Electronic Supplementary Information

C-reactive protein-directed immobilization of phosphocholine ligands on a solid surface

Eunjoo Kim, Hyun-Chul Kim, Se Geun Lee, Sung Jun Lee, Tae-Jung Go, Chul Su Baek, Sang Won Jeong*

Nano & Bio Research Division, Daegu Gyeongbuk Institute of Science and Technology (DGIST), Daegu 711-873, Korea

* Corresponding author. Tel.: +82 53 7852510; fax: +82 53 7853559
E-mail address: sjeong@dgist.ac.kr (S.W. Jeong).

1. Materials and characterization

All chemicals used for synthesis were purchased from Sigma-Aldrich or Fluka. Styrene was purified by distillation under reduced pressure and divinylbenzene (DVB) was purified according to a literature procedure.[1] The purified styrene and DVB were stored at 4 ^{o}C. Standard buffer solution (pH 7.0) was purchased from Samchun Co., Ltd. (Pyeongtaek, Korea). Phosphate buffered saline (PBS, HyClone®) was purchased from Thermo Scientific Co. Ltd (Rochester, USA). Human plasma CRP, monoclonal mouse anti-human CRP (anti-CRP), goat anti-mouse IgG alkaline phosphatase conjugate (anti-IgG-AP), bovine serum albumin (BSA), p-nitrophenyl phosphate (p-NPP) substrate solution, and phosphocholine chloride calcium salt tetrahydrate were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, USA). Polyclonal goat anti-human CRP-horseradish peroxidase conjugate (anti-CRP-HRP) and tetramethylbenzidine (TMB) substrate were purchased from Koma Biotech Inc. (Seoul, Korea). CRP ELISA kit was purchased from Alpha Diagnostic International (San Antonio, USA). Human CRP-positive and CRP-negative serum samples were the components of CRPA Latex Test Set purchased from Cenogenics corp. (Morganville, USA). All other chemicals were of analytical grade and used as received.

^{1}H NMR and ^{13}C NMR spectra were obtained in $CDCl_3$ using a Bruker NMR spectrometer (AVANCE 400). FT-IR spectra were measured by a ThermoNicolet IR spectrometer (IR380). Electrospray ionization mass spectrometry was performed on a Waters Micromass ZQ (MM1) mass spectrometer. Immunoassay absorbance was read with a Thermo automatic ELISA reader (Multiskan EX). Computational simulation of the CRP-VPC binding was carried out using CAChe (Scigress Explorer Ultra v7.7.0.36, Fujitsu). The critical micelle concentration (CMC) of VPC was determined by a Varian

fluorescence spectrophotometer (CARY Eclipse) using pyrene as a probe.

2. Synthesis of 3(4)-vinylbenzyl 12-phosphorylcholinedodecanoate (VPC)

To a mixture of 12-hydroxydodecanoic acid (3.00 g, 13.9 mmol) and potassium carbonate (2.30 g, 16.6 mmol) in DMF (100 mL) was added 3(4)-vinylbenzyl chloride (1.96 mL, 13.9 mmol). The mixture was stirred at 80 ^{o}C for 24 h. After concentration in vacuo the residue was diluted with dichloromethane. The organic layer was washed with water and dried over $MgSO_4$. Solvent was removed on a rotovap to yield the crude product, which was purified by flash column chromatography, eluting with hexane/ethyl acetate (3:1) to give 3.30 g of a white solid **1** (72% yield): R_f = 0.48 in hexane/ethyl acetate (2:1); ^{1}H NMR ($CDCl_3$) δ (ppm) 7.41 (m, CH_2=CH$C_4H_4CH_2$-), 6.72 (dd, J_1 = 17.6 Hz, J_2 = 10.8 Hz, CH_2=$CH$$C_4H_4CH_2$-), 5.763, 5.761 (dd, J_1 = 17.6 Hz, J_2 = 4.0 Hz, CH_2=$CH$$C_4H_4CH_2$-), 5.269, 5.267 (dd, J_1 = 10.8 Hz, J_2 = 4.0 Hz, CH_2=$CH$$C_4H_4CH_2$-), 5.108, 5.094 (s, =$CHC_4H_4CH_2$OC(O)-), 3.64 (t, J = 6.4 Hz, -$CH_2CH_2CH_2$OH), 2.36, 2.35 (t, J = 7.6 Hz, -CH_2OC(O)CH_2CH_2-), 1.64 (m, -OC(O)-$CH_2CH_2CH_2$-, -$CH_2CH_2CH_2$OH), 1.27 (m, -$CH_2(CH_2)_7CH_2$-); ^{13}C NMR ($CDCl_3$) δ (ppm) 173.74, 137.90, 137.52, 136.47, 136.40, 136.35, 135.62, 128.77, 128.47, 127.60, 126.36, 126.05, 126.01, 114.39, 114.30, 65.97, 65.83, 63.11, 34.35, 32.81, 29.56, 29.49, 29.41, 29.24, 29.12, 25.74, 24.96; FT-IR (cm^{-1}) 3328, 2914, 1728, 1628, 1462; ESI-MS *m/z* 355 (M + Na^+) calcd for $C_{21}H_{32}O_3Na$ 355.5.

Compound **1** (1.50 g, 4.51 mmol) and triethylamine (2.09 mL, 15.0 mmol) were dissolved in dichloromethane (20 mL) and cooled in ice bath, and then 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP) (0.68 mL, 7.44 mmol) was added. The reaction solution was stirred at ice bath temperature for 1h and at room temperature overnight. After

concentration in vacuo the resulting residue was diluted with chloroform and filtered through a glass filter. The filtrate was concentrated in vacuo and the crude product was separated by flash column chromatography, eluting with hexane/ethyl acetate (1:2) to give 0.86 g of an viscous oily **2** (44% yield): R_f = 0.35 in hexane/ethyl acetate (1:1); ^{1}H NMR ($CDCl_3$) δ (ppm) 7.41 (m, CH_2=CH-C_4H_4-CH_2-), 6.72 (dd, CH_2=CH-C_4H_4-CH_2-), 5.77 (dd, CH_2=$CHC_4H_4CH_2$-), 5.27 (dd, CH_2=$CHC_4H_4CH_2$-), 5.11, 5.10 (s, =$CHC_4H_4CH_2OC(O)$-), 4.45 (m, -CH_2-OP(O)(O-CH_2)$_2$), 4.15 (t, J = 6.4 Hz, -CH_2-CH_2-OP(O)O-), 2.36, 2.35 (t, J = 7.6 Hz, -CH_2-OC(O)-CH_2-CH_2-), 1.64 (m, -OC(O)-CH_2-CH_2-CH_2-, -CH_2-CH_2-CH_2-OH), 1.25 (m, -CH_2-$(CH_2)_7$-CH_2-).

In a pressure bottle, compound **2** (0.86 g, 1.96 mmol) dissolved in acetonitrile (5 mL) was cooled to -20 °C and trimethylamine (0.55 mL, 5.86 mmol) was added. The reaction mixture was stirred at 60 °C for 24 h. After concentration in vacuo the crude product was purified by flash column chromatography, eluting with chloroform/methanol/water (65:25:4). Solvent was removed in vacuo. The resulting residue was dissolved in chloroform, filtered through a 0.22 μm PTFE filter, and reduced to give 0.24 g of a sticky solid VPC (25% yield): R_f = 0.12 in chloroform/methanol/water (65:25:4); ^{1}H NMR ($CDCl_3$) δ (ppm) 7.40 (m, CH_2=CH-C_4H_4-CH_2-), 6.71 (dd, J_1 = 17.6 Hz, J_2 = 10.8 Hz, CH_2=$CHC_4H_4CH_2$-), 5.753, 5.751 (dd, J_1 = 17.6 Hz, J_2 = 4.0 Hz, CH_2=$CHC_4H_4CH_2$-), 5.258, 5.256 (dd, J_1 =10.8 Hz, J_2 = 4.0 Hz, CH_2=$CHC_4H_4CH_2$-), 5.09, 5.08 (s, -$CHC_4H_4CH_2OC(O)$-), 4.26 (m, -CH_2-CH_2-$N^+(CH_3)_3$), 3.80 (m, -CH_2-CH_2-CH_2-OP, -CH_2-CH_2-$N^+(CH_3)_3$), 3.42 (s, -CH_2-CH_2-$N(CH_3)_3$), 2.332, 2.325 (t, J = 7.6 Hz, -OC(O)-CH_2-CH_2-), 1.58 (m, -OC(O)-CH_2-CH_2-CH_2-, -CH_2-CH_2-CH_2-OH, 4H), 1.25 (m, -CH_2-$(CH_2)_7$-CH_2-); ^{13}C NMR ($CDCl_3$) δ (ppm) 173.68, 137.87, 137.50, 136.46, 136.39, 136.33, 135.61, 128.77, 128.44, 127.57,

126.35, 126.01, 125.99, 114.39, 114.30, 66.25, 65.95, 65.81, 65.66, 65.60, 59.20, 54.29, 34.32, 31.07, 31.00, 29.70, 29.64, 29.53, 29.49, 29.32, 29.16, 25.92, 24.96. FT-IR (cm^{-1}) 3367, 2923, 1732, 1483, 1232; ESI-MS *m/z* 520 ($M + Na^+$) calcd for $C_{26}H_{44}NO_6PNa$ 520.3; CMC 45 μM.

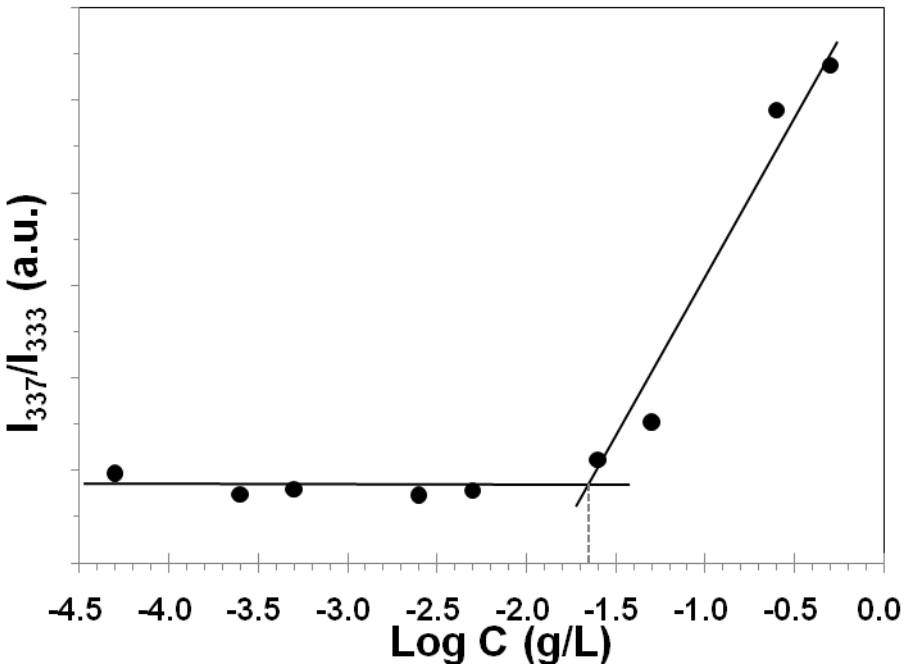

Fig. S1 Plot of I_{337}/I_{333} ratios versus the logarithm of VPC concentrations.

3. Preparation of the MIP

In a borosilicate scintillation vial (volume of 50 mL, radius of 1.25 cm), standard buffer solution (9.0 mL) was purged with nitrogen. A mixture of CRP (1.0 nmol) and VPC (5.0 nmol) in binding buffer (0.1 M Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 8.0; 1.0 mL) was pre-incubated at ice bath temperature for 30 min and added to standard buffer solution. Then, a mixture of styrene (1.1 mL, 9.6 mmol), DVB (0.15 mL, 1.1 mmol), and dibenzylketone (DBK) (22 μL, 0.11 mmol) was placed carefully on top of the aqueous solution. The polymerization was performed at 37 ^oC for 2 h and allowed to

proceed at 90 ^{o}C for 2 h by a standard laboratory UV light source.

The obtained film of 2.5 cm diameter and 1 mm thickness was immersed in EDTA-containing elution buffer (0.1 M Tris/HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0; 3.0 mL) for 20 min at room temperature for dissociation of the template CRP. The MIP was washed twice with TBST buffer (10 mM Tris/HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and PBS, respectively, with absolute ethanol and acetone, and then dried. A non-imprinted polymer (NIP) was synthesized in the same manner but without the addition of the template CRP. A control polymer (CP) was synthesized without both CRP and VPC. To reduce nonspecific binding of protein, all the films were treated with 0.1% BSA solution for 30 min at room temperature and washed twice with TBST and PBS, respectively. All the washing steps were performed for 5 min with mild shaking at room temperature.

4. CRP rebinding to the MIP and determination of bound CRP

The MIP, the NIP, and the CP films were reacted with CRP in binding buffer (6.7 nM, 3.0 mL) for 30 min at room temperature. The films were washed with TBST three times and PBS twice. For ELISA analysis, the films were immersed in elution buffer (2.0 mL) for 20 min at room temperature. CRP in the buffer solution was quantitatively determined by a commercial ELISA kit according to the manufacturer's instruction.

For the direct determination of CRP bound, MIP-based sandwich-type immunoassay (MSIA) methods were developed as follows.[2]

MSIA-1 method: CRP bound to the MIP was treated with anti-CRP-HRP in binding buffer (0.1 μg/mL, 3.0 mL) for 30 min at room temperature. Nonspecifically bound antibodies were washed away with TBST three times and PBS twice. The film was

developed by immersion in TMB substrate solution (2.0 mL) for 10 min according to the manufacturer's instruction. 2 N H_2SO_4 (100 μL) as a stop solution was added to aliquots of the substrate solution (100 μL) in a 96-well plate. The absorbance was measured at 450 nm with an ELISA reader.

MSIA-2 method: CRP bound to the MIP was reacted with anti-CRP antibody (10 μg) and anti-IgG-AP (1:30,000 titer) for 30 min with mild shaking at room temperature. Nonspecifically bound antibodies were washed away with TBST three times and PBS twice. To the MIP immersed in developing buffer (0.1 M Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5, 2.0 mL) was added p-NPP substrate solution (1.0 mL). Development was continued for 20 min at room temperature and 3 N NaOH (100 μL) was added to aliquots (100 μL) of all the samples to stop development at the same time. The absorbance was measured at 405 nm with an ELISA reader.

Data were averaged from three separate films. After measurements, the films were regenerated for repeated use by treating with EDTA, washing, and drying as described in Section 3.

5. Effect of free PC and other serum proteins on CRP binding to the MIP

To the MIP, the NIP, and the CP immersed in binding buffer (3.0 mL) containing CRP (2.0 nM) was added free PC (0, 0.1 and 100 μM) and the reaction proceeded for 30 min at room temperature. The MSIA-2 method was used for determination of CRP binding. The OD values of the MIP and the NIP subtracted by those of the CP were averaged from three separate films.

The CRP-negative serum (397 mg/mL of total protein) was diluted 100-fold with binding buffer. To the MIP, the NIP, and the CP immersed in binding buffer (3.0 mL)

containing CRP (3.3 nM) was added the diluted CRP-negative serum (10 µL, 39.7 µg of serum proteins). The reaction of CRP binding proceeded for 30 min at room temperature. The MSIA-1 method was used for analysis. The OD values of the MIP and the NIP subtracted by those of the CP were averaged from three separate films.

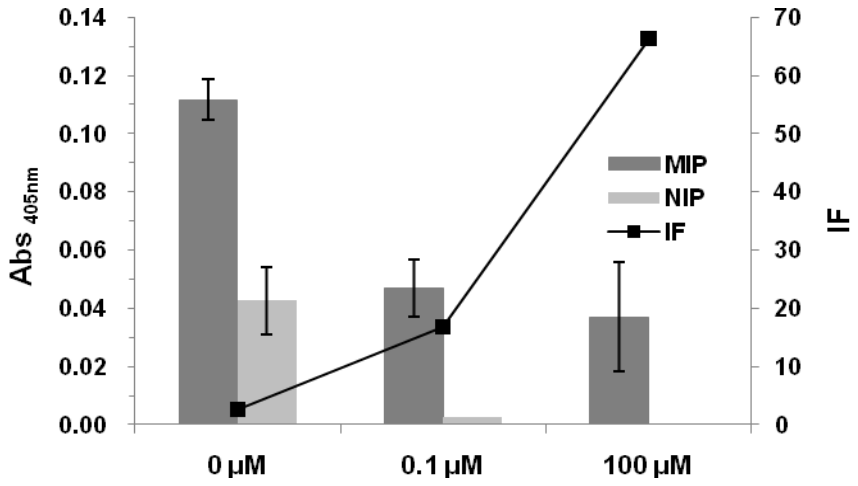

Fig. S2 CRP binding to the MIP and the NIP upon addition of free PC on ([CRP] = 2.0 nM). Each column represents the mean value of three experiments with error bars indicating ±SD.

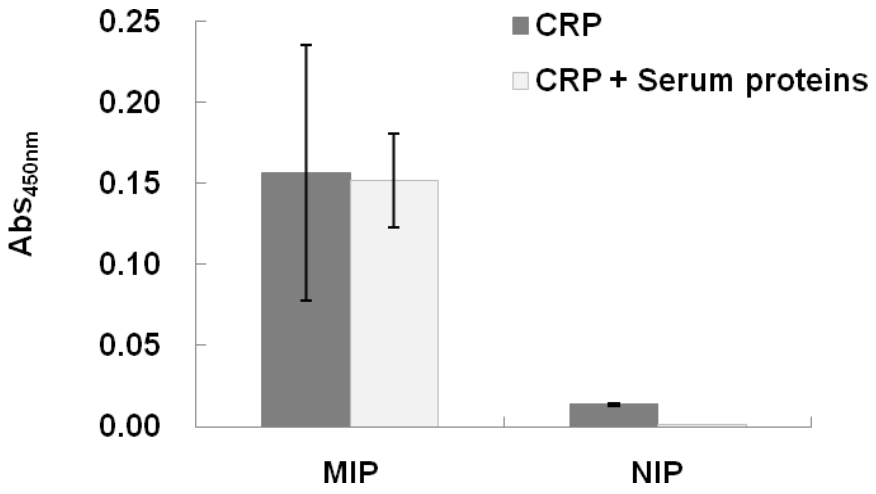

Fig. S3 CRP binding to the MIP and the NIP in the presence of other serum proteins ([CRP] =

3.3 nM, other serum proteins/CRP (w/w) = 35). Each column represents the mean value of three experiments with error bars indicating ±SD.

6. Langmuir Adsorption isotherm

The binding of CRP to the MIP was performed in the CRP concentration range of 0.2 to 10.0 nM in binding buffer (3.0 mL). The reaction of CRP binding proceeded for 30 min at room temperature, followed by measurement using the MSIA-2 method. The OD values of the MIP subtracted by those of the CP from three separate films were used to calculate the binding constant.

7. Serum CRP determination using MSIA

The serum CRP sample solution was prepared by dilution of the human CRP-positive serum into binding buffer and the CRP concentration was 3.67±0.28 mg/L as determined by ELISA. The MIP was reacted with the prepared CRP solution (10 μL) in binding buffer (3.0 mL) for 30 min at room temperature before the MSIA-1 measurement. A calibration curve for the MSIA-1 was obtained from standard CRP solutions (10 μL) in the concentration range of 2.4 to 120 mg/L in binding buffer (3.0 mL). The CRP concentration was calculated by the semi-log standard curve-fitting method from the three separate MIP films.

References

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2. J. Pultar, U. Sauer, P. Domnanich and C. Preininger, *Biosens. Bioelectron.*, 2009,

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