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A synthetic polymer ligand-grafted plasmonic chip was fabricated and demonstrated highly sensitive detection of C-reactive protein (CRP) by grating-coupled surface plasmon field-enhanced fluorescence. Poly(2-methacryloyloxyethyl-phosphorylcholine) was used as a CRP-specific polymer ligand layer and was grafted on the plasmonic chip using surface-initiated controlled/living radical polymerization (limit of detection: ca. 10 pM).

Coronary heart disease is a group of diseases that include heart attack and angina, and is a major cause of death in industrialized countries. Atherosclerosis is a principal cause of coronary heart disease and therefore methods for its early diagnosis are urgently required. C-reactive protein (CRP) is an important inflammation maker protein; for example, the blood concentration of CRP rapidly increases to over 30 nM in response to tissue destruction and cancer.[1-5] It was recently reported that the blood concentration of CRP slightly increases in patients with atherosclerosis, and a CRP level above 3.9 nM may be indicative of arteriosclerosis.[6-9] The CRP concentration of neonates is lower than that of adults because CRP is only gradually produced in neonates after birth. If highly sensitive CRP detection tools were available, changes in the blood CRP levels of neonates with time could be measured to determine if they are infected.

Highly sensitive and versatile CRP detection methods have been developed based on antigen-antibody interactions and include enzyme-linked immunosorbent assays (ELISA) and antibody-immobilized magnetic particles/quantum dots, where the limit of detection (LOD) were 88 pM - 44 nM.[10-14] We previously reported highly sensitive immunoassays for CRP using surface plasmon resonance (SPR) and reflectometric interference spectroscopy (RIfS), where LOD were ca. 175 pM and ca. 560 pM, respectively.[15,16] We recently demonstrated the highly sensitive detection of CRP based on non-immunological interactions.[17,18] The phosphorylcholine group is a specific ligand for CRP in the presence of Ca^{2+} ions[19-21] and thus our methodology utilizes protein-ligand interactions between a polymeric ligand, poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC), and CRP.[17, 18, 22] PMPC has been reported to exhibit low non-specific protein binding[23-25] which can be decreased further. The selective binding capability of PMPC toward CRP compared with other proteins has been investigated.[17, 18, 22] Highly sensitive detection of CRP (440 pM and 39 pM) was achieved using localized surface plasmon resonance (LSPR) based sensing using PMPC-grafted-gold nanoparticles[17] and a PMPC-grafted-SPR sensor chip,[18] respectively.

Plasmonic chips are prepared by coating thin metal films on a periodic structure and have been developed as a powerful sensing tool for grating-coupled surface plasmon field-enhanced fluorescence (GC-SPF).[26,27] Grating-coupled-SPR (GC-SPR) field can enhance the fluorescence emitted by bound fluorescent substances on these plasmonic chips nonlinearly when the GC-SPR field is used as an excitation field. This allows GC-SPF without the need for expensive instruments. Plasmonic chips are advantageous because of their low mass production cost, short operation time, and ease of use. Highly sensitive immunoassays for various proteins have been reported in which antibodies are immobilized on plasmonic chips.[28,29] For example, α-fetoprotein (AFP) was detected with high sensitivity by sandwich immunoassays using anti-AFP antibody immobilized on a plasmonic chip; the LOD was reported to be 55 fM.[30]

We here demonstrate the highly sensitive detection of CRP by GC-SPF using bio/chemical hybrid plasmonic chips. This approach uses PMPC as a synthetic polymeric ligand, in contrast to conventional immunosensing chips which use immobilized antibodies to capture target analytes. PMPC was

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Electronic Supplementary Information (ESI) available: [materials, apparatus, preparation of PMPC-grafted-plasmonic chips, XPS and XRR data, the SPR angle on the plasmonic chips, preparation of Cy5-anti-CRP and biotin-anti-CRP, conditions of fluorescence measurements using fluorescent microscopy, procedures and calibration curves of the sandwich assays with Cy5-anti-CRP and biotin-anti-CRP/Cy5-streptavidin, and a fluorescent microscope image of the PMPC-grafted-plasmonic chip]. See DOI: 10.1039/x0xx00000x

grafted on the plasmonic chips by a surface-initiated activator generated by electron transfer atom transfer radical polymerization (AGET ATRP).[31-33] CRP in buffer solution or diluted human serum was captured by the PMPC layer on the chip, then biotinylated anti-CRP antibody and fluorescently-labelled streptavidin were added to detect the captured CRP on the chip in the conventional sandwich manner.

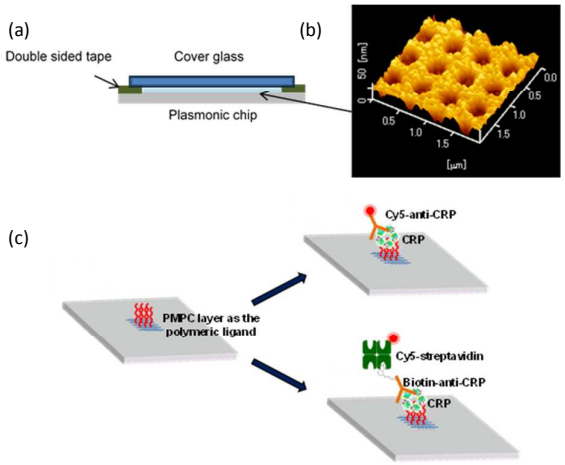

Fig. 1. Schematic illustration of cell for measurement on the plasmonic chip (a), AFM image of the plasmonic chip surface (b) and the two CRP detection systems based on the sandwich assay using PMPC-grafted-plasmonic chip with Cy5-anti-CRP and biotin-anti-CRP/Cy5-streptavidin (c).

The present plasmonic chip comprised four layers (Ti, Ag, Ti, and SiO_2) on a nanoimprinted two-dimensional periodic structure (Fig. 1). The two-dimensional periodic structure on the plasmonic chip enabled us to use a low incident light angle and allowed target protein sensing using a fluorescence microscope.[30] NH_2-functionalized plasmonic chips were prepared by a silane coupling reaction using APTES and the silanol groups of the SiO_2 layer. Amination was confirmed by XPS measurements and a new peak assigned to the N1*s* orbital appeared following amino functionalization (Fig. S1). Further modification to introduce bromo groups was carried out using 2-bromoisobutyryl bromide, resulting in a Br-functionalized plasmonic chip. The introduced bromo groups were used to surface-initiate AGET ATRP of MPC with $CuBr_2$/PMDETA as a catalyst in deionized water. XPS measurements of the resultant PMPC-grafted-plasmonic chip showed a P2*p* orbital, confirming the formation of the PMPC layer on the plasmonic chip (Fig. S2), and also indicated that the thickness and density of the PMPC layer were approximately 4.4 nm and 0.7 g/cm^3, respectively (Table S1).

SPR must be conducted at a low incident angle in order to measure GC-SPF by fluorescence microscopy. We determined if SPR on the PMPC-grafted-plasmonic chip occurred at a low incident angle by conducting SPR spectral measurements of the chip at different azimuthal angles (Fig. S3). Three SPR angles were obtained, and the smallest resonance angle for all azimuthal angles tested was below 17 ° (7 °– 8 °) (Fig. S4), where 17 ° is the incorporation angle of the objective lens used. Therefore, it should be possible to detect fluorescence excited by GC-SPR field on the PMPC-grafted-plasmonic chip by using a fluorescence microscope. In this study, the PMPC-grafted-plasmonic chip was single-use for each condition and three chips were tested for the same condition to check the reproducibility.

Cy5-anti-CRP was synthesized using a coupling reaction between the side chain amino groups of lysine residues on anti-CRP and Cy5-NHS. MALDI-TOF-MS measurements indicated approximately 14 conjugated Cy5s on each anti-CRP (Fig. S5). CRP sensing was demonstrated by a sandwich assay with Cy5-anti-CRP as the detection antibody on the PMPC-grafted-plasmonic chips. CRP was incubated with the chips in 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl and 2 mM $CaCl_2$; $CaCl_2$ was added because CRP shows calcium-dependent binding to phosphorylcholine. Cy5-anti-CRP was then placed on the chip to detect the captured CRP by the PMPC layer on the plasmonic chips. The fluorescence emitted by the bound Cy5-anti-CRP in the presence of 1 nM CRP was clearly observed using a fluorescence microscope (Fig. S6) and was found to be much more intense at the inside of the gratings than at the outside of the PMPC-grafted-plasmonic chip.

We demonstrated the sensitive detection of CRP using a sandwich assay with Cy5-anti-CRP as a detection antibody on the PMPC-grafted-plasmonic chips at various concentrations of CRP (0 -1000 pM): as the CRP concentrations increased, the F-F_0 values also gradually increased and was essentially saturated at 100 pM CRP (Fig. S7). However, the error bars were large, indicating that the reproducibility of CRP sensing by the chip is low, perhaps due to non-specific binding of CRP or Cy5-anti-CRP. Indeed, a high F_0 value was obtained in the sandwich assay even in the absence of the target CRP (Fig. 2), clearly indicating that highly sensitive detection of CRP would require mitigating non-specific binding.

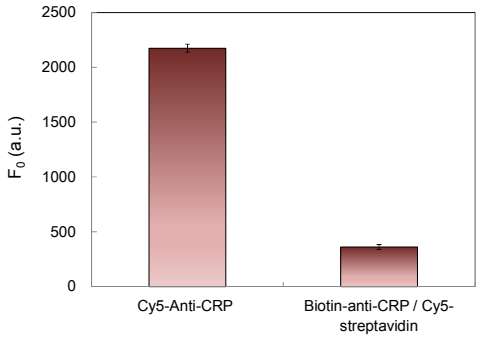

Fig. 2. Background fluorescent intensity observed without CRP (F_0) in the Cy5-anti-CRP and the biotin-anti-CRP/Cy5-streptavidin systems with 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl and 2 mM $CaCl_2$.

To improve the reproducibility, we examined a biotinylated anti-CRP/Cy5-streptavidin system in place of Cy5-anti-CRP as streptavidin exhibits low non-specific binding because of its neutral p*I* and the absence of sugar chains. Biotin-anti-CRP was synthesized using a coupling reaction between amino groups on the anti-CRP lysine side chains and biotin-PEG12-NHS. MALDI-TOF-MS measurements indicated approximately 1 biotin on each anti-CRP (Fig. S5).

The F_0 value obtained using the biotin-anti-CRP/Cy5-streptavidin system was 6 times smaller than that using Cy5-anti-CRP, indicating significantly decreased non-specific binding (Fig. 2). Non-specific binding was further reduced by using bovine serum albumin (BSA) as a blocking agent. CRP sensing with the biotin-anti-CRP/Cy5-streptavidin system was conducted in 10 mM Tris-HCl buffer (pH 7.4) containing 1 wt% BSA, 140 mM NaCl, and 2 mM $CaCl_2$. (Fig. 3) The F_0 value in the presence of BSA was approximately 70 times smaller than that without BSA, revealing that BSA effectively worked as a blocking agent to suppress non-specific binding of fluorescently-labelled streptavidin.

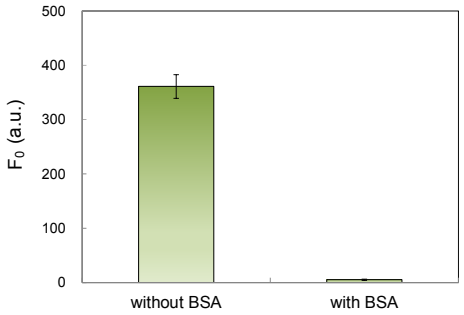

Fig. 3. Background fluorescence intensity (F_0) of the biotin-anti-CRP/Cy5-streptavidin system with 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl and 2 mM $CaCl_2$ in the presence and the absence of 1wt% BSA.

The grating effect of the plasmonic chip on fluorescence intensity enhancement at 1 nM CRP was investigated by comparing F-F_0 values at the inside of the grating, the outside of the grating, and at a glass substrate with the grating but without the Ag layer; in all cases, the substrates were grafted with PMPC using the same procedure (Fig. 4). The intensity at the inside of the grating was approximately 6.3 times greater than that on the outside, and approximately 30 times higher than that on the grating glass substrate, indicating that fluorescence emitted from bound biotin-anti-CRP/Cy5-streptavidin was successfully enhanced by GC-SPF on the PMPC-grafted-plasmonic chip.

Highly sensitive detection of CRP with the sandwich assay was demonstrated using the PMPC-grafted-plasmonic chip and biotin-anti-CRP/Cy5-streptavidin system with various concentrations of CRP (Fig. 5): the results showed that the F-F_0 values were proportional to the CRP concentration (R^2=0.957), and the estimated LOD was approximately 10 pM, where the LOD is determined in traditional binding studies from a concentration corresponding to an average of three standard deviations for the fluorescence intensities (F) of non-specific binding given without CRP (Fig. S8). The obtained LOD was much lower than those of previously reported PMPC-based SPR, LSPR and RIfS sensing systems.[10, 12-18] (Table S2)

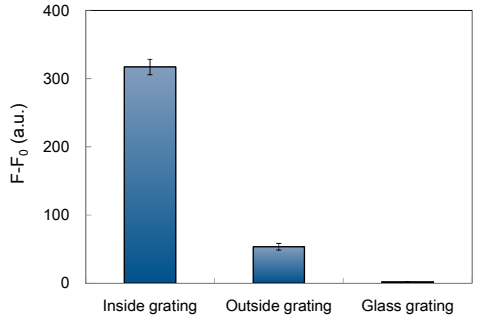

Fig. 4. Fluorescence intensities of the inside and the outside of the grating on the PMPC-grafted-plasmonic chip, and the PMPC-grafted-glass substrate bearing the grating without the Ag layer in the biotin-anti-CRP/Cy5-streptavidin system with 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl, 2 mM $CaCl_2$ and 1 wt% BSA (CRP: 1 nM).

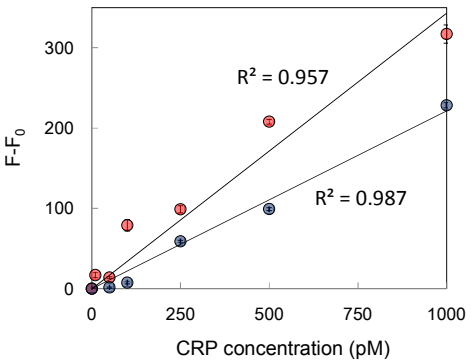

Fig. 5. Detection of CRP using the sandwich assay with the biotin-anti-CRP/Cy5-streptavidin system on the PMPC-grafted-plasmonic chips in 10 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl, 2 mM $CaCl_2$ and 1wt% BSA (red), and in human serum diluted 100 times with the buffer (blue).

The utility of the present sandwich assay-based bio/chemical hybrid sensing system for clinically-relevant samples was demonstrated by sensing CRP in human serum diluted 100 times with the buffer (Fig. 5). The F-F_0 values lineally increased with increasing CRP concentrations (R^2=0.987), although the values were slightly lower than those obtained in the absence of human serum components, which may be due to non-specific binding by serum components. The linearity appeared to be slightly better, for which the reason may be the hindrance of unfavorable binding sites by serum components. The LOD (ca. 10 pM) was comparable to that of

CRP in standard buffer solution, which is sufficient for the diagnosis of arteriosclerosis (3.9 nM).

Since the chip was single-use for each condition and the response form three chips were highly reproducible, *i.e.* the coefficient of variation values (%) for 10 pM and 50 pM or more of CRP were 19 % and less than 10 %, respectively, and a linear correlation between the concentration and the response was observed, the in-between reproducibility of the chip production was confirmed to be high enough for the disposable use. These results suggest that the present CRP sensing system based on the PMPC-grafted-plasmonic chip can readily detect CRP in clinically-relevant samples.

We here demonstrated a highly sensitive sandwich assay-based bio/chemical hybrid sensing system for CRP detection. This system utilizes PMPC as a CRP capturing synthetic polymer ligand grafted on a plasmonic chip and achieves highly sensitive and selective GC-SPF detection of CRP. Binding is observed using a fluorescence microscope. The PMPC (ca. 4 nm)-grafted plasmonic chip was prepared by surface-initiated AGET ATRP of MPC from a Br-functionalized plasmonic chip. The biotin-anti-CRP/Cy5-streptavidin system was found to be more suitable than the Cy5-anti-CRP system because Cy5-streptavidin showed lower non-specific binding than Cy5-anti-CRP. Furthermore, the use of BSA as a blocking agent further decreased non-specific binding, making the system more sensitive, even in the presence of diluted human serum (LOD: approximately 10 pM).

Consequently, the present sandwich assay-based bio/chemical hybrid sensing system based on this synthetic polymer ligand-grafted plasmonic chip has great potential for detecting proteins with high sensitivity and reproducibility. The use of artificial polymeric ligands instead of antibodies as sensing layers on the chips can suppress the cost and extend the self-life. We anticipate that this novel system will provide the foundation for developing affordable protein sensing systems for the diagnosis of diseases, for proteomics applications, and for use in other medical and pharmaceutical fields.

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Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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