Talanta 81 (2010) 1402–1408

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

High sensitive detection of C-reactive protein by total internal reflection fluorescence microscopy on rapidly making nanoarray protein chip

Md. Shahinul Islam[a], Hee Gu Lee[b], Jaebum Choo[c], Joon Myong Song[d], Seong Ho Kang[a,*]

[a] Department of Chemistry and Research Institute of Physics and Chemistry (RINPAC), Chonbuk National University, Jeonju 561-756, South Korea
[b] Medical Genomic Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-333, South Korea
[c] Department of Applied Chemistry, Hanyang University, Ansan 426-791, South Korea
[d] Research Institute of Pharmaceutical Science and College of Pharmacy, Seoul National University, Seoul 151-742, South Korea

ARTICLE INFO

Article history:
Received 12 December 2009
Received in revised form 12 February 2010
Accepted 15 February 2010
Available online 23 February 2010

Keywords:
C-reactive protein
Nanoarray protein chip
Surface patterning tool
Single-molecule detection
Total internal reflection fluorescence microscopy (TIRFM)

ABSTRACT

This study investigated a method for the high sensitivity detection and quantification of the C-reactive protein (CRP) in human serum using total internal reflection fluorescence microscopy (TIRFM) on a rapidly made nanoarray protein chip. The nanoarray biotin-probe was patterned onto 3-mercaptopropyl trimethoxysilane-coated cover glass with a spot diameter of ~400 nm within 1 min using a NanoeNabler™-based surface patterning tool. The unlabeled CRP molecules were detected in human sera using TIRFM, based on a sandwich fluorescence immunoassay. The linear regression for standard CRP in the range of 50 zM–1 fM was determined using the equation $y = 0.437x + 84.991$ ($R = 0.9993$). This proposed method was ~2000 times faster than conventional atomic force microscopy based dip-pen nanolithography in terms of the chip manufacturing process. Additionally this method was 6×10^6 times more sensitive than enzyme-linked immunosorbent assay and exhibited a wide dynamic linear range (50 zM–1 fM).

1. Introduction

C-reactive protein (CRP) is well recognized as a non-specific acute phase marker that is found in blood serum, in response to inflammations ranging from atherosclerosis and infections to malignant growths [1]. Abundant epidemiologic studies have revealed that the high-sensitivity CRP levels in blood serum provide a strong and independent indication of the risk of future heart attacks, ischemic stroke and peripheral arterial disease, even among individuals who are free from vascular disease [2]. Other studies have shown that elevations in the CRP in blood are related to future major cardiovascular risk, where a CRP concentration of less than 1 mg/L indicates low risk, 1–3 mg/L an intermediate risk, and 3–10 mg/L a high risk [3]. This concentration can rise up to 20–40 mg/L for viral infections or even 500 mg/L for bacterial infections [4].

In the clinical arena, the typical enzyme-linked immunosorbent assay (ELISA) [5] has been widely been used for the detection and quantification of CRP biomarkers. Although ELISA is a long standing standard for quantitative analysis of CRP, it suffers from a relatively low throughput because of its lack of multiplexing ability and high reagent and sample consumption [6]. Biochip-based detection techniques offer the distinctive advantages of a highly parallel format and a high throughput capacity. Many arraying technologies have been developed to design biochips using a broad range of supporting substrates, based on microarray and nanoarray techniques in order to overcome the limitations of the conventional ELISA based method. These microarray based techniques are a useful high-throughput screening tool for the detection of biomarkers and molecular diagnosis [7]. However, microarrays must have relatively large sample volumes and additional capture materials. Additionally, these microarrays have a high limit of detection (LOD) and a long incubation time [8]. Consequently, nanoarray techniques have been recommended to overcome the limitations of microarray technology. Several methods, such as micro-contact printing [9], ink-jet printing [10], electrospray deposition [11], mechanical pin-tool deposition [12], dip-pen nanolithography (DPN) [13], have been used to create ultra-miniaturized nanoarray. Recently, proteins have been detected in the zeptomolar concentration range (zM, $\times 10^{-21}$ M) using a mass spectrometer [14], gold nanoparticles [15] or atomic force microscopy (AFM)-based DPN [16,17]. In particular, AFM-based DPN is a well recognized nanoarray system that is used to write molecules onto surfaces using scanning probe tips. Nevertheless, the application of AFM-based DPN is limited to situations where the scale and throughput are not of vital importance, but rather the flexibility of design and material choice is of

* Corresponding author. Tel.: +82 63 270 3421; fax: +82 63 270 3408.
E-mail addresses: shkang@jbnu.ac.kr, shkang@chonbuk.ac.kr (S.H. Kang).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.02.042

interest. A relatively long scan time is required in order to detect complete protein molecules on array chips [18].

This study demonstrated a technique for the quantitative detection of the CRP in human blood serum at the single-molecule level using a total internal reflection fluorescence microscopy (TIRFM) system on fast patterning nanoarray biochips. A NanoeNabler™-based surface patterning tool (SPT) was used as the nanoarrayer, and this SPT offered over a 2000 times faster patterning rate than the DPN method in terms of a single-pin rather than multiple pins [19]. Moreover, previous reports have shown that TIRFM is a compatible optical sensor that can be used to investigate interfacial bimolecular interactions [16,17,20]. Therefore, TIRFM can be used to capture images and monitor the CRP molecules within an evanescent wave (~300 nm) that were produced on the chip surface at a high signal-to-noise ratio [21,22]. Additionally, the proposed chip-based method more accurately estimated the CPR values in human sera with ultra-sensitivity compare to the well-established ELISA technique.

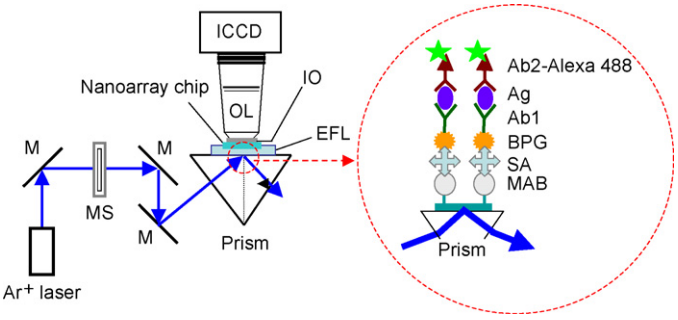


Fig. 1. Schematic diagram of the laboratory-built TIRFM system that was used to detect the CRP based on a sandwich fluorescence immunoassay using a nanoarray protein chip. The right circle represents the overall single-molecule sandwich immunoassay. M: mirror; MS: mechanical shutter; OL: objective lens; EFL: evanescence field layer; IO: immersion oil; ICCD: intensified charge-coupled device; MAB: maleimide–PEO_2–biotin; SA: streptavidin; BPG: biotin protein G; Ab1: first monoclonal antibody; Ag: antigen; Ab2: second antibody.

2. Experimental

2.1. Chemical and reagents

EZ-Link®maleimide–PEO_2–biotin was purchased from Dojindo (Dojindo Laboratories, Japan). 3-Mercaptopropyl trimethoxysilane (MPTMS), streptavidin, biotinylated protein G (BPG) and Tween-20 were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA). The blocking reagent, mPEG-maleimide (5K), was obtained from ID Biochem Inc. (Seoul, Korea). The recombinant human CRP, the monoclonal anti-human CRP antibody, the biotin affinity purified polyclonal antibody to the human CRP, and the ELISA kit (Cat. No. SCRP00) were purchased from R&D systems (Minneapolis, MN, USA). Streptavidin-Alexa Fluor®488 was obtained from Molecular Probes (Eugene, OR, USA). All of the solutions from the ELISA kit were diluted with 1× phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and pH 7.4). The buffer solutions were prepared with ultra-pure water (>18 MΩ), filtered through a 0.2-μm membrane filter (Whatman International Ltd., Maidstone, England) and photobleached overnight using a UV-B lamp (G15TE, Philips, The Netherlands).

2.2. Preparation of human serum samples

The human blood samples were collected from five consenting patients suffering from heart disease, inflammation and tissue injury and five consenting healthy individuals of different ages. The experiments were performed using serum samples after the blood samples were drawn from the individuals into anticoagulant-free vacutainers. The serum was separated using the standard clinical procedure. Briefly, the samples were centrifuged at 1500 rpm and 2 °C for 10 min in order to isolate the serum samples, and then the samples were stored at −20 °C without further dilution in 1× PBS until the experiments were performed.

2.3. Laboratory-built TIRFM system for the detection of CRP

The basic experimental setup of the TIRFM system is shown in Fig. 1. An argon ion laser (30 mW at 488 nm; Melles Griot, Irvin, CA, USA) was used as the excitation light source. A Pentamax 512-EFT/1EIA intensified CCD (ICCD, Roper Scientific, Princeton, NJ) camera was mounted on top of a Zeiss Axioskop-2 upright microscope. The filter cube consisted of a fixed mirror, a dichroic filter (565dcxr, Chroma technology Co., Rockingham, USA) and an emission filter (D535/40, Chroma Technology Co.). A Zeiss 100 × /1.3 N.A. Plan-Neofluar® microscope objective lens (Zeiss, Germany) was used. The experimental timing was controlled using a Stanford Research System model DG-535 four channel digital delay/pulse generator (Stanford Research Systems, Inc., Sunnyvale, CA, USA), and the ICCD camera was triggered at time of 0 ms with a TTL pulse duration of 10 ms. The sampling frequency was 10 Hz, and the shutter driver was set to a 10 ms exposure and a 90 ms delay. The nanoarray protein chip was placed onto the hypotenuse face of a right-angle fused silica prism (Melles Griot, Irvine, CA; $A = B = C = 2.54$ cm, refractive index, $n = 1.463$), in Fig. 1 (circular). Then the laser beam was directed through the prism toward the protein chip/media interface at an incidence angle (θ_i) that was slightly greater than 72°. A Uniblitz mechanical shutter (Model LS3Z2, Vincent Associates, Rochester, NY, USA) was used to block the laser beam when the camera was switched off in order to reduce the amount of photobleaching. The fluorescence from the individual protein molecules was passed through a filter box in order to eliminate any unnecessary light and to obtain images of the immobilized single CRP molecules that were labeled with fluorescent dyes. All of the images were collected using WinView/32™ (Version 2.5.14.1, Downingtown, PA, USA) and analyzed with MetaMorph (Version 7.3.1.0 software, Universal Imaging Co., Downingtown, PA, USA).

2.4. Chip substrate preparation and nano-patterning

The Corning cover glass substrates (No.1, 22 mm sq.) were prepared and silanized using MPTMS according to a standard procedure that was described elsewhere [16]. The contact angles of bared and MPTMS-coated glasses were measured using a SEO Contact Angle analyzer (Phoneix 300, Gyunggido, Korea). Then the MPTMS-coated substrates were arrayed with maleimide–PEO_2–biotin (1 mg/mL) at room temperature using a NanoeNabler™-based SPT (silicon dioxide cantilever, SPT-S-C10S, BioForce Nanosciences, Ames, IA, USA). The SPT device can be used to rapidly and precisely dispense aL- to fL-sized (10^{-18}–10^{-15} L) sample volumes onto a wide variety of surfaces (Fig. 2A). The following fabrication procedure was used in this study (Fig. 2B). Briefly, a 0.5 μL volume of enzyme linked maleimide–PEO_2–biotin containing 0.20% Tween-20 in 1× PBS was loaded into the reservoir on the backside of the cantilever of SPT using a micropipette. The NanoeNabler™ system was operated in the non-laser mode using a contact speed of 0.05 μm/ms, a force constant (k) of 0.05 N/m, a contact time of 0.5 s and a waiting time of 0.5 s with a resolution of 20 nm at room temperature. The relative humidity (RH) was adjusted to 50%. All of the stage movement and patterning parameters were controlled through a custom software package called NanoWare™.

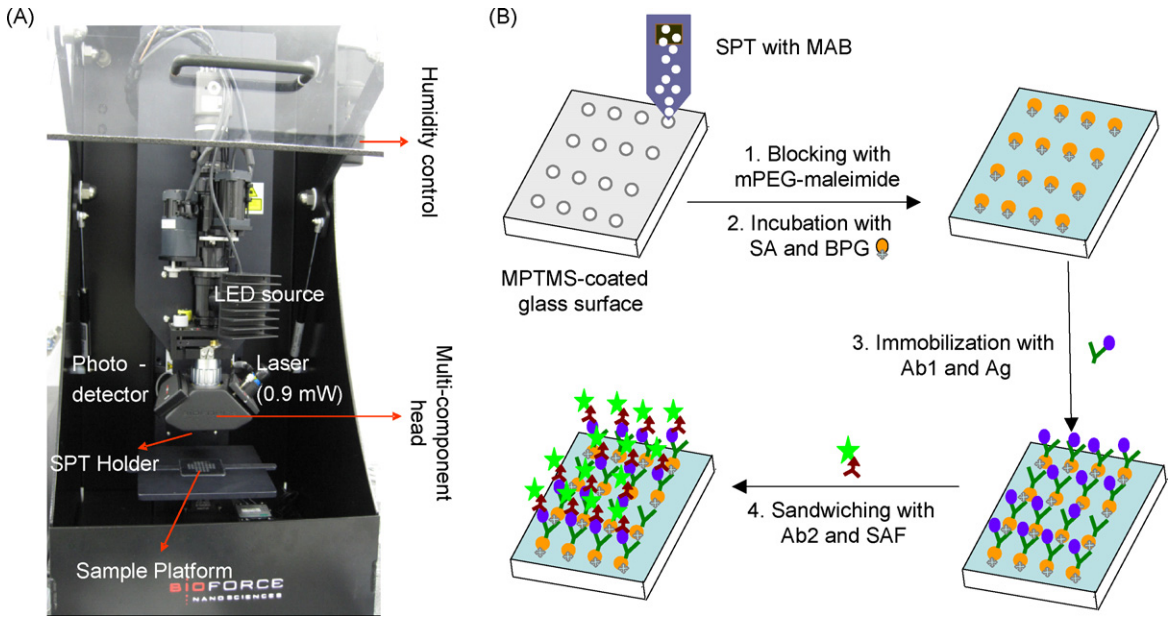


Fig. 2. (A) NanoeNabler™ system with a high resolution (20 nm) optical microscope. This system had a red diode laser beam that tracks the position of the SPT. An optical lever was used to monitor the deflections of the surface. A 1/3″ color CCD camera with a 640 × 480 pixels resolution was used to capture and save the images and videos. A UV-tip cleaner was facilitated for cleaning the SPT for 30 min before use in the patterning. (B) SPT generated nanoarray of maleimide–PEO_2–biotin and sandwich immunoassay procedure that was used for the detection of the CRP molecules on the nanoarray chip. The acronyms used were the same as in Fig. 1.

Nano-patterning procedure using the AFM-based DPN method was similar to the procedure that was described in a previous report [23]. Briefly, 2 μL of MPTMS was evaporated onto the substrate using a clean AFM tip (a silicon nitride cantilever with a force constant (k) of 42 N/m) with a radius of curvature of ≤10 nm at 120 °C over a period of 30 min. Then the substrate was further coated with DPN ink for 10 min in maleimide–PEO_2–biotin (1 mg/mL) containing 0.05% Tween-20 in 1× PBS, and blow-dried with nitrogen. The DPN experiments were performed using a Bio-AFM (atomic force microscope, NanoScope IIIa controller, Trondheim, Norway) from Digital Instruments. The RH was adjusted to 60%, and all of the DPN experiments were performed in the tapping mode at room temperature.

2.5. Sandwich fluorescence immunoassay of human CRP on nanoarray chips

The fabricated chips were incubated with a blocking solution, mPEG-maleimide (5K) (25 μL volume of 10 μg/mL, in 1× PBS), for 10 min so that they were resistant to nonspecific adsorption. Then the chips were allowed to react with streptavidin for 10 min, with BPG for 1 h and with a monoclonal antibody to the human CRP (M_w = 150 kDa) for 1 h. For all three reactions, 25 μL of the reactant was used at a concentration of 2 μg/mL. Subsequently, the chips were reacted with a wide range of concentrations (50 zM–1 pM) of the standard CRP antigens or human serum samples for 1 h, and then the chips were incubated with 25 μL of 2 μg/mL human CRP detection antibody for 1 h. Finally, the same chips were treated with streptavidin-Alexa Fluor®488 (25 μL volume of 2 μg/mL) for 10 min. The chips were washed in 100 mL of 1× PBS-T for 1 min after each step and then washed for an additional 2 min with PBS before the images were acquired.

An atomic force microscope (NanoScope microscope, Digital Instruments, Trondheim, Norway) that was equipped with a type-J scanner (scan size, 100 μm), was operated in the tapping mode in order to obtain the topographic scan of the maleimide–PEO_2–biotin that was fabricated on the MPTMS-coated glass surface using both DPN and SPT. The resonance frequency was 300 kHz for the silicon tips (Olympus Co. Ltd., Japan). These short cantilevers were 150–160 μm and had a nominal force constant (k) of 42 N/m and a radius of curvature of ≤10 nm. The AFM images were obtained using NanoScope software (Version 6.13, Veeco Instruments Inc. NY, USA).

Then the average intensities of the array spots were detected and measured using the TIRFM system. A small drop of immersion oil (n = 1.518, Immersol™ 518F, Zeiss, Germany) was placed onto the surface of the right-angle fused silica prism, and then the nanoarray protein chip was placed on top of the oil (Fig. 1). The 100× objective lens was matched to the nanoarray chips with another drop of the immersion oil. The ICCD camera gain was 75. A fixed exposure time (10 ms exposure and 90 ms delay) was applied for all of the samples so that a direct comparison could be made between the fluorescence data [24]. All of the images were collected using WinView/32™ (Version 2.5.14.1, Downingtown, PA, USA), and the spot average intensities were measured with MetaMorph (Version 7.3.1.0 software, Universal Imaging Co., Downingtown, PA, USA).

2.6. ELISA of human CRP

The sandwich enzyme immunoassay type ELISA was used for the quantitative analysis of the human CRP in the standards and the sera samples according to the standard protocol [25]. The serum samples needed to be diluted 100-fold using calibrator diluent RD5P (1×). Then 50 μL of the standard, the control and/or the samples were added to each well and incubated for 2 h at room temperature. A CRP 2-fold dilution series in the range of 0.0079–2 ng/mL (0.30–76.93 pM) was used as the standard, and a horseradish peroxidase (HRP) conjugated monoclonal antibody was used as a CRP conjugate. At the end of the reaction, 50 μL of the stop solution (2 M H_2SO_4) was added in each well, and the color of the mixture changed from blue to yellow. The UV absorbance was measured at 540 nm using a Thermoreader (Variskan® Flash, Thermoscientific, Waltham, MA. USA).

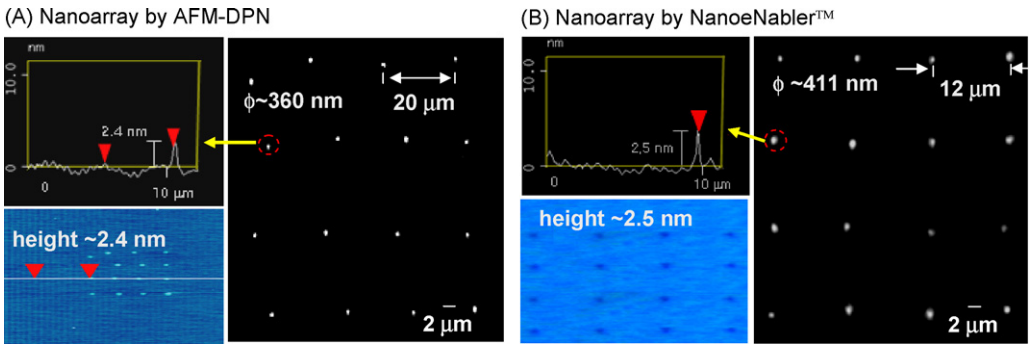


Fig. 3. AFM tapping mode images with the spot heights of maleimide–PEO_2–biotin and TIRFM images after the deposition of the 4 × 4 nanoarray using (A) AFM-DPN and (B) the NanoeNabler™-SPT.

3. Results and discussion

3.1. Evaluation of nanoarray chips and antigen-antibody binding assay

The MPTMS-coated glass surface was partially hydrophilic (contact angle = 49 ± 1.2°) after silanization (Supporting information Fig. S1), which was in agreement with a previous report [26]. The patterning of maleimide–PEO_2–biotin on this glass surface was evaluated using the AFM tapping mode images and the spot height measurements. The measured spot height of maleimide–PEO_2–biotin was 2.4 nm with a diameter of 360 and 2.5 nm with a diameter of ~400 nm diameters for DPN and SPT, respectively (Fig. 3), whereas the theoretical spacer length of maleimide–PEO_2–biotin was 2.9 nm. Therefore, maleimide–PEO_2–biotin was most likely deposited as a monolayer on the MPTMS-coated surface [23]. The effect of the tip-surface contact time in the range of 0.5–2.5 s on the spot diameter of maleimide–PEO_2–biotin was examined at a RH of 50% and 0.2% Tween-20 in 1× PBS (pH 7.4) (Fig. 4A). The SPT transferred the submicron domain of the proteins onto the substrates surfaces in less than 100 ms [19]. In this study, the spot diameter increased up to a value of 7.41 μm (at 2.5 s) as the contact time increased, possibly because a large amount of ink material was transferred over the increased time and immediately spread over the surface. Fig. 4B shows the effect of the Tween-20 concentration on the spot diameter when maleimide–PEO_2–biotin was printed onto the partially hydrophilic MPTMS-coated glass surface (contact angle = 49 ± 1.2°). The addition of a small amount of nonionic surfactant, Tween-20, that was dissolved in PBS activated the transfer of the biotin linker molecules onto the MPTMS-coated substrate by increasing their wettability [23]. In this study, various concentrations of Tween-20 ranging from 0.05 to 0.4% in 1× PBS were used. Tween-20 concentrations less than 0.2% resulted in a very irregular spot size, and array spots were frequently missing, even though the RH and contact time were unchanged. This phenomenon might have been caused by the very low concentration of Tween-20 (such as 0.05–0.1%), which did not sufficiently enhance the wettability of the maleimide–PEO_2–biotin ink. Therefore, the spot diameter at the submicron level (~400 nm) was obtained using 0.2% Tween-20. Above this concentration, the size of the array spots gradually increased, reaching a value of 6.67 μm at 0.4% Tween-20. Consequently, a contact time of 0.5 s and a concentration of 0.2% for Tween-20 in 1× PBS (pH 7.4) with a RH of 50% were selected as the optimal conditions in order to minimize the spot diameter at the submicron level during the formation of the nanoarray of biotin linker molecules using the SPT.

DPN is a widely used nanoarray patterning technique that is capable of creating a submicron-50 nm spot size with wide range of multiplexing abilities [27,28]. However DPN generally requires a relatively long time to array the ink materials (proteins or organic molecules). A contact time of up to 250 s per micron was required in order to transfer biomolecules such as proteins [29], whereas the SPT required a transfer time of less than 100 ms per micron in order to create a 1 μm diameter spot size for the protein molecules [19]. Therefore, the patterning rate of the SPT was 2500 times faster than the DPN method. A previous report showed that the almost 30,000 protein sample spots of could be molecularly printed within 1 h on wide ranges of substrates using a NanoeNabler™ [30]. Meanwhile, Lee et al. used a multiple-pen cantilever (26-pen called A-26) to pattern the protein A/G onto gold-coated substrates with a diameter ranging from 150 to 650 nm [31]. Although the multiple-pen based DPN perform rapidly, micro- and nanometer scale work was not easily and rapidly finished with a single experiment. Addition-

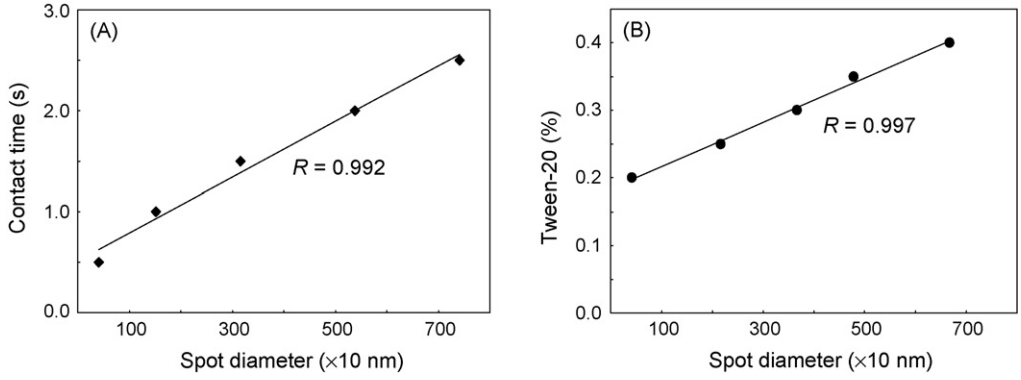


Fig. 4. Dependence of the spot diameter of maleimide–PEO_2–biotin on the MPTMS-functionalized glass (A) on the indicated tip-substrate contact time in the range of 0.5–2.5 s at 50% RH and 0.2% Tween-20 and (B) on the concentration (%) of Tween-20. The spots diameter was minimized at the submicron level (410 ± 5.37 nm or 420 ± 4.61 nm) using 0.2% of Tween-20 in 1× PBS with a contact time of 0.5 s.

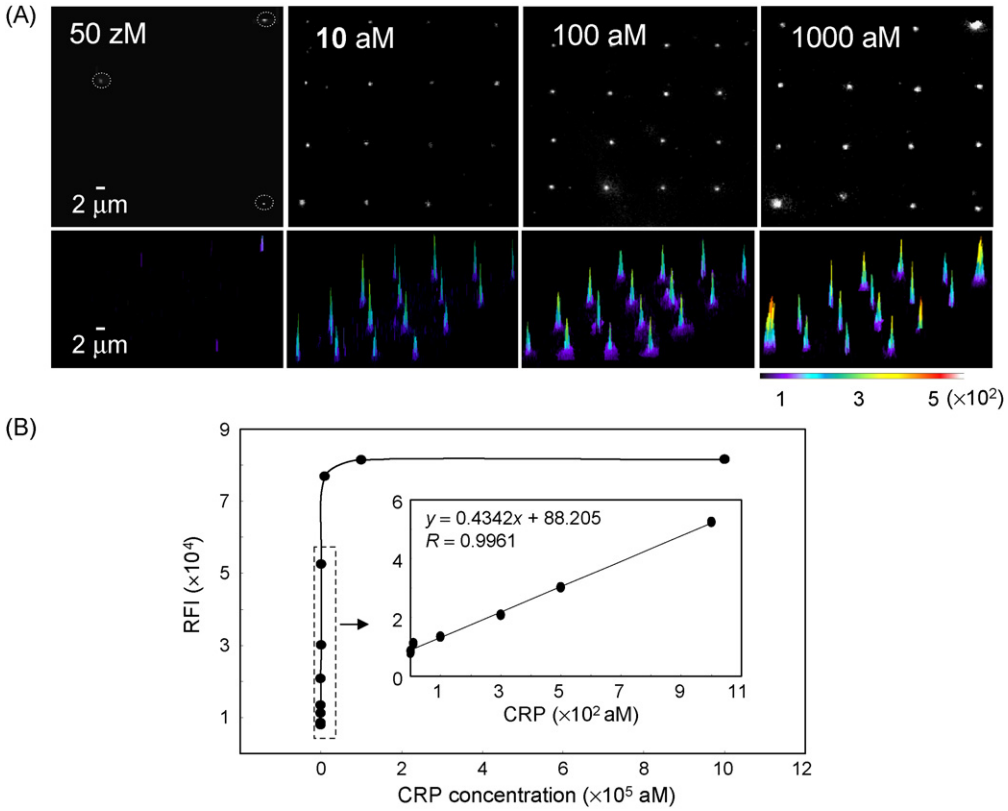


Fig. 5. (A) Representative TIRFM images of the blank chip (negative control) and array spots that were produced using the standard CRP on the biotin probe array chips followed by the sandwich immunoassay method. (B) Calibration curve of the human standard CRP. The inset shows the linear range of the calibration curve. The average relative fluorescence intensities (RFI) of all of the spots were corrected through background subtraction ($n = 5$). Error bar represents standard deviation. RFI: relative fluorescence intensity.

ally, fragile and costly two–dimensional (2D) cantilever arrays are required for large area patterning [32]. Practically, large molecules patterning was observed using a single-pen cantilever in this study, and DPN comparatively required more time than the SPT. A 4×4 dot array of maleimide–PEO_2–biotin ink was form on the MPTMS-coated glass surface within 1 min for the SPT. Conversely, the DPN method took almost 1 h to produce the same array pattern. Although, the spot size using the SPT was larger than that DPN, the ultra-fast patterning rate without any misalignment encouraged its potential application in clinical and biological research.

After patterning, the array chips were tested in order to determine whether the ink material (*i.e.*, maleimide–PEO_2–biotin) retained its biological activities using a sandwich fluorescence immunoassay. Polymer-coated cover glasses were used as the substrate, and multi-step immunoassay procedure was used. The MPTMS polymer formed a self-assembled monolayer on the glass surface and provided thiol groups as an active functional group that bound with the maleimide groups of maleimide–PEO_2–biotin to form a biotin terminated layer that was used for the orientation of streptavidin in the next step (circle in Fig. 1) [33]. Streptavidin was uniformly oriented in the direction of the biotin groups of maleimide–PEO_2–biotin after incubation for 10 min because of its strong binding affinity (dissociation constant of the order = 0.1 fM). The other side of the oriented streptavidin reacted with BPG in order to immobilize the monoclonal CRP antibody. The antibody was immobilized on protein G, which facilitates the binding of antigen with the Fc regions of antibody. After the first antibody reacted with BPG on the nanoarray protein chips for 1 h, the chip was incubated for another hour with various concentrations of the standard CRP antigens and the human serum samples.

3.2. Quantification of CRP on nanoarray chip by TIRFM

The fluorescence images (Fig. 5A) were captured using TIRFM, and the average intensities of the array spots (Table 1) on the nanoarray chip were obtained using a digital image processing program. The TIRFM fluorescence intensities increased with increasing standard CRP concentration (Fig. 5). However, at a CRP concentration exceeding 1 fM, the fluorescence intensity no longer increased because of its dependence on the number of CRP molecules at an antibody concentration of 2 μg/mL (=13.3 nM) on the nanoarray chip. At CRP concentrations in the range from 1 aM to 1 pM, the optimal incubation time of the antigen with the antibody was 1 h,

Table 1
Values of spot fluorescence average intensities and theoretical number of molecules based on the concentration of human standard CRP on nanoarray chips.

CRP conc. (aM)	0	0.05	1	10	10^2	3×10^2	5×10^2	10^3
Theoretical mol. no.[a]	0	0.75	15	150	1505	4517	7528	15,057
RFI (a.u.)[b]	0[c]	79 ± 12.4	85 ± 7.6	98 ± 6.1	134 ± 8.6	208 ± 6.6	301 ± 9.7	525 ± 23.2

[a] The value indicates the theoretical number of CRP molecules in 25 μL sample volume.
[b] Relative fluorescence intensity (arbitrary unit).
[c] The RFI is zero because the TIRF intensity was equivalent with background intensity and RFI was calculated by subtracting from background intensity (RFI = TIRF intensity – background intensity).

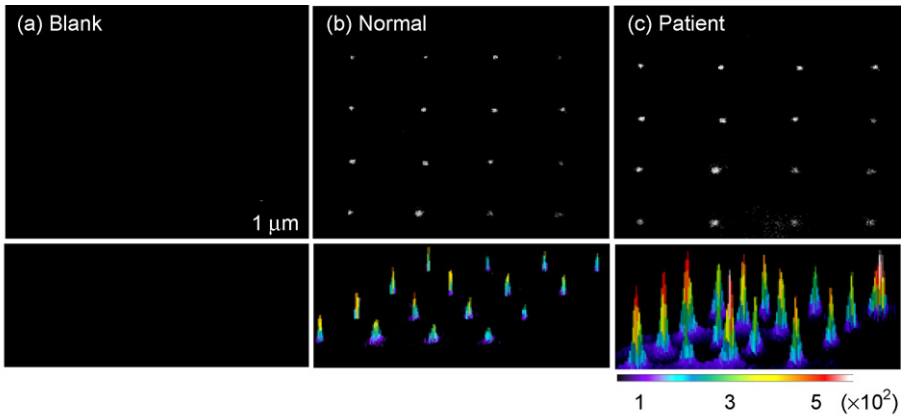
and the required volume of the standard CRP solution was 25 μL. However, in 25 μL volume of 50 zM concentration, the theoretical number of CRP molecule is 0.75 (~1). It was very confusing and uncertain to detect any spot on our nanoarray chip using the small volume (25 μL) of sample which theoretically contains only one CRP molecule. To avoid this uncertainty, we used 100 μL volume of standard CRP sample instead of 25 μL and could detect 3 spots on the nanoarray chip. The average spot intensity was 79 ± 12.4 (Table 1). As increasing the CRP concentration, the number of molecules as well as the spot fluorescence intensities also increased. Therefore, we measured the spot fluorescence intensities against various concentrations of CRP. In the case of 50 zM concentration, we assumed these 3 spots represent 3 individual CRP molecules in 100 μL volume which was possible only at this type of ultra-low concentration. Beside this, admirable light transmittance efficiency (99.4%) of the MPTMS-coated glass substrate and the high signal-to-noise ratio of the TIRFM made it possible to detect individual CRP molecules on the chip. Moreover, boundaries were shaped by etching a cross-pattern onto the cover glass surface before the sample was patterned in order to determine the exact positions of the array and the CRP-binding spots. These experiments were repeated at least five times in order to confirm that the individual CRP molecules were only detected in the specific array region of the glass surface. Single-CRP molecules were detected under these conditions, especially when the concentration of the human standard CRP was 50 zM. When a negative control experiment carried out without using any CRP solution or serum sample, no detectable spot was found on nanoarray chip and the average intensity was very low (21 ± 2.3) which was equivalent with the background intensity. For all concentrations of CRP, the relative fluorescence intensity (RFI) was calculated by subtracting from background intensity (RFI = TIRF intensity – background intensity). Therefore in the negative control, the RFI was zero (Table 1). Finally, we determined the LOD at 50 zM concentration.

The number of CRP molecules was calculated based on the molecular weight of the recombinant human standard CRP (M_w = 26 kDa) and Avogadro's number ($N = 6.023 \times 10^{23}$) (Table 1). Additionally, the incubation time of the antigen-antibody interaction was increased to a maximum of 2.5 h under agitation in order to attain a sufficient binding probability for the individual CRP molecules and the antibody molecules. In general, the fluorescence intensity increases with increasing reaction time because more antigens are bound to the antibody on the nanoarray chips [34]. Practically, the TIRFM fluorescence intensity stopped increasing at reaction times exceeding 1 h, suggesting that an interaction time of 1 h was sufficient in order to bind the individual protein molecules onto the nanoarray protein chip. These results demonstrated the successful detection of the antigen at the single-molecule level on the nanoarray protein chip at an ultra-low concentration. The linear range of the plot extended from 50 zM to 1 fM (0.05–10^3 aM) (correlation coefficient, $R = 0.9993$) in the assay of the nanoarray protein chip (Fig. 5B). The average fluorescence intensities for all the spots were corrected by subtracting them from the background intensity.

3.3. Clinical applications of the nanoarray CRP chip

Fig. 6A shows the representative TIRFM images of the observed CRP molecules for both the healthy individual (normal) and the

(A)



(B)

Samples	Nanoarray chip (pM)	ELISA (pM)	Difference
1	768.9	769.2	–0.3
2	512.6	502.5	10.1
3	530.9	524.7	6.2
4	132.7	135.8	–3.1
5	292.9	280.3	12.6
6	1897.0	1902.5	–5.5
7	1759.8	1758.0	1.8
8	1311.3	1313.6	–2.3
9	1164.8	1169.2	–4.4
10	1203.7	1202.5	1.2

Mean of differences = 1.63; Std. dev.= 6.2; $t_{calculated} = 0.84$

Fig. 6. (A) Representative TIRFM images of (a) the blank (=only PBS buffer), (b) the serum sample from normal individuals and (c) the serum sample from known heart disease patients on the nanoarray protein chips when exposed to 13.3 nM of the first monoclonal antibody. (B) Comparative features of the CRP level in the blood serum of healthy individuals and heart disease patients using the nanoarray chip and the ELISA.

patient samples. No fluorescence spots were observed the blank sample solution that was simply a 1× PBS solution in the place of the antigen. Fig. 6B shows the estimated CRP levels in the blood sera of healthy individuals and patients using the proposed nanoarray chip-based technique and conventional ELISA. The first five samples were obtained from the individuals with no-known diseases that are associated with CRP elevation. Samples 6 and 7 were collected from reported heart disease patients. Similarly, samples 8 and 9 were obtained from patients with tissue wounds, and the last sample was taken from an individual suffering from a viral infection. In all of the cases, the linear equations $y = 0.437x + 84.991$, which was obtained from the representative calibration curve in Fig. 5B, and $y = 0.0009x + 0.3978$, which was obtained from the ELISA calibration curve (Supporting information Fig. S2), were used to calculate the CRP concentration in the blood serum of the clinical samples using the spot fluorescence intensities and optical densities (Supporting information Table S1). The blood sera required a 100-fold dilution in order to estimate the CRP concentration using the dilution factor (Fig. 6B). The paired Student's *t*-test was used to determine if there was a significance difference between the results of the two methods [35]. The value of $t_{calculated}$ was 0.84 for 10 ($n = 10$) samples according to the formula: $t_{calculated}$ = (absolute value of the mean difference/standard deviation) × $(n)^{1/2}$. The proposed nanoarray chip-based method and ELISA were not significantly different at a 95% confidence level. These results showed a better accuracy for the chip-based detection compare to the well-established ELISA technique. The average CRP concentration in the patients was almost three times higher than the normal individuals (Fig. 6B). The highest CRP level was quantified as ~2 nM in the serum of one of the heart disease patient. This value was 3–15 times higher than the normal individuals. According to a previous report [3], the calculated CRP concentrations in the blood serum of the patients were indicators of low risk cardiovascular diseases.

4. Conclusions

High sensitivity CRP assays have been distinctly researched in order to establish well defined devices and methods for the fast and precise diagnosis at ultra-low concentrations. As a continuation of these efforts, this study demonstrated a fast patterning method for nanoarray biochips using a NanoeNabler™-based surface patterning tool and a high sensitivity detection method at zeptomolar (50 zM) concentrations of CRP using a TIRFM system. A 4 × 4 nanoarray chip with a spot diameter of 400 nm was formed within 1 min using the SPT. This array time for the single-pen nanoarray was much faster than DPN. The nanoarray CRP chip exhibited an ultra-low LOD of 50 zM, and only three CRP molecules were observed on the chip. Compared to the conventional ELISA technique, the proposed chip-TIRFM method exhibited a 6×10^6 times higher detection sensitivity with a wide dynamic linear range (50 zM–1 fM). The CRP was quantified in the human blood sera of normal individuals and patients in order to demonstrate the potential diagnosis of clinical samples. Therefore, this approach is a forthcoming analytical technique for the detection of biological samples with ultra-low concentrations at the single-molecule level in clinical investigations and biological recognition processes.

Acknowledgements

This work was supported by a grant from the Korea Ministry of Science and Technology (M1053608003-05N3608-00310) and the Korea Science & Engineering Foundation (R01-2007-000-20238-0).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.02.042.

References

[1] M.B. Pepys, G.M. Hirschfield, J. Clin. Invest. 111 (2003) 1805.
[2] N. Rifai, P.M. Ridker, Curr. Opin. Lipidol. 13 (2002) 383.
[3] A. Pfützner, T. Forst, Diabetes Technol. Ther. 8 (2006) 28.
[4] C. Albrecht, N. Kaeppel, G. Gauglitz, Anal. Bioanal. Chem. 391 (2008) 1845.
[5] R.M. Lequin, Clin. Chem. 51 (2005) 2415.
[6] M.F. Elshal, J.P. McCoy, Methods 38 (2006) 317.
[7] H. Andresen, C. Grötzinger, K. Zarse, M. Birringer, C. Hessenius, O.J. Kreuzer, E. Ehrentreich-Förster, F.F. Bier, Sens. Actuators B: Chem. 113 (2006) 655.
[8] G. Russo, C. Zegar, A. Giordan, Oncogene 22 (2003) 6497.
[9] A. Kumar, G.M. Whitesides, Appl. Phys. Lett. 63 (1993) 2002.
[10] B.D. Martin, B.P. Gaber, C.H. Patterson, D.C. Turner, Langmuir 14 (1998) 3971.
[11] V.N. Morozov, T.Y. Morozova, Anal. Chem. 71 (1999) 1415.
[12] M. Schena, D. Shalon, R.W. Davis, P.O. Brown, Science 270 (1995) 467.
[13] K.-B. Lee, S.-J. Park, C.A. Mirkin, J.C. Smith, M. Mrksich, Science 295 (2002) 1702.
[14] S.-Y. Hou, H.-K. Chen, H.-C. Cheng, C.-Y. Huang, Anal. Chem. 79 (2007) 980.
[15] Y. Shen, N. Tolic, C. Masselon, L. Pasa-Tolic, D.G. Camp II, M.S. Lipton, G.A. Anderson, R.D. Smith, Anal. Bioanal. Chem. 378 (2004) 1037.
[16] S. Lee, S. Lee, Y.-H. Ko, H. Jung, J.D. Kim, J.M. Song, J. Choo, S.K. Eo, S.H. Kang, Talanta 78 (2009) 608.
[17] S. Lee, N.-P. Cho, J.D. Kim, H. Jung, S.H. Kang, Analyst 134 (2009) 933.
[18] W.T. Huck, Angew. Chem. Int. Ed. 46 (2007) 2754.
[19] M. Lynch, C. Mosher, J. Huff, S. Nettikadan, J. Johnson, E. Henderson, Proteomics 4 (2004) 1695.
[20] S.H. Kang, M.S. Islam, Biochip J. 3 (2009) 97.
[21] A.N. Asanov, W.W. Wilson, P.B. Oldham, Anal. Chem. 70 (1998) 1156.
[22] Y.K. Yen, J.Y. Lee, M.C. Kuo, L.S. Huang, Proceedings of the 2005 IEEE, International Conference on Mechatronics, Taipei, Taiwan, 2005, p. 280.
[23] H. Jung, C.K. Dalal, S. Kuntz, R. Shah, C.P. Collier, Nano Lett. 4 (2004) 2171.
[24] S.H. Kang, M.R. Shortreed, E.S. Yeung, Anal. Chem. 73 (2001) 1091.
[25] www.rndsystems.com.
[26] R. Lenigk, M. Carles, N.Y. Ip, N.J. Sucher, Langmuir 17 (2001) 2497.
[27] J. Haaheim, O.A. Nafday, Scanning 30 (2008) 137.
[28] B. Li, G. Lu, X. Zhou, X. Cao, F. Boey, H. Zhang, Langmuir 25 (2009) 10455.
[29] D.L. Wilson, R. Martin, S. Hong, M. Cronin-Golomb, C.A. Mirkin, D.L. Kaplan, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 13660.
[30] J. Xu, M. Lynch, J.L. Huff, C. Mosher, S. Vengasandra, G. Ding, E. Henderson, Biomed. Microdevices 6 (2004) 117.
[31] S.W. Lee, B.-K. Oh, R.G. Sanedrin, K. Salaita, T. Fujigaya, C.A. Mirkin, Adv. Mater. 18 (2006) 1133.
[32] F. Huo, Z. Zheng, G. Zheng, L.R. Giam, H. Zhang, C.A. Mirkin, Science 321 (2008) 1658.
[33] A.S. Anderson, A.M. Dattelbaum, G.A. Montano, D.N. Price, J.G. Schmidt, J.S. Martinez, W.K. Grace, K.M. Grace, B.I. Swanson, Langmuir 24 (2008) 2240.
[34] J.-W. Choi, B.S. Chun, B.-K. Oh, W. Lee, W.H. Lee, Colloids Surf. B Biointerf. 40 (2005) 173.
[35] D.C. Harris, Quantitative Chemical Analysis, 6th ed., W.H. Freeman and Company, New York, 2003.