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New antibody purification procedure using a thermally responsive poly(*N*-isopropylacrylamide)–dextran derivative conjugate

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Abstract

Through their specificity and affinity, antibodies are useful tools in research and medicine. In this study, we investigated a new type of chromatographic method using a thermosensitive polymer for the purification of antibodies against a dextran derivative (DD), as a model. The thermally reversible soluble–insoluble poly(*N*-isopropylacrylamide)–dextran derivative conjugate, named poly(NIPAAm)–DD, has been synthesized by conjugating amino-terminated poly(*N*-isopropylacrylamide) to a DD via ethyl-3-(3-dimethylaminopropyl)-carbodiimide. On one hand, this report describes the two steps of poly(NIPAAm)–DD conjugation and characterization. On the other hand, the poly(NIPAAm)–DD conjugate was used as a tool to purify polyclonal antibodies in serum samples from rabbits subcutaneously immunized with the derivatized dextran. Antibodies were purified and quantified by immunoenzymatic assays. Our results indicate that antibodies recognized both DD and poly(NIPAAm)–DD. In contrast, they did not bind to native poly(NIPAAm) or poly(NIPAAm) conjugated with another anionic dextran. We conclude that the conjugation of a polysaccharide to poly(NIPAAm) leads to an original and efficient chromatographic method to purify antibodies. Moreover, this novel method of purification is rapid, sensitive, inexpensive and could be used to purify various types of antibodies.

Keywords: Antibody purification; Poly(*N*-isopropylacrylamide); Dextran

1. Introduction

Due to their specificity and affinity, antibodies are useful tools in medical diagnostics or to elucidate the mechanism of action of bioactive macromolecules. Polysaccharides are T-cell independent antigens and are generally poor immunogens [1]. Rohova and Riha have studied the immunogenicity properties of synthetic or natural polysaccharides and showed that immunogenicity increased with their molecular mass and their heterogeneity [2]. In a previous study, we have purified anti-dextran antibodies by affinity chromatography from normal human serum [3]. The data indicated that purified antibodies recognized with a high affinity the native dextran, and they cross-reacted with various dextran derivatives (DD)

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synthesized from native dextran by successive random substitutions [4,5]. Interestingly, purified antibodies did not bind to anionic polysaccharides such as the sulfated dextran or carboxymethylated dextran, or to structurally unrelated sulfated polysaccharides such as heparin. Nowadays, studies are devoted to improve the understanding about their specific mechanisms of action using different biochemical tools.

Various reports have described the biological properties of DD, including anticoagulant and anticomplementary activities, modulation of cell proliferation and extracellular matrix protein biosynthesis, and their protective effect on growth factors [6–8]. In order to develop specific antibodies, CarboxyMethyl Dextran Benzylamide Sulfonate/Sulfate (CMDBS) was chosen as a polysaccharide antigen to give an antibody response. Consequently, anti-CMDBS serum was prepared by subcutaneous immunizations of rabbits and precipitation [9]. In this study, we have then prepared a thermosensitive poly(NIPAAm)–CMDBS conjugate.

Thermosensitive poly(NIPAAm) is a soluble polymer in aqueous solution at room temperature but precipitates at higher temperatures. The precipitation temperature is called the lower critical solution temperature (LCST) or cloud point. Thermo-responsive polymers have been investigated because of their ease of recovery and their potential for many applications [10–14]. The interest in this method for antibody purification lies in the thermally-dependent recovery process, the recycling of the polymer conjugate, the rapidity and sensitivity of the procedure.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (NIPAAm, Eastman Kodak, Rochester, NY) was purified by recrystallization from *n*-hexane. *N*,*N*-Dimethylformamide (DMF), 1-ethyl-3-[3-dimethylamino) propyl] carbodiimide (EDC), *N*,*N*′-dicyclohexyl carbodiimide (DCC), and dimethylsulfoxide (DMSO) were purchased from Sigma (St Louis, MO). EDC, DCC, DMF and DMSO were used without further purification.

2.2. Polymers

Poly(NIPAAm) with one terminal amino group was synthesized by chain transfer free-radical polymerization of NIPAAm in DMF, using 2,2′-azobisbutyronitrile and 2-aminoethanethiol hydrochloride as an initiator and a chain transfer reagent [15]. The poly(NIPAAm) was purified by precipitating the reaction solution into diethyl ether and its number average molecular mass (Mn) was determined by terminal functional group titration to be 2100. The derivatized dextran called CMDBS was synthesized from native 40 kDa dextran (Pharmacia, St Quentin-en-Yvelines, France) as previously described [7]. Briefly, the CMDBS synthesis involves the three following reactions. The first is a random carboxymethylation (CM) of hydroxyl groups on D-glucose monomers, using monochloroacetic acid in alkaline medium at 60°C for 1 h. Secondly, the substitution of some of the CM groups with benzylamine to form benzylamide (B) units, was performed with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as coupling agent in water/ethanol (v/v) solution at room temperature. Finally, sulphonation of some aromatic rings and sulphatation (S) of free hydroxyls were carried out by use of chlorosulphonic acid on the polymer in dried dichloromethane. The CMD compound was obtained after the carboxymethylation step. The CMD and CMDBS were characterized as follows: the carboxylic content was determined by acidimetric titration in water/acetone (v/v). Nitrogen and sulphur contents were determined by microanalysis. The carboxylic contents were 103 and 60% for CMDBS and CMD, respectively. Benzylamide and sulfur contents of CMDBS were 23 and 21%, respectively.

The chromatographic molecular mass (Mc) of CMD and CMDBS polymers was determined by high-performance steric exclusion chromatography in 0.15 *M* sodium chloride buffered at pH 7 with NaH_2PO_4 using a Lichrospher Si300 Diol column (Merck, Nogent-Sur-Marne, France) connected with a HEMA Sec Bio 40 column (Interchim, Montluçon, France). The columns were calibrated with pullulan

standards (Interchim). Both Mc for CMD and CMDBS were of 50 kDa.

2.3. Synthesis and purification of poly(NIPAAm) conjugates

For poly(NIPAAm)–CMD conjugation, CMD was protonated in aqueous solution at pH 2.9, and collected by precipitating in methanol. The protonated CMD was then dissolved in hot DMSO. To this solution, amino-terminated poly(NIPAAm) was added. The molar ratio of $(-COOH)/(-NH_2)$ was 18. DCC dissolved in DMSO was dropped into the solution at (DCC/poly(NIPAAm) ratio of 1. The conjugation solution was stirred at room temperature for 17 h and then dropped into diethylether to precipitate the poly(NIPAAm)–CMD conjugates and poly(NIPAAm) oligomer [16–18]. On the other hand, CMDBS was partially protonated (–COOH and $-SO_3^-$ in aqueous solution at pH 1.9) and was collected from the solution. During the conjugation step, partially protonated CMDBS was dissolved in hot DMSO. Amino-terminated poly(NIPAAm) was dissolved in the solution. The molar ratio of poly(NIPAAm)/CMDBS was 11.9. EDC dissolved in DMSO was then added to the solution. EDC was present at a twofold excess of poly(NIPAAm) to maximize the conversion of poly(NIPAAm). The conjugation solution was stirred at room temperature for 18 h. Both poly(NIPAAm)–CMDBS and poly(NIPAAm)–CMD solutions were dissolved in water and then dialyzed against water using cellulose tubing with a molecular mass cut-off of 12 000 for 48 h. Finally, the dialyzates were lyophilized.

2.4. Physico-chemical characterization of poly(NIPAAm) conjugates

The lower critical solution temperature (LCST) of the polymers was determined by cloud point measurement. Samples of poly(NIPAAm), poly(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates were prepared at a concentration of 5 mg/ml in water at 4°C. LCST determination was performed on a Perkin-Elmer UV/Vis spectrophotometer. The absorbance at 500 nm was measured when the temperature raised from 25 to 50°C. The LCST was defined as the temperature at the inflection point in the absorbance versus temperature curve. Gel permeation chromatography (GPC) of both poly(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates was conducted before and after dialysis. GPC was carried out in sodium acetate and DMF mobile phase for poly(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates, respectively.

2.5. Immunizations and bleeding

Two female ''Fauve de Bourgogne'' rabbits (Baylu farm, Val d'Oise, France), weighing 2.5–3 kg were administered CMDBS. The antigen administration was carried out subcutaneously at approximately 10 sites into the back of rabbits. First, animals were immunized with 5 n*M* CMDBS dissolved in 1 ml 0.15 *M* phosphate buffered saline (PBS), pH 7.2, emulsified with an equal volume of Freund's adjuvant. After 5 weeks, animals were boosted with a mixture of 2.5 n*M* CMDBS and Freund's adjuvant incomplete (final volume was 2 ml). The animals were then allowed to rest for 2 months. Blood samples were taken through a sterile syringe introduced into the ear vein of rabbits before and after each immunization.

2.6. Antibody purification through poly(NIPAAm) conjugates

Antibodies from immunized serum were precipitated at 4°C by 50% saturated ammonium sulfate. Collected antibodies at a concentration of 0.5 m*M* in 0.15 *M* PBS (pH 7.2) were separately incubated at room temperature for 90 min with both poly(NIPAAm) conjugates (v/v). The first purification cycle (P_1) was achieved as follows: solution was incubated at 37°C for 5 min and centrifuged (1800 *g*, 37°C, 5 min). Two other similar purification cycles were performed and named P_2 and P_3, respectively. Recovery of conjugates was performed by lowering the temperature of the solution. Adsorbed antibodies were then eluted with 0.1 *M* glycine–HCl (pH 2.6) and 0.1 *M* glycine–NaOH (pH 9.8) buffers. Collected fractions were dialyzed overnight at 4°C against 0.15 *M* PBS (pH 7.2). Finally, the desorbed proteins were quantified and analyzed by ELISA and a commercially available micro BCA protein assay reagent kit from Pierce (Interchim, Montluçon,

France) according to the manufacturer's protocol. The minimum detection level by BCA is 0.5 p*M* for bovine serum albumin, used as a standard protein. Concentration of each sample was determined from the standard curve.

2.7. ELISA

Microplate wells (Costar, Guyancourt, France) were coated with 100 μl antigen solutions (poly-(NIPAAm) oligomer, poly(NIPAAm)–CMD, poly-(NIPAAm)–CMDBS, CMD or CMDBS) at a concentration of 100 μg/ml in 0.1 *M* carbonate–bicarbonate buffer (pH 9.6). The plates were left to incubate overnight at 4°C. The wells were then washed three times with 0.15 *M* PBS (pH 7.2) containing 0.05% Tween (PBS–Tween). Overcoating was performed with 100 μl/well of 0.5% teleostean gelatin (Sigma) in PBS at room temperature for 90 min. Then, 50 μl of diluted purified rabbit antibodies prepared in PBS–Tween were added in duplicate and incubated at room temperature for 90 min. The wells were washed three times with PBS–Tween and incubated at room temperature for 90 min with 100 μl of Fc specific monoclonal anti-rabbit IgG biotin-conjugated (Sigma) diluted in PBS–Tween at 1/20 000. After incubation, wells were washed three times in PBS–Tween and further incubated with 100 μl of streptavidin-coupled horseradish peroxidase at 1/50 (Sigma) at room temperature for 15 min. After washing six times with 0.1 *M* citrate buffer (Sigma), 100 μl of 0.1% of 3,3′,5,5′-tetramethyl benzidine (Sigma) prepared in citrate buffer plus 0.05% hydrogen peroxide at 30% (Sigma) were added in each well. Finally, the reaction was stopped by adding 100 μl/well of 1 *M* H_2S0_4 and the absorbance was measured at 450 nm using an automated microplate reader (Model EL 311, Bio-Tek, Winnoski, VT, USA).

3. Results

3.1. Preparation of poly(NIPAAm) conjugates

The preparation of conjugates included three main steps: protonation, conjugation with purification, and then characterization. In order to use organic solvents as conjugation medium, CMD and CMDBS were protonated in aqueous solution, collected through different procedures, and redissolved in DMSO. Poly(NIPAAm) conjugation to CMD and CMDBS was carried out in DMSO with DCC and EDC, respectively (Fig. 1). GPC profiles indicated that there was no poly(NNIPAAm) oligomer peak in both conjugates (data not shown), indicating that dialysis was sufficient to remove the oligomers from the conjugates. Characterization of both conjugates was then achieved by LCST determination. The LCSTs of poly(NIPAAm)–CMD and poly-(NIPAAm)–CMDBS in comparison to that of poly-(NIPAAm) are shown in Fig. 2. When the temperature is raised above their LCST, the conjugates undergo a phase transition to a hydrophobic state. The transition range was broader for poly-(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates than for poly(NIPAAm) oligomer. The LCSTs of the conjugates were 33°C, while that of poly(NIPAAm) was between 31 and 32°C. It was then interesting to evaluate the ability of poly-

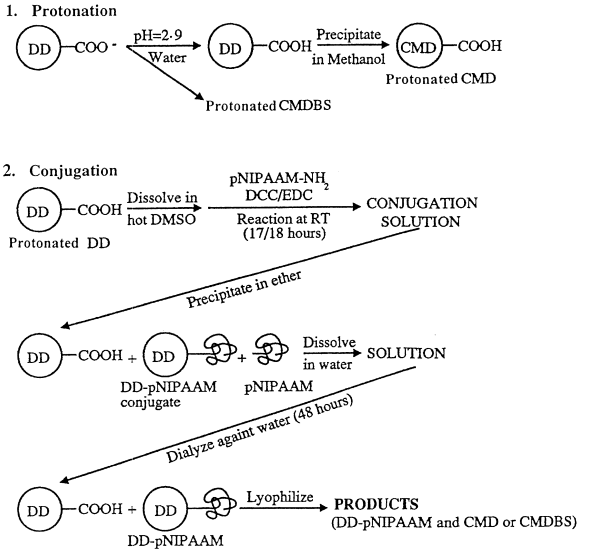

Fig. 1. Scheme for the synthesis of poly(NIPAAm)–DD conjugates. Firstly, the dextran derivatives (DD), i.e. CMD or CMDBS were protonated. Conjugation steps were then performed using as coupling agents, DCC and EDC dissolved in DMSO, for poly-(NIPAAm)–CMD and poly(NIPAAm)–CMDBS, respectively.

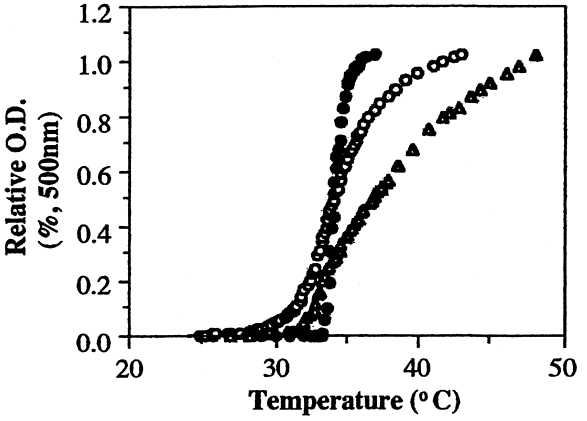

Fig. 2. LCSTs of poly(NIPAAm), poly(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates. The LCSTs of the polymers were measured by spectrophotometric determination of the absorbance at 500 nm of the polymers in PBS buffer. Turbidity of the solution at various temperatures ranging from 25 to 50°C was spectrophotometrically measured: (●), native poly(NIPAAm); (○), poly(NIPAAm)–CMD; (△), poly(NIPAAm)–CMDBS. The results display the LCSTs of both conjugates at 33°C and that of poly(NIPAAm) oligomer between 31 and 32°C.

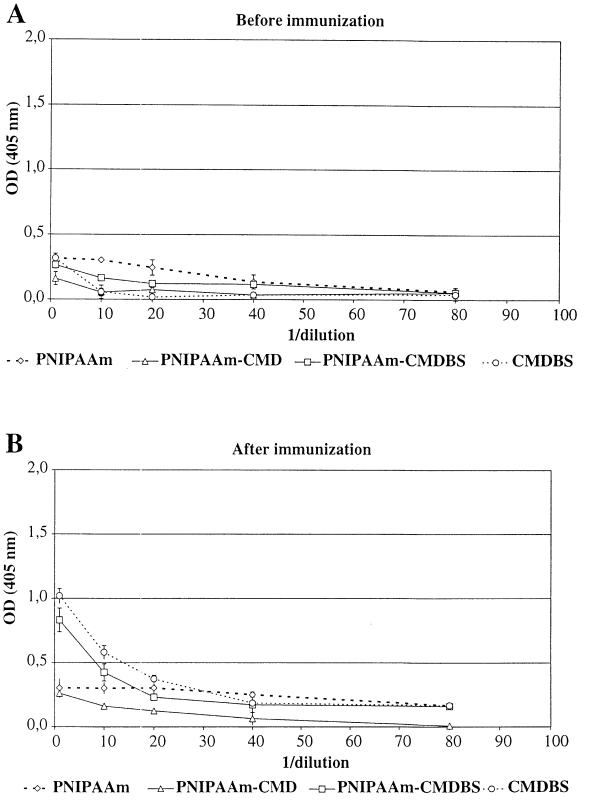

Fig. 3. Analysis by ELISA of sera before and after rabbit immunizations. Four antigens were coated into microplates as described in Material and methods. (◇), poly(NIPAAm); (△), poly(NIPAAm)–CMD, (□), poly(NIPAAm)–CMDBS, and (○), CMDBS. As expected, antibodies from control serum recognized no antigen (A). In contrast, antibodies from immunized serum bind to CMDBS and poly(NIPAAm)–CMDBS, but did not bind to poly(NIPAAm) or poly(NIPAAm)–CMD (B). Results are means±S.E.M. of two experiments.

(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates to purify anti-CMDBS antibodies.

3.2. Antibody purification

The rabbit serum was tested by ELISA for the presence of antibodies prior to purification of antibodies using the polymer conjugates. As shown in Fig. 3A, polyclonal antibodies from serum obtained before immunization were used and a background signal was observed regardless of the coated antigen: poly(NIPAAm) oligomer, poly(NIPAAm)–CMD, poly(NIPAAm)–CMDBS or CMDBS. In contrast, antibodies from serum of rabbits immunized with CMDBS exhibited affinity to both poly(NIPAAm)–CMDBS conjugate and CMDBS, but did not bind to poly(NIPAAm)–CMD or poly(NIPAAm) oligomer (Fig. 3B). Although two immunizations were performed, the titer was low.

We then tested the capacity of the two synthesized conjugates to purify antibodies. Purification was based on temperature sensitivity of conjugates. Fig. 4 displays the thermosensitive procedure for antibody purification, using poly(NIPAAm)–DD conjugate. To separate specific antibodies and recycle the poly(NIPAAm)–DD conjugate, the temperature must be raised to precipitate the complex, which is above the LCST of the conjugate. Antibodies eluted with 0.1 *M* glycine–HCl (pH 2.6) and 0.1 *M* glycine–NaOH (pH 9.8) buffers were thus analyzed by the BCA protein assay method. The assay results showed progressively decreasing amounts of proteins in supernatants of the three successive purification cycles. The results also indicated the presence of proteins in 0.1 *M* glycine–HCl, and no detectable proteins in 0.1 *M* glycine–NaOH when the poly-

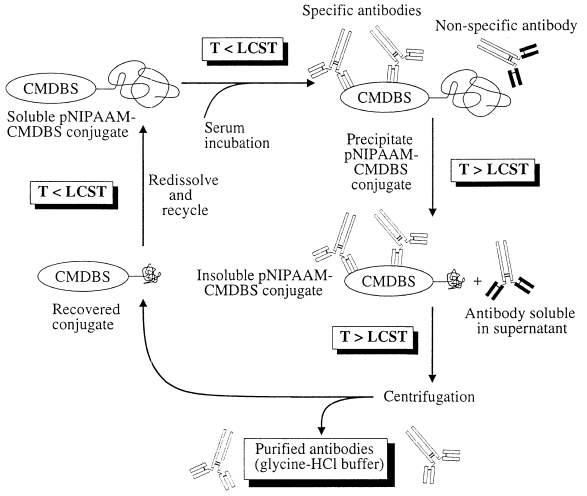

Fig. 4. Thermal-sensitive process allows antibody purification. CMDBS antibodies were purified after binding to poly(NIPAAm)–CMDBS by complexation. Representation of temperature-dependent recycling process for purifying CMDBS antibodies and recycling the poly(NIPAAm)–CMDBS bioconjugate as described in Material and methods. No specific antibodies were eliminated in supernatants. After centrifugation, specific antibodies were eluted and the conjugate recovered.

(NIPAAm)–CMDBS conjugate was used. As confirmed by ELISA, purified antibodies eluted with glycine–HCl contained antibodies which specifically

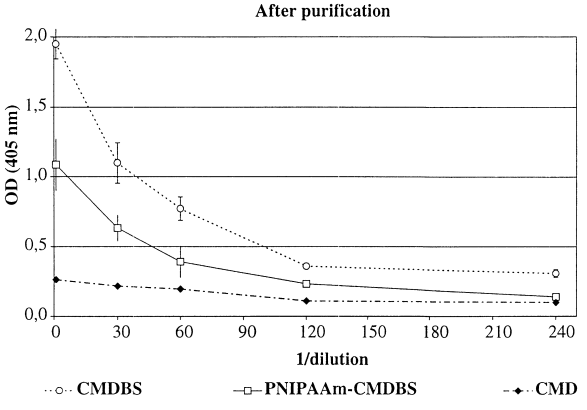

Fig. 5. ELISA analysis of eluted antibodies with 0.1 *M* glycine–HCl buffer. Antibodies from rabbit immunized serum were purified through poly(NIPAAm)–CMDBS conjugate. The results indicated that purified antibodies bound to CMDBS (○) and poly(NIPAAm)–CMDBS (□), but did not recognize other antigen such as the carboxymethyl dextran, CMD (◆). Results are means±S.E.M. of three experiments.

recognized CMDBS, and the poly(NIPAAm)–CMDBS conjugate, but did not bind to CMD, the poly(NIPAAm) oligomer or poly(NIPAAm)–CMD (Fig. 5). Moreover, no antibodies were purified through the poly(NIPAAm)–CMD conjugate (data not shown). Taken together, these results indicated that poly(NIPAAm)–CMDBS conjugate was able to specifically bind to CMDBS antibodies at room temperature (or lower) and release at 37°C, bound CMDBS antibodies. In contrast, the poly(NIPAAm)–CMD exhibited temperature-sensitive properties but was not able to interact with CMDBS antibodies.

4. Discussion

In the present study, we have grafted a synthetic poly(*N*-isopropylacrylamide) to dextran derivatives. Using two derivatized dextrans (CMD and CMDBS), we have succeeded in synthesizing poly(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates. It is well established that poly(NIPAAm) is a thermoreversible polymer which is water soluble at room temperature and precipitates when it is heated above its LCST [19]. Various researchers have described its conjugation to biological molecules such as extracellular matrix, streptavidin, immunoglobulin and enzymes [19–23]. Ding et al. have previously prepared a poly(NIPAAm)–trypsin conjugate able to catalyze enzymatic reactions in solution with a minimal activity loss [12]. They have also demonstrated that the conjugate can efficiently separate the enzyme from the substrate, by thermal cycling through the LCST. As documented in this report, the preparation of conjugates should follow two main steps (Fig. 1): protonation of carboxylic groups on DD, and conjugation to amino-terminated poly(NIPAAm). Their ability to purify CMDBS antibodies was then investigated.

Widespread applications using immunological recognition methods remain an important goal for medical diagnostics. Three types of diagnostic assay systems are available: homogeneous, heterogeneous involving a surface, and a combination of both. After comparing their characteristics, Hoffman et al. have developed three novel immunoassays which are rapid, sensitive and combine many of advantages of homogeneous and heterogeneous immunoassays,

without their disadvantages. All of these assays involve the use of a chemical conjugate of poly-(NIPAAm) with a monoclonal antibody [24,25]. In our case, the preformed polymer–antigen conjugates (pNIPAAm–CMDBS and pNIPAAm–CMD) are soluble at room temperature (under the LCST of both conjugates at 33°C). When the polymer–antigen conjugate is incubated with the antibodies such as the rabbit serum containing antibodies, the immune complexes remain soluble in solution (Fig. 4). Furthermore, if the temperature is raised above the LCST, the conjugate rapidly precipitates in seconds to minutes, due to the precipitation of poly-(NIPAAm).

We have generated antibodies that might be reactive with the sugar backbone by immunizing rabbits with CMDBS polymer. Specific CMDBS antibodies of the IgG class were detected by an ELISA method in which poly(NIPAAm) oligomer, poly(NIPAAm)–CMD and poly(NIPAAm)–CMDBS conjugates, and CMD or CMDBS were coupled to wells in a microtiter plate. This protocol is similar to previous work by Chen et al. who have described the purification of human immunoglobulin G via a poly(NIPAAm)–protein A conjugate [23]. As demonstrated in our study, this affinity precipitation could be successfully applied to polysaccharides (Fig. 5). Proteins bound to the conjugate were then eluted in solution. Two negative controls were also performed: the poly(NIPAAm)CMD conjugate and the non-immunized rabbit serum. Our results demonstrate that antibodies were specific for CMDBS and were purified by this technique.

Immunogenicity of polysaccharides has been extensively reviewed with regard to the production of antibodies. Different authors analyzed the parameters affecting the immunogenicity of synthetic and natural polysaccharides, i.e. their composition, size and steric conformation, electrical charge and degradability. The presence of more than two types of determinants was found to modulate antibody production. By increasing the molecular mass of polysaccharides, their immunogenicity was enhanced. Moreover, poorly degradable polymers with repetitive units are often tolerogenic rather than immunogenic by persisting for a long period of time in the organism [2]. Our method is rapid and sensitive for detection/titration of antibodies directed against different polysaccharides and may help in the general understanding of the immune response in humans.

5. Conclusion

In this study, we have prepared a new specific tool to purify antibodies with a temperature responsive polymer. Bioseparations, diagnostics, enzyme processes, and targeted delivery of drugs or chemical agents were already achieved with thermo-sensitive polymer. In our study, antibodies directed against a dextran derivative were purified by a thermally responsive poly(NIPAAm) conjugated to CMDBS polymer which bound to specific antibodies by CMDBS complexation. This technique may help to obtain new data regarding the reactivity and physicochemical properties of immunological recognition mediated by antibodies. The purified antibodies may also represent valuable tools for the understanding of cellular processes involved with these polysaccharides [26].

Acknowledgements

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