hydroxyphenyl)propan-2-one as substrate and (1*S*,2*S*)-3-(2-amino-1-hydroxypropyl)phenol as product from 1 h to 20.5 h reaction time at 35 °C and 20 °C in HEPES buffer. The fold-change (secondary axis, orange) gives the quotient between the activity at 35 °C and 20 °C and is a measure for enzyme activity change upon change of temperature ($n = 3$). b) Production of (1*S*,2*S*)-3-(2-amino-1-hydroxypropyl)phenol (3OH-AHP) per mg *Bm*TA at 20 °C (left) or 35 °C (right) over the course of 50 h in HEPES buffer. *Bm*TA was either used as free enzyme in solution or immobilized in poly(*N*-acryloyl glycinamide) (pNAGA) hydrogels that were loaded via encapsulation (loading with 10 µg protein/mg gel dry weight or 20 µg protein/mg gel dry weight) ($n = 3$).

- change in substrate/product diffusion due to differences in water content changed accessibility of the active site for the substrate;
- changed enzyme conformation and moving flexibility due to the packing of enzyme into the hydrogel meshes;
- change in enzyme environment (hydrophilicity, presence of charged groups).

To ascertain the deactivation of enzymes in response to hydrogel deswelling, further basic studies ideally including enzymes undergoing stronger conformational changes during catalysis have to be conducted. Such enzymes might react more sensitive towards changes in swelling degree than *Bm*TA and are therefore interesting candidates for enzyme activity control. Furthermore, other UCST-type materials have to be tested, as pNAGA only showed a weak temperature response in buffered solution, and a stronger deactivation is expected to be associated with a higher difference between swollen and shrunken state. Also, a sudden change of swelling could be beneficial over gradual changes.

Conclusion

In this contribution, we investigated upper critical solution temperature (UCST)-type hydrogels of poly *N*-acryloyl glycinamide (pNAGA) as a smart matrix for biocatalysis and enzyme activity modulation. In general, pNAGA gels were found to be suitable for the immobilization of *Bacillus megaterium* transaminase (*Bm*TA) with high immobilization efficiency (>89%) and high activity (97%) by adding the enzyme to the hydrogel pre-gel solution prior to free radical polymerization. Still, due to the physical encapsulation within the gel, significant protein loss occurred during washing and repetitive use in biocatalytic reactions. The swelling of pNAGA gels was strongly reduced by adding buffer and substrates to the swelling medium, which is unfortunate, as a high swelling at high temperature is expected to go hand in hand with high enzyme activity. Still, the preferred temperature-sensitiveness of the gel swelling was also maintained in buffered solutions. pNAGA gels showed a gradual deswelling upon temperature reduction regardless of whether *Bm*TA was immobilized. The enzymatic product formation was reduced upon temperature reduction from 35 °C to 20 °C, as expected from the Arrhenius plot. When comparing the deactivation for free and immobilized *Bm*TA (free enzyme ≈44%; immobilized enzyme ≈62–68%), deactivation appeared to be more pronounced in the immobilizates, indicating an additional activity decrease upon hydrogel deswelling.

To the best of our knowledge, this is the first report on the application of UCST gels to modulate enzyme activity by changing the degree of swelling. Even though pNAGA gels are certainly not ideal for application in biocatalysis up to now, as they shrink strongly in buffered and especially substrate-containing solution, and additionally show considerable leakage of the enzyme, the authors believe that they are a starting point for future investigations. Since research on UCST-type hydrogels and their application for catalysis is still in the very beginning, improved properties for biocatalysis, i.e. lower response to electrolytes, and improved immobilization properties, e.g. by including affinity binding sites, are expected in the future.

In order to exploit the full potential of UCST-type hydrogels for modulating enzyme activity, especially the relationship between hydrogel network structure (i.e. mesh size and swelling degrees), enzyme size (hydrodynamic diameter), enzyme flexibility and resulting enzyme activity have to be elucidated. This knowledge would then help to rationally design not only pNAGA gels, but also other UCST-type as well as LCST-type hydrogels for enzyme activity modulation.

Experimental Section

Materials

Alpha-methylbenzylamine (α-MBA), dimethyl sulfoxide (DMSO), hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone, pyridoxal-5'phosphate (PLP) and urea were purchased from Sigma-Aldrich (Germany). Acetonitrile, pyruvic acid, trifluoroacetic acid (TFA) were purchased from Carl Roth (Germany). Other reagents were purchased from the following sources: Bovine serum albumin (BSA) from Fluka (Germany), 3-(2-amino-1-hydroxypropyl)phenol ((*R,S*)-3OH-AHP) from Toronto Research Chemicals (Canada).

The UV lamp VL-UVA135.M (365 nm, 135 mm reflector, intensity 45 mW/cm^2 at 38 cm distance; Vilber Lourmat, Germany) was used for the production of hydrogels by photopolymerization. A temperature-controlled rocker (Enviro-Genie, Scientific Industries, USA) was used for washing and swelling of the hydrogels. Absorbance measurement for protein determination *via* Bradford assay was performed using the UV1800 spectrophotometer (Shimadzu, Japan). HPLC-analysis was performed using the Ultimate 3000 system (Thermo-Fisher Scientific, USA) with a LiChrospher® 100 RP-18 column (Merck, Germany).

Hydrogel preparation and loading of *Bm*TA

The transaminase from *Bacillus megaterium* (*Bm*TA) and the *N*-acryloyl glycinamide (NAGA) monomer used as building block for hydrogel synthesis were produced and purified according to the procedures given in the Supporting Information (section 1 in case of *Bm*TA; section 2 in case of NAGA).

Preparation of poly(*N*-acryloyl glycinamide) hydrogels: Hydrogels were synthetized with *N*-acryloyl glycinamide (NAGA) by free radical photopolymerization. Stock solutions of the monomer NAGA (25 wt.% in ddH_2O) and of the photoinitiator hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (10 wt.% in DMSO) were prepared. 800 μL NAGA stock, 60 μL hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone stock and 140 μL ddH_2O were combined to prepare a pre-gel solution containing 20% NAGA and 3% hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (relative to NAGA). The pre gel solution was transferred into a polytetrafluoroethylene (PTFE) mold with indentations of 3 mm diameter and 1 mm depth. Hydrogels were cross-linked by UV irradiation at 365 nm for 2 min. Six hydrogel pellets were washed in ddH_2O for 24 h at 30 °C in a rocker, water was changed every hour during the first three hours of washing.

Loading of BmTA by diffusion: Loading of *Bm*TA *via* diffusion was done by placing six washed and swollen hydrogel pellets *Bm*TA solution (1.3 mg lyophilizate per mL in ddH_2O, protein concentration determined by Bradford assay) and shaking for 24 h at 35 °C. The applied loading volume was adjusted to be 30 μL mg^{-1} gel dry

weight, so that loading corresponded to approximately 20 μg protein per mg gel dry weight. After loading, the supernatant was removed and hydrogels were washed for 24 h in ddH_2O at 30 °C while shaking.

Loading of BmTA by encapsulation: For the encapsulation approach, *Bm*TA was added to the pre-gel solution prior to irradiation. A stock solution of *Bm*TA was prepared in ddH_2O (100 mg lyophilizate per mL, protein concentration determined by Bradford assay). The amount of *Bm*TA stock solution was adjusted to yield final concentrations of 20 μg protein per mg gel dry weight (pNAGA-*Bm*TA-20), or 10 μg protein per mg gel dry weight (pNAGA-*Bm*TA-10) or, respectively. After loading hydrogels were washed for 24 h in ddH_2O at 30 °C while shaking.

Characterization of hydrogel loading

Immobilization efficiency: Protein concentrations were determined by Bradford protein assay. The Bradford reagent was prepared and Bradford assay for free enzyme was carried out according to previous publications[4,31] (see Supporting Information section 3.1 & 3.2). Protein concentration within the hydrogels was determined with an adapted Bradford assay for dissolved hydrogels. In this method, the gels were first dissolved in ddH_2O by adding urea and heating. Details on the procedure can be found in the Supporting Information section 3.3. Immobilization efficiency was determined comparing the amount of protein applied for loading with the protein amounts in the hydrogels directly after loading, after washing, and after their use in an activity assay.

Activity of Immobilizates: reaction conditions of the enzymatic transformation

Immobilizates were used in two different enzymatic transformations as described in the following:

Initial rate activity determination: Initial rate activity was measured with an HPLC-based assay following the conversion from pyruvate and α-MBA to (*S*)-alanine and acetophenone similar to a previous publication reporting a photometric assay for transaminase activity.[32] The reaction was carried out in 10 mM HEPES buffer at pH 7.5 supplemented with 0.1 mM PLP as cofactor, 10 mM α-MBA and 10 mM pyruvic acid as substrate. The enzyme/immobilizates were applied at a concentration of 5 μg mL^{-1} free *Bm*TA as non-immobilized control or the immobilizates pNAGA-encapsBmTA-10 (66–79 μg mL^{-1}). Details can be found in the Supporting Information section 4.1.

The initial rate activity was calculated from the slope in the linear range of the acetophenone concentration (max. 10% conversion) plotted against time according to Equation (1):

$$\textit{specific activity}\ (\mu mol\ min^{-1}\ mg^{-1}) = \frac{\textit{slope}(\mu mol\ min^{-1})}{\textit{total protein in reaction}\ (mg)} \quad (1)$$

To determine the specific activity the amount of protein in the reaction was determined by the Bradford protein assay.

Time-course conversion experiments: Activity of the immobilizates was furthermore determined in small scale conversion experiments (1 mL) following the reductive amination of (1*S*)-1-hydroxy-1-(3-hydroxyphenyl)propan-2-one ((*S*)-3OH-PAC) (synthesis see Supporting Information section 5) to the aromatic amino alcohol (1*S*,2*S*)-3-(2-amino1-hydroxypropyl)phenol ((*S*,*S*)-3OH-AHP) over time, with α-MBA as co-substrate analogue to a reaction published before.[4] The reaction was performed in 10 mM HEPES buffer at pH 7.5 supplemented with 0.2 mM PLP as cofactor, 20 mM (*S*)-3OH-PAC as substrates and 40 mM α-MBA as co-substrate. The enzyme/immobilizates were applied at concentrations of 100 μg mL^{-1} free *Bm*TA (control) and the immobilizates pNAGA-encapsBmTA-10 (56–84 μg mL^{-1}) and pNAGA-encapsBmTA-20 (114–144 μg mL^{-1}). Details can be found in the Supporting Information section 4.2.

The specific product formation was calculated from the product formation between 1 h and 20.5 h reaction time. The amount of protein in the reaction was determined by the Bradford protein assay.

Storage stability of immobilizates: The storage stability of the immobilizates was tested by leaving free and immobilized transaminase in 10 mM HEPES buffer rocking in a thermoshaker for 2 weeks at pH 7.5 and 30 °C. The protein content and the initial rate activity were determined prior to incubation, after one week and after two weeks of storage.

Reusability of immobilizates in consecutive syntheses: Whether the immobilizates were reusable was tested with three consecutive time-course conversion experiments. The reaction mixture was prepared as described above and reactions were allowed to proceed for 24 h at 35 °C. After 24 h, the immobilizates were removed from the reaction solutions, rinsed with ddH_2O and added to fresh substrate solution to start the next synthesis. As a control, syntheses with free enzyme were conducted accordingly. Free enzyme was isolated from the reaction solution by filtration in a spin filter (Amicon Ultra MWCO 30 kDa), washed with ddH_2O and added to fresh substrate solution to start the next synthesis.

Temperature influence

Swelling degree of hydrogels: The influence of temperature on the swelling degree of hydrogels (with and without *Bm*TA) was investigated in 10 mM HEPES buffer in the temperature range between 35 °C and 20 °C. Temperature was decreased in 5 °C steps; then, hydrogels were allowed to equilibrate at the new temperature for 1.5 h. After equilibration the hydrogels were taken out of the solution, then excessive liquid was removed with paper towel and gels were weighed ($m_{swollen}$). The swelling degree was calculated according to Equation (2):

$$\textit{swelling degree} = \frac{m_{swollen} - m_{dry}}{m_{dry}} x100\,\% \quad (2)$$

The dry mass of the gels (m_{dry}) was calculated from the mass of the unswollen, unwashed hydrogels with the polymer content of 20%.

Dependency of enzyme activity on temperature: The influence of temperature on activity of immobilizates was investigated in HEPES buffer at 35 °C and 20 °C (see section activity of immobilizates). As a control, free enzyme was investigated in the same way.

Statistical analysis

Statistical analysis was done using a two-sided Student's T-test. At *p* values less than 0.05 data were considered statistically significantly different. All data are presented as mean ± standard deviation. The value of *n* is defined as the number of independently performed replications (biological replicates). All experiments were conducted with one *Bm*TA batch and two NAGA syntheses. Preparation and characterization of hydrogels with and without immobilized enzyme was typically done with *n* = three; *n* values are given in the results part.

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Conflict of Interest

The authors declare no conflict of interest.

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