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Quantitative analysis of human serum leptin using a nanoarray protein chip based on single-molecule sandwich immunoassay

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ABSTRACT

We report a method for the quantitative analysis of human serum leptin, which is a protein hormone associated with obesity, using a nanoarray protein chip based on a single-molecule sandwich immunoassay. The nanoarray patterning of a biotin-probe with a spot diameter of 150 nm on a self-assembled monolayer functionalized by MPTMS on a glass substrate was successfully accomplished using atomic force microscopy (AFM)-based dip-pen nanolithography (DPN). Unlabeled leptin protein molecules in human serum were detected based on the sandwich fluorescence immunoassay by total internal reflection fluorescence microscopy (TIRFM). The linear regression equation for leptin in the range of 100 zM–400 aM was determined to be $y = 456.35x + 80{,}382$ ($R = 0.9901$). The accuracy and sensitivity of the chip assay were clinically validated by comparing the leptin level in adult serum obtained by this method with those measured using the enzyme-linked immunosorbent assay (ELISA) performed with the same leptin standards and serum samples. In contrast to conventional ELISA techniques, the proposed chip methodology exhibited the advantages of ultra-sensitivity, a smaller sample volume and faster analysis time.

1. Introduction

Leptin is a protein hormone associated with obesity, appetite regulation, energy expenditure, and reproduction in animals and humans. Noticeably higher leptin levels have been observed in obese humans than in non-obese humans [1–3]. Leptin is thought to contribute to body weight regulation by controlling food intake and energy expenditure at the hypothalamic level [4]. It has been suggested that abnormalities in its level increase the propensity to obesity. In addition to its role in metabolic disorders and obesity, leptin also has an important regulatory effect on bodily hormonal [5,6] and gonadal [7] functions. So far, the capillary electrophoresis [8–10], immunofunctional assay [11], enzyme-linked immunosorbent assay (ELISA) [12,13], radioimmunoassay [14–18] and Western blotting [19–21] techniques have been conventionally used to determine human leptin. Although they are reliable, these methods are relatively expensive and are restricted to the determination of single target specificity.

The possible application of microarray biochips to the analysis of various cytokines, including leptin, in serum samples, was demonstrated by Du et al. [22]. The ability to fabricate highly robust microarrays in which a thousand samples are immobilized enables the generation of massive amounts of biologically relevant data using low sample quantities. Recently, nanoarray technology has been suggested as a means of overcoming the problems of microarray technology, such as the relatively large sample volumes and long incubation times that are required, and the high limits of detection (LOD). A new generation nanoarray biochip has also been described which is capable of supporting high-throughput and multiplexed ELISA. Despite its advantages, the nanoarray assay is not able to achieve the femtomolar (fM, $\times 10^{-15}$ M) LOD of ELISA, which limits its applications in immunoassays [23,24]. However, the detection of proteins in the zeptomolar concentration range (zM, $\times 10^{-21}$ M) has recently been demonstrated using a mass spectrometer or gold nanoparticles conjugated with antibodies [25,26]. Proteins in the zM concentration range exhibit almost no detectable, nonspecific binding to the passivated portions of nanoarrays, even when they are present in the form of complex mixtures, and therefore provide the opportunity to study a variety of surface-mediated, biological recognition processes.

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This paper reports the development of a nanoarray protein chip for the quantitative analysis of human leptin in serum at the zM–aM concentration level. The leptin protein nanoarrays formed via atomic force microscopy (AFM)-based dip-pen nanolithography (DPN) were detected using a total internal reflection fluorescence microscopy (TIRFM) system at the single-molecule level based on a single-molecule sandwich fluorescence immunoassay. Leptin protein molecules at the 100 zM concentration level in human serum were detected on the nanoarray protein chip without any nonspecific binding of protein molecules. The nanoarray protein chip assay was further clinically validated by comparing the leptin level in adults obtained by this method with that measured using the conventional ELISA performed with the same leptin standards and serum samples.

2. Experimental

2.1. Chemical and reagents

(3-Mercaptopropyl)trimethoxysilane (MPTMS), streptavidin, biotinylated protein G (BPG) and Tween-20 were obtained from Sigma–Aldrich (St. Louis, MO). mPEG–maleimide (5K), used as a blocking solution, was purchased from IDB (ID Biochem Inc., Seoul, Korea), streptavidin-Alexa Fluor® 488 from Molecular Probes (Eugene, OR, USA), and Ez-Link®maleimide–PEO_2–biotin from Dojindo (Dojindo Laboratories, Japan). Rabbit polyclonal antibody to human leptin (Abcam, Cambridge, UK) was used as the first antibody on the nanoarray protein chip. All samples were diluted with a 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) buffer solution. All buffer solutions were prepared with ultra-pure water (>18 MΩ) and filtered through a 0.2-μm membrane filter (nylon membrane filter, Whatman International Ltd., Maidstone, England) and photo-bleached overnight using a UV–B lamp (G15TE, 280–315 nm, Philips, The Netherlands). A human leptin ELISA kit (cat. no. EZHL-80SK) was purchased commercially from Millipore Co. (Charles, MO, USA).

2.2. Preparation of human serum samples

Blood samples were obtained from five healthy humans with a body mass index (BMI) range of 19.1–30.5 kg/m^2. The serum samples were isolated from the blood by centrifuging at 2000 rpm for 15 min at 2 °C and stored at −20 °C prior to the experiment. The concentrations of human leptin in the serum samples were quantified using the conventional sandwich ELISA and the nanoarray protein chip.

2.3. Nano-patterning on the MPTMS-coated cover glass

The following, two-step silanization procedures were performed for the fabrication of the nano-pattern [27]. Briefly, a Corning glass cover slip (No. 1, 22 mm^2) was silanized by evaporating 2 μL of neat liquid MPTMS onto it at 120 °C for 30 min and then gently washing with deionized water. After drying in nitrogen, the resulting, MPTMS-coated substrate was arrayed with maleimide–PEO_2–biotin (1 mg/mL) at room temperature by DPN. The DPN nano-pattern was produced using an evaporation method [28]. Briefly, 2 μL of MPTMS was evaporated onto the substrate using a clean AFM tip (silicon nitride cantilever, force constant (k) of 42 N/m) with a radius of curvature of ≤10 nm at 120 °C for 30 min and then 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) from Digital Instruments. The relative humidity was adjusted to 60% by purging with nitrogen gas that had been bubbled through water in a large glove box. All DPN experiments were performed in tapping mode at room temperature.

2.4. Nanoarray protein chip assay of human leptin

The MPTMS-coated cover glasses were nano-patterned with biotin molecules using the DPN technique. mPEG–maleimide (5K) (1 mg/mL in 1× PBS), which is resistant to nonspecific adsorption, was incubated on the biotin nano-pattern chip for 10 min, because the maleimide groups of mPEG–maleimide (5K) react efficiently and specifically with free sulfhydryl groups at pH 6.5–7.5 to form stable thioether bonds. Streptavidin (33.4 nM) was incubated with the biotin nano-pattern chip for 10 min. The streptavidin-bound biotin chip was allowed to react for 1 h with 50 μL of 2 μg/mL BPG to facilitate the binding of the antibody Fc regions, after which it was reacted for 1 h with 50 μL of rabbit polyclonal antibody to human leptin (150 kDa; 13.3 nM). The chip was incubated again for 1 h with various concentrations of standard leptin antigens (100 zM–7.8 pM) or human serum samples and then for 1 h with 50 μL of human leptin detection antibody (i.e., the biotinylated mouse anti-human leptin antibody in the ELISA kit). Finally, streptavidin-Alexa Fluor® 488 was reacted leptin antigens for 10 min to obtain the TIRFM images. The chip was washed by dipping it in 100 mL of 1× PBS-T for 2 min at each step, and washed for 2 min with PBS before image acquisition. All reactions were carried out under agitation at 25 °C.

2.5. Laboratory-built TIRFM system

The basic experimental set-up of the lab-built, prism-type, TIRFM system (Fig. 1) used to quantitatively analyze the human leptin on the nanoarray protein chip was similar to that described in previous reports [29,30]. Briefly, an argon ion laser (model 532-LAP-431-220; Melles Griot, Irvin, CA; output 40 mW at 488 nm) was used as the excitation source. TIRFM was performed using an upright Olympus BX51 microscope (Olympus Optical Co. Ltd., Shinjuku-ku, Tokyo) with an Olympus 100x UPLFL objective lens (oil type, 1.3 N.A., W.D. 0.1 mm). The objective lens was matched to the nanoarray chip with an immersion oil (Immersol™ 518F, Zeiss, n = 1.518). A CCD camera (QuantEM 512SC, Photometrics, Tucson, AZ) was used as a detector with an exposure time of 100 ms. A filter cube with a 488-nm notch filter (Korea Electro Optics, Korea) and a 530-nm band-pass filter (FWHM 20 nm, Olympus Optical Co. Ltd., Shinjuku-ku, Tokyo) was installed between the CCD camera and the objective lens. After the completion of all reactions, the nanoarray

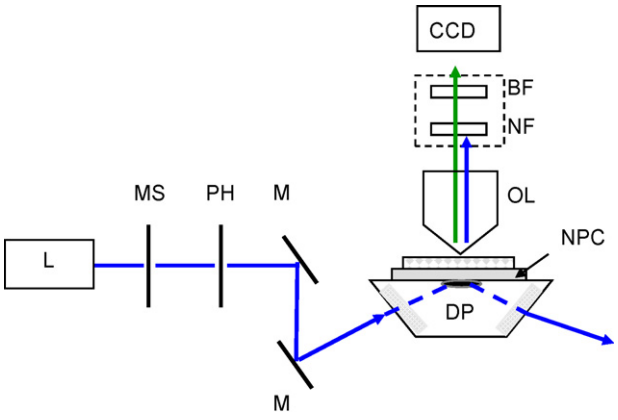

Fig. 1. Schematic diagram of the TIRFM system used for the quantitative analysis of human leptin. The dotted box indicates the filter cube with NF and BF. The following acronyms are used: L, laser; MS, mechanical shutter; PH, pinhole; M, mirror; DP, dove prism; NPC, nanoarray protein chip; OL, objective lens; NF, notch filter; BF, band-pass filter.