VERSATILITY OF THERMOSENSITIVE PARTICLES

Haruma Kawaguchi* Kohki Kisara, Tomomi Takahashi, Kohji Achiha, Mamoru Yasui, and Keiji Fujimoto

Department of Applied Chemistry, Faculty of Science & Technology, Keio University, Yokohama 223-8522 Japan

Abstract: Poly(N-isopropylacrylamide) (PNIPAM) particles are highly swollen below the transition temperature in water so that they become very soft and hydrophilic, but collapsed above the transition temperature to become hard and hydrophobic. Such changes bring about the discontinuous temperature dependence of adsorbability of protein, activity of immobilized enzyme, cell activating ability of the particles. The transition temperature of PNIPAM was changed by binding something to the chain end and this property was used to control the enzyme activity with temperature. Sudden change in the topology of the particle surface by quick temperature change gave excess stimulus to cells when the change was transmitted straightly to the cells.

INTRODUCTION

"Thermosensitive polymer" is defined as a polymer which exhibits discontinuous response to temperature change. Many polymers such as acrylamide derivatives, polyethyleneglycol-containing polymers, and cellulose derivatives satisfy this criterion (1). Among them, poly(N-isopropylacrylamide) (PNIPAM) is the most representative thermosensitive polymer, having its transition temperature or the lower critical solution temperature (LCST) at 32℃. Microspheres composed of PNIPAM could be prepared by precipitation polymerization (2) or soap-free emulsion copolymerization (3). The microspheres and their dispersions can be used for various purposes such as an adsorbent, a transducer, a coagulant, etc. whose performance is controlled with temperature. When biospecific components are combined to such microspheres, the resulting hybrid microspheres gain more functions. In this paper the versatility of PNIPAM microspheres is discussed focusing on their biomedical applications.

PROTEIN ADSORPTION ON THERMOSENSITIVE PARTICLES

PNIPAM beads have been used for protein concentration and separation (4). Proteins that have no affinity with PNIPAM can be concentrated in its aqueous solution because water is selectively absorbed into the polymer. Specific proteins which have affinity with PNIPAM can be separated from others by being selectively absorbed into the polymer. After these operations the PNIPAM gel beads are heated to be collapsed so that they can be re-used.

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Adsorption of any protein onto PNIPAM microspheres is more or less sensitive to temperature. It would be attributed mainly to the change in hydrophilicity/hydrophobicity of the surface with temperature because proteins are adsorbed on hydrophobic surfaces preferably in general and thus adsorbed more on PNIPAM microspheres at a temperature above 32℃ than below it. Therefore, the proteins once adsorbed on PNIPAM microspheres at a high temperature could be desorbed more or less when the system is cooled down to room temperature as shown in Table 1 (5). This was not the case when polystyrene microspheres are used as an adsorbent on which a large amount of proteins were adsorbed regardless of the temperature. Polystyrene microspheres did not release the once-adsorbed proteins when the temperature was decreased.

Table 1 Adsorption of protein at 37℃ and desorption at 25℃ on two kinds of particles

Particle	Protein	Isoelectric point	Amount (A) of protein adsorbed at 37℃ (μg/g particle)	Amount (B) of protein desorbed when cooled to 25℃ (μg/g particle)	B/A
PSt	Globulin	6.8	8.5	<0.1	
PNIPAM	Globulin	6.8	2.4	1.0	0.42
PNIPAM	Albumin	5	1.9	1.5	0.79
PNIPAM	Lysozyme	11	0.40	0.06	0.15

Particle: PSt Polystyrene, PNIPAM Poly(N-isopropylacrylamide). Both are anionic particles
Experiment was carried out at pH 7.0.
The temperature was down to 25℃ after 30 min. incubation at 37℃.

Albumin was adsorbed appreciably at 37℃ and most of them is desorbed on cooling. In contrast, hydrophilic proteins such as lysozyme was adsorbed in a small amount at 37℃, even though the electrostatic force between the surface and protein was favorable for the adsorption, and desorbed a little when cooled. The adsorption and desorption depending on the temperature can be used in protein collection on and release from the adsorbent microspheres. Enzymes were collected after the enzymatic reaction and released for the re-use in PNIPAM dispersion. Because PNIPAM surface is much softer than polystyrene surface even above the transition temperature, an enzyme desorbed from PNIPAM microsphere retained higher activity than that from polystyrene microspheres.

It should be mentioned that there is another factor to strongly control the protein adsorption at different temperatures. That is the electrostatic force which comes from the difference between the surface potentials of PNIPAM microspheres and protein. Therefore the adsorption and desorption were affected by pH, ionic strength and temperature. Lowering temperature decreased the charge density in PNIPAM shell and so the apparent surface potential.

THERMAL BEHAVIOR OF ENZYME CARRIER

An enzyme, trypsin, was immobilized chemically onto different kinds of PNIPAM shell particles and the temperature dependence of the activity of immobilized enzyme was studied. The enzyme activity is affected by the diffusivity of substrate through PNIPAM layer as well as by the immobilized mode and density of enzyme. The temperature dependence of the diffusion of low molecular weight materials through PNIPAM layer has been studied by measuring the reaction rates of reduction and oxidation between oxidant (or reductant) in the core and reductant (or oxidant) in the medium as a function of temperature. For example, ascorbic acid in water diffused through PNIPAM shell layer to reach ubiquinone in the core (6). The diffusion rate was high at low temperature and decreased with increasing temperature. This could be explained by the temperature dependent density of PNIPAM layer. Namely, above the transition temperature, the dense PNIPAM layer rejected the permeation of water-soluble reductant or oxidant. It must be mentioned that rise in temperature also brought about hydrophobization of PNIPAM layer and consequently decreased the solubility of water-soluble molecules in PNIPAM.

In the system of trypsin-immobilizing crosslinked PNIPAM microsphere, the activity of trypsin was measured as a function of temperature(7). The relative enzyme activity decreased with increasing temperature. This was attributed to the diffusion effect above mentioned.

But the situation was drastically changed when the enzyme carrier had different architectures. PNIPAM bearing carboxyl group(s) at the chain end(s) was prepared in solution polymerization and chemically bound to latex particle. Trypsin was immobilized at the other end of some PNIPAM chains. Coexisting two kinds of PNIPAM chains, trypsin-carrying and free chains, on the microspheres had different transition temperatures as shown in Fig. 1 A (8). Therefore, free chains collapse at lower temperature than trypsin-carrying chains and so trypsin is exposed toward the aqueous medium above the transition temperature of free-end PNIPAM chain as shown in Fig. 1B. Trypsin can encounter the substrate easily under a such condition and exhibit higher enzymatic activity. Expected results were obtained as shown in Fig. 2. However, the enzymatic activity started to increase from a temperature slightly lower than the transition temperature of free-end PNIPAM chain. This temperature shift was supposed to result from n-clustering of free-end PNIPAM chains (9).

Such an increase in reactivity with increasing temperature could be observed in the system of large size substrate / concentrated PNIPAM chains on microspheres. Small size substrates / thinly bound PNIPAM system do not suffer such a steric effect caused by coexisting free-end PNIPAM chains. The substrate-size effect was clearly shown with two curves in Fig. 2. That is,

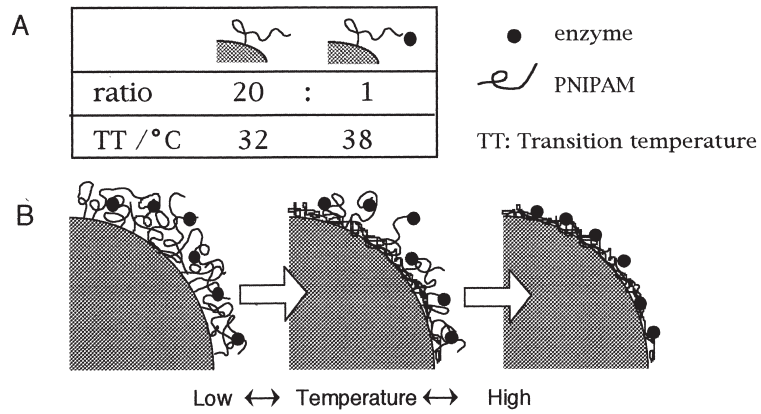

Fig. 1 State of surface on which two kinds of PNIPAM chains exist.

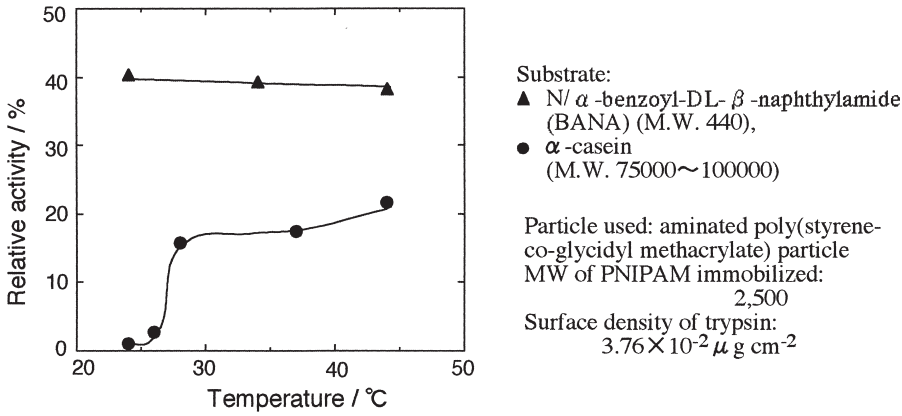

Fig. 2 Effect of temperature and molecular size of substrate on enzymatic activity.

the lower curve for large substrate presented significant temperature dependence but the upper curve for small substrate did not.

CELL ACTIVATION IN PNIPAM MICROSPHERES DISPERSION

When granulocytes were stimulated by external materials, the cells consume oxygen dissolved in the medium and produce active oxygen. The cells were incubated in a dispersion of polymeric microspheres and the amount of active oxygen was measured to assess the cell-stimulating power of polymeric microspheres (10). The cells produced a large amount of active oxygen when mixed with polystyrene microspheres dispersion. On the contrary, PNIPAM microspheres system presented quite different performance.

The microspheres were almost inert at room temperature and gave a slight stimulus to cells at 37 ℃. The stimulus at 37℃ in PNIPAM microspheres dispersion was, however, much smaller than in polystyrene system. These results seem to be brought about by the differences in hydrophobicity and/or hardness of the particles at different temperatures. No excess response was observed when the temperature of the system was suddenly changed from room temperature to 37℃.

CELL ACTIVATION BY 2-DIMENSIONAL MICROSPHERES ASSEMBLY

PNIPAM microspheres were deposited on a plate to produce a 2-dimensional array of the microspheres. Once the plate of microspheres assembly was dried, the microspheres were never detached even the plate was shaken in water. Differing from floating microspheres, the fixed microspheres gave stronger stimulus to the cells when contacted (11).

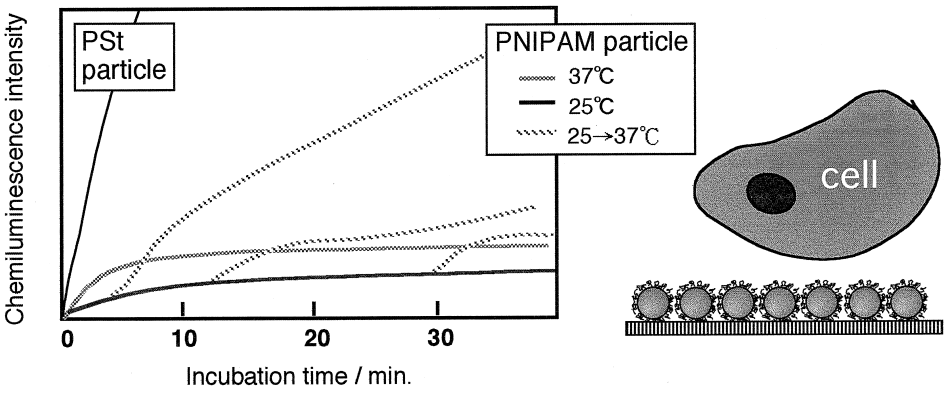

Fig. 3 Cell activation on PNIPAM particles assembly at constant and varied temperatures

Unique phenomenon was observed when the temperature was suddenly changed from 25℃ to 37℃, that is, cells released an excess amount of oxygen as shown with dotted lines in Fig. 3, which did not approach the curve for 37℃ but passed over it. It was attributed to the stimulus by dynamic motion of PNIPAM shell of microspheres resulting from PNIPAM's volume phase transition. Therefore, such phenomenon was not observed on a flat PNIPAM surface or an array of non-thermosensitive polystyrene microspheres.

CELL ACTIVATION WITH AID OF LIGAND / RECEPTOR INTERACTION

Different types of thermosensitive microspheres were prepared by precipitation polymerization of NIPAM with a small amount of acrylamide and methylenebisacrylamide. The diameter of a dry microsphere was 300 nm. The hydrodynamic diameter of the microsphere changed significantly with temperature through the transition temperature. The microspheres were aminated by the Hoffman reaction and then a cell-adhesive tetrapeptide, RGDS, was bound chemically to the thermosensitive microspheres. The transition temperature of RGDS-carrying microspheres were eventually the same as that of the parent microspheres. The hybrid microspheres thus obtained (coded as NA-RGDS) were incubated with cells at different temperatures to examine the quality and quantity of the stimulus which the hybrid microspheres give to the cells.

When NA-RGDS microspheres were incubated with cells, the former attached the latter through RGDS / integlin binding. The cells then started to produce active oxygen in response to the stimulus transferred to the cell skeleton.

No detection of active oxygen from NA-RGDS / cell dispersion in Mg-free medium is one of strong evidences for the contribution of RGDS-integlin interaction to active oxygen production. Microspheres having no RGDS gave no stimulus to cells at 25℃ and a little at 37℃ as supposed from the results presented in the previous section.

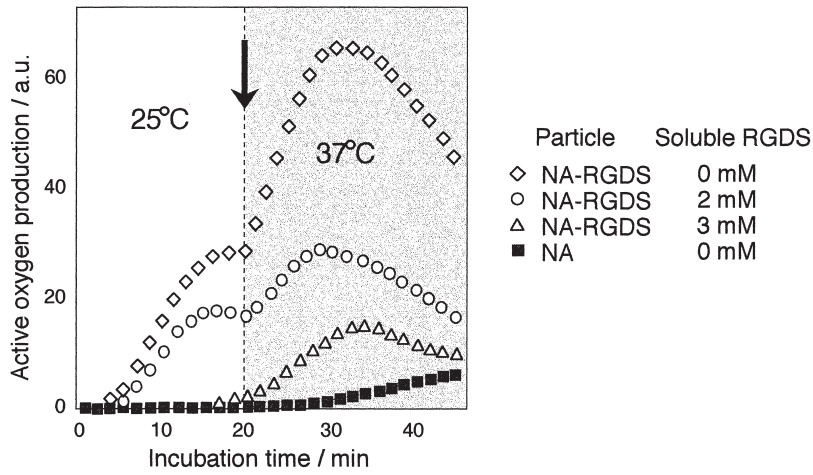

Fig. 4 Effect of temperature and soluble RGDS on active oxygen production caused by NA-RGDS / cell interaction.
The temperature was changed from 25℃ to 37℃ at 20 min.

The amount of released active oxygen resulting from NA-RGDS stimulation was dependent on the temperature and the existence of soluble free RGDS which competed with the on-particle RGDS in binding integlin but did not cause the production of active oxygen. The effect of the competitor on cell activation is shown in Fig. 4. The results in Fig. 4 indicate that the free RGDS effectively blocked the integlin from the attack by NA-RGDS.

Interesting phenomena were observed when the temperature of NA-RGDS microspheres / cell dispersion was changed from 25℃ to 37℃. As shown by the tracks with diamonds in Fig. 4, NA-RGDS microspheres induced excessive activation of cells when heated. The excessive activation in present system was attributed to thermosensitive microsphere-modulating stimulus, that is, sudden conformational change of thermosensitive polymer chains carrying RGDS caused clustering of receptor or gave mechanical stress to cell skeleton as shown in Fig. 5.

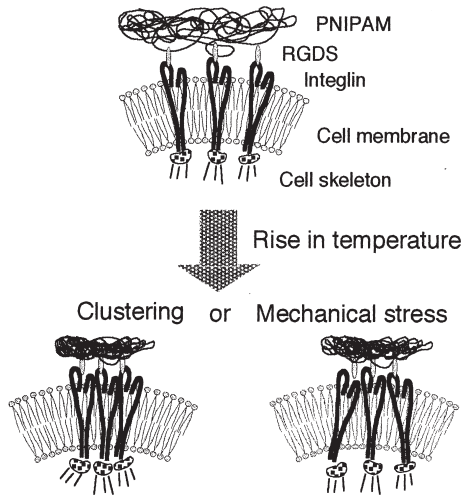

Fig. 5 Possible mechanisms for signal transduction through RGDS/ thermosensitive polymer - integlin hybrid.

CONCLUSIONS

Adsorption of proteins on poly(N-isopropylacrylamide) (PNIPAM) microspheres was controlled with temperature. Activity of enzyme immobilized in or on the PNIPAM shell layers was also controlled with temperature. The enzyme immobilized at the chain end of PNIPAM hair showed unique temperature-dependent activity due to the different thermal properties of PNIPAM hairs having different substituents at their chain ends. Three kinds of PNIPAM microsphere systems, that is, PNIPAM microspheres dispersion, 2-dimensional PNIPAM microspheres assembly, and cell-adhesive peptide-, RGDS-, carrying PNIPAM microspheres, were contacted with cells. The latter two gave an excess stimulus to the cells when heated suddenly because sudden topological change of particle surface is transmitted straightly to the cells.

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