In Situ Entrapment of Urease in Cryogels of Poly(*N*-isopropylacrylamide): An Effective Strategy for Noncovalent Immobilization of Enzymes

Petar Petrov,[1] Severina Pavlova,[1,2] Christo B. Tsvetanov,[1] Yana Topalova,[2] Raycho Dimkov[2]

[1]*Institute of Polymers, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[2]*Faculty of Biology, "St. Kliment Ohridsky" Sofia University, 1164 Sofia, Bulgaria*

Received 2 December 2010; accepted 4 January 2011
DOI 10.1002/app.34063
Published online 9 June 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Supermacroporous poly(*N*-isopropylacrylamide) (PNIPAAm) cryogels containing urease were prepared via UV irradiation technique and hydrogen peroxide as initiator. Specifically, due to the cryostructuration phenomenon urease molecules were embedded into the dense cryogel walls. Thus, although the enzyme is physically entrapped, the system exhibited remarkable resistance against leaking due to the dense polymer network formed in the cryogel walls. The immobilized urease can catalyze the hydrolysis of urea in a broad temperature range in both batch and flow regime. The interconnected macropores assist for unhindered diffusion of the substrate and reaction products through the gel, thus, paving the way for consecutive reuse at a constant activity, in contrast to the conventional PNIPAAm hydrogel. Due to the spongy-like morphology PNIPAAm cryogels containing urease can be exploited as highly permeable membrane for direct removal of traces of urea from continuously flowing feed solutions.
 J Appl Polym Sci 122: 1742–1748, 2011

Key words: macroporous hydrogels; enzymes; PNIPAAm; urease; irradiation

INTRODUCTION

Enzymes are being used intensively as biocatalysts in biochemical processes due to their potential applications in different biomedical and biotechnological fields. The immobilization of enzymes on support matrix is among the preferred techniques developed to improve almost all enzyme properties: stability under both storage and operational conditions, activity, specificity and selectivity, reduction of inhibition, minimizing or eliminating protein contamination of the product etc.[1] The main advantage of enzyme immobilization is the possibility for industrial reuse for many reaction cycles.

Generally, the immobilization of biocatalysts onto matrix is carried out by entrapment, physical adsorption, encapsulation or covalent attachment.[2,3] Immobilization of enzymes via formation of covalent bonds is among the most widely used. A variety of synthetic and natural polymers such as chitosan, polyacrylonitrile, polystyrene, nylon, and cellulose derivatives[4–8] have been used as matrix and support material for covalent immobilizing of enzymes. An advantage of this method is that the enzyme cannot be released into the solution upon use due to the stable nature of the bonds formed between biomolecules and matrix. However, to achieve high levels of activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support. In addition, due to the structural changes induced by the interaction of the macromolecules with the support, a fall of activity has been observed.[8,9] Another convenient method is the entrapment of enzymes into polymer gels. This method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through, but retains the enzyme.[10–12] In this case, the performance of the system depends strongly on the enzyme retention, its specific activity, and the diffusibility of the substrates and products.

Recently, special attention has been paid to the immobilization of enzymes in matrices based on the so called "smart" polymers.[13,14] These polymers reversibly undergo dramatic conformational changes in aqueous media in response to an external stimulus such as change of pH, ionic strength, temperature, or addition of chemical species. One of the most studied representatives of this family is the thermo-responsive and biocompatible poly(*N*-isopropylacrylamide) (PNIPAAm). In aqueous solution PNIPAAm exhibits a lower critical solution temperature (LCST) while the chemically crosslinked

Correspondence to: P. Petrov (ppetrov@polymer.bas.bg).
Contract grant sponsor: National Science Fund of Bulgaria; contract grant number: BY-X-302/07.

PNIPAAm hydrogel undergoes reversible volume phase transition (VPT) from hydrophilic to hydrophobic state at about 32°C. Hence, the temperature of enzyme reaction can be tuned to ensure the best operation conditions.[15,16] However, due to the appearance of a thick, dense skin layer on the hydrogel surface during the shrinking process,[17] the response rate to external temperature changes of conventional PNIPAAm hydrogel is slow and restricts some applications where a fast response rate is needed. To increase the response rate one may use the method of cryotropic gelation, where the system of large interconnected pores alleviates the temperature exchange.[18]

This article aims at reporting on a novel and effective approach for enzyme immobilization, based on entrapment into the walls of poly(*N*-isopropylacrylamide) cryogels. The macroporous PNIPAAm cryogels possess chemical and biological stability, very good physicomechanical characteristics that provide high exploitation life of these materials. Urease, a nickel-dependent metalloenzyme which catalyzes the hydrolysis of urea to ammonia and carbon dioxide, was selected as the model enzyme.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide (M_W = 113.16 g/mol) and poly(ethylene glycol) diacrylate ($M_W \sim$ 575 g/mol) were purchased from Aldrich and used without purification. Urease (EC 3.5.1.5 from Jack Bean) and H_2O_2 (30 vol % water solution) were received from Merck.

Synthesis of PNIPAAm cryogel with *in situ* immobilized urease

About 0.35 g NIPAAm was dissolved in 5 mL deionized water at room temperature and 0.0105 g urease, previously dissolved in 2 mL deionized water using ultrasound bath, was added. Then, 0.035 g crosslinking agent (PEGDA) and 0.06 mL initiator (H_2O_2) were added under stirring to obtain a homogeneous aqueous solution. One milliliter of solution was poured into Teflon dishes (six dishes with diameter of 20 mm) forming a thick layer, which were frozen at minus 20°C for 2 h. The dishes were then irradiated with full spectrum UV-vis light with a "Dymax 500-EC" UV curing equipment for 5 min (irradiation dose = 28.5 J/cm^2). The samples were then immersed in deionizied water for 3 days at room temperature and the water was replaced every 24 h to wash out the unreacted chemicals.

Synthesis of PNIPAAm cryogel

PNIPAAm cryogel was synthesized and treated by the same procedure described above, except adding urease.

Synthesis of PNIPAAm hydrogel

About 0.35 g NIPAAm was dissolved in 7 mL deionized water at room temperature. Then, 0.035 g PEGDA and 0.06 mL H_2O_2 were added under stirring to obtain homogeneous aqueous solution. The latter was poured into Teflon dishes (20 mm diameter) and directly irradiated with UV-light for 5 min (irradiation dose = 28.5 J/cm^2) at temperature 18°C.

Immobilization of urease into the preformed PNIPAAm hydro- and cryogels

Discs (diameter 20 mm, thickness 5 mm) of freeze dried PNIPAAm hydro- and cryogels were immersed in 3 mL urease solution (1.5 g/L) for 30 min at given temperature. Then, the samples were immersed for 30 s in deionized water to wash out the free enzyme adsorbed onto the outer surface of the support and incubated in 10 mL urea solution (3.2 g/L) for 24 h.

Measurements of gel fraction and degree of swelling

Gel fraction (GF) yield and degree of swelling (DS) of the hydro- and cryogels were determined gravimetrically. The GF was estimated by weighing the insoluble part after extraction for a few days at room temperature.

$$\text{GF yield } (\%) = \frac{\text{weight of dried sample}}{\text{initial weight of polymer}} \times 100$$

The degree of swelling was determined gravimetrically at equilibrium water uptake:

$$\text{DS} = \frac{\text{weight of swollen sample}}{\text{weight of dried sample}}$$

Surface morphology

The extracted cryogels were frozen in liquid nitrogen, fractured and freeze dried in an "Alpha 1-2 Freeze drier" (Martin Christ) at minus 55°C and 0.02 mbar for 24 h. Then the specimens were fixed on a glass substrate and coated with gold for 60 s. The interior morphology of the gels was studied by using a JEOL JSM-6390 scanning electron microscope operating at 5 kV.

Protein assay

The amount of urease released by the hydro- and cryogels was determined by the Biuret method. The method is based on spectrophotometric measurement at 330 nm of the blue–purple color resulting from the complex formed between the peptide bonds of protein and copper ions from Benedict reactant, in alkaline medium.[19]

Activity assay of free and immobilized urease

The activities of the free and immobilized urease were determined using Nessler's method.[9] The method is based on the ammonium liberated from the urea hydrolysis, determined spectrophotometrically at wavelength of 405 nm by measuring the intensity of the yellow-colored compound formed after the addition of Nessler's reagent.

Free urease activity

About 0.1 mL of enzyme solution (1.5 g/L) was added to 0.9 mL of phosphate buffer (50 m*M*, pH = 7.4) and incubated with 1 mL of urea solution (3.2 g/L) at given temperature and time. The enzymatic reaction was terminated by addition of 1 mL 10% trichloroacetic acid. An aliquot (1 mL) of the reaction mixture was transferred to a 50-mL volumetric flask and 1 mL Nessler's reagent was added and then the volume was made up to 50 mL with distilled water.

Immobilized urease assay

For the determination of immobilized urease activity in batch regime, discs (diameter 20 mm, thickness 5 mm) of hydro- and cryogels containing urease were incubated in the presence of 1.8 mL phosphate buffer and 2 mL urea solution (3.2 g/L) at given temperature and time. After given time, the hydro- and cryogels with immobilized enzyme were separated from the reaction mixture and kept in a freezer. An aliquot of the reaction mixture was assayed analogical as the free urease.

For the determination of immobilized urease activity in flow regime, four discs of PNIPAAm cryogels with *in situ* immobilized urease (diameter 20 mm, thickness 5 mm) were placed on the bottom of column and 10 mL urea solution (3.2 g/L) were passed through the column at a flow rate of 10 mL/h and temperature of 37°C. An aliquot of the reaction mixture was assayed analogical as the free urease.

Calculation:

$$\mathrm{EA}(\mu\mathrm{M.\,min^{-1}}) = \frac{\Delta \mathrm{A.V.MA}}{\mathrm{t.v.l}} \times 1000$$

where EA, enzyme activity; ΔA, absorbance change; V, total reaction volume; MA, molar absorptivity; v, volume of sample used; t, incubation time; l, length of the cuvette.

RESULTS AND DISCUSSION

Supermacroporous PNIPAAm cryogels with *in situ* immobilized urease (PNIPAAm-CryoUrW) were synthesized by UV irradiation of moderately frozen aqueous systems employing a procedure described elsewhere.[18] To embed the urease molecules predominantly into the cryogel walls, the enzyme was mixed with the aqueous solution of reagents prior freezing. It is known that in the process of cryostructuration the soluble substances are accumulated into a nonfrozen liquid microphase[20] where the formation of PNIPAAm network takes place upon irradiation with UV light. Thawing of the frozen system resulted in opalescent heterogeneous supermacroporous cryogel of high GF yield, comprising interconnected pores surrounded by thin compact walls containing physically entrapped urease [Fig. 1(a)].

One has to mention that the applied irradiation dose did not decrease noticeably EA of immobilized urease, as confirmed by comparing native urease (Table I, NU1) and urease extracted from frozen PNIPAAm/urease sample irradiated with UV light for 5 min (Table I, UV3). This test aimed to roughly get an idea whether the UV irradiation with a dose of 28.5 J/cm^2, employed to obtain the cryogels, influence the enzyme activity. In general, the UV light decreased EA of urease in aqueous media, except the frozen system containing PNIPAAm, which seems to act as a shell and protect the enzyme. (Linear PNIPAAm was used instead of crosslinked one for simplicity in the work up procedure of enzyme extraction).

To evaluate the efficacy of urease retention by the cryogel, PNIPAAm-CryoUrW was compared with another two different systems prepared: PNIPAAm cryogel with enzyme entrapped only into the pores (PNIPAAm-CryoUrP) and conventional PNIPAAm hydrogel containing similar amount of urease (PNIPAAm-HydroUr) [Fig. 1(b,c)]. For that purpose, PNIPAAm hydrogels and PNIPAAm cryogels were synthesized, lyophilized and, then, immersed in the urease solution. Thus, the enzyme solution filled the interconnected pores of swollen cryogel, while the structure of hydrogel allowed a homogeneous distribution of urease molecules within the whole sample. Figure 2 shows the gel fraction yield of cryo- and hydrogels obtained by UV irradiation. Overall, the gels have very high GF yield and, in the case of cryogels, nearly quantitative monomer conversion was achieved. It seems that the presence of given amount of urease (up to 3 wt % to monomer) in the initial solution have only marginal effect on the crosslinking efficacy. Although the GF yield is

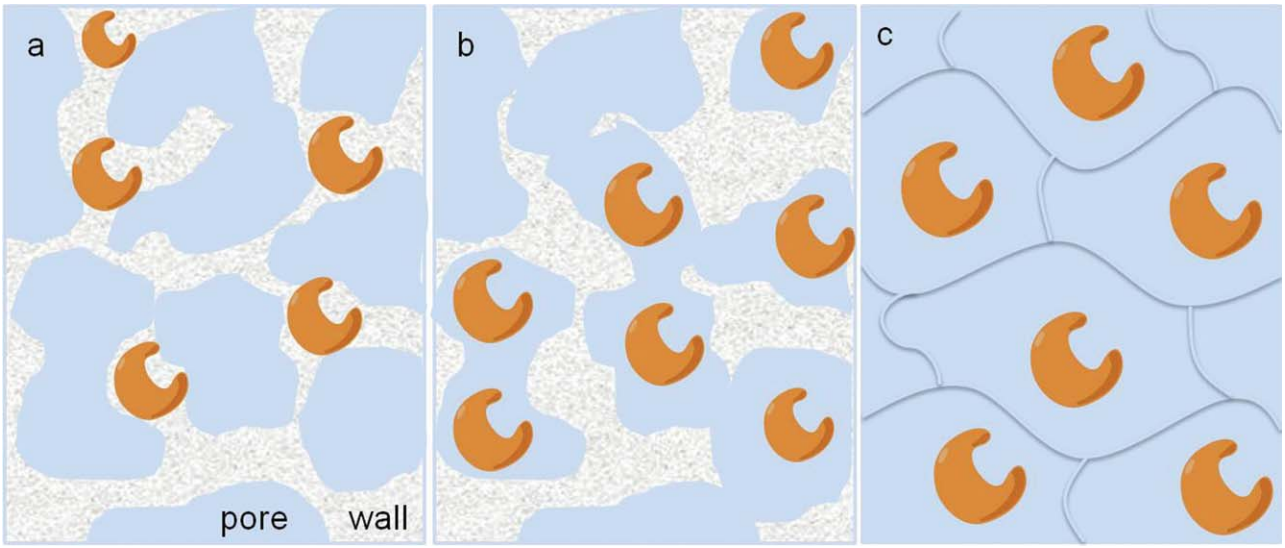

Figure 1 Schematic representation of urease entrapment into: (a) walls of PNIPAAm cryogel (PNIPAAm-CryoUrW), (b) pores of PNIPAAm cryogel (PNIPAAm-CryoUrP), and (c) PNIPAAm hydrogel (PNIPAAm-HydroUr). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

very high, all cryo- and hydrogels were thoroughly extracted with deionized water before use.

Crucial feature of the systems based on physically entrapped enzyme is to ensure effective barrier against the leakage of enzyme from the polymer matrix. Concerning the conventional hydrogels, the nature and density of polymer network are main factors that can be exploited to tune the permeability of polymer matrix. Taking into account that the preparation of cryogels involves a procedure of freezing and cryo-concentration, respectively,[20] one can accept that the density of polymer network formed is higher when compared with the hydrogel obtained at equal concentration of monomer in the initial aqueous solution. In other words, we suggested that the *in situ* entrapment of urease into the dense walls of cryogel could lead to efficient retention into polymer carrier, while the interconnected pores will assist for the free diffusion of the lower molecular mass urea and catalytic products.

As already mentioned, PNIPAAm is a temperature-responsive polymer and the cryo- and hydrogels obtained undergo a reversible volume phase transition from highly swollen to collapsed state at around 32°C.[18] In particular, PNIPAAm-CryoUrW decreased its apparent degree of swelling from 19 at

TABLE I
Comparison of Urease Activity at Different Experimental Conditions

Sample code	Sample type	Incubation temperature (°C)	Enzyme activity (μmol/min) × 10^{-3}
NU1	Native urease solution	20	10.2
UV1	Urease solution irradiated with UV light for 5 min at 25°C	20	0.6
UV2	Frozen urease solution (−20°C) irradiated with UV light for 5 min	20	0.9
UV3	Frozen PNIPAAm[a]/urease solution irradiated with UV light for 5 min	20	10.2
NU2	Native urease solution	37	20.1
NU3	Native urease solution[b]	50	54.7
PCr1	PNIPAAm-CryoUrW[c]	50	0.8
PHy1	PNIPAAm-HydroUr[d]	50	1.7

[a] Experiment was performed with linear PNIPAAm (M_W ca. 10 Kg/mol) to allow full extraction of the enzyme after thawing.
[b] Dissolved in deionized water (1.5 g/L).
[c] Maximum value reached for 24 h incubation.
[d] Maximum value reached for 1 h incubation.

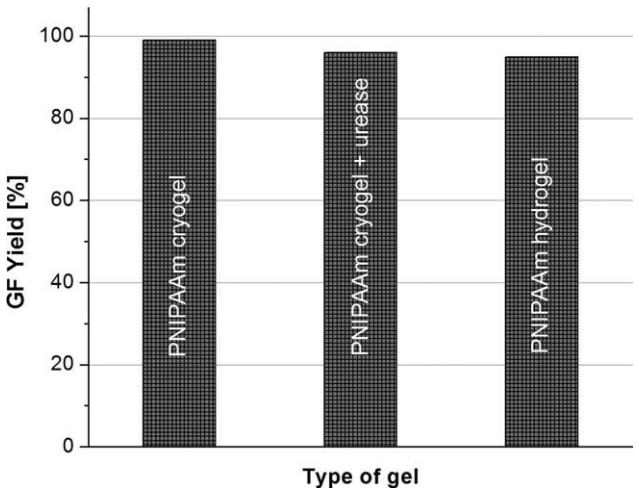

Figure 2 Gel-fraction yield of PNIPAAm cryogels with/without urease (frozen at −20°C for 2 h) and PNIPAAm hydrogel synthesized by irradiation with UV light for 5 min.

20°C to 6 at 50°C [Fig. 3(a)]. This result is attributed to a significant shrinkage of polymer network and cryogel walls, respectively, which reduced the size of the pores. However, the material maintained its open-porous structure at temperature above VPT [Fig. 3(b)] which, as described below, is an advantage concerning the unhindered diffusion of species within the cryogel.

The efficiency of urease retention by the polymer network of the three systems prepared was quantified by incubation of PNIPAAm cryo- and hydrogels, containing urease, in water for 24 h and a subsequent determination of the protein content in the extract. Thus, one may estimate the amount of urease, if any, released by the polymer carrier. As expected for an open-porous structure, PNIPAAm-CryoUrP exhibited significant and continues release of urease within the time interval studied (Fig. 4). This, undoubtedly, is due to the big difference in the size of enzyme molecules and size of interconnected pores of cryogel where the urease is located. Obviously, even at temperature above VPT of polymer network the diffusion through the channels is not restricted and, roughly, more than 1/3 of enzyme was released for 24 h. In sharp contrast, the extracts from PNIPAAm-CryoUrW specimens showed very low absorbance corresponding to 1–2 wt % leakage of urease, when compared with the initial amount, at both temperatures studied. In other words, more than 98 wt % of enzyme is permanently entrapped into the dense walls of PNIPAAm cryogel which is a direct evidence for the high efficiency of method employed. Definitely, the dense polymer network of cryogel is nonpermeable for urease molecules and act as barrier against release.

Conventional PNIPAAm hydrogels containing urease exhibited a slight release of enzyme in the initial 10–20 min (Fig. 4). Noteworthy, at 20°C the polymer network is highly swollen (DS = 40) and most probably this is the reason for release of ~ 10 wt % of enzyme for 24 h. At 50°C the polymer network is in its hydrophobic state (DS = 4) which resulted in decreased leaking out of urease (<5 wt %). Consequently, concerning the immobilization strategies utilized in this study, one may conclude that the entrapment of urease inside the interconnected macroscopic pores of cryogel is not effective method for permanent immobilization into the gel. On the other hand, the entrapment of urease into

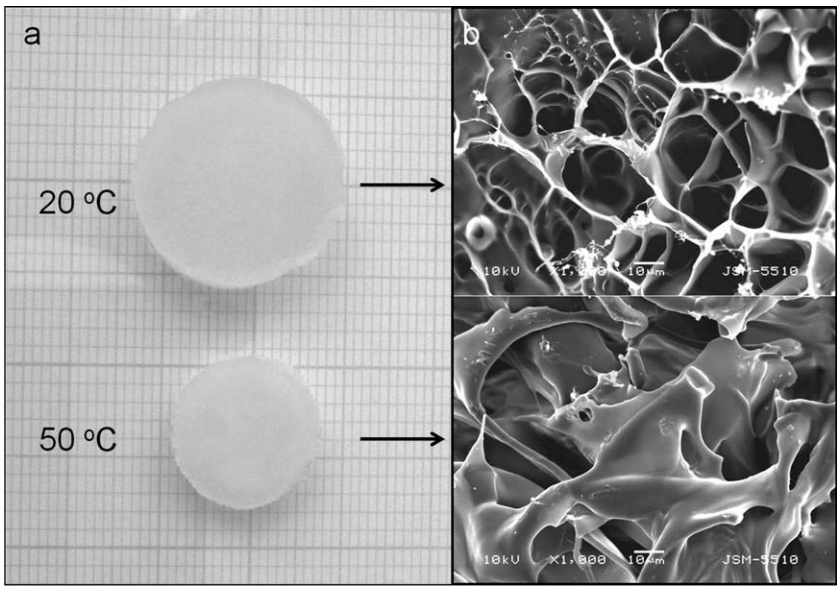

Figure 3 Digital picture (a) and SEM micrographs (b) of PNIPAAm-CryoUrW below and above the temperature of volume phase transition.

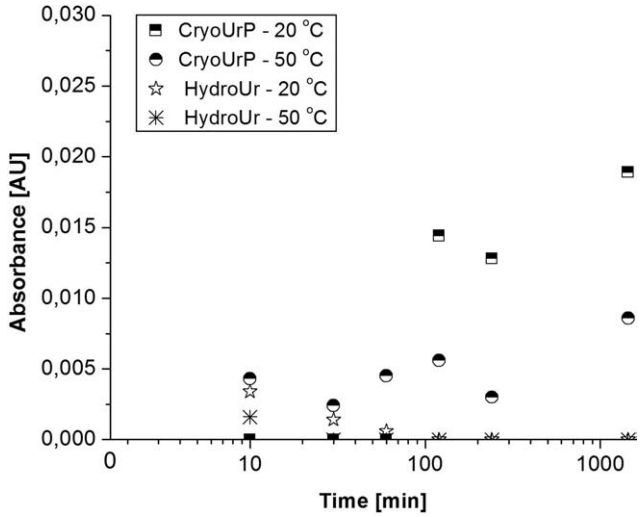

Figure 4 Absorbance at 330 nm of the complex formed between the protein, released from the cryo- and hydrogels, and the copper ions from the Benedict reactant.

the walls of PNIPAAm cryogel leads to nearly quantitative retention of enzyme into the carrier, below and above the temperature of VPT, while the PNIPAAm hydrogel has better retention ability in the case of collapsed polymer network (50°C). Taking into consideration the abovementioned results, our further experiments were focused on the assessment of catalytic degradation of urea by two of the systems prepared, PNIPAAm-CryoUrW and PNIPAAm-HydroUr, at temperatures above VPT. Noteworthy, it was confirmed experimentally that EA of native urease increases with the increase of temperature from 20 to 50°C (Table I, NU1-NU3).

One of the properties of enzyme that have been considered for improvement via immobilization is its stability under storage and operation conditions.[1] Although the immobilization generally decreases the urease activity compared to the native urease (Table I), important benefits of it are the facile separation from the reaction media and possibility for reuse which reflect in higher catalyst productivities. On the other hand, EA values are order of magnitude higher when compared with some data reported for covalently immobilized urease.[8] Since we found that the urease entrapped into PNIPAAm-CryoUrW and PNIPAAm-HydroUr do not leak out notably, the next step was to assess the performance of both systems in a batch reactor after several continuous hydrolysis reactions (cycles). The test involved consecutive incubation of gels in 10 mL urea solution (3.2 g/L) for three cycles of 1 h, followed by another three cycles of incubation for 24 h. After every cycle the specimens were placed immediately in a fresh reaction medium. Figure 5 shows the relative activity of both systems (the highest activity was taken as 100%) at repeated use.

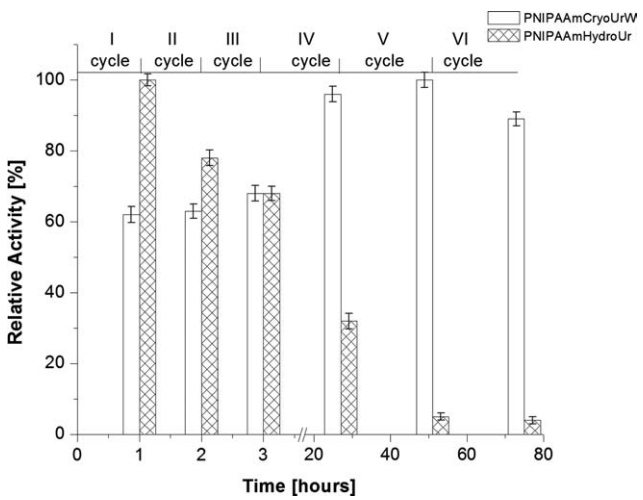

Figure 5 Effect of reaction time and number of cycles on the enzymatic activity of PNIPAAm-CryoUrW and PNIPAAm-HydroUr incubated in 10 mL urea solution (3.2 g/L) at 50°C.

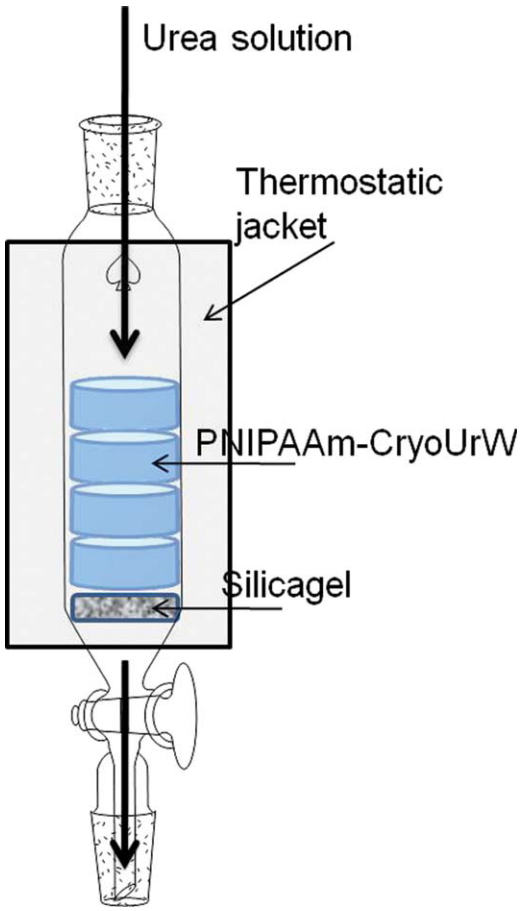

Figure 6 Schematic representation of a model column-type reactor containing several reactive membranes of PNIPAAm-CryoUrW and "Silicagel" layer on the bottom. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Both systems exhibited activity of the same order, however, PNIPAAm-HydroUr reached the maximum value in the first 1 h/cycle (Table I, PHy1) and, then, a gradual decrease was observed. Such behavior might be attributed to a progressive accumulation of reaction products into the hydrogel that leads to inhibition and decreased reaction rate, respectively. The hydrolysis of urea by PNIPAAm-CryoUrW seems to be unaffected by the reaction product obtained for incubation periods of 1 h and 24 h, respectively. Evidently, the existence of interconnected macroscopic pores alleviates the diffusion and, thereby, the removal of ammonium ions. However, the system has higher activity at cycles of 24 h incubation (Table I, PCr1) when compared with the cycles of 1 h (Fig. 5). One may speculate that urea molecules need more time to access urease located into the dense cryogel walls. Overall, the system based on cryogel exhibited better potential for hydrolysis of urea by means of repeated use than the hydrogel containing the same amount of enzyme. Importantly, the cryogels remained compact after repeated use and no ruptures were observed at all. Moreover, the freeze dried system was very stable with time and regained the same performance after several weeks' storage.

One substantial difference between the properties of cryo- and hydrogel is the rate of water diffusion through the gel. A simple test performed by placing the gels as a membrane (diameter = 60 mm; thickness = 5 mm) on a filtration set showed that the spongy-like PNIPAAm cryogel has a debit of 150 ± 10 mL/h at 20°C, which is orders of magnitude higher than the debit of hydrogel. Therefore, the ability of PNIPAAm-CryoUrW to hydrolyze urea in a continuous flow regime was of particular interest. In this study, the urea solution (3.2 g/L) was added continuously to the column-type reactor (Fig. 6) at a flow rate of 10 mL/h at physiological temperature. The activity, calculated on the bases of ammonium liberated after urea hydrolysis by the specimens containing totally ~ 6 mg urease, was equal to 0.9×10^2 μmol/min at the experimental conditions reported. Since the ammonium ions are toxic and nondesired species, to remove them an additional layer of "Silicagel" was placed on the bottom of reactor. Thus, the positively charged NH^{4+} was quantitatively absorbed, as confirmed by measuring a zero absorbance value.

This simple experiment shows that PNIPAAm-CryoUrW can be used as membrane for direct removal of urea form the feed solution in a continuous flow regime which make this material attractive for treatment of contaminated water, blood detoxication, dialysate regeneration system of artificial kidneys, removal of urea from beverages, etc.

One should mention, that the method of *in situ* immobilization reported in the present work is not restricted only to the entrapment of urease. It could work with other enzymes which have molecular weight (hence, hydrodynamic volume) significantly higher when compared with the substrate and reaction products.

CONCLUSION

In this study, we demonstrated a facile strategy for noncovalent entrapment of urease into PNIPAAm cryogel. The method of cryotropic gelation allowed immobilization of enzyme into the dense cryogel walls of the spongy-like matrix, thus, achieving a nearly quantitative retention of urease within the carrier. The system exhibited a constant enzyme activity after several hydrolysis reactions in batch reactor, in contrast to conventional hydrogel, which is attributed to the unhindered diffusion of substrate and reaction products through the interconnected macroscopic pores. Moreover, the open-porous structure of PNIPAAm-CryoUrW gave the opportunity to tune an appropriate debit of liquids in a continuous-flow column reactor, which make this material attractive for up-scale and industrial use.

Thanks to Prof. Bojidar Chorbanov for fruitful discussion.

References

1. Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. Enzyme Microb Technol 2007, 40, 1451.
2. Brena, B. M.; Batista-Viera, F. In Immobilization of Enzymes and Cells, 2nd ed.; Guisan, J. M., Ed.; Humana Press: Totowa, NJ, 2006; p 15.
3. Sheldon, R. A. Adv Synth Catal 2007, 349, 1289.
4. Gabrovska, K.; Georgieva, A.; Godjevargova, T.; Stoilova, O.; Manolova, N. J Biotechnol 2007, 129, 674.
5. Lehtonen, O.; Viljanen, M. J Immunol Methods 1980, 34, 61.
6. Chellapandian, M.; Sastry, C. A. Bioprocess Eng 1994, 11, 7.
7. Chellapandian, M.; Krishnan, M. R. V. Process Biochem 1998, 33, 595.
8. Dumitriu, S.; Popa, M.; Artenie, V.; Dan, F. Biotechnol Bioeng 1989, 34, 283.
9. Tyagi, C.; Tomar, L. K.; Singh, H. J Appl Polym Sci 2009, 111, 1381.
10. O'Driscoll, K. F. In Methods in Enzymology; Mosbach, K., Ed.; Academic Press: New York, 1976, p 169.
11. Uhlich, T.; Ulbricht, M.; Tomaschewski, G. Enzyme Microb Technol 1996, 19, 124.
12. Kara, F.; Demirel, G.; Tümtürk, H. Bioprocess Biosyst Eng 2006, 29, 207.
13. Gil, E. S.; Hudson, S. M. Prog Polym Sci 2004, 29, 1173.
14. Dimitrov, I.; Trzebicka, B.; Mueller, A. H. E.; Dworak, A.; Tsvetanov, C. B. Prog Polym Sci 2007, 32, 1275.
15. Chen, J.-P.; Chiu, S.-H. Enzyme Microb Technol 2000, 26, 359.
16. Dong, L. C.; Hoffman, A. S. J Controlled Release 1986, 4, 223.
17. Zhang, X.-Z.; Yang, Y.-Y.; Chung, T.-S. J Colloid Interface Sci 2002, 246, 105.
18. Petrov, P.; Petrova, E.; Tsvetanov, C. B. Polymer 2009, 50, 1118.
19. Herbert, D.; Phipps, P. J.; Strange, R. E. In Methods in Microbiology; Norris, J. R.; Ribbons, D. W., Eds.; Academic Press, London/New York, 1971, p 209.
20. Lozinsky, V. I. Russ Chem Rev 2002, 71, 489.