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A Novel Immunoassay System and Bioseparation Process Based on Thermal Phase Separating Polymers

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ABSTRACT

Poly-*N*-isopropylacrylamide (poly*N*IPAAm), a water-soluble, thermally precipitating synthetic polymer, has been conjugated together with a monoclonal antibody (MAb) and utilized in a novel separation method for an immunoassay. The Poly*N*IPAAm precipitates out of water above a critical temperature of 31°C, enabling a polymer-bound immune complex to be separated from the solution. The principal advantages of this method are that it utilizes a homogeneous incubation for the antigen–antibody reaction, plus, it has the ability to assay large-molecular-weight antigens with sensitivities equivalent to other nonisotopic heterogeneous immunoassays. In addition, since the polymer–immune complex may be reversibly redissolved by cooling, the method may be used both to concentrate the signal and isolate the analyte. This general technique may also be used for a wide variety of separation processes in addition to immunoassays, in which a specific component in a biological fluid, industrial process stream, or body of water is to be isolated for analysis, recovery, or disposal. Thus, product recovery and/or toxin or pollutant removal processes are possible with this methodology.

Index Entries: Immunoassay; antibody-polymer conjugates; thermal phase separating polymers; poly*N*-isopropyl acrylamide; antigen isolation; bioseparation; competitive enzyme immunoassay; sandwich enzyme immunoassay; antigen capture fluorescence immunoassay.

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INTRODUCTION

Immunoassays have found widespread applications in the field of clinical diagnostics for the detection and measurement of drugs, vitamins, hormones, proteins in general, metabolites, microorganisms, and other substances of interest in biological fluids, as well as in process streams and environmental waters (*1–3*). Immunoassays can be divided into two general categories, homogeneous and heterogeneous.

In a homogeneous immunoassay, the signal emitted by the specifically bound, labeled reactant in solution is different from the signal emitted by the free, labeled reactant, also in solution. Hence, bound and free reactant (or analyte) can be distinguished without physical separation. A typical homogeneous assay is the enzyme mediated immunoassay test (EMIT®). Homogeneous immunoassays have the advantages of being rapid, easy to perform, and readily amenable to automation. Their principal disadvantages are that they are prone to interferences, are relatively low in sensitivity, and are mainly limited to lower-molecular-weight analytes (*4*).

In a heterogeneous immunoassay, on the other hand, the signal emitted by the bound, labeled reactant is indistinguishable from the signal emitted by the free, labeled reactant; therefore, a separation step is required to distinguish between the two. Typical heterogeneous immunoassays include the solid-phase radioimmunoassay (RIA) and the enzyme-linked immunosorbant assay (ELISA) (*1–3*). Most of the heterogeneous immunoassays employ at least one reactant immobilized on a solid phase. Since the kinetics of reaction between an immobilized antibody (or antigen) and its binding partner tend to be slower than the kinetics of the same reaction occurring in solution, relatively long incubation times are frequently required. Heterogeneous assays also tend to be time consuming and labor-intensive because of the requirement of multiple wash steps. However, they are generally more sensitive than homogeneous assays, less prone to inferences, and can be used with both low- and high-molecular-weight analytes. Solids used to immobilize reactants in immunoassays have included controlled pore glass and preformed polymers, such as polyacrylamides, polysaccharides (dextrans), and polystyrenes (*3*).

Numerous separation methods have been used in heterogeneous immunoassays. These include centrifugation, microfiltration, affinity chromatography, and gel-permeation chromatography (*3, 4*). Most of these are costly and time consuming. There is still a need for a heterogeneous immunoassay that is sensitive, rapid, economical, and readily amenable to automation. We describe here our novel, polymer-based immunoassay, called "precipitation immunoassay," or "PRECIPIA," which fulfills many of these requirements (*5*).

BASIS OF IMMUNOASSAY BIOSEPARATION TECHNIQUE

Certain water-soluble polymers are known to phase separate or precipitate when the temperature is raised to a critical solution temperature. This temperature, at which demixing occurs, is referred to as the lower critical solution temperature (LCST) (*6*, *7*). Among polymers that exhibit a lower critical solution temperature, poly-*N*-isopropylacrylamide(poly*N*IPAAm) has an LCST of about 31–33°C throughout a wide concentration range (up to ca. 5%). We have utilized this demixing behavior of poly*N*IPAAm at or above the LCST as the separation technique in our new immunoassay. The essential features of our method in a double antibody, antigen-capture assay include: (1) conjugating a first monoclonal antibody (MAb_1), which is specific to one epitope of an antigen (Ag), to the backbone of poly*N*IPAAm; (b) conjugating a second antibody (MAb_2), which is specific to a different epitope of the antigen, to a signal molecule, such as an enzyme [e.g., horseradish peroxidase (HRP)] or a fluorophore (e.g., fluorescein or phycoerythrin); (c) admixing in solution the polymer/first antibody conjugate, the biological fluid sample suspected of containing the Ag, and the second antibody/signal conjugate at a temperature below the polymer's LCST to form a "sandwich"-type immune complex in solution; (d) raising the temperature of the solution above the polymer's LCST to cause the polymer/immune complex sandwich to precipitate; and (e) measuring the amount of signal found in the precipitate to determine the concentration of the antigen. The precipitated polymer–immune complex may also be redissolved in cold PBS, and the signal in this solution is then assayed. A brief sketch of our assay is shown in Fig. 1.

MATERIALS AND METHODS

Preparation of Monomer Conjugated and Fluorescein-Labeled Monoclonal Antibody

A mouse MAb to the kappa light chain of human IgG was purified from ascites fluid by ion exchange chromatography and dialyzed overnight against 0.29*M* carbonate buffer, pH 9.3. To the dialyzed, 5-mg/mL solution, an eightfold molar excess of the *N*-acryloxysuccinimide (*N*ASI) was added (2 mg active ester/mL DMSO) by the method of Pollack et al. (*8*). The resultant mixture was incubated for 60 min at 36°C, then passed through a column of Sephadex G-25 that had been equilibrated with phosphate-buffered saline (PBS), pH 7.4. The fractions of the eluate that contained antibody were pooled and stored at −20°C.

An aliquot of this monomer-conjugated MAb (MAb_M) was further reacted with a 25-fold molar excess of fluorescein isothiocyanate, FITC

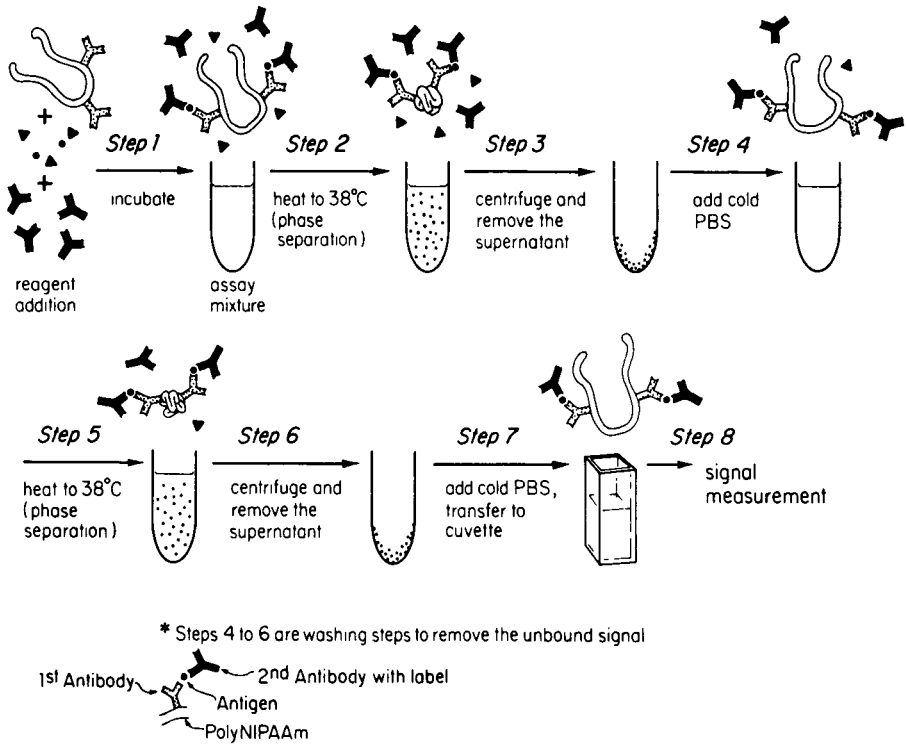

Fig. 1. Thermally induced precipitation immunoassay procedure ("PRECIPIA").

(isomer II, 10 mg/mL in DMSO). Excess, unconjugated FITC was removed by gel filtration on a Sephadex G-25 column. The resultant, double-conjugated MAb ($MAb_{M,F}$) was stored at −20°C.

Copolymerization of $MAb_{M,F}$ with *N*IPAAm

To 1.6 mL of 1.25% *N*IPAAm (w/v) in PBS, 0.2 mL of $MAb_{M,F}$ (8.4 mg/mL) was added. Polymerization was initiated using 0.1 mL of 100 m*M* ammonium persulfate (APS) and 0.1 mL of 0.8*M* *N,N,N′,N′*-tetramethyl ethylenediamine (TEMED). The reaction mixture was incubated for 3 h at room temperature, at which time the poly*N*IPAAm/MAb_F conjugate was isolated by one of two different procedures, as follows: (a) Isolation of poly*N*IPAAm/MAb_F conjugate by gel-permeation chromatography; the reaction mixture was applied to a Sephacryl S-300 gel permeation column (1.0 × 50 cm) to separate the polymer-conjugated MAb_F from the unbound $MAb_{M,F}$. The void volume fractions that contained poly*N*IPAAm/MAb_F conjugate (>10^6 molecular weight) were pooled and used for subsequent studies. (b) isolation of poly*N*IPAAm/MAb_F conjugate by serial precipitation; the poly*N*IPAAm/MAb_F conjugate was isolated by serially precipitating it from solution as follows: The reaction mixture (1% in poly*N*IPAAm) was diluted tenfold with PBS and aliquoted into 1.5-mL Eppendorf tubes. The tubes were incubated for 10 min at

37°C to precipitate the polymer, then centrifuged for 5 min at 4000*g* in a 37°C water bath. The precipitate was then redissolved in ice-cold PBS and the cycle repeated for a total of three times. After the third precipitation, the precipitate was dissolved in 1/10 the original volume of PBS. The contents of the tubes were pooled and stored at 4°C (1% poly*N*IPAAm/MAb$_F$).

Incorporation of MAb$_{M,F}$ into poly*N*IPAAm After a Single Thermal Precipitation

The MAb$_{M,F}$ was copolymerized with 1% *N*IPAAm under the same conditions as described above, except the antibody concentration was reduced to 100 μg/mL and the copolymerization time was increased to 24 h. The reaction mixture was incubated at 37°C for 10 min and then centrifuged for 5 min at 8000*g* at 37°C. The supernatant was withdrawn and the amount of fluorescence remaining in the solution determined. The difference in the amount of fluorescence before and after precipitation was expressed as %MAb$_{M,F}$ incorporated into the polymer.

Entrapment of Fluorescein-Labeled Bystander Monoclonal Antibody (MAb$_F$)

Concentrations used were the same as in the above experiment. The FITC conjugation of the MAb was carried out similar to the method described in the preparation of MAb$_{M,F}$, also described above.

Competitive Enzyme Immunoassay for the Quantitation of Mouse IgG

Mouse IgG standards were prepared in PBS/1% (w/v) BSA to the following concentrations: 0, 0.5, 5.0, and 50.0 μg/mL. A sheep anti-mouse IgG labeled with horseradish peroxidase (sαmIgG/HRP) was diluted 1:1000 in PBS/BSA. The Poly*N*IPAAm/MAb$_F$ conjugate was prepared as described above.

Fifty microliters each of mouse IgG standard, sαmIgG/HRP, and poly*N*IPAAm/MAb$_F$ were admixed with 350 μL of PBS/BSA. The resultant mixture was incubated for 30 min at room temperature to allow specific binding to occur. The polymer was then precipitated by incubating for 10 min at 37°C. The resultant precipitate was pelleted by centrifugation at 4000*g* for 5 min at 37°C, the supernatant was withdrawn, and the pellet redissolved in 1 mL of ice-cold PBS. This procedure was repeated two times, and the last pellet was redissolved in 200 μL of ice-cold PBS. A 25-μL aliquot of this solution was transferred to a microtiter well. One hundred microliters of substrate solution (1 mg *o*-phenylene diamine, OPD/mL in citrate–phosphate buffer, pH 5.0, containing 0.03% H_2O_2) was then added. The reaction mixture was incubated for 15 min at room temperature, and the reaction was stopped by

the addition of 50 μL 2.5*N* H_2SO_4. Absorbance was determined at 490 nm using a microELISA reader. The calibration curve for mouse IgG is expressed in terms *%B/Bo*. *B* is the fraction of the label bound to mouse IgG/poly*N*IPAAm conjugate in the presence of the indicated amount of mouse IgG. *Bo* is the fraction of the label bound to mouse IgG/poly-*N*IPAAm conjugate in the absence of mouse IgG.

Antigen Capture Fluorescence Immunoassay for Human IgG

A series of human IgG standards were prepared in PBS/BSA to the following concentrations: 0, 0.05, 0.10, 0.19, 0.375, and 0.75 μg/mL. A poly*N*IPAAm/α-human K MAb (2H1) conjugate (MAb unlabeled) was prepared as described above. An MAb specific for the gamma chain of human IgG, designated 3F6, was labeled with phycoerythrin (PE) (MAb_F).

The assay was performed as follows: To 300 μL of PBS/BSA was added the following reagents: 50 μL of poly*N*IPAAm/2H1 conjugate (4.5 μg MAb), 50 μL of 1% of poly*N*IPAAm (as a co-precipitating agent), 50 μL of IgG standard, and 100 μL of 3F6/PE (1 μg MAb). The reaction mixture was incubated for 60 min at room temperature to allow specific binding to occur. The temperature was then raised to 45°C for 10 minutes to precipitate the polymer. The resultant precipitate was pelleted by centrifugation at 4000*g* for 5 min at 37°C. The supernatant was withdrawn, and the precipitate was redissolved in 1 mL of ice-cold PBS. The temperature was again raised to 45°C to precipitate the polymer, the resultant precipitate was pelleted by centrifugation, the supernatant was withdrawn, and the pellet redissolved in 200 μL of ice-cold PBS. A 150-μL aliquot of the resultant solution was diluted into 1350 μL of PBS and the fluorescence measured in a fluorimeter (λ_{ex} 545 nm, λ_{em} 575 nm).

Sandwich Enzyme Immunoassay for Rabbit Anti-Mouse IgG

A series of rabbit anti-mouse (rαmIgG) standards was prepared by diluting an antiserum 1:20, 1:100, 1:500, 1:2,500, or 1:12,500 in PBS/BSA. A poly*N*IPAAm/2H1 conjugate (MAb 2H1 unlabeled) was prepared as described above.

The assay was performed as follows: To 300 μL of PBS/BSA was added 50 μL of poly*N*IPAAm/2H1 conjugate (100 μg MAb/mL), 50 μL of 1% poly*N*IPAAm (as coprecipitating agent), and 100 μL of a dilution of rαmIgG. The reaction mixture was incubated for 30 min at room temperature to allow specific binding to occur. The reaction mixture was then diluted to 1mL by the addition of 500 μL of PBS. The temperature was raised to 37°C for 10 min to precipitate the polymer. The resultant precipitate was pelleted by centrifugation for 5 min at 4000*g* at 37°C. The supernatant was withdrawn, and the precipitate was redissolved by the addition of 1 mL of ice-cold PBS. The polymer was again precipitated by raising the temperature to 37°C, the precipitate was pelleted by

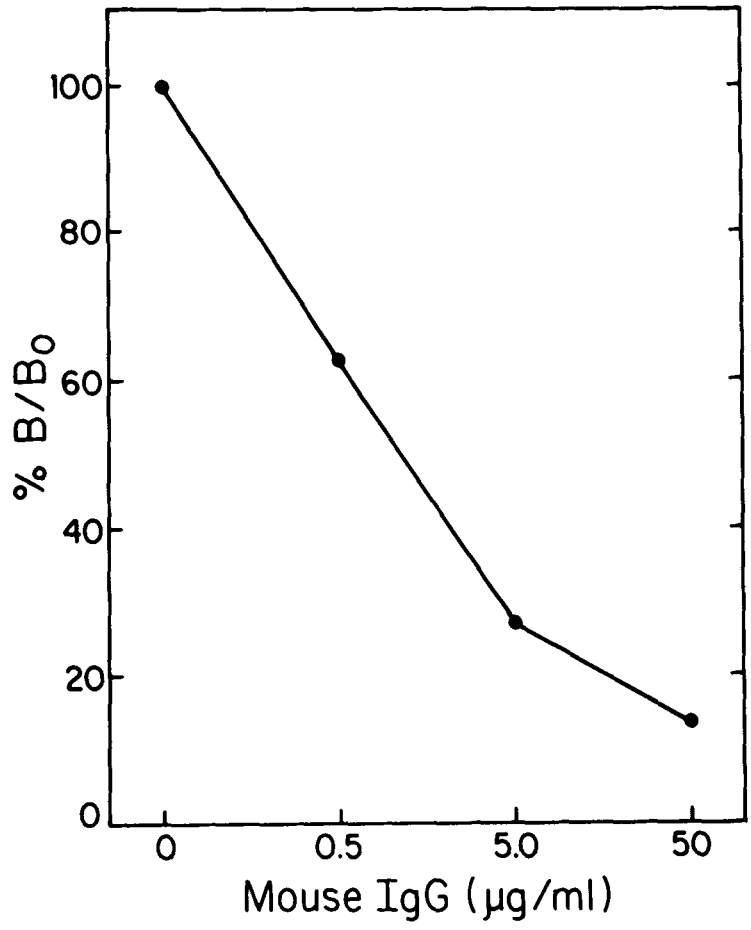

Fig. 2. Competitive enzyme immunoassay for mouse IgG using a poly*N*IPAAm/MAb conjugate (*see* text for details).

centrifugation, the supernatant withdrawn, and the precipitate dissolved in 100 µL of goat anti-rabbit IgG/HRP (Cappel Laboratories, diluted 1:1000 in PBS/BSA). The reaction volume was brought to 0.5 mL by addition of 400 µL of PBS/BSA. Incubation was continued for 30 min at room temperature, at which time the reaction volume was brought to 1 mL by addition of 500 µL of PBS. The reaction mixture was incubated at 37°C for 10 min to precipitate the polymer. The resultant precipitate was pelleted by centrifugation at 4000*g* for 5 min at 37°C, the supernatant was withdrawn, and the pellet was redissolved in 1 mL of ice-cold PBS. This cycle was repeated one more time, the final precipitate was dissolved in 200 µL of ice-cold PBS, and a 15-µL aliquot was transferred to a microtiter well containing 50 µL of PBS/BSA. One-hundred microliters of substrate solution prepared as in Fig. 2 was added, and the reaction was incubated for 10 min at room temperature. The reaction was terminated by the addition of 100 µL of 2.5*N* H_2SO_4. The absorbance of the resultant solution was measured at 490 nm on a microELISA reader.

RESULTS AND DISCUSSION

In order to prepare a conjugate of MAb and poly*N*IPAAm, the MAb was first conjugated with an acrylamide monomer using *N*ASI, as shown in Fig. 3. We found, using isoelectric focusing, that about six monomers were conjugated per antibody. We then labeled the MAb_M with fluorescein. The monomer and fluorescein conjugated antibody ($MAb_{M,F}$) was then copolymerized with N*I*PAAm monomer in a free radical copolymerization using the redox initiator system of ammonium persulfate and TEMED. We have also prepared antibody-poly*N*IPAAm conjugates using a prepolymerized copolymer of NIPAAm with *N*ASI active ester monomer (*9*). This polymer was then reacted with the antibody, yielding the poly*N*IPAAm-MAb conjugate.

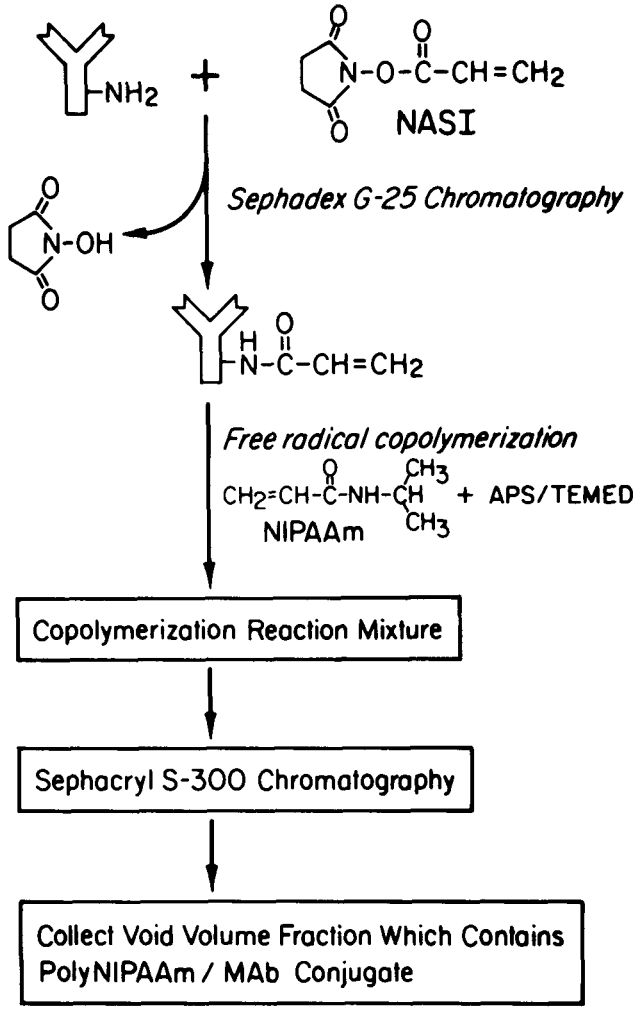

Fig. 3. Synthesis and purification of antibody conjugated poly*N*IPAAm.

When the poly*N*IPAAm–MAb_F conjugate was thermally precipitated, it was found that about 85% of the monomer-conjugated antibody had been incorporated into the precipitated MAb-polymer conjugate (as shown in the left side of Fig. 4). When unconjugated poly*N*IPAAm was thermally precipitated in the presence of free ("bystander") antibody, which had been fluorescein labeled (but not monomer conjugated), less than 5% of the antibody was entrapped in the precipitating polymer (see right side of Fig 4.). These experiments demonstrate the feasibility of the PRECIPIA immunoassay technique.

In order to improve the recovery of the poly*N*IPAAm/immune complex sandwich after precipitation, we also carried out recovery studies of poly*N*IPAAm conjugated with a fluoresceinated monoclonal antibody as a model, using three cycles of centrifugation, redissolution, and reprecipitation. We separately added surfactant, albumin (BSA), and serum to test the effects of these components on recovery of the precipitated signal. As shown in Table 1, the recovery of fluorescence is greater than

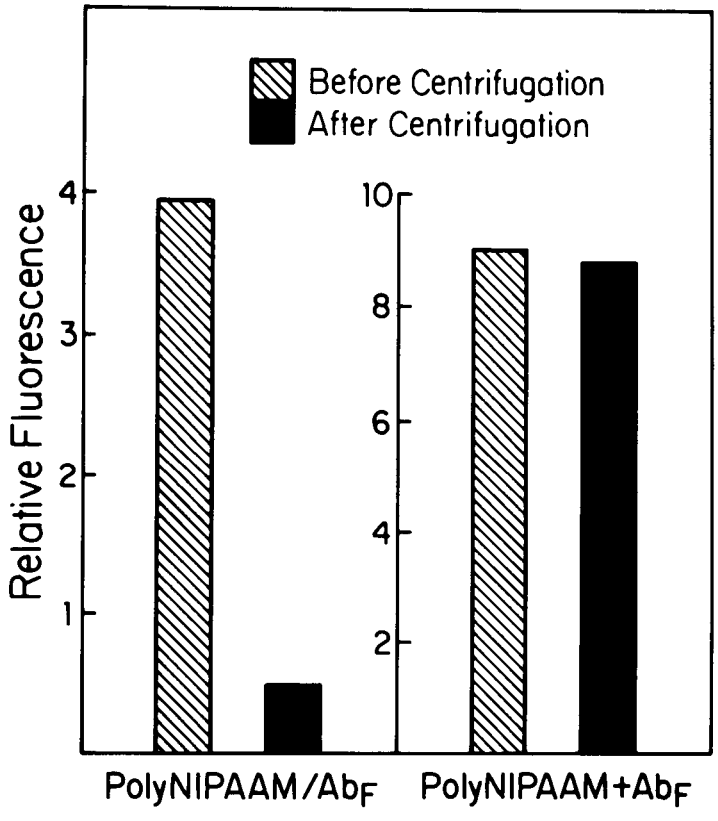

Fig. 4. Incorporation of fluorescein-labeled, monomer-conjugated antibody into precipitated poly*N*IPAAm after polymerization (left) in contrast to incorporation of free, fluorescein labeled "bystander" antibody into precipitated poly*N*IPAAm (right).

TABLE 1
Recovery of PolyNIPAAm/MAb_F from Various Assay Solutions After Serial Precipitations[a]

Assay solution	% Fluorescence recovered
PBS/Tween	83
BPS/BSA	85
PBS	88
Normal human serum, 33% in PBS/BSA	90

[a]One hundred microliters of polyNIPAAm/MAb_F was diluted 1:4 with either PBS, PBS containing 0.05% (w/v) Tween (PBS/Tween), PBS containing 1% BSA (PBS/BSA), or normal human serum (diluted 1:3 in PBS/BSA). The mixtures were incubated for 10 min at 37°C. The supernatants were removed and 500 μL of ice-cold PBS was added to each to dissolve the polyNIPAAm/MAb_F precipitate. This cycle was repeated three times. After final precipitation, the precipitate was redissolved in 500 μL of ice-cold PBS and 150 μL of PBS. The fluorescence of this solution was determined and expressed ase-cold PBS and 150 μL of recovered.

80% in the presence of 0.05% Tween 20, 1% BSA, or 33% human serum, even after these three cycles of redissolution and reprecipitation. We have also examined the influence of a variety of other substances on the LCST of polyNIPAAm. Only SDS was found to have a significant influence (Table 2).

In order to demonstrate the immunoassay technique itself, polyNIPAAm was conjugated with unlabeled, MAb_1. After the free-radical copolymerization, the resultant mixture was fractionated on Sephacryl S-300. The polyNIPAAm/MAb_1 conjugate came out in the void volume from the column and was used for the thermally induced phase-separation immunoassay. We have found that this thermally phase separating polymer–antibody conjugate may be utilized in a competitive enzyme immunoassay for mouse IgG (Fig. 2A), an antigen capture fluorescence immunoassay for human IgG (Fig. 5), and a second antibody, sandwich enzyme immunoassay for rabbit anti-mouse IgG (Fig. 6). The sensitivities of these assays are in the range of typical nonisotopic heterogeneous assays. Indeed, this system has been used to develop an assay for hepatitis B surface antigen having detection levels in serum of 0.5 ng/mL (*10*).

Our thermally induced phase-separation immunoassay system offers several advantages over typical heterogeneous immunoassays. First, the specific binding reactions occur in solution rather than on a solid phase. Hence, the reaction kinetics are more favorable, leading to reduced incubation times. Second, nonspecific binding or entrapment is very low, as noted in the very low nonspecific signal given by a goat anti-rabbit IgG/HRP for the sample containing polyNIPAAm alone, instead of polyNIPAAm/Mouse IgG (Fig. 6). This is probably related to the fact that the polymers that may be used are water soluble and, thus, reasonably hydrophilic, unlike conventional solid-phase systems, which utilize

TABLE 2
Effect of Added Substances on the Phase Separation of poly*N*IPAAm

Substance added	Maximum concentration studied that did not cause any change in the poly*N*IPAAm phase separation process[a]
Surfactants	
Tween 20 (v/v)	>1.0%
Nonidet P-40 (v/v)	>1.0%
Triton X-100 (v/v)	>1.0%
Sodium deoxycholate (w/v)	>1.0%
Sodium cholate (w/v)	>1.0%
Zwittergent 3-14 (w/v)	>1.0%
Sodium dodecylsulfate (w/v)	0.025%
Ions	
SO_4^{-2}	0.25*M*
CO_3^{-2}	0.25*M*
PO_4^{-2}	0.25*M*
Ca^{+2}	0.125*M*
Mg^{+2}	0.06*M*
Li^{+1}	0.06*M*
Na^{+1}	>1.0*M*
$C1^{-1}$	>1.0*M*
HPO_4^{-1}	0.5*M*
HCO_3^{-1}	0.5*M*
Other substances	
Na Citrate	0.25*M*
Urea	0.5*M*
N-acetylcysteine	0.25*M*
Creatinine	0.125*M*
Polyethylene glycol	5.0%
Ethylendiamine tetraacetic acid (Na_2)	0.05*M*
KSCN	>0.5*M*
Serum	undiluted
Urine	undiluted
Bovine serum albumin	>5.0%

[a]Effect of additives on phase separation of poly*N*IPAAm was examined as follows: poly*N*IPAAm at the concentration of 0.02% was dissolved in distilled water. One milliliter of the poly*N*IPAAm solution was added to 1.0 mL of the solution containing various amounts of each of the reagents listed in Table 2. The reagents were prepared either in % (v/v or w/v; serial twofold dilution starting from 1%, except BSA and polyethylene glycol, which started from 10%, or in molarity (serial twofold dilution starting from 1*M*). The tube was heated to 45°C, and the tubidity of the solution was measured at 600 nm using a Perkin-Elmer spectrometer model 35. One concentration level before the concentration that showed a change in turbidity was designed as the maximum concentration that did not cause a change in the phase separation process.

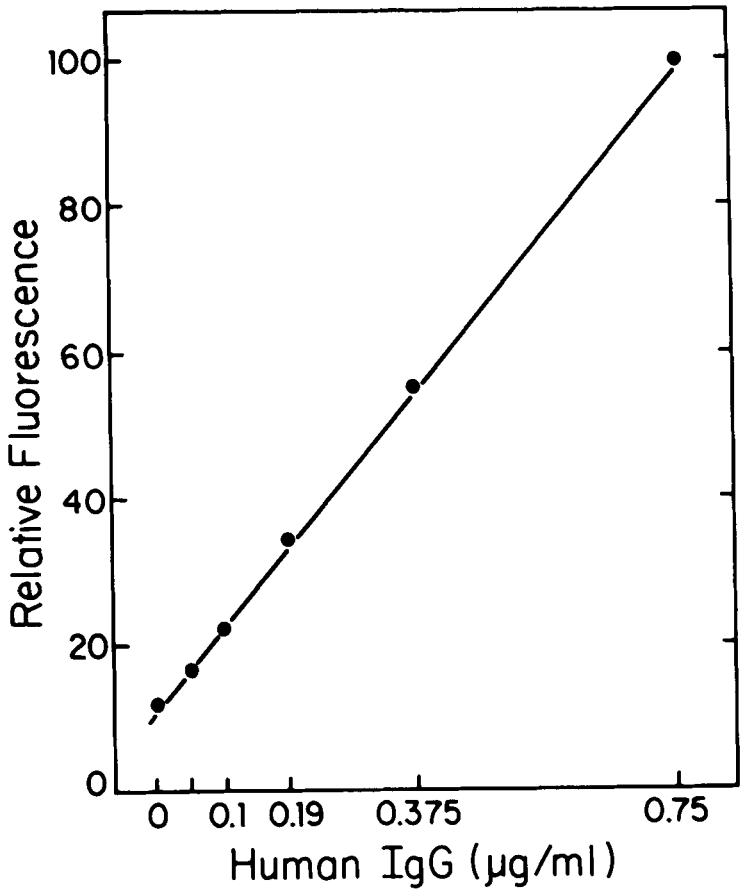

Fig. 5. Antigen capture fluorescence immunoassay for human IgG using a poly*N*IPAAm/MAb conjugate (*see* text for details).

polystyrene or other hydrophobic polymers. Third, the signal may be concentrated by redissolving the precipitated polymer in a substantially smaller volume than the original volume of the assay. For example, in all of the assays described in this paper the final pellet was redissolved into a 200-μL volume of PBS instead of the original 500-μL volume. Finally, it should be noted that we have used centrifugation in our assay; however, other separation methods, such as filtration, are equally possible. Although we have utilized repeated precipitation, washing, and redissolution steps in some of our examples, our assay may be run with relatively high sensitivity in a simple two-step process, incubation followed by precipitation and assaying the epifluorescence in the precipitate.

CONCLUSIONS

We have demonstrated a novel immunoassay using thermally phase-separating polymer–antibody conjugates. This assay utilizes a homogeneous incubation for the antigen–antibody reaction, and it may also

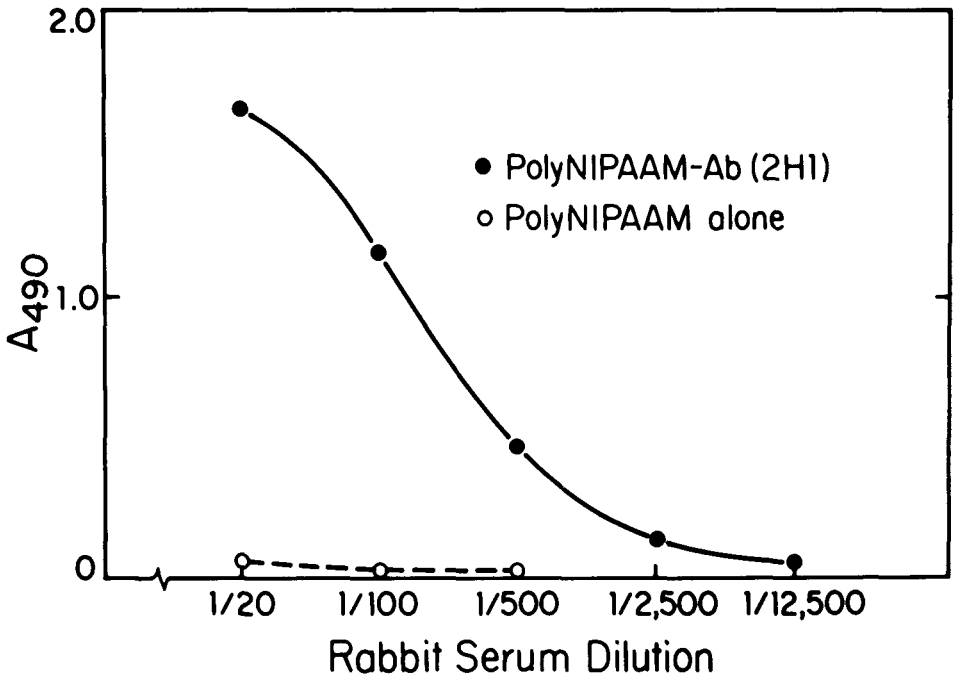

Fig. 6. Sandwich enzyme immunoassay for rabbit anti-mouse IgG (*see* text for details).

be used for high-molecular-weight antigens. It is capable of achieving sensitivities equivalent to nonisotopic heterogeneous immunoassays. Thus, it combines some of the advantages of both homogeneous and heterogeneous assays. This general technique may be used for a wide variety of separation processes in addition to immunoassays, in which a specific component in a biological fluid, industrial process stream, or body of water is desired to be isolated for recovery or removal. Thus, product recovery and/or toxin or pollutant removal processes are possible with this methodology.

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