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Mendeleev Commun., 2023, **33**, 559–561

Mendeleev Communications

Temperature behavior of glucose oxidase immobilized into surface-attached stimuli-sensitive copolymer microgel

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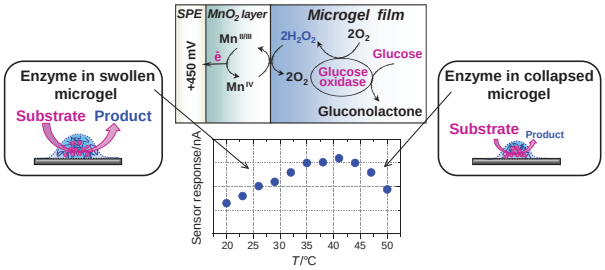
DOI: 10.1016/j.mencom.2023.06.038

Electrochemical responses of glucose oxidase loaded (*via* electrostatic immobilization) into a surface-attached pH- and temperature-sensitive copolymer microgel were examined. The observed temperature behavior of the immobilized enzyme provides evidence that such systems enable pH-dependent regulation of activity of glucose oxidase by a (repeated) temperature cycling, which reversibly transforms the polymeric (microgel) matrix from the swollen state to the collapsed one.



Keywords: microgel, stimuli-sensitivity, poly(*N*-isopropylacrylamide-*co*-*N*-(3-aminopropyl)methacrylamide), enzyme immobilization, enzymatic activity regulation, glucose oxidase.

Enzymes immobilized into polymeric matrices are widely involved in green and sustainable biotechnological production of various fine chemicals, used in biosensing technologies, and applied as food additives, gentle washing agents, *etc*.[1,2] Among various polymers exploited as matrices for enzyme immobilization, stimuli-sensitive microgels based on poly(*N*-isopropylacrylamide) (PNIPAM)[3,4] – a polymer exhibiting a lower critical solution temperature behavior in aqueous solutions[5] – are of particular interest as they can reversibly change their properties and characteristics (*e.g.*, size, swelling, and mesh size) upon temperature variation.

When PNIPAM-based microgels are functionalized with chargeable moieties, they are sensitive not only to temperature variations but also to changes in the pH of the surrounding aqueous solution, thereby becoming double stimuli-responsive.[6,7] More specifically, such microgels demonstrate pronounced pH-dependent temperature-sensitivity, wherein their charging strongly suppresses the response to temperature changes.[8] Furthermore, microgels with chargeable moieties (including but not limited to the PNIPAM-based ones) can (reversibly) bind considerable amounts of various oppositely charged payloads, possessing a high capacity towards both low- and high-molecular-weight guests.[9–15]

Due to their highly hydrated interior, PNIPAM and PNIPAM-based microgels are desired polymeric matrices for secure immobilization of enzymes, preserving (and in some cases even enhancing) their enzymatic activities.[8,16–20] In this context, one can mention our works on capacious electrostatic loading of PNIPAM-based microgels with enzymes, which enabled fabrication of thin microgel–enzyme films and development of advanced enzymatic biosensor systems.[8,18–20]

It is worth noting that secure immobilization of enzymes into polymeric (micro)gels in most cases happens gently (without any loss of enzymatic activity), that is, without deformation of the enzyme's active site or dissociation of a multi-subunit enzyme into subunits. However, this appears to be not sufficient to meet the ever-increasing biological and industrial demands. Even more important and highly needed nowadays is reversible temperature-mediated regulation of enzymatic activity during typical enzymatic applications in bioengineering and chemical industry.[21] Some examples of such regulation in temperature-sensitive (micro)gel matrices in aqueous media include (but not limited to) β-galactosidase,[22] α-chymotrypsin,[23,24] alkaline phosphatase,[24] pepsin,[25] albumin,[26] lipase B,[27] glucose oxidase and complex systems derived therefrom.[28,29]

Herein, we demonstrate that the PNIPAM-based microgel functionalized with weak cationic moieties can be successfully exploited for engineering of microgel–enzyme systems with regulated enzymatic activity. Electrostatic attraction can be used as a binding principle for immobilization of enzymes, provided that the enzyme is oppositely charged with respect to the microgel, while temperature can be applied to induce a collapse of the microgel, which is expected to block an active site of the immobilized enzyme to a substrate. Specifically, we highlight pH-dependent temperature regulation of enzymatic activity of glucose oxidase (GO), which is immobilized (*via* electrostatic immobilization) into the surface-attached pH- and temperature-sensitive cationic PNIPAM-based microgel. The microgel, P(NIPAM-*co*-APMA), was synthesized *via* precipitation polymerization of *N*-isopropylacrylamide (NIPAM) and a cationic comonomer *N*-(3-aminopropyl)methacrylamide (APMA) in the presence of a cross-linker *N*,*N*′-methylene bisacrylamide as described elsewhere[30] and contains about 16 mol% of the cationic comonomer (APMA) units.

According to potentiometric titration, the P(NIPAM-*co*-APMA) microgel transforms from a fully protonated (charged) state to a fully deprotonated (uncharged) state upon increasing pH from pH 5.2 ($\alpha = 1$) to pH 10.3 ($\alpha = 0$),[30] where α denotes the degree of protonation. The hydrodynamic size of the P(NIPAM-*co*-APMA) microgel in aqueous media (measured by means of dynamic light scattering) is determined by the pH and temperature. Even a partial deprotonation of the P(NIPAM-*co*-

APMA) microgel from $\alpha = 1$ to $\alpha = 0.45$ results in a pronounced decrease in its hydrodynamic radius (R_h) from 113 ± 4 nm to 101 ± 2 nm.[30] A rise of temperature induces a collapse of the P(NIPAM-*co*-APMA) microgel, which becomes more and more pronounced upon decreasing the charge of the microgel. Indeed, the temperature change from 20 to 50 °C causes R_h-value of the P(NIPAM-*co*-APMA) microgel to decrease by about 20 nm (≅ 15%) at $\alpha = 1$ and by about 35 nm (≅ 30%) at $\alpha = 0.45$.[30] These findings indicate that at low pH and low temperatures the microgels are hydrophilic and highly swollen, while they are considerably dehydrated and collapsed at high pH and elevated temperatures.

At elevated temperatures, the P(NIPAM-*co*-APMA) microgels being considerably dehydrated and collapsed can aggregate provided that their aqueous dispersions are sufficiently concentrated. Such aggregation is manifested by a clear decrease in transmittance of the dispersions as evidenced by means of turbidimetric measurements (Figure 1). It is worth noting that the observed decrease in transmittance appears to be fully reversible if the P(NIPAM-*co*-APMA) microgel retains a considerable charge ($\alpha > 0.45$). When the microgels are sufficiently discharged, their aqueous dispersions exhibit incomplete restoring of transmittance as clearly demonstrated in Figure S1 (Online Supplementary Materials) by a heating–cooling curve at pH 9.5 and even irreversible macroscopic phase separation (precipitation) could be observed at higher pH values.

The GO (MW = 160 kDa) from *Aspergillus niger* (E.C. 1.1.3.4, activity 168 100 U g^{-1} of solid) was taken as a model enzyme because of its high thermal stability and a low isoelectric point (pI = 4.2).[31] At pH > pI, globules of GO bear a total negative charge and therefore can be loaded into the P(NIPAM-*co*-APMA) microgel due to the electrostatic interaction with its cationic comonomer (APMA) units.

The enzymatic response of GO immobilized into the P(NIPAM-*co*-APMA) microgel was measured electrochemically as described elsewhere.[8] Shortly, P(NIPAM-*co*-APMA)/GO constructs were fabricated onto graphite-based screen-printed electrodes (modified by a thin mediator layer composed of manganese dioxide nanoparticles)[32] *via* a two-step procedure. First, the microgel was adsorbed onto the electrode's surface at high pH (pH 9.5) and elevated temperature (50 °C). As we showed previously,[30] these conditions enable superior surface modification of the graphite-based screen-printed electrodes. Then, GO was loaded into the microgel film under 'mild' (non-destructive) conditions (pH 7, 25 °C), thereby building up the microgel–enzyme construct. The enzymatic response of GO was assayed by recording the oxidative current in response to addition of β-D-glucose at an applied potential of +450 mV *versus* Ag/AgCl[8] (more details can be found in Online Supplementary Materials).

The temperature dependences of the enzymatic responses of GO for the fabricated P(NIPAM-*co*-APMA)/GO constructs at three different pH-values and the corresponding Arrhenius plots are presented in Figure 2. In the low-temperature range, they increase with temperature as expected because elevating temperature accelerates the enzymatic reaction [Figure 2(*a*)]. From the Arrhenius plots [Figure 2(*b*)], one clearly sees that the low-temperature parts of these dependences can be linearized and their slopes, which are related to activation energies of the enzymatic reaction, decrease with increasing pH of the electrochemical assay. This decrease can result from possible pH-induced changes of conformation of the enzyme's active site, thereby influencing the enzymatic activity and its temperature behavior.

Much more remarkable for the temperature dependences of the enzymatic responses of GO immobilized into the P(NIPAM-*co*-APMA) microgel is their distinct decrease with temperature in the high-temperature range. As is seen, it happens when temperature exceeds a certain value [Figure 2(*a*)], which corresponds to a breakpoint (T_{bp}) in the corresponding Arrhenius plot [Figure 2(*b*)]. Such "unexpected" behavior can reasonably be associated with transformation of the microgel from the swollen state to the collapsed one. At temperatures close to T_{bp}, the microgels are found to be collapsed (shown by means of dynamic light scattering)[30] and at sufficient concentration can undergo aggregation (shown by means of turbidimetry) (Figure 1).

Furthermore, the values of T_{bp} shift to lower temperatures at higher pH values, while the linearized high-temperature parts in the Arrhenius plots gradually become steeper with increasing pH of the electrochemical assay [Figure 2(*b*)]. Hence, the effect of the microgel collapse on the enzymatic responses of GO becomes more pronounced with increasing pH. These findings allow us to conclude that the observed deviations from the Arrhenius

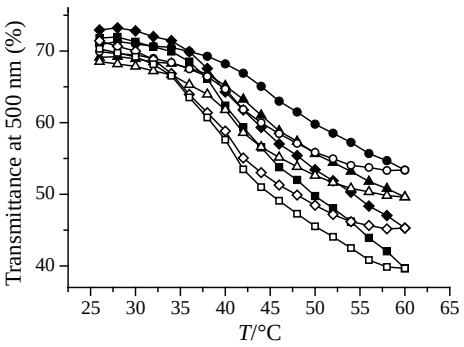


Figure 1 Temperature-induced changes in transmittance of a 0.5 g dm^{-3} P(NIPAM-*co*-APMA) microgel dispersion in 10 mM TRIS at different pH values: pH 7.5, T_{CP} = 44 °C, $\alpha = 0.89$ (circles); pH 8, T_{CP} = 43 °C, $\alpha = 0.84$ (triangles); pH 8.5, T_{CP} = 40 °C, $\alpha = 0.76$ (diamonds); pH 9, T_{CP} = 39 °C, $\alpha = 0.62$ (squares). Solid and open symbols correspond to heating and cooling, respectively. The cloud point (T_{CP}) for each pH was determined as a minimum of the derivative of the heating part of the corresponding transmittance-temperature curve. The values of the degree of protonation (α) were extracted from the potentiometric titration data.[30]

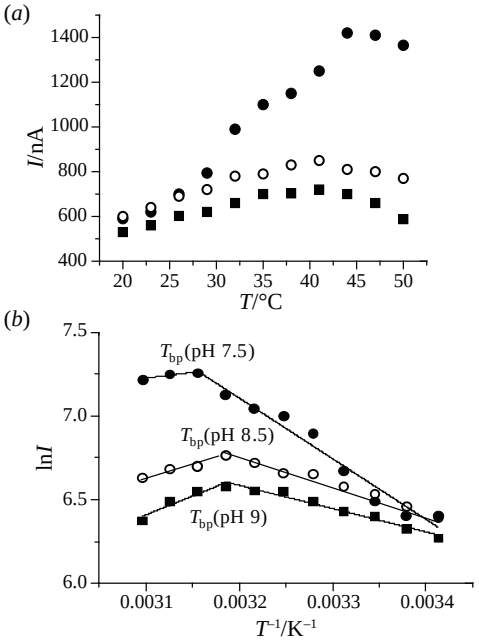


Figure 2 (*a*) Temperature dependences of the electrochemical responses of the P(NIPAM-*co*-APMA)/GO constructs on addition of a 0.2 mM β-D-glucose solution at pH 7.5, $\alpha = 0.89$ (solid circles), pH 8.5, $\alpha = 0.76$ (open circles), pH 9, $\alpha = 0.62$ (solid squares) and (*b*) the corresponding Arrhenius plots. The values of T_{bp} were found to be 44, 41 and 40.5 °C at pH 7.5, 8.5 and 9, respectively.

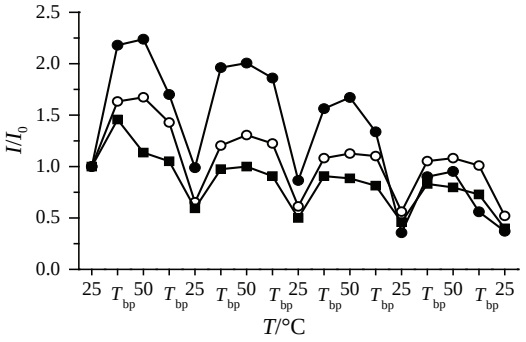


Figure 3 Evolution of the electrochemical responses of the P(NIPAM-*co*-APMA)/GO constructs measured on addition of a 0.2 mM β-D-glucose solution at pH 7.5, α = 0.89 (solid circles), pH 8.5, α = 0.76 (open circles), pH 9, α = 0.62 (solid squares) upon repeated temperature cycling 25 °C → T_{bp} → 50 °C → T_{bp} → 25 °C. The electrochemical responses were normalized by the first measurement.

behavior of the enzymatic responses of GO (which are proportional to its enzymatic activity) for the fabricated P(NIPAM-*co*-APMA)/GO constructs is first and utmost caused by temperature-sensitivity of the polymer (microgel) matrix.

To perform repeated temperature regulation of enzymatic activity of GO immobilized into the P(NIPAM-*co*-APMA) microgel and reveal if such regulation is reversible at different pH values, the enzymatic responses were measured in a multiple cyclic mode by varying temperature as follows 25 °C → T_{bp} → → 50 °C → T_{bp} → 25 °C. As is seen, the results given in Figure 3 conceptually (a proof-of-principle) confirm that such repeated regulation of the enzymatic activity is possible. The suppression of the Arrhenius behavior of the enzymatic responses of GO immobilized into the P(NIPAM-*co*-APMA) microgel at high temperatures is reproduced in each temperature cycle. These findings strongly suggest that the temperature effect on the enzymatic activity is modulated by temperature-induced deswelling–swelling of the polymer (microgel) matrix.

A certain gradual lowering of the corresponding enzymatic responses in each temperature cycle is found with the rising number of repetitions. Most probably, this lowering of the enzymatic responses from one to the next temperature cycle and further can be caused by slow thermal inactivation of the enzyme during such long-time experiments. A release of a certain fraction of immobilized GO from the microgel film at higher pH values (pH 9 and pH 8.5) or desorption of a certain faction of the microgels loaded with enzymes from the electrode's surface at a lower pH value (pH 7.5) might be also feasible and could explain the observed overall decrease in the enzymatic responses upon the performed multiple repetitions.

Thus, we highlight herein the beneficial application of the stimuli-sensitive P(NIPAM-*co*-APMA) microgel for engineering of microgel–enzyme systems with regulated enzymatic activity. Such systems enable control of the immobilized enzyme's activity by a (repeated) temperature cycling, which reversibly transforms the polymeric (microgel) matrix from a swollen state to a collapsed one. When the microgel changes to the collapsed state ($T > T_{bp}$), the enzymatic activity decreases, while it appears to restore (albeit partially for the considered system) upon lowering temperature.

This work was supported by the Russian Science Foundation (RSF) within project no. 22-24-00424. We are grateful to Dr. M. Brugnoni (Institute of Physical Chemistry II, RWTH Aachen University, Germany) for the synthesis of the P(NIPAM-*co*-APMA) microgel, which was funded by the Deutsche Forschungsgemeinschaft (DFG) within SFB 985 'Funktionelle Mikrogele und Mikrogelsysteme' (projects A3 and A6).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.06.038.

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Received: 19th January 2023; Com. 23/7086