ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

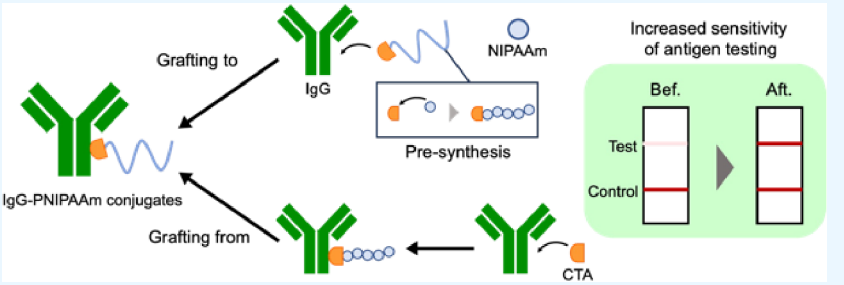
A Comparative Study of "Grafting to" and "Grafting from" Conjugation Methods for the Preparation of Antibody-Temperature-Responsive Polymer Conjugates

Erika Yoshihara, Ahmed Nabil,* Michihiro Iijima, and Mitsuhiro Ebara*

Cite This: *ACS Omega* 2024, 9, 22043−22050

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

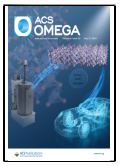


ABSTRACT: Early diagnosis of infectious diseases is still challenging particularly in a nonlaboratory environment or limited resources areas. Thus, sensitive, inexpensive, and easily handled diagnostic approaches are required. The lateral flow immunoassay (LFIA) is commonly used in the screening of infectious diseases despite its poor sensitivity, especially with low pathogenic loads (early stages of infection). This article introduces a novel polymeric material that might help in the enrichment and concentration of pathogens to overcome the LFIA misdiagnosis. To achieve this, we evaluated the efficiency of introducing poly(*N*-isopropylacrylamide) (PNIPAAm) into immunoglobulin G (IgG) as a model antibody using two different conjugation methods: grafting to (GT) and grafting from (GF). The IgG−PNIPAAm conjugates were characterized using SDS-PAGE, DLS, and temperature-responsive phase transition behavior. SDS-PAGE analysis revealed that the GF method was more efficient in introducing the polymer than the GT method, with calculated polymer introduction ratios of 61% and 34%, respectively. The GF method proved to be less susceptible to steric hindrance and more efficient in introducing high-molecular-weight polymers into proteins. These results are consistent with previous studies comparing the GT and GF methods in similar systems. This study represents an important step toward understanding how the choice of polymer incorporation method affects the properties of IgG−PNIPAAm conjugates. The synthesized polymer allowed binding and enrichment of mouse IgG that was used as a model antigen with a clear LFIA band. On the basis of our findings, this system might help in improving the sensitivity of simple diagnostics.

INTRODUCTION

Various studies including ours have reported the severe drawbacks of the current commercially available SARS-CoV-2 diagnostic kits, as most of them have a high false negative rate and poor sensitivity, and even the gold standard polymerase chain reaction (PCR) is recommended to be repeated to overcome this misdiagnosis.[1−5] Many factors were reported to contribute to this problem, including fluctuation in the viremia load of nasopharyngeal and oropharyngeal swaps (NP/OP) commonly used for SARS-CoV-2 diagnosis,[6−8] virions colonize the lower respiratory tract while these swaps are collected from the upper respiratory tract,[9,10] and finally, the dynamics of the viral load of SARS-CoV-2 where the viral load peaked on day 10 after the onset of infection, approximately.[11−13] On the other hand, PCR testing requires sophisticated equipment, high cost, time, and experts to collect mucosa samples and detect viral load and other antigenic targets. To solve these issues, point-of-care testing (POCT) has been developed, which is a simple real-time test that can be performed by healthcare professionals themselves at the patient's side using a small analyzer or rapid diagnostic kits. Our team focused on the lateral flow immunoassay (LFIA) as one of the POCT approaches. LFIA is superior because it is cheap, portable, fast, and widely used in diagnostics including influenza detection kits and pregnancy tests. Moreover, the result of the LFIA test can be easily determined visually.[14] However, the main problem with LFIA is its limited detection sensitivity.[15] For example, lipoarabinomannan (LAM) is

Received: January 4, 2024
Revised: March 1, 2024
Accepted: March 12, 2024
Published: May 10, 2024



secreted in the urine of tuberculosis (TB) patients and is a biomarker of TB, but LAM is currently difficult to detect by LFIA due to its limited concentration in urine samples, which is below the detection limit of LFIA (several μg/mL). Similarly, in the diagnosis of SARS-CoV-2, low viral antigenic concentrations (<ng/mL) in (NP/OP) samples make visualization of positive results challenging. Therefore, it is now evident that our primary challenge is to improve the limited sensitivity of LFIA. This can be achieved through the enrichment of biomarkers in the specimens tested before their application to LFIA. Chromatography and ultrafiltration are common antigen concentration methods[16] but are not suitable for POCT due to their sophisticated and expensive equipment. Therefore, we focused on poly(*N*-isopropylacrylamide) (PNIPAAm), which is a temperature-responsive polymer, as an alternative method for antigen purification and easy enrichment easily.

PNIPAAm is water-soluble at room temperature and has a lower critical solution temperature (LCST), around 32 °C, so it aggregates and precipitates at temperatures above 32 °C.[17] Taking advantage of this property, PNIPAAm and its derivatives can be introduced into antibodies to provide ON−OFF switching functionality upon temperature stimulation. Antigen enrichment is expected by its trapping with specific antibody-temperature-responsive polymer conjugates. Many previous studies, including ours, that introduced PNIPAAm and PNIPAAm-derived polymers into various proteins, including antibodies, have already been reported. Hoffman et al. have successfully prepared conjugates with a series of amine-containing proteins, including lysozyme, myoglobin, protein A, hemoglobin, albumin, and γ-globulin by using NIPAAm copolymer with *N*-hydroxysuccinimide (NHS).[18,19] Okano et al. have also synthesized semitelechelic PNIPAAm with NHS groups at the ends of the polymer and successfully conjugated it with antibodies.[20,21] Recently, our group reported the preparation of polymeric antibody conjugates in real biological samples by adding functional groups to proteins by modifying PNIPAAm-derived copolymers using click chemistry.[1,10] These temperature-responsive protein-polymer conjugates were prepared by a method called "grafting-to" (GT), in which a presynthesized polymer is conjugated to a functionalized protein by a coupling reaction. Although this method seems to be simple and convenient, it requires a complicated process that includes the synthesis of PNIPAAm-derived polymers in multiple steps, in addition to the functionalization of the polymer end groups and the purification that accompanies this process. In addition, the efficiency of the introduction of polymers into protein is low due to steric obstacles; therefore, the addition of excess free polymers is necessary to improve its enrichment efficiency.[22]

In addition to the GT method, there is another "grafting-from" (GF) method, which is also used for introducing polymers into proteins. The GF method is a technique that has been attracting attention in recent years because it introduces polymers directly by polymerizing monomers in solution using functionalized proteins as the starting point for polymerization, thus overcoming the previously mentioned issues of the GT method, including steric hindrance and excess addition of free polymers.[23,24] Studies comparing the GT and GF methods of introducing polymers into RNA, cellulose, and graphene have generally concluded that the GF method is the most efficient way to introduce polymers.[25−27] GF basically uses living-radical polymerization in water to introduce the polymer. In some reported studies, a chain transfer agent (CTA) was introduced into bovine serum albumin (BSA) and lysozyme by reversible addition−fragmentation chain transfer polymerization (RAFT).[28−30] This demonstrated that the protein functionality of the conjugates was retained after the NIPAAm polymerization from proteins and the introduction of PNIPAAm by living radical polymerization. We have also succeeded in preparing antibody-temperature-responsive polymer conjugates by introducing CTA into antibodies and performing NIPAAm polymerization with antibody as the initiation point of polymerization.[31] This conjugate that was prepared by the GF method showed nanoparticle morphology, which was also reported to be useful in improving various physical properties including stability against enzymes, high antigen−antibody reaction activity, and applicability for different medical applications, including drug delivery systems (DDSs). However, there are no previous comparative studies between the GT and GF methods, which can give an indication whether antibody-polymer conjugates produced by the GF method are inferior to antibody-polymer conjugates produced by the GT method or vice versa.

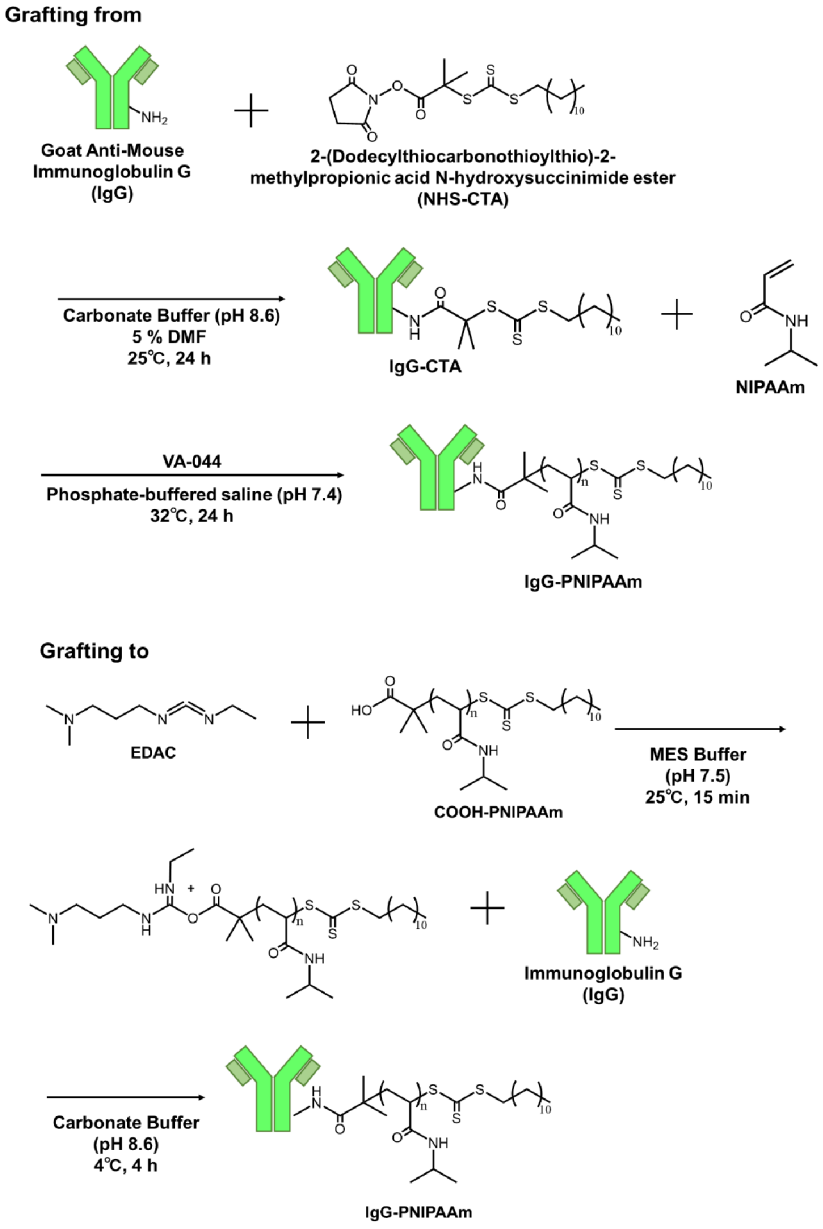
Therefore, the objective of this study is to clarify the performance of antibody polymer conjugates (APCs) produced by the GF method compared to the GT method by introducing thermoresponsive polymers to antibodies using each of the GT method and the GF method, which were established in our previous studies, and by comparing and evaluating the polymeric introduction capacity and the polymeric conjugate efficiency (thermal precipitation efficiency, activity against antigens, etc.). In addition, this study aimed to improve the medical applications of GF by increasing the sensitivity and accuracy of antigen testing by antigen enrichment using the IgG−PNIPAAm conjugate. To the best of our knowledge, this report may be the first to conduct a comparative study between GT and GF methods in addition to evaluation of their biomedical performance.

MATERIALS AND METHODS

Materials. *N*-Isopropylacrylamide (NIPAAm, Fujifilm Fujifilm Wako Pure Chemical, 97%) was recrystallized from hexane and dried under vacuum prior to use. 2,2′-Azobis [2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044, Tokyo Kasei, 98.0%) was recrystallized from methanol and dried under vacuum before use. 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid *N*-hydroxysuccinimide ester (NHS-CTA, Sigma-Aldrich, St. Louis, MO, USA), poly(*N*-isopropylacrylamide), *N*-hydroxysuccinimide (NHS) ester terminated (PNIPAM-NHS, , Mn 2,000, Sigma-Aldrich, St. Louis, MO, USA), poly(*N*-isopropylacrylamide), *N*-hydroxysuccinimide (NHS) ester terminated (NHS-PNIPAAm, Mn 2000, Sigma-Aldrich, St. Louis, MO, USA), *N*,*N*-dimethylformamide (DMF, 99.5%, Fujifilm Wako Pure Chemical), dulbecco phosphate buffered saline (PBS, Aldrich), goat polyclonal secondary antibody to mouse IgG−H&L (HRP) (IgG, abcam), dimethyl sulfoxide (DMSO, Fujifilm Wako Pure Chemical, 99.0%), DL-2-aminobutyric acid (Tokyo Kasei, 99.0%), fluorescamine (Tokyo Kasei), sodium ascorbate (Aldrich), tris (hydroxymethyl) amino methane (Tris, 99.8%, Aldrich), hydrochloric acid (1.0 mol/L, Fujifilm Wako Pure Chemical), methanol (99.8%, Fujifilm Wako Pure Chemical), 10 × tris/glycine/SDS buffer (BIO-RAD), coomassie brilliant blue *R*-250 (CBB, BIO-RAD), laemmli sample buffer (BIO-RAD), 2-mercaptoethanol (Fujifilm Wako Pure Chemical, 99%), precision plus protein

https://doi.org/10.1021/acsomega.4c00103
ACS Omega 2024, 9, 22043−22050

Scheme 1. Synthesis of Antibody-Temperature-Responsive Polymer Conjugates by the "Grafting to" and "Grafting from" Methods



unstained standards (BIO-RAD), goat antibody to mouse IgG (1.96 mg/mL, abcam), goat antibody to mouse IgG−H&L (2.06 mg/mL, abcam), mouse IgG (antigen, abcam), hydrochloric acid (Stop solution, Goat antibody to mouse IgG (1.96 mg/mL, abcam), 1.0 mol/L), goat anti-mouse IgG H&L (Biotin) (2 mg/mL, abcam), streptavidin (HRP) (1 mg/mL, abcam), 3′,5,5′-tetramethylbenzidine (TMB) solution (BIO-RAD), and the QuickQuant Mouse IgG Quantification Kit (funakoshi) were purchased and used as received. Poly(oxyethylene sorbitan monolaurate) (Tween 20, Tokyo Kasei) was used after diluting to a concentration of 0.5% in PBS after purchase. This is a purified goat polyclonal antibody (IgG), prepared by injecting whole mouse IgG into a healthy goat. The product specifically targets mouse IgG. This antibody has been shown to react with mouse IgG in ELISA (1:10000) and has been evaluated for activity using our previous reports.[10,31] ELISA coating buffer (abcam) was used after diluting 10 times with ultrapure water after purchase.

Methods. *IgG−PNIPAAm Conjugate Preparation by the GF Method.*[30,31] Synthesis was performed according to our previously established reports, as we allowed conjugation between NHS-CTA and IgG, and an alkaline buffer was used to facilitate carbodiimide chemistry (Scheme 1). The buffer was prepared by dissolving $NaHCO_3$ (105 mg, 1.25 mmol) in ultrapure water (resistivity value: 18.2 Ω cm, 20 mL). Subsequently, an aqueous NaOH solution (1 mol L^{-1}) was added dropwise to the buffer until the pH of the solution reached 8.6. Before conjugation, IgG (400 μg, 0.0026 μmol) was diluted to 1 mg/mL in $NaHCO_3$ buffer, then NHS-CTA dissolved in DMF (0.26 μmol, 123.1 μg) (21 μL) was added, and the reaction was continued for 24 h in a 25 °C block bath shaker. After the reaction, the byproducts were removed by ultrafiltration (Amicon Ultra Centrifugal Filter, MWCO

https://doi.org/10.1021/acsomega.4c00103
ACS Omega 2024, 9, 22043−22050

10000, 0.5 mL), and the IgG conjugates were washed three times with PBS. The synthesized IgG-CTA (400 μg, 0.0026 μmol) was mixed with 200 μL of PBS (pH 7.5) in a 1.5 mL microtube. Then NIPAAm (18 mg, 160 μmol) and 200 μL of PBS containing (3.6 μmol, 1.2 mg) of the initiator VA-044 were added and dissolved in the previous solution. The reaction was then carried out in a shaking water bath at 32 °C for 24 h.

IgG−PNIPAAm Conjugate Preparation Using the GT Method.[20] IgG (400 μg, 0.0026 μmol) was diluted to 1 mg/mL by replacing the solution with $NaHCO_3$ buffer. PNIPAAm with an NHS end group (NHS-PNIPAAm) (0.26 μmol) was dissolved in DMF (21 μL) and added to the IgG solution (Scheme 1). The solution was then reacted at 4 °C for 12 h, purified by centrifugal dialysis, and the solution was replaced with PBS. For molecular weights of 25,000 and 50,000, COOH-terminal PNIPAAm was synthesized and conjugated to the antibody by active esterification to form amide bonds. The synthesized polymers are mentioned in the Supporting Information.

Sodium Dodecyl Sulfate-Polyacrylamide (SDS-PAGE) Measurement. The IgG polymeric conjugates were then characterized by SDS-PAGE compared to IgG and the polymer solution, alone as previously mentioned in our previous publications.[1,10]

DLS Measurement. The conjugate solutions prepared were diluted and adjusted with PBS to the IgG concentration of 0.05 mg/mL and measured using a Malvern Zetasizer-Nano ZSP at λ = 633 nm, scattering angle 173°, and temperature 25 °C. The diameter and aggregation state of the samples were evaluated.

Lower Critical Solution Temperature (LCST) Measurement. The temperature dependence of the transmittance in the prepared samples was measured by using a spectrophotometer. The prepared conjugate solution was diluted and adjusted to 0.5 mg/mL IgG concentration with PBS. The sample solution and the stirring bar were subjected to absorbance measurement using a spectrophotometer in a nitrogen atmosphere at a wavelength of 450 nm, a temperature range of 25−40 °C, and a temperature increase rate of 0.2 °C/min.

Evaluation of the IgG Recovery Ratio. The conjugate solutions prepared were diluted with PBS to an IgG concentration of 0.5 mg/mL, and then 300 μL was added to a 1.5 mL microcentrifuge tube and centrifuged at 37 °C and 15,000 rpm for 15 min. After 240 μL of the supernatant was aliquoted, 240 μL of PBS was added, and the solution was redissolved. The IgG concentration in the solution was then measured by the BCA method, and the recovery ratio was calculated.

BCA Method for the IgG Concentration Assay. The conjugate solutions prepared were diluted with PBS and adjusted to a concentration of IgG of 0.05 mg/mL. Then 25 μL of this solution and 200 μL of working solution (Micro BCATM Protein Assay Kit) were added to 96 well plates and incubated at 37 °C for 30 min. The absorbance of each sample was measured at a wavelength of 562 nm, and the BSA concentration was calculated according to the manufacturer's instructions based on the IgG calibration curve (0, 0.0125, 0.025, 0.05, and 0.1 mg/mL).

Evaluation of the Antibody Temperature Responsive Polymeric Conjugate Binding Constant to Antigen. A sandwich enzyme-linked immunosorbent assay (ELISA, developed in-house) was used to measure the apparent binding affinities of the IgG-CTA and IgG−polymer conjugates. The primary antibody (1.0 mg mL^{-1}) was first stabilized into a 96-well plate and incubated overnight. Following stabilization, the plate was washed five times with PBS containing 0.5% Tween 20. Second, the plate was blocked with 200 μL of blocking buffer and incubated for 30 min. The plate was then washed five times with PBS containing 0.5% Tween 20. Third, 100 μL of antigen was added to the 96-well plate by varying the antigen concentration from 10 to 118 pg mL^{-1} and incubated for 1 h. Mouse IgG was used as the model antigen. In the next step, the plate was washed five times with PBS containing 0.5% Tween 20. Thereafter, 1 mg mL^{-1} IgG-CTA biotin-labeled or conjugates were added and incubated for 30 min. After that, HRP-conjugated streptavidin was added, and the mixture was incubated for 30 min. The binding affinity was evaluated. After five washes with PBS containing 0.5% Tween 20, TMB was added. The final assay signals were recorded by measuring the absorbance at 450 nm after 10 min of incubation with an acid treatment to stop the enzymatic reaction.

Evaluation of the Antibody Temperature-Responsiveness of Polymer Antigenic Enrichment Efficacy Using a Lateral Flow Immunoassay Strip (LFIA). Different concentrations of mouse IgG were tested by using LFIA as a model antigen. QuickQuant Mouse IgG Quantification Kit (funakoshi) was used. Antigen concentrations were (200, 100, 50, 25, 10, and 5 ng/mL) to determine the lower detection limit of this LFIA. Samples that were negative tested using the LFIA QuickQuant Mouse IgG Quantification Kit (funakoshi) were used and were retested again after polymeric enrichment (polymer prepared by the GF method). Mouse IgG purified (10 ng/mL) 900 μL in PBS was mixed with antibody-polymer conjugate and incubated for 1 h, then 3 mg of free polymer was added and centrifuged in microtubes at 37 °C and 13 000*g* for 15 min. The supernatant (900 μL) and the precipitate (100 μL) were collected, and the concentrated portion was again evaluated by LFIA.

RESULTS AND DISCUSSION

Evaluation of the Efficiency of Introduction of Immunoglobulin G (IgG)−poly(*N*-Isopropylacrylamide) (PNIPAAm). IgG−polymeric conjugate products were applied to SDS-PAGE to confirm the molecular weight shift due to the introduction of the polymer, as shown in Figure 1. In the case of IgG−PNIPAAm that was prepared by the GT method, a molecular weight shift was observed with smeared migration

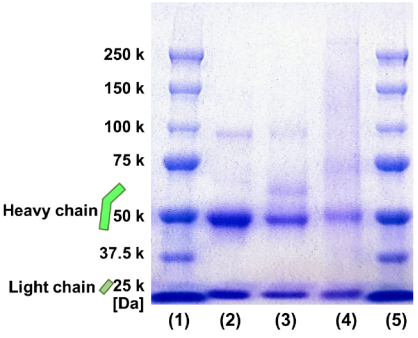


Figure 1. SDS-PAGE analysis for IgG−PNIPAAm conjugates: (lanes: (1) protein standard, (2) IgG, (3) to 2,500, (4) from 400 mM, and (5) protein standard).

https://doi.org/10.1021/acsomega.4c00103
ACS Omega 2024, 9, 22043−22050

patterns between 50 and 75 kDa, confirming the increase in molecular weight due to the introduction of the polymer. The ratio of polymer introduction to antibody was calculated from the area of the unreacted 50 kDa band and the shifted band of polymeric conjugates using ImageJ and was found to be 34% as shown in Table 1. This value is relatively close to the

Table 1. Characterization of Conjugates of IgG−PNIPAAm

sample name	introduced molecular weight in GT method (g/mol)	monomer concentration for synthesis in GF method (mM)	conjugation yield (%)	LCST (°C)	IgG recovery ratio (%)
GT-2,500	2,500	–	34	40	–
GT-25,000	25,000	–	–	30.5	37.6
GT-50,000	50,000	–	–	29	72.8
GF-250	–	250	–	31	45.3
GF-400	–	400	61	31	66.5
GF-550	–	550	–	30	71.3

introduction efficiency of conventionally used polymers with NHS groups at the ends, suggesting that conjugation at the polymeric ends by the GT method has low introduction efficiency. On the other hand, in IgG−PNIPAAm prepared by the GF method, a wide band widening range appeared between 50 and 250 kDa, as mentioned before in our previous studies.[1,10,30,31] Similarly, the polymer introduction ratio was calculated to be 61%, which is higher than that of the GT method. Compared to the GT method, GF is less affected by steric hindrance and more efficient in polymer introduction, as polymers are directly introduced by polymerization from monomers. Furthermore, in the 400 mM band, a higher molecular weight shift was shown when compared to the GT method, suggesting that the GF method can introduce high molecular weight polymers to proteins. The band visibility in our SDS-PAGE results and the polymer introduction efficiency differences between GT and GF conjugates were consistent with the previous report by Lin et al. They tried to compare the GT and GF methods in small interfering ribonucleic acid conjugation to polymer in a similar PPC system.[25] Another couple of interesting studies had compared GT and GF methods for the introduction of polymers from cellulose substrates or graphene surfaces and both concluded that the GF method was superior to controlling the surface distribution of polymers than the GT method.[26,27]

Temperature-Responsive Phase Transition Behavior. To confirm whether the introduction of PNIPAAm into the antibody provided temperature responsiveness, we measured LCSTs and observed the temperature-responsive phase transition. LCST was defined as the temperature at which 50% transmission was observed. The LCST of IgG−PNIPAAm produced by the GT method decreased with increasing molecular weight: 40 °C, 30.5 °C, and 29.5 °C when the length of the polymer chain introduced into the antibody was changed to 2,500, 25,000, and 50,000, respectively (Figure 2). These findings were parallel with Hazer et.al., previous study as they reported that the LCST of the PNIPAAm-PEG copolymer showed a significant increase as the percentage of PEG in the copolymer increased, indicating that the percentage of the hydrophilic conjugate to PNIPAAm affects LCST.[32]

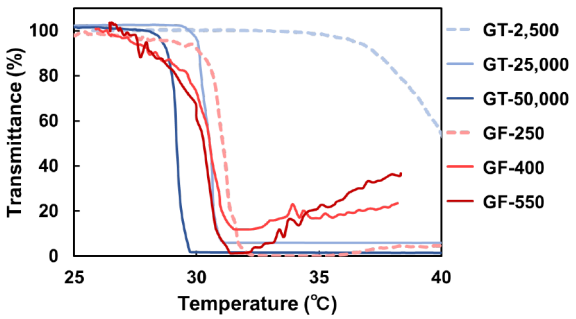


Figure 2. Transmittance of the IgG−PNIPAAm solutions against temperature (thermal-response; solvent: PBS (pH: 7.4), IgG concentration: 0.5 mg/mL, heating rate: 0.2 °C/min, wavelength: 450 nm).

The LCST of IgG−PNIPAAm produced by the GF method was 31 °C, which was close to that of IgG−PNIAAm produced by the GT method, which introduced a polymer with a chain length of tens of thousands. Field flow fractionation measurements from our previous case study, combined with the results of this experiment, suggest that the molecular weight of the polymer in the conjugate, prepared using the GF method, is in the tens of thousands.[31]

Characterization of IgG−PNIPAAm Using DLS. DLS measurements were performed to confirm the change in particle size as a result of polymer conjugation. The sample with a polymer chain length of 50,000 prepared by the GT method showed a particle size of 29.2 ± 10.1 nm below LCST, which is larger than the other samples (Figure 3A). This result suggests that amide hydrogen bonds were induced because the distance between the chain lengths of PNIPAAm was closer.[33] This leads to a larger particle size. We reported that the IgG-CTA and IgG−PNIPAAm precursor prepared by the GF method had particle sizes of 100−200 nm in our previous study (Figure 3B).[31] The investigation approved the formation of 100−200 nm particle size by the GF method in APCs.

Thermal Precipitation and Recovery Ratios of IgG−PNIPAAm Temperature-Responsive Polymeric Conjugates. To compare the effect of different conjugation methods on the thermal precipitation efficiency, we evaluated the recovery ratio of IgG. All conjugates were thermally stimulated, followed by centrifugation to allow the sedimentation of the polymeric conjugates. IgG concentration in the supernatant was measured to calculate the IgG recovery ratio. In the sample with a molecular weight of 2,500 prepared by the GT method, IgG could not be recovered because thermal precipitation did not occur (Figure 4A). In samples with molecular weights of 25,000 and 50,000, IgG recovery was about 40% and 70%, respectively, and the recovery ratio increased with increasing molecular weight. For samples prepared using the GF method, the recovery ratios were approximately 45% at 250 mM and approximately 70% at 400 and 550 mM, respectively (Figure 4B). All samples prepared by the GF method, as well as those with a polymer chain length of 50,000 prepared by the GT method, exhibited high recovery values, which can indicate the formation of particles. The investigation implies an improvement of the thermal precipitation efficiency that could have been caused by entanglement between chain lengths when the PNIPAAm chain length introduced and the amount of free PNIPAAm in solution were increased.

Evaluation of the IgG−PNIPAAm Antigen Binding Constant. The apparent binding affinities of the sample with a

https://doi.org/10.1021/acsomega.4c00103
ACS Omega 2024, 9, 22043−22050

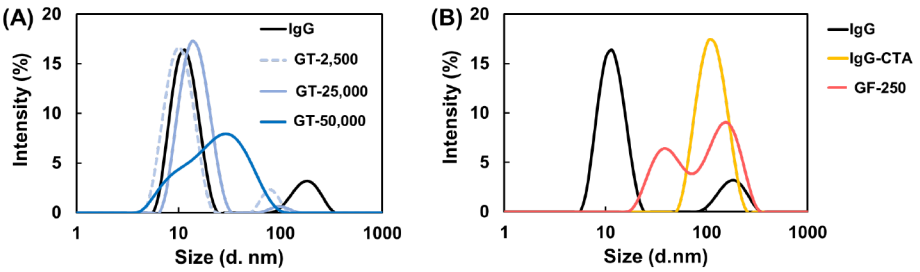


Figure 3. Confirmation of the particle size of IgG−PNIPAAm conjugates using DLS at 25 °C and IgG concentration of 0.05 mg/mL. (A) Samples produced by the GT method. (B) samples by the produced GF method.

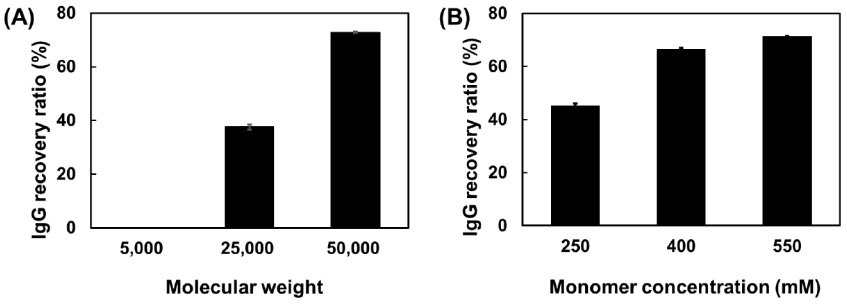


Figure 4. Effects of different conjugation methods on the IgG recovery ratio (mean ± SD, $n = 3$). (A) Samples produced using the GT method. (B) Samples produced by the GF method.

polymer chain length of 20,000 prepared by the GT method (GT-25,000) and prepared by the GF method at 400 mM conjugates (GF-400) were evaluated using a sandwich ELISA (developed in-house). The results of binding affinities to the free antigen are listed in Figure 5. When the maximum binding

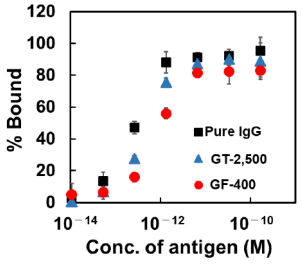


Figure 5. Effect of antigen concentration on antigen−antibody affinities measured by ELISA (mean ± SD, $n = 3$).

ratio of native IgG to the antigen was 100%, the maximum binding ratio of the GT-2,500 conjugate and the GF-400 conjugates was 90% and 80%, respectively. The maximum binding ratio of the GF-400 conjugate was similar to that of our previous study, suggesting that the introduction of polymers slightly decreased the binding affinity of the antibody. However, in the GT-2,500 conjugate, the maximum binding ratio was slightly lower than the free antibody and as good as the GF method one, presumably due to the close structure of the conjugate produced by the GT and GF methods. Both the GT and GF methods, in most cases, result in similar cyclic peptide−polymer conjugates. This has been discussed in studies where peptide−polymer conjugates were systematically compared using both the GT synthesis route and the GF polymer synthesis route.[34]

Previously, our team succeeded in the introduction of polymeric antibodies by click reaction in a GT strategy with high polymer introduction efficiency, but the maximum antigenic binding ratio decreased markedly to approximately 30% after polymeric conjugation.[10] These findings suggest that antibody modification at the polymer end is less affected by the antigen−antibody reaction than polymer modification at the polymer side chain.

Evaluation of Antigen Enrichment Using a Lateral Flow Immunoassay Strip. Antibody-temperature-responsive polymer conjugates prepared by the GF method were used to enrich antigens to evaluate their antigen enrichment capacity and their potential in improving the diagnostic sensitivity of LFIA. Mouse IgG was used as the model antigen. The minimum detection limit of mouse IgG using LFIA was 25 ng/mL, as shown in Figure 6A while lower concentrations, including 10 and 5 ng/mL, showed false negative results. After polymer introduction, antigen−antibody reaction was allowed, and antigens were enriched in the misdiagnosed sample of 10

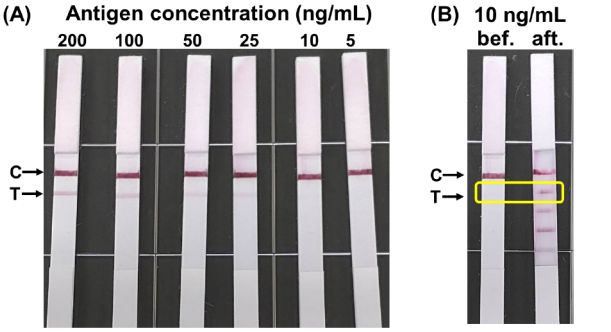


Figure 6. Lateral flow immunoassay (LFIA) evaluation for mouse IgG detection using our developed antibody-temperature-responsive polymer enrichment strategy. (A) Different concentrations of mouse IgG were tested using a lateral flow immunoassay. (B) Comparison of misdiagnosed samples before and after polymeric enrichment using the mouse antibody-temperature-responsive polymer.

https://doi.org/10.1021/acsomega.4c00103
ACS Omega 2024, 9, 22043−22050

ng/mL by thermal stimulation followed by centrifugation. The lateral flow band of the 10 ng/mL misdiagnosed sample before and after enrichment, which was not visible before enrichment, became clearly visible after enrichment (Figure 6B). Interestingly, polymeric enrichment allowed different subclasses of mouse IgG to be enriched and visualized for the first time compared to the highest concentration of the antigen that did not appear without our strategy. The enrichment capacity in this experiment was up to 10 times that of the original concentration. The sample with an antigen concentration of 10 ng/mL, which was misdiagnosed without polymeric enrichment, was enriched to an antigen concentration equivalent to 100 ng/mL and the test line became visible after applying our strategy. These data showed good agreement with our previous LFIA results, as we succeeded in antigenic enrichment and avoided LFIA misdiagnosis in our previous reports.[1,10] Furthermore, there are no other reports of attempts to enhance the sensitivity of lateral flow immunoassays by enriching antigens using antibody-PNIPAAm conjugates produced by the GF method. In addition, it is known that immobilization of antibodies on the surface of nanoparticles enhances the diagnostic performance of ELISA. This trend is also observed in nanoparticle-produced antibody-PNIPAAm conjugates produced by the GF method reported in our previous study.[31] From this, it is suggested that the GF method is a superior technique in terms of enhancing the diagnostic performance of lateral immunoassays after antigen enrichment. Finally, what makes the GF method superior to the GT method is that in the GF method, antibodies are conjugated to responsive polymers to produce PPCs before antigen−antibody interactions, which allow its involvement in a variety of applications.[1,10] On the basis of the above, the GF method is expected to highly contribute to LFIA diagnostic sensitivity improvement by antigen enrichment.

CONCLUSIONS

This research describes the evaluation of IgG−PNIPAAm conjugates produced by two different methods, GT and GF, with respect to their efficiency and temperature-responsive phase transition behavior. The efficiency of polymer introduction to antibody was evaluated by SDS-PAGE analysis, and the ratio of polymer introduction to antibody was calculated. The GT method showed a lower introduction efficiency than the GF method. LCSTs were measured to observe the temperature-responsive phase transition, and the LCST of IgG−PNIPAAm produced by the GF method was found to be close to that of IgG−PNIPAAm produced by the GT method, which introduced a polymer with a chain length of tens of thousands. DLS measurements were performed to confirm the change in particle size as a result of polymer conjugation. The particle size of the IgG−PNIPAAm conjugate produced by the GT method increased with an increasing molecular weight of the polymer chain length. However, the IgG−PNIPAAm conjugate produced by the GF method had a particle size of 100−200 nm, confirming the formation of APCs. In summary, both methods offer unique advantages and challenges. The GT method provides simplicity but demands careful design and purification, whereas the GF allows precise control but requires appropriate surface chemistry for initiation. We need to select the most appropriate method based on specific applications and desired surface modifications. In general, these findings provide important insight into the design and development of novel biomaterials that respond to temperature.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c00103.

^{1}H NMR spectrum of COOH-PNIPAAm in DMSO (Figure S1) and GPC results of COOH-PNIPAAm (Figure S2 and Table S1) (PDF)

AUTHOR INFORMATION

Corresponding Authors

Mitsuhiro Ebara − *Research Center for Macromolecules and Biomaterials, National Institute for Materials Science (NIMS), Tsukuba 305-0044, Japan; Graduate School of Pure and Applied Sciences, University of Tsukuba, Tsukuba 305-8577, Japan; Graduate School of Industrial Science and Technology, Tokyo University of Science, Shinjuku, Tokyo 162-0825, Japan;* orcid.org/0000-0002-7906-0350; Email: ebara.mitsuhiro@nims.go.jp

Ahmed Nabil − *Research Center for Macromolecules and Biomaterials, National Institute for Materials Science (NIMS), Tsukuba 305-0044, Japan; Biotechnology and Life Sciences Department, Faculty of Postgraduate Studies for Advanced Sciences (PSAS), Beni-Suef University, Beni-Suef 62511, Egypt; Egyptian Liver Research Institute and Hospital (ELRIAH), El Mansoura 35511, Egypt;* orcid.org/0000-0002-5617-4726; Email: tolba.ahmednabil@nims.go.jp

Authors

Erika Yoshihara − *Research Center for Macromolecules and Biomaterials, National Institute for Materials Science (NIMS), Tsukuba 305-0044, Japan; Graduate School of Pure and Applied Sciences, University of Tsukuba, Tsukuba 305-8577, Japan*

Michihiro Iijima − *Department of Materials Chemistry and Bioengineering, National Institute of Technology, Oyama College (NIT, Oyama College), Oyama 323-0806, Japan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c00103

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors of this manuscript express their great gratitude to Nanotechnology Innovation Station at NIMS for their support. The authors are grateful to Prof. Allan S. Hoffman (University of Washington) for his continued and valuable discussions.

REFERENCES

(1) Nabil, A.; Yoshihara, E.; Hironaka, K.; Hassan, A. A.; Shiha, G.; Ebara, M. Temperature Responsive Smart Polymer for Enabling Affinity Enrichment of Current Coronavirus (SARS-CoV-2) to Improve Its Diagnostic Sensitivity. *Comput. Struct. Biotechnol. J.* **2021**, *19*, 3609−3617.

(2) Chen, Z.; Li, Y.; Wu, B.; Hou, Y.; Bao, J.; Deng, X. A Patient with COVID-19 Presenting a False-Negative Reverse Transcriptase Polymerase Chain Reaction Result. *Korean J. Radiol.* **2020**, *21* (5), 623−624.

(3) Nabil, A.; Uto, K.; Elshemy, M. M.; Soliman, R.; Hassan, A. A.; Ebara, M.; Shiha, G. Current Coronavirus (Sars-Cov-2) Epidemio-

logical, Diagnostic and Therapeutic Approaches: An Updated Review until June 2020. *Excli J.* **2020**, *19*, 992−1016.
(4) Winichakoon, P.; Romanee, C.; Liwsrisakun, C.; Salee, P.; Goonna, A.; Limsukon, A.; Kaewpoowat, Q. Negative Nasopharyngeal and Oropharyngeal Swabs Do Not. *J. Clin. Microbiol.* **2020**, *58* (5), 19−20.
(5) Zou, L.; Ruan, F.; Huang, M.; Liang, L.; Huang, H.; Hong, Z.; Yu, J.; Kang, M.; Song, Y.; Xia, J.; Guo, Q.; Song, T.; He, J.; Yen, H.-L.; Peiris, M.; Wu, J. SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. *N. Engl. J. Med.* **2020**, *382* (12), 1177−1179.
(6) Wang, X.; Tan, L.; Wang, X.; Liu, W.; Lu, Y.; Cheng, L.; Sun, Z. Comparison of Nasopharyngeal and Oropharyngeal Swabs for SARS-CoV-2 Detection in 353 Patients Received Tests with Both Specimens Simultaneously. *Int. J. Infect Dis.* **2020**, *94*, 107−109.
(7) Ai, T.; Yang, Z.; Hou, H.; Zhan, C.; Chen, C.; Lv, W.; Tao, Q.; Sun, Z.; Xia, L. Correlation of Chest CT and RT-PCR Testing for Coronavirus Disease 2019 (COVID-19) in China: A Report of 1014 Cases. *Radiology* **2020**, *296* (2), No. E32−E40.
(8) Nabil, A.; Elshemy, M. M.; Uto, K.; Soliman, R.; Hassan, A. A.; Shiha, G.; Ebara, M. Coronavirus (Sars-Cov-2) in Gastroenterology and Its Current Epidemiological Situation: An Updated Review until January 2021. *Excli J.* **2021**, *20*, 366−385.
(9) Fang, Y.; Zhang, H.; Xie, J.; Lin, M.; Ying, L.; Pang, P.; Ji, W. Sensitivity of Chest CT for COVID-19: Comparison to RT-PCR. *Radiology* **2020**, *296* (2), No. E115−E117.
(10) Hironaka, K.; Yoshihara, E.; Nabil, A.; Lai, J. J.; Kikuchi, A.; Ebara, M. Conjugation of Antibody with Temperature-Responsive Polymer: Via in Situ Click Reaction to Enable Biomarker Enrichment for Increased Diagnostic Sensitivity. *Biomater. Sci.* **2021**, *9* (14), 4870−4879.
(11) Peeri, N. C.; Shrestha, N.; Siddikur Rahman, M.; Zaki, R.; Tan, Z.; Bibi, S.; Baghbanzadeh, M.; Aghamohammadi, N.; Zhang, W.; Haque, U. The SARS, MERS and Novel Coronavirus (COVID-19) Epidemics, the Newest and Biggest Global Health Threats: What Lessons Have We Learned? *Int. J. Epidemiol.* **2021**, *49* (3), 717−726.
(12) Prompetchara, E.; Ketloy, C.; Palaga, T. Immune Responses in COVID-19 and Potential Vaccines: Lessons Learned from SARS and MERS Epidemic. *Asian Pacific J. Allergy Immunol.* **2020**, *38* (1), 1−9.
(13) Peiris, J. S. M.; Chu, C. M.; Cheng, V. C. C.; Chan, K. S.; Hung, I. F. N.; Poon, L. L. M.; Law, K. I.; Tang, B. S. F.; Hon, T. Y. W.; Chan, C. S.; Chan, K. H.; Ng, J. S. C.; Zheng, B. J.; Ng, W. L.; Lai, R. W. M.; Guan, Y.; Yuen, K. Y. Clinical Progression and Viral Load in a Community Outbreak of Coronavirus-Associated SARS Pneumonia: A Prospective Study. *Lancet* **2003**, *361* (9371), 1767−1772.
(14) Liu, J.; Mazumdar, D.; Lu, Y. A Simple and Sensitive "Dipstick" Test in Serum Based on Lateral Flow Separation of Aptamer-Linked Nanostructures. *Angew. Chem.* **2006**, *118* (47), 8123−8127.
(15) Tabarsi, P.; Marjani, M.; Moniri, A.; Farnia, P.; Dizaji, M. K.; Garssen, J.; Adcock, I. M.; Mortaz, E. Investigation of Urine Lipoarabinomannan in Human Immunodeficiency Virus Patients with or without Coinfection with Tuberculosis in Iran. *Int. J. Mycobact.* **2016**, *5*, S186−S187.
(16) Oehm, A.; Behrmann, I.; Falk, W.; Pawlita, M.; Maier, G.; Klas, C.; Li-Weber, M.; Richards, S.; Dhein, J.; Trauth, B. C.; Ponsting, H.; Krammer, P. H. Purification and Molecular Cloning of the APO-1 Cell Surface Antigen, a Member of the Tumor Necrosis Factor/Nerve Growth Factor Receptor Superfamily. Sequence Identity with the Fas Antigen. *J. Biol. Chem.* **1992**, *267* (15), 10709−10715.
(17) Heskins, M.; Guillet, J. E. Solution properties of poly (N-isopropylacrylamide). *J. Macromol. Sci., Part A: Chem.* **1968**, *2*, 1441−1455.
(18) Chen, G.; Hoffman, A. S. Preparation and Properties of Thermoreversible, Phase-Separating Enzyme-Oligo(N-Isopropylacrylamide) Conjugates. *Bioconjugate Chem.* **1993**, *4* (6), 509−514.
(19) Ding, Z.; Chen, G.; Hoffman, A. S. Synthesis and purification of thermally sensitive oligomer−enzyme conjugates of poly(*N*-isopropylacrylamide)-trypsin. *Bioconjugate Chem.* **1996**, *7*, 121−125.
(20) Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Sakurai, Y.; Okano, T.; Matsukata, M.; Kikuchi, A. Temperature-Responsive Bioconjugates. 3. Antibody-Poly(N-Isopropylacrylamide) Conjugates for Temperature-Modulated Precipitations and Affinity Bioseparations. *Bioconjugate Chem.* **1994**, *5* (6), 577−582.
(21) Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. Temperature-Responsive Bioconjugates. 1. Synthesis of Temperature-Responsive Oligomers with Reactive End Groups and Their Coupling to Biomolecules. *Bioconjugate Chem.* **1993**, *4* (1), 42−46.
(22) Cobo, I.; Li, M.; Sumerlin, B. S.; Perrier, S. Smart Hybrid Materials by Conjugation of Responsive Polymers to Biomacromolecules. *Nat. Mater.* **2015**, *14* (2), 143−149.
(23) Purohit, P.; Bhatt, A.; Mittal, R. K.; Abdellattif, M. H.; Farghaly, T. A. Polymer Grafting and Its Chemical Reactions. *Front. Bioeng. Biotechnol.* **2023**, *10*, 1−22.
(24) Mathieu-Gaedke, M.; Böker, A.; Glebe, U. How to Characterize the Protein Structure and Polymer Conformation in Protein-Polymer Conjugates − a Perspective. *Macromol. Chem. Phys.* **2023**, *224* (3), 1−18.
(25) Lin, E. W.; Maynard, H. D. Grafting from Small Interfering Ribonucleic Acid (SiRNA) as an Alternative Synthesis Route to SiRNA-Polymer Conjugates. *Macromolecules* **2015**, *48* (16), 5640−5647.
(26) Hansson, S.; Trouillet, V.; Tischer, T.; Goldmann, A. S.; Carlmark, A.; Barner-Kowollik, C.; Malmström, E. Grafting Efficiency of Synthetic Polymers onto Biomaterials: A Comparative Study of Grafting- from versus Grafting- To. *Biomacromolecules* **2013**, *14* (1), 64−74.
(27) Rubio, N.; Au, H.; Leese, H. S.; Hu, S.; Clancy, A. J.; Shaffer, M. S. P. Grafting from versus Grafting to Approaches for the Functionalization of Graphene Nanoplatelets with Poly(Methyl Methacrylate). *Macromolecules* **2017**, *50* (18), 7070−7079.
(28) Li, H.; Bapat, A. P.; Li, M.; Sumerlin, B. S. Protein Conjugation of Thermoresponsive Amine-Reactive Polymers Prepared by RAFT. *Polym. Chem.* **2011**, *2* (2), 323−327.
(29) De, P.; Li, M.; Gondi, S. R.; Sumerlin, B. S. Temperature-Regulated Activity of Responsive Polymer-Protein Conjugates Prepared by Grafting-from via RAFT Polymerization. *J. Am. Chem. Soc.* **2008**, *130* (34), 11288−11289.
(30) Yoshihara, E.; Sasaki, M.; Nabil, A.; Iijima, M.; Ebara, M. Temperature Responsive Polymer Conjugate Prepared by "Grafting from" Proteins toward the Adsorption and Removal of Uremic Toxin. *Molecules* **2022**, *27* (3), 1051.
(31) Yoshihara, E.; Nabil, A.; Mochizuki, S.; Iijima, M.; Ebara, M. Preparation of Temperature-Responsive Antibody−Nanoparticles by RAFT-Mediated Grafting from Polymerization. *Polymers* **2022**, *14* (21), 4584.
(32) Hazer, B.; Ayyıldız, E.; Bahadır, F. Synthesis of PNIPAM−PEG Double Hydrophilic Polymers Using Oleic Acid Macro Peroxide Initiator. *JAOCS J. Am. Oil Chem. Soc.* **2017**, *94* (9), 1141−1151.
(33) Onogi, S.; Lee, S. H.; Fruehauf, K. R.; Shea, K. J. Abiotic Stimuli-Responsive Protein Affinity Reagent for IgG. *Biomacromolecules* **2021**, *22* (6), 2641−2648.
(34) Larnaudie, S. C.; Brendel, J. C.; Jolliffe, K. A.; Perrier, S. Cyclic Peptide-Polymer Conjugates: Grafting-to vs Grafting-From. *J. Polym. Sci., Part A: Polym. Chem.* **2016**, *54* (7), 1003−1011.

https://doi.org/10.1021/acsomega.4c00103
ACS Omega 2024, 9, 22043−22050