**Electronic Supplementary Information**

**A Plasmonic Chip-Based Bio/Chemical Hybrid Sensing System for the Highly Sensitive Detection of C-Reactive Protein**

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**Materials**

Sodium hydrogen carbonate, copper (II) bromide (CuBr₂), acetic acid, sodium carbonate, tetra-sodium ethylenediaminetetraacetate (EDTA-4Na), and L-ascorbic acid were purchased from Nacalai Tesque, Inc. (Japan). 2-Methacryloyloxyethyl phosphorylcholine (MPC) and \(N,N',N''\)-pentamethyldiethylenetriamine (PMDETA) were purchased from Sigma-Aldrich Co. LLC. (USA). Ethanol, dimethylformamide (DMF), potassium chloride, disodium hydrogenphosphate, sodium dihydrogenorthophosphate, 2-amino-2-hydroxymethyl-1,3-propanediol, 1 M hydrochloric acid, albumin from bovine serum (BSA), and calcium chloride (CaCl₂) were purchased from Wako Pure Chemical Industries, Ltd. (Japan). 3-Methacryloxypropyltrimethoxysilane was purchased from Shin-Etsu Chemical Co., Ltd. (Japan). UV-curable resin (PAK-02-A) was provided by Toyo Gosei Co., Ltd (Tokyo, Japan). 3-Aminopropyltriethoxysilane (APTES), \(N,N''\)-diisopropylethylamine (DIEA), and 2-bromoisobutyryl bromide were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). C-reactive protein mouse monoclonal antibody (anti-CRP) was purchased from HyTest Ltd. (Finland). Sodium chloride (NaCl) was purchased from Tomita Pharmaceutical Co., Ltd (Japan). CRP was purchased from Merck Millipore Corporation (Germany). Cy5-streptavidin (approximately four Cy5 were modified on the streptavidin) and EZ-Link NHS-PEG12-Biotin were purchased from Thermo Fisher Scientific Inc. (USA). Human Serum (pool) was purchased from Cosmobio Co., Ltd. (Japan). Cy5 Mono-Reactive Dye Pack was purchased from General Electric Company (USA). Deionized water used was obtained from a Millipore Milli-Q purification system.

**Characterizations**

X-ray photoelectron spectroscopy (XPS) measurements were carried out using a PHI X-tool (ULVAC-PHI, Japan). Fluorescence measurements were carried out using IX-73 (Olympus Corporation, Japan), U-HGLGPS (Olympus, Japan), Zyla sCMOS and SOLIS (Andor Technology Ltd, UK). X-ray reflectometer measurement (XRR) for thickness, density and surface roughness characterizations of PMPC layer were carried out using MARY-102FM (Five Lab Co., Ltd., Japan). Atomic force microscope (AFM) observations were carried out using SPI-3800 and SPA-400 (Seiko...
Instruments Inc., Japan). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) measurements were carried out using a Voyager-DE-1000 (Applied Biosystems, USA). Surface plasmon resonance (SPR) measurements were carried out by using an instrument based on the previously reported system [Cui, X. Q.; Tawa, K.; Hori, H.; Nishii, J. Adv. Funct. Mat. 2010, 20, 546-553; Cui, X. Q.; Tawa, K.; Kintaka, K.; Nishii, J. Adv. Funct. Mat. 2010, 20, 945-950.].

Preparation of PMPC-grafted-plasmonic chips by surface-initiated AGET ATRP (Scheme S1)

**Plasmonic chip with a thin Ag layer**

Glass substrates (Matsunami Glass Ind., Ltd, 25 × 25 mm) were immersed in a solution of 3-methacryloxypropyltrimethoxysilane dissolved in a mixture of water, ethanol and acetic acid (50:25:25 vol%). After reaction at 40 °C for 1 h, the methacryloyl-functionalized glass substrates were rinsed with ethanol, then a UV-curable resin, PAK-02-A, was dropped on the surface of the substrate and covered with a quartz mold (size: 4 × 4 mm, period: 500 nm, groove depth: 30 nm, duty ratio: 1:1). The UV-resin was irradiated from behind the mold using a deep-UV lamp (Ushio, Spot Cure) at an energy level of 100 mJ cm⁻². Following this UV-nanoimprint lithography (NIL) step, the chips were ultrasonically cleaned in ethanol for 3 min and dried in vacuo at 55 °C for 1 h. The glass substrates imprinted with a periodic structure were subjected to rf sputtering (Cryo-vac, custom-made) at room temperature under an Ar atmosphere to deposit an approximately 3 nm thick layer of Ti. Subsequently, a layer of silver approximately 140 ± 15 nm thickness was deposited, an approximately 3 nm thick layer of Ti was deposited on the thin silver thin film, and finally an approximately 20 nm thick layer of SiO₂ was deposited for the suppression of fluorescence quenching.
Scheme S1. Generation of PMPC-grafted-plasmonic chips by surface-initiated AGET ATRP

NH₂-functionalized plasmonic chip
Plasmonic chips with a surface SiO₂ layer were rinsed with ethanol and deionized water. A silane coupling reaction for the preparation of NH₂-functionalized plasmonic chip was conducted by mixing APTES (10.6 μL, 45.5 μmol) in deionized water (100 μL) and dropping the solution on the plasmonic chips. After 1 h at room temperature, the chips were rinsed with deionized water.

Br-functionalized plasmonic chip
DIEA (13.9 μL, 81 μmol) was dissolved in DMF (1.62 mL), then 2-bromoisobutyryl bromide (10 μL, 81 μmol) was mixed in under a N₂ atmosphere. The solution was dropped on NH₂-functionalized plasmonic chips, reacted at room temperature for 2 h under a N₂ atmosphere, then the Br-functionalized plasmonic chips were rinsed with deionized water.

PMPC-grafted-plasmonic chip
MPC (13.29 mg, 45 μmol, 500 mM), CuBr₂ (0.201 mg, 0.9 μmol, 10 mM), and PMDETA (0.188 μL, 0.9 μmol, 10 mM) were dissolved in deionized water (80 μL), and this solution was dropped on the Br-functionalized plasmonic chips. After ten N₂/degas cycles, ascorbic acid dissolved in deionized water (10 μL, 5 mM) was added to the remaining solution to reduce the copper species. After ten further N₂/degas cycles, surface-initiated AGET ATRP was started by conducting the polymerizations at 25 °C for 15 min. Following polymerization, the copper species were removed by washing with a 1 M EDTA-4Na aqueous solution at room temperature overnight, then the PMPC-grafted plasmonic chips were rinsed with deionized water.

XPS measurements for the plasmonic chips
XPS measurements were conducted using the following conditions: X-ray source: AlKα; X-ray conditions: 20 kV 101 W; take off angle: 45 °; initial survey scans: 0-1000 eV binding energy. Compositional narrow scans for C 1s, O 1s, N 1s, P 2p, Br 3d, and Si2p were carried out using a detector pass energy of 112 eV.

XRR measurements for the plasmonic chips
The thickness of the PMPC layer grafted on a plasmonic chip was evaluated by XRR measurements using the following conditions: source: CuKα₁ radiation, λ=0.154 nm; measured area: 1.0 (cm²); angle range (2θ): 0.0-6.0°. The thickness of the PMPC layer was estimated by fitting an analysis curve to each X-ray reflective profile. The fitting equations and the detailed data were in the supporting information.

Measurement of SPR angle on the plasmonic chips
SPR measurements were conducted using a previously reported experimental setup.²⁶
Reflectivity measurements were performed on the PMPC-grafted-plasmonic chips using previously reported experimental procedures and a p-polarized He–Ne laser (λ= 633 nm). SPR angles were investigated using an azimuthal angle set in 0, 10, 20, 30, 40, 45, 50, 60, 60, 70, 80, 90°.

Cy5 labelling to anti-CRP (Cy5-anti-CRP)

Monoclonal mouse anti-Human C-reactive protein (anti-CRP; 7.1 mg/mL (50 μL)) in 10 mM phosphate buffer saline (PBS) was diluted with 100 mM carbonate buffer (pH 9.2) (274.5 μL) to provide a 1 mg/mL anti-CRP solution (319.5 μL). This solution was added to a vial containing an excess of Cy5 N-hydroxysuccinimide ester (Cy5-NHS) from a Cy5 labeling kit (Cy5 Mono-Reactive Dye Pack) and stirred at room temperature for 30 min. Non-reacted Cy5-NHS was removed by dialysis (molecular weight cut-off < 3,500) in PBS; the PBS was changed after 1 h, 3 h and 5 h, then the sample was dialyzed overnight.

Biotin labelling to anti-CRP (Biotin-anti-CRP)

Anti-CRP (1 eq., 4.2 nmol) was added to NHS-PEG12–biotin dissolved 10 mM PBS solution (3 eq., 12.6 nmol) and reacted at 4 °C for 1 h. The obtained Biotin-anti-CRP was purified by dialysis (molecular weight cut-off < 3500) in PBS; the PBS was changed after 1 h, 3 h and 5 h, then the sample was dialyzed overnight.

Fluorescence measurements on the PMPC-grafted plasmonic chips using fluorescence microscopy

For this purpose, an inverted epifluorescence microscope (IX73, Olympus) equipped with a Hg lamp, scientific complementary metal oxide semiconductor (sCMOS) camera (Zyla sCMOS, ANDOR), a 10 × objective lens (NA 0.30), and a fluorescence filter (exciter 600-650 nm, emitter 675-725 nm), was used. Fluorescence microscopic images were observed under illumination with a Hg lamp (exposure time: 0.1s or 0.5 s) using a 10× objective lens (NA 0.30) and a restricted illumination angle of 0 °-17 ° (Figure S4, yellow region).

The camera images obtained were analyzed using SOLIS software. Light intensity was defined as the mean of intensity inside the red frame in Figure S6. Images obtained in the absence of excitation light were defined as blank (b) and images obtained with excitation light were defined as measured values (m). Light intensity (L) is defined as:

\[
L = \frac{(m - b)}{e} \quad e: \text{exposure time (s)} \quad \cdots(1)
\]

L after interaction with fluorescent molecules and before interaction with CRP (background) is defined as \(L_{\text{after}}\) and, \(L_{\text{before}}\) respectively. Fluorescence intensity (F) due to fluorescent molecules is defined as:

\[
F = L_{\text{after}} - L_{\text{before}} \quad \cdots(2)
\]

F in the absence of interaction with CRP was defined as \(F_0\). The relative fluorescence intensity was defined as \((F-F_0)/F_0\).
CRP sensing using the sandwich assay with Cy5-anti-CRP

For all experiments, the PMPC-grafted-plasmonic chip was disposable use for each condition and three chips were tested for the same condition to obtain statistical data. A round cover glass was attached to a PMPC-grafted-plasmonic chip with double-sided tape and fluorescence intensity was measured after injecting buffer solution (10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 2 mM CaCl₂, 40 μL) into the space between the PMPC-grafted-plasmonic chip and the cover glass. Background measurements were performed using a fluorescence microscope. Next, buffer solution (40 μL) containing different concentrations of CRP (0, 0.05, 0.1, 1, 10, 100, 1000 pM) were sequentially injected and allowed to interact with the PMPC layer for 10 min. After washing three times with 40 μL buffer solution, buffer solution (40 μL) containing 100 nM Cy5-anti-CRP was injected and allowed to interact for 10 min with the CRP adsorbed on the PMPC-grafted-plasmonic chip. The chip was again washed three times with 40 μL buffer solution, then the fluorescence intensity arising from the remained Cy5-anti-CRP was measured using a fluorescence microscope.

CRP sensing using the sandwich assay with Biotin-anti-CRP and Cy5-streptavidin

For all experiments, the PMPC-grafted-plasmonic chip was disposable use for each condition and three chips were tested for the same condition to obtain statistical data. A round cover glass was attached to a PMPC-grafted-plasmonic chip with double-sided tape. A buffer comprising 10 mM Tris-HCl solution (pH 7.4) containing 140 mM NaCl and 2 mM CaCl₂ was prepared. First, the buffer solution (40 μL) was injected between the PMPC-grafted-plasmonic chip and the round cover glass and the fluorescence background was measured using a fluorescence microscope. Next, buffer solution (40 μL) containing different concentrations of CRP was injected, and the CRP was allowed to interact with the PMPC layer on the plasmonic chip for 10 min. After washing three times with 40 μL buffer, 40 μL buffer containing 100 nM biotin-anti-CRP was injected and allowed to interact with the CRP adsorbed on the PMPC layer for 10 min. After repeating the washing step, 40 μL 100 nM Cy5-streptavidin in buffer was injected and incubated for 10 min. The reaction surfaces were rinsed three times with 40 μL buffer and the fluorescence intensity arising from the remained Cy5-streptavidin was measured using a fluorescence microscope.

Fluorescence intensities of the inside and the outside of the grating on the PMPC-grafted-plasmonic chip, and the PMPC-grafted-glass substrate bearing the grating without the Ag layer was carried out in the same manner for 1 nM CRP in 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl, 2 mM CaCl₂ and 1 wt% BSA. CRP sensing experiments using 1 wt% BSA as a blocking agent and 1 vol% human serum as a model sample were also conducted in the same manner.
Figure S1. XPS spectra (N 1s orbital) before (top) and after (bottom) silane coupling reaction with APTES to the plasmonic chip.

Figure S2. XPS spectra (P 2p orbital) before (top) and after (bottom) surface-initiated AGET ATRP of MPC to the Br-functionalized plasmonic chip.
Figure S3. SPR spectra of the PMPC grafted plasmonic chip per azimuthal angle.

Figure S4. SPR angle of the PMPC-grafted-plasmonic chip per azimuthal angle. SPR was caused on the PMPC-grafted-plasmonic chip because NA of fluorescence microscope object lens contains SPR angle 1 and 2 (yellow region).
(a) anti-CRP
(b) Cy5-anti-CRP
Figure S5. MALDI-TOF-MS spectra of anti-CRP (a), Cy5-anti-CRP (b) and Biotin-anti-CRP (c)
Figure S6. Fluorescent microscope image of the PMPC-grafted-plasmonic chip after the sandwich assay for 1 nM CRP (red frame: region for the measurement of fluorescence intensity).

Figure S7. Detection of CRP using the sandwich assay with Cy5-anti-CRP as the detection antibody on the PMPC-grafted-plasmonic chips in 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl and 2 mM CaCl₂. CRP concentration: 0, 0.05, 0.1, 1, 10, 100, 1000 pM.
Figure S8 Calibration curve (F) by the sandwich assay with the biotin-anti-CRP/Cy5-streptavidin system for the calculation of LOD in 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl, 2 mM CaCl₂ and 1wt% BSA (red). CRP concentration: 0, 50, 100, 250, 500, 1000 pM.
**XRR measurements**

An X-ray reflectivity profile of PMPC-grafted plasmonic chip was obtained (Figure S9). X-ray reflectivity is defined as $|R_{n,n+1}|^2 = \frac{1}{I_0}$. Reflectivity ($R_{j,j+1}$) of each layer was calculated by the following equations.

\[
R_{j,j+1} = \frac{(R_{j-1,j}+F_{j,j+1})}{R_{j-1,j}×F_{j,j+1+1}} × a_{j+1}^4 \quad \cdots (S1)
\]

\[
F_{j,j+1} = \frac{g_{j+1}-g_j}{g_{j+1}+g_j} × \exp\left(-8\pi^2 g_j g_{j+1} \sigma_j^2 / \lambda^2\right), \quad \cdots (S2)
\]

\[
a_{j+1} = \exp\left(-i\pi g_j d_{j+1} / \lambda\right), \quad \cdots (S3)
\]

\[
g_j = \sqrt{n_j^2 - \cos^2 \theta} \quad \cdots (S4)
\]

$\theta$ is the X-ray incident angle, $\lambda$ is the X-ray wavelength, $I_0$ is the X-ray incident intensity, $I$ is the X-ray reflected intensity, $n^*_i$ is the refraction index of each layer, calculated according to the formula $n^*_i = 1 - \delta_i + \beta_i$, where $\delta_i$ and $\beta_i$ are shifts from the refraction index (=1). $\delta_i$ and $\beta_i$ are defined as follows.

\[
\delta_i = r e^2 p N_A / 2\pi \frac{\Sigma[x_i (Z_i f_i (\lambda))]}{\Sigma[x_i A_i]}, \quad \beta_i = r e^2 p N_A / 2\pi \frac{\Sigma[x_i (Z_i f_i (\lambda))]}{\Sigma[x_i A_i]} \quad \cdots (S5)
\]

$\sigma_j$ is the interface roughness of each layer and $d_i$ is the thickness of each layer. The optimal values of these four parameters ($\delta_i$, $\beta_i$, $d_i$, and $\sigma_i$) were calculated by minimizing $\chi^2$ and reliability factor (R(%)). $\chi^2$ represents logarithmic error sum of the squares between the experimental value and calculated value via non-linear least-squares method.

\[
\chi^2 = \Sigma_{i=1}^{N_p} \left[\log(I_{exp}(\alpha_i)) - \log(I_{cal}(\alpha_i))\right]^2 \quad \cdots (S6)
\]

\[
R\% = \sqrt{\frac{\chi^2}{\Sigma_{i=1}^{N_p} \left[\log(I_{exp}(\alpha_i))\right]^2}} × 100 \quad \cdots (S7)
\]

The thickness of the PMPC polymer layer was calculated by fitting an analysis curve to each X-ray reflective profile (Figure S9, Table S1).
Figure S9 XRR experimental and simulated curve of PMPC-grafted-plasmonic chip

Table S1 Physical property of each layer on the PMPC grafted plasmonic chip

<table>
<thead>
<tr>
<th></th>
<th>Thickness (nm)</th>
<th>Density (g/cm³)</th>
<th>Roughness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMPC</td>
<td>4.4</td>
<td>0.7</td>
<td>2.0</td>
</tr>
<tr>
<td>SiO₂</td>
<td>20.6</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Ti</td>
<td>3.6</td>
<td>4.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ag</td>
<td>137.2</td>
<td>10.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Ti</td>
<td>3.2</td>
<td>4.1</td>
<td>1.8</td>
</tr>
<tr>
<td>SiO₂</td>
<td>0.0</td>
<td>2.2</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table S2 Comparison with other methods for the determination of CRP

<table>
<thead>
<tr>
<th>Method</th>
<th>LOD</th>
<th>Media</th>
<th>Ref. # in the text</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmonic chip</td>
<td>10 pM (1.0 ng/mL)</td>
<td>1 vol% serum with Tris-HCl</td>
<td>the present work</td>
</tr>
<tr>
<td>ELISA</td>
<td>0.03 μg/mL</td>
<td>serum prediluted 100× with 1% BSA-PBS</td>
<td>10</td>
</tr>
<tr>
<td>Superparamagnetic nanoparticles</td>
<td>0.2 μg/mL</td>
<td>Blood patient samples</td>
<td>12</td>
</tr>
<tr>
<td>Magnetic nanoparticles</td>
<td>0.12 μg/mL</td>
<td>Serum</td>
<td>13</td>
</tr>
<tr>
<td>Antibody-coated latices</td>
<td>5 mg/mL</td>
<td>Buffer (pH 7.4) which consisted of 20 mM Tris, 20 mM CaCl₂, 300 mM NaCl, 0.05% Tween 20, 0.2% BSA, 0.002% normal mouse immunoglobulins, and 0.09% NaN₃</td>
<td>14</td>
</tr>
<tr>
<td>SPR sensing chip</td>
<td>19.9 ng/mL</td>
<td>HBS-P buffer (pH 7.4) containing 2 mM CaCl₂</td>
<td>15</td>
</tr>
<tr>
<td>RIFS-based immunosensing</td>
<td>60 ng/mL</td>
<td>HBS-P containing 2 mM CaCl₂</td>
<td>16</td>
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<tr>
<td>LSPR of PMPC-grafted AuNPs</td>
<td>50 ng/mL</td>
<td>1% human serum diluted by 10 mM Tris-HCl buffer solution containing 140 mM NaCl and 20 mM CaCl₂</td>
<td>17</td>
</tr>
<tr>
<td>PMPC thin layers grafted from gold substrate</td>
<td>4.4 ng/mL</td>
<td>10 mM Tris-HCl buffer pH 7.4 containing 140 mM NaCl and 2 mM CaCl₂</td>
<td>18</td>
</tr>
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