J. Biol. Macromol., **17** (1), 15-21 (2017)

Article

High-sensitivity measurement of immunoglobulin G (IgG) using HRP-IgG-phospholipid polymer conjugate

Shujiro Sakaki[1,*], Tomomichi Nishino[1], Megumi Ito[2], Kouichi Maruyama[1] and Kai Sasaki[1]

[1]Department of Applied Chemistry, Akita National College of Technology, 1-1 Iijima Bunkyo, Akita 011-8511

[2]Technology Education Support Center, Akita National College of Technology, 1-1 Iijima Bunkyo, Akita 011-8511

Received February 10, 2017; Accepted June 5, 2017

Key words: horseradish peroxidase, HRP, 2-methacryloyloxyethyl phosphorylcholine, MPC, ELISA

ABSTRACT

This report describes the high-sensitivity measurements of immunoglobulin (IgG) with the help of a phospholipid polymer conjugate, 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer-IgG-horseradish peroxidase (HRP) conjugate. The phospholipid polymer, poly(MPC-*co*-2-aminoethyl methacrylate) (PMAE), was synthesized. To prepare the 1-step conjugate, IgG and PMAE were chemically modified with HRP at the same time. To prepare the 2-step conjugate, PMAE was first chemically modified with HRP, and then IgG was chemically modified. The activities of 1-step and 2-step conjugates significantly decreased compared with that of a conventional IgG conjugated with HRP (conventional conjugate) ($p < 0.05$). The detection sensitivity of the 1-step and 2-step conjugates was also compared with that of conventional conjugate. When 0.5 μg/mL anti(goat IgG)IgG was used as an analyte, compared with a conventional conjugate, the values of Δ absorbance of the 1-step and 2-step conjugates were 4.8 and 9.8 times respectively. These results demonstrate that high-sensitivity measurements are possible using HRP-IgG-PMAE conjugate.

INTRODUCTION

Enzymes used as a label molecule is more advantageous than the other kinds of label molecules with respect to immunohistochemistry and immunoassay purposes. In immunohistochemistry, an immunoglobulin G (IgG) conjugated with an enzyme (enzyme-IgG conjugate) permits localization and observation of cellular antigens by optical and electron microscopy in relation to tissue structures [1–3]. Immunologic measurement

*To whom correspondence should be addressed: sakaki@akita-nct.jp, Tel: +81-18-847-6065

is useful for the detection or quantification of an analyte (a very small amount of a physiologically active molecule). The use of an enzyme-linked immunosorbent assay (ELISA), an immobilized antibody, and an enzyme-antibody conjugate for the measurement of antigens is known as the sandwich immunoassay technique [4, 5]. However, for immunohistochemistry and immunoassay, a high-sensitivity analysis is necessary for the early detection and diagnosis of diseases.

Various enzymes can be modified chemically by synthetic polymers having phospholipid polar groups. 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers are excellent for the enhancement of enzymatic functions and stability. Miyamoto et al. reported an MPC polymer with a molecular weight of 5×10^3 that showed no adverse effect on papain after conjugation.[6] In another study, Miyamoto et al. compared the stability of papain–poly(MPC) and papain–PEG conjugates and reported that the former exhibited higher stability at 40 °C in buffer.[7] Depp et al. applied protein-initiated atom transfer radical polymerization to synthesize grafts of high Mw poly(MPC), poly(*N*-2-hydroxypropylmethacrylamide (HPMA)), and poly(monomethoxy-polyethyleneglycol-methacrylate) with chymotrypsin.[8] Oupicky et al. synthesized chymotrypsin-poly(HPMA)) conjugates that showed improved proteolytic stability and reduced immunogenicity.[9] Phospholipid polymers composed of MPC units have excellent biocompatibility and thrombogenicity properties and so are well-suited as biomaterials.[10]

For the present study, we synthesized a water-soluble MPC polymer with primary amino groups to bind IgG and horseradish peroxidase (HRP). We also investigated the relative enzyme activity and the chemical modifications between the MPC polymer, IgG, and HRP. Finally, we demonstrated a high-sensitivity measurement using MPC polymer-IgG-HRP conjugate.

Materials and Methods

Synthesis of water-soluble MPC polymer

A random copolymer of MPC and 2-aminoethyl methacrylate (AEMA) hydrochloride (Polysciences Inc., PA, U.S.A.) was prepared using the conventional radical polymerization technique in H_2O with 2,2'-azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044) (Wako Pure Chemical Industries Ltd., Osaka, Japan) as the initiator. All other reagents were of an extra-pure grade. To prepare a polymerization mixture, 1187.5 μmol MPC, 62.5 μmol AEMA, and 12.5 μmol VA-044 were dissolved in 20 mL of H_2O. After purging with Ar gas to remove oxygen in the aqueous solution, the solutions were put into glass tubing with sealed tubing and then mixed. The polymerization was carried out at 50 °C for 90 h.

The chemical structure of poly (MPC-*co*-AEMA) (PMAE) was confirmed using ^{1}H-NMR (JNM-AL400; JEOL Ltd., Tokyo, Japan) in D_2O. Gel permeation chromatography (TSKgel G5000PW$_{XL}$ and TSKgel G4000PW$_{XL}$; Tosoh Corp., Tokyo, Japan) was used to determine the number-average molecular weight (M_n) and weight-average molecular weight (M_w) of

Table 1 Synthetic results of poly(MPC-*co*-AEMA) (PMAE)

Mole fraction of MPC		Yield (%)	M_n [b]	M_w [b]
In feed	In polymer[a]			
0.95	0.99	42	> 15 × 10^6	> 15 × 10^6

a: Unit of mole fraction of MPC in the copolymer was determined by ^{1}H-NMR in D_2O.

b: Determined by GPC with the poly(ethylene oxide) standard, M_n and M_w represent the number-average and weight-average molecular weight, respectively.

PMAE in H_2O with poly (ethylene oxide) standards. To prepare the propionic acid succinimide ester, 0.5 mmol of propionic acid, 0.6 mmol *N,N'*-carbonyldiimidazole, and 0.6 mmol of *N*-hydroxysuccinimide were dissolved in 1.3 mL of *N*,*N*-dimethylformamide and incubated at 25 °C for 3 h. Then, 20 mg of PMAE was dissolved in 0.6 mL of the propionic acid succinimide ester solution and incubated overnight at 4 °C to allow the reaction between the amino group and propionic acid succinimide ester. After the chemical structure was confirmed, the MPC polymer with the amino group was purified by dialysis against H_2O. Figure 1 shows the chemical structure of poly(MPC-*co*-AEMA) (PMAE). Table 1 presents the synthesis results.

Preparation of conjugates

To prepare the 1-step conjugate, 1000 μL of a 10 mg/mL horseradish peroxidase (HRP, Wako Pure Chemical Industries Ltd., Osaka, Japan) aqueous solution and 200 μL of 250 mM sodium periodate aqueous solution were mixed and stirred slowly at 25 °C for 20 min. Then, they were dialyzed against 1.0 mM acetate buffer (pH 4.5) at 4 °C for 24 h. To form a Schiff base, 20 μL of a 200 mM carbonate buffer (pH 10.0), 500 μL of a 1.0 mg/mL PMAE solution (10 mM carbonate buffer, pH 10.0) and 500 μL of a 4.0 mg/mL IgG from goat serum (IgG, Sigma-Aldrich Corp. LLC, MO, U.S.A.) solution (10 mM carbonate buffer, pH 10.0) were added to the solution. Then, this solution was stirred slowly at 25 °C for 2 h. To reduce the Schiff base, 100 μL of 10 mg/mL sodium tetrahydroborate aqueous solution was added and then stirred slowly at 4 °C for 2 h in the dark. For purification, the solution was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) at 4 °C for 24 h.

The same method was used for the preparation of the 2-step conjugate, except that only PMAE solution was added to form the Schiff base. After slow stirring of the solution at 25 °C for 2 h, the IgG solution was added and stirred slowly at 25 °C for another 2 h.

The same method was also followed to prepare the conventional conjugate, except that 1000 μL of a 20.0 mg/mL IgG solution was added to form the Schiff base.

The 1-step, 2-step, and conventional conjugates were isolated from unreacted IgG and HRP with the help of size exclusion chromatography using Bio-Gel P-100 Gel (Bio-Rad Laboratories Inc., CA, U.S.A.), (15 × 260 mm, flow rate 6.0 mL/h, 10 mM sodium phosphate buffer (pH 7.0) as the mobile phase). A schematic representation of the synthesis of the 1-step conjugate and 2-step conjugate is shown in Figure 2.

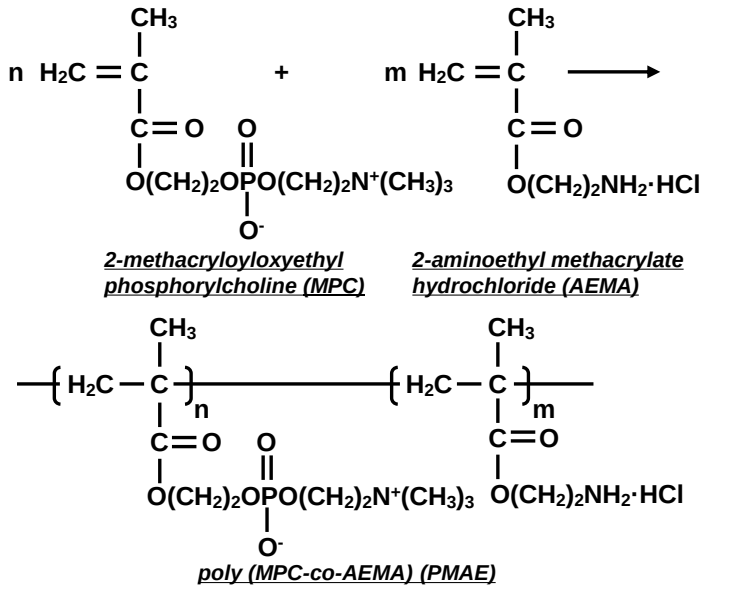

Figure 1 Chemical structure of poly(MPC-*co*-AEMA)

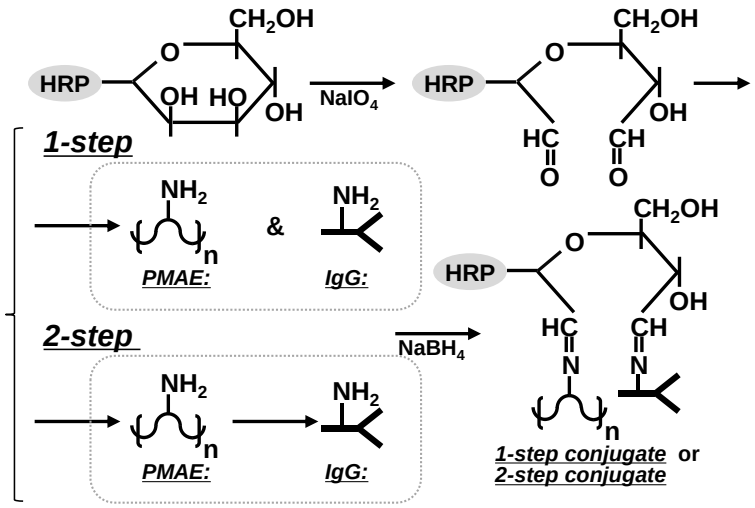

Figure 2 Schematic representation of synthesis of 1-step and 2-step conjugates

Molecular weight evaluation using SDS-PAGE

The molecular weight of the 1-step, 2-step, and conventional conjugates were evaluated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were prepared by direct mixing of each solution of the 1-step, 2-step, or conventional conjugates and the Sample Treatment for Tris solution (Cosmo Bio Co. Ltd., Tokyo, Japan).–Then, they were kept at 100 °C for 5 min. The ratio of each solution to the Tris SDS solution was 1:1 (vol/vol). Each sample was electrophoresed on polyacrylamide gel (7.5%T) for 80 min at 20 mA. A staining solution (EzStain Aqua; Atto Corp., Tokyo, Japan) was added to the gel to ascertain the location of the 1-step, 2-step or conventional conjugates.

Measurement of relative enzyme activity

Each solution of the 1-step, 2-step, and the conventional conjugates were diluted to 0.1 μg/mL HRP using 10 mM sodium phosphate buffer (pH 7.0). To measure the relative enzyme activity, 50 μL of these diluted solutions and 100 μL of ABTS 1-Component Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories Inc., SF, U.S.A.) solution were mixed at 25 °C for 10 min. The absorbance at 405 nm was measured (Model 680 Microplate Reader; Bio-Rad Laboratories Inc., CA, U.S.A.).

Measuring sensitivity for immunoassay

Figure 3 shows the procedure for measuring sensitivity. To immobilize rabbit anti (goat IgG) antibody (Rockland Immunochemicals Inc., PA, U.S.A.) on the surface of an ELISA plate H (Sumitomo Bakelite Co. Ltd., Tokyo, Japan), 100 μL of 0, 0.5 and 1.0 μg/mL rabbit anti (goat IgG) antibody solution (Dulbecco's phosphate buffer saline, D-PBS) were pipetted into a well. Then, the ELISA plate was sealed with an adhesive strip and incubated at 4 °C for 24 h

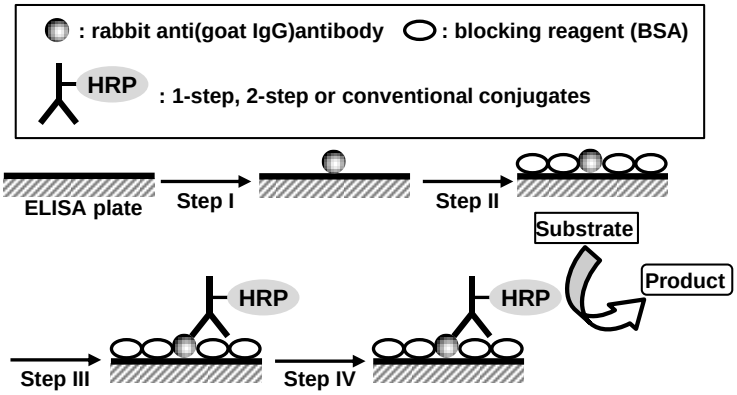

Figure 3 Procedure for measuring sensitivity

(step I). After incubation, the rabbit anti (goat IgG) antibody solution was aspirated from the well. To remove the unimmobilized antibody, the well was washed three times with 200 µL of D-PBS. After washing, 200 µL of 1% bovine serum albumin (BSA, Sigma-Aldrich Corp. LLC, MO, U.S.A.) and D-PBS were pipetted into the well. Then, the ELISA plate was sealed with an adhesive strip and incubated at 4 °C for 24 h (step II). After incubation, the BSA solution was aspirated from the well. Each solution of the 1-step, 2-step, and conventional conjugates was diluted to 20.0 µg/mL IgG using 1% BSA solution, and D-PBS was pipetted into the well. Then, the ELISA plate was sealed with an adhesive strip and incubated at 4 °C for 24 h (step III). After incubation, each solution was aspirated from the well. Then, the wells were washed three times with 200 µL of D-PBS. Later, 100 µL of ABTS 1-component microwell peroxidase substrate solution was pipetted into the well of the ELISA plate and incubated at 25 °C for 10 min (step IV). Absorbance at 405 nm was measured using the microplate reader.

Results and Discussion

Synthesis of water-soluble MPC polymer

Copolymerization proceeded homogeneously in H_2O. The PMAE chemical structure was confirmed using ^{1}H-NMR, i.e., the peaks assigned to α-CH_3 (0.95 ppm), β-CH_3 (2.0 ppm), -$N^+(CH_3)_3$ (3.1 ppm), -CH_2N- (3.3 ppm), -CH_2PO_2- (3.6 ppm), –OCH_2- (4.3 and 4.7 ppm) were observed. The mole fraction of the MPC unit in the polymer was 0.99, which was determined with the help of NMR spectroscopy, specifically taking into account the ratio of integration of -N^+ $(CH_3)_3$ (3.1 ppm) peak and -CH_2N- (3.3 ppm) peak.

Preparation of conjugates

The 1-step, 2-step, and conventional conjugates did not precipitate. Furthermore, to prepare the modified conventional conjugate, 1000 µL of a 2.0 mg/mL IgG solution was used instead of 1000µL of a 20.0 mg/mL IgG solution. The authors could not use the precipitate modified conventional conjugate in the following procedures in this experiment.

The fractions containing the 1-step, 2-step, and conventional conjugates were collected before the fraction containing unreacted IgG and HRP. In order to measure the HRP and the IgG concentration of each conjugate, absorbance at 280 nm and 403 nm was measured using a UV–VIS spectrophotometer (UV-VIS Spectrophotometer V-560, JASCO Corp.) as absorption coefficients (mg^{-1} cm^2); HRP (280 nm) = 1.209, HRP (403 nm) = 2.275, IgG (280 nm) = 1.285, IgG (403 nm) = 0.000. Concentration of HRP and IgG was 0.29 and 0.14 mg/mL, 0.29 and 0.12 mg/mL, and 0.27 and 0.93 mg/mL for 1-step conjugate, 2-step conjugate and conventional conjugate, respectively.

Molecular weight evaluation using SDS-PAGE

Figure 4 shows an SDS-PAGE image. The molecular weights of 1-step, 2-step, and conventional conjugates were over 400,000 because the stacking gel was 5%T. The SDS-PAGE result indicates that the 1-step, 2-step, and conventional conjugates were isolated from unreacted IgG and HRP. In case of molecular weight measurement by gel permeation chromatography, the peak of the 1-step and 2-step conjugates was out of the calibration curve because the size of these conjugate was too large to permeate the pores (Date not shown).

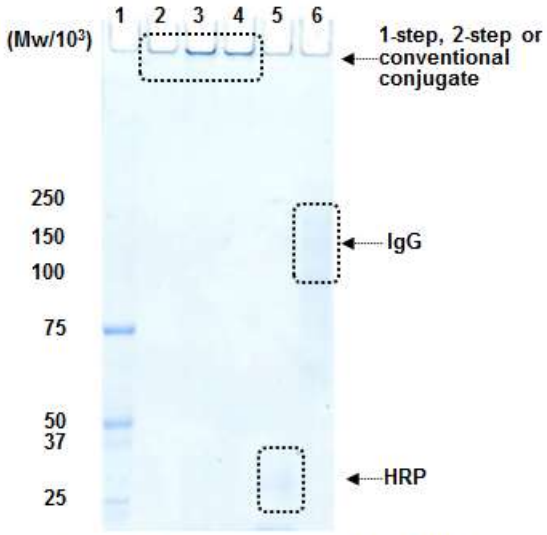

Figure 4 Molecular weight evaluation using SDS-PAGE
[polyacrylamide gel] = 7.5%T, 2.4 μg protein/lane: lane 1, molecular weight indicator; lane 2, conventional conjugate; lane 3, 1-step conjugate; lane 4, 2-step conjugate; lane 5, IgG; lane 6, HRP

Measurement of relative enzyme activity

As the absorbance of native HRP was 100%, the relative enzyme activities (%) of 1-step, 2-step, and conventional conjugates were determined. To determine if the two sets of data were significantly different from each other, the student *t*-test was used, and the *p*-value was obtained from relative enzyme activity. Figure 5 presents the relative enzyme activity results. In comparison with native HRP, the relative enzyme activities of the 1-step and 2-step and conventional conjugates were in the range of 60-77% because of chemical modification using sodium periodate and sodium tetrahydroborate. No significant difference was recorded ($p > 0.05$) between 1-step conjugate and 2-step conjugate, but a significant difference was observed ($p < 0.05$) between 1-step or 2-step conjugate and conventional conjugate.

Measuring sensitivity for immunoassay

As the absorbance of 0 μg/mL rabbit anti (goat IgG) antibody solution was blank, Δ absorbance of the 1-step, 2-step, and conventional conjugates were determined. Figure 6 presents results of the sensitivity of the 1-step, 2-step, and conventional conjugates. The Δ absorbance values of the 1-step conjugate and 2-step conjugate, using 1.0 μg/mL anti (goat IgG) antibody, were 2.8 and 6.5 times higher than that of the conventional conjugate. The Δ absorbance values of the 1-step and 2-step conjugate, using 0.5 μg/mL anti (goat IgG) antibody, were 4.8 and 9.8 times higher than that of the conventional conjugate.

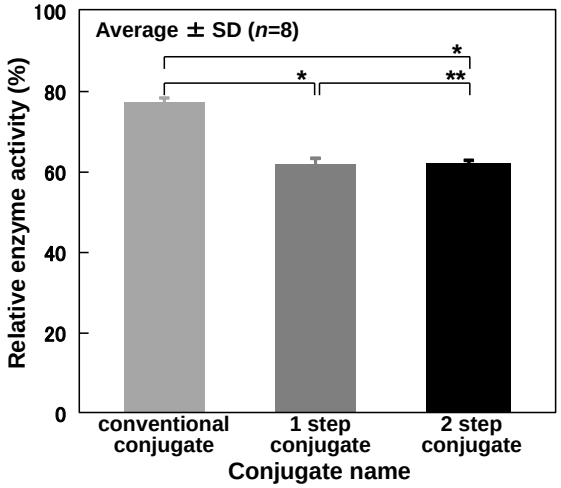

Figure 5 Relative enzyme activity of conventional, 1-step and 2-step conjugates.
Absorbance of Native HRP is 100%.
*: $p < 0.05$, **: $p > 0.05$ (Student *t-test*)

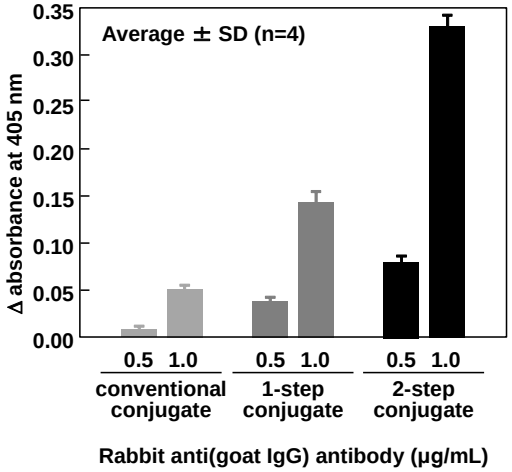

Figure 6 Measuring sensitivity of conventional, 1-step and 2-step conjugates.
The absorbance of 0 μg/mL rabbit anti(goat IgG) antibody solution is blank.

CONCLUSIONS

An amphiphilic phospholipid polymer with an amino group was synthesized using conventional radical polymerization technique. The periodic acid-Schiff reaction was used for the synthesis of HRP-IgG-poly (MPC-*co*-AEMA) (PMAE) conjugates. The enzyme-IgG-PMAE conjugate is superior to conventional enzyme-IgG conjugate with respect to high-sensitivity measurements.

ACKNOWLEDGMENTS

The authors are grateful to NOF Corp. for their assistance with regard to the provision of materials. We also thank Dr. Katsuhiko Takenaka of the Nagaoka University of Technology for his aid in relation to ^{1}H-NMR analysis.

REFERENCES

1. Osborn, M., Webster, R. E. & Weber, K., *J. Cell Biol*. **77**, R27-R34 (1978).
2. Farr, A. G. & Nakane, P. K. *J. Immunol. Meth.* **47**, 129-144 (1981).
3. Orci, L. *Diabetologia* **28**, 528-546 (1985).
4. Anderton, B. H. & Thorpe, R. C. *Immunol. Today* **2**, 122-127 (1980).
5. Wisdom, G. B. *Clin. Chem*. **22**, 1243-1255 (1976).
6. Miyamoto, D., Watanabe, J. & Ishihara, K. *J. Appl. Polym. Sci*. **91**, 827-832 (2004).
7. Miyamoto, D., Watanabe, J. & Ishihara, K. *Biomaterials* **25**, 71-76 (2004).
8. Depp, V., Alikhani, A., Grammer, V. & Lele, S. B. *Acta Biomaterialia* **5**, 560-569 (2009).
9. Oupicky, D., Ulbrich, K. & Rihova, B. *J. Bioactive Compat. Polym*. **14**, 213-231 (1999).
10. Ishihara, K. *Trend. Polym. Sci*. **5**, 401 (1997).

Communicated by Mihara Hisaaki