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Simultaneous detection of platelet-specific antibodies based on a photonic crystal-encoded suspension array

Xin Kong [a], Baofen Ye [b], Zixue Yang [b,c], Baoan Chen [c], Yun Ling [a,c,*]

[a] The Third Affiliated Hospital of Soochow University, The First People's Hospital of Changzhou, Changzhou, Jiangsu 213000, China
[b] State Key Laboratory of Bioelectronics Southeast University, Nanjing 210096, China
[c] Department of Hematology, Zhongda Hospital, Medical School, Southeast University, Nanjing 210009, China

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ABSTRACT

Background: The appearance of antibodies to platelets in the blood is an important cause of immune thrombocytopenia (ITP), and platelet glycoprotein (GP)-specific antibody detection may be helpful to diagnose this condition.
Methods: Photonic crystal microspheres with different distinct reflection spectra were coated with anti-GPIIb, -GPIIIa, -GPIb and -GPIX monoclonal antibodies (MoAbs) to create a photonic crystal-encoded suspension array (PCSA). Fluorescein isothiocyanate-labelled goat anti-human IgG was added to detect human IgG simultaneously. The detection results were analysed by fluorescence microscopy. Parallel MoAb immobilization of platelet antigen (MAIPA) was used as a reference test. Both methods were used to analyse 63 clinical samples including serum from 32 ITP patients and 31 healthy humans.
Results: The PCSA showed greater sensitivity than MAIPA in detecting anti-GPIIb (75.0% vs 31.1%) and GPIIIa (84.4% vs 40.6%) antibodies and similar sensitivity as MAIPA in detecting anti-GPIb (37.5% vs 34.4%) and GPIX (50.0% vs 40.8%) antibodies. The MAIPA and PCSA tests had similar specificity. The PCSA detected higher dilutions of serum containing anti-GPIIIa antibody or anti-GPIIb antibody than did MAIPA. The entire testing process was controlled within 3.5 h.
Conclusions: The PCSA assay described has comparable or better sensitivity and specificity compared to the MAIPA and is more rapid.

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1. Introduction

Platelet-specific antibody can cause platelet destruction, thrombocytopenia and megakaryocyte maturation disorder. Detection of platelet-specific antibodies is important for the diagnosis, treatment and prognosis of patients with immune thrombocytopenia (ITP) [1]. Researchers have shown that platelet-specific antibodies can be detected in the serum of patients with ITP [2,3], post-transfusion thrombocytopenic purpura or drug-induced thrombocytopenia [4,5]. Platelet-specific antibodies are generated against platelet membrane glycoproteins (GPs) such as GPIIb/IIIa, Ib/IX, Ia/IIa, IV and V. One or more specific type of antibody may exist in one patient at the same time, and the type of antibodies can differ between individuals [6,7]. The main platelet-specific antibody is anti-GPIIb/IIIa, followed by anti-GPIb/IX, anti-GPIa/IIa and anti-GPIV antibodies.

The platelet immunofluorescence test is the main assay used for the early and sensitive detection of platelet antibodies [8]. The targets of the method are platelet-associated antibodies, including platelet-specific antibodies and HLA antibodies, which are suitable for screening. Monoclonal antibody (MoAb) immobilization of platelet antigen (MAIPA), the gold standard method, was reported in 1987 [9] and was improved in 1997 by the addition of the indirect detection of platelet-specific antibodies in serum [8]. The improved MAIPA is now the most sensitive and specific assay, and is used in specialized laboratories throughout the world. Simultaneous analysis of specific platelet antibodies (SASPA) [10] is a multiplex assay that also uses polystyrene microspheres. SASPA has a faster reaction and greater flexibility compared with MAIPA. However, the specificity of SASPA is slightly lower than that of MAIPA. There is at present no suitable detection method for clinical application.

Photonic crystal microspheres (PCMs) are used as reflection peaks of the encoding element and are applied as biomolecular supports [11,12]. The encoding signal of PCMs depends on the periodic arrangement structure of nanoparticles, which makes them stable and unlikely to influence the detection signal. The use of PCMs has attracted attention among researchers in the fields of biology and biochemistry [13,14]. Photonic crystals have been applied to optical switches, microlasers and efficient microwave antennas over the past few decades [15,16]. Photonic crystal-encoded bead arrays have been shown to be suitable for the sensitive multiplex detection of tumour markers [17]. The photonic crystal-encoding technique is an optical encoding technique

* Corresponding author at: The Third Affiliated Hospital of Soochow University, The First People's Hospital of Changzhou, Changzhou, Jiangsu, 213000, China.
E-mail address: a110426007@hotmail.com (Y. Ling).

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based on the unique reflection spectra rather than the emission spectra of fluorescent dyes [18].

2. Materials and methods

2.1. Sample collection

Serum samples were obtained from 32 patients with newly diagnosed ITP treated at the Third Affiliated Hospital of Soochow University, China). All ITP patients that enrolled have met the practice guidelines from the American Society of Hematology and no history of blood transfusion. Their actual platelet count range was from 2×10^9/l to 30×10^9/l, normal or increased bone marrow megakaryocytes without morphological evidence of dysplasia, and no other diseases that could account for their thrombocytopenic state. Thirty-one healthy donors, who had never received a transfusion and whose platelet counts were in the normal range, were also enrolled. The platelets used for the PCSA and MAIPA were obtained after differential centrifugation of blood taken from healthy adult donors with type O red cells. The platelets were washed twice with ethylenediaminetetraacetic acid (EDTA)–phosphate-buffered saline (PBS), resuspended in EDTA–PBS at a concentration of 5×10^8 ml^{-1} and used for the PCSA and MAIPA within 8 h.

2.2. Antibodies and other materials

MoAbs recognizing GPIX (SZ1, anti-GPIX), GPIb (SZ2, anti-GPIb), GPIIb (SZ22, anti-GPIIb) and GPIIIa (SZ21, anti-GPIIIa) were obtained from the Key Lab of Thrombosis and Hemostasis (First Affiliated Hospital of Soochow University). All MoAbs have been previously characterized and reported to have good specificity and sensitivity [19–22]. Fluorescein isothiocyanate (FITC)-labelled goat anti-human IgG (FITC-GAH) and FITC-labelled goat anti-mouse IgG (FITC-GAM) polyclonal antibodies were from Biodee Biotechnology Co.

Silica PCMs were made in the State Key Laboratory of Bioelectronics (Southeast University). Monodisperse silica nanoparticles were used for the production of photonic crystal-encoded silica microspheres and were synthesized using the Stober method [23]. Epichlorohydrin (ECH) was purchased from Bodi Chemical Co. bovine serum albumin (BSA) was obtained from Sigma-Aldrich. PBS (pH 7.4) and PBS-Tween-20 wash buffer (PBST, PBS containing 0.05% Tween-20) were made in our laboratory. Other reagents were purchased from Sinopharm Chemical Reagent Co. Ultra-pure water was obtained by treatment in a Milli-Q system and was used in all experiments.

The reflection spectra of the PCSA were recorded on a metallographic microscope (Olympus BX51) equipped with a fibre-optic spectrometer (Ocean Optics, USB2000-FLG). The intensity of the emission fluorescence was recorded using an upright fluorescence microscope (IX71, Olympus) equipped with a digital imaging system (DP73, Olympus). PCSA images were captured under bright field and dark field by the same microscope.

2.3. PCSA

Four kinds of silica PCMs were fabricated using well-controlled droplets of perfect size (diameter 250 μm ± 10 μm) with a microfluidic device (Fig. 1). The mechanical stability of the microspheres was improved by calcination. PCMs were added into a melting 4% agarose hydrogel over an 80 °C water bath to form photonic crystal hydrogel microspheres (PCHMs). The agarose hydrogel was cooled at room temperature and allowed to solidify, and the PCHMs were rubbed out of a massive piece of hydrogel by hand. PCHMs were observed under a low-power microscope to determine whether the agarose hydrogel had been removed completely. The silica was then corroded with 10% hydrofluoric acid to confirm whether the PCM perfusion was completed. PCHMs were activated by ECH based on the principle that many of the hydroxyl groups on the surface of PCHMs can react with

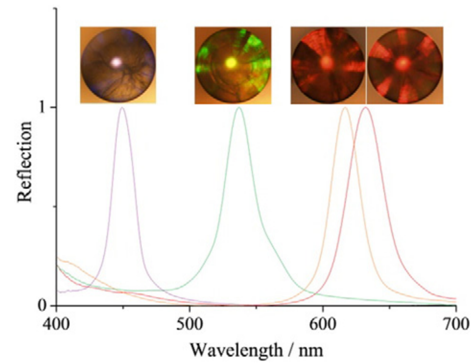

Fig. 1. Photonic crystal-encoded suspension array (PCSA). a: Four kinds of photonic crystal hydrogel microspheres (PCHMs) under bright field. b: Reflection peaks of the four PCHMs. The reflection peaks of a, b, c and d in image A are 630 nm, 615 nm, 540 nm and 450 nm, respectively.

ECH to form active epoxy groups in an alkaline environment [24,25]. Microspheres in batches of 10 were soaked in 100 μl containing a mixture thereof with 50 μl of 20% ECH and 50 μl of 2.0 mol/l NaOH for 3 h at 37 °C and then washed five times with ultra-pure water.

The PCSA was conducted in experiments that included four PCMs with reflection peak wavelengths at 630, 615, 540 and 450 nm as the encoding carriers. The PCMs were coated with MoAbs recognizing GPIX (SZ1), GPIb (SZ2), GPIIIa (SZ21) or GPIIb (SZ22). The MoAbs were fixed onto the surface of the PCHMs through the combination of active epoxy groups on the surface of activated PCHMs and amino groups. Ten microliters of MoAbs diluted in 0.2 mol/l $NaHCO_3$ was mixed with activated PCHMs in a flat-bottom vessel and maintained at 25 °C overnight. After immobilization of the MoAbs, the PCHMs were washed successively with ultra-pure water, 0.5 mol/l NaCl and ultra-pure water. The unbound active sites on PCHMs were blocked with 5% BSA at 37 °C for 2 h, washed 3 times with PBST and stored at 4 °C in PBS buffer containing 0.02% sodium azide for later multiplex detection. FITC-GAM IgG was used to detect the activity of MoAbs on the surface of PCHMs that had been stored for 0, 7, 14, 28, 60 and 120 days. FITC-GAH IgG was used as the blank control.

2.4. Detection of PCSA and MAIPA

A schematic illustration is shown in Fig. 2. The serum samples from ITP patients and healthy donors were incubated with an equal volume of platelets (5×10^8 ml^{-1}) for 60 min at room temperature. After incubation, the platelets were washed three times with EDTA–PBS, solubilized in Triton X-100 lysis buffer for 30 min and centrifuged at 13,000*g* for 40 min. The supernatant was diluted 1:5 in PBS to obtain the lysates used in the MAIPA. One unit of PCSA (1 multiplex detection and 4 single MoAb assays) was incubated with 20 μl of lysate in a flat-bottom vessel at room temperature for 30 min to capture the platelet GP–human antibody complexes, and the vessel was washed three times with PBST followed by three times with PBS. Twenty microliters of 0.1 mg/ml FITC-GAH IgG was used as the secondary antibody to detect human antibodies bound to PCHMs. The PCSA was observed under bright field and dark field using an upright fluorescence microscope equipped with a digital imaging system, as described above.

The multiplex detection and single MoAb assays were compared to investigate whether there was mutual interference of the detection of different targets. In a separate test in different flat-bottom vessels, PCHMs coated with different MoAbs were used to analyse the serum samples. All 32 of the serum samples from ITP patients were detected using the single MoAb. The volume of platelet lysates was proportional to the number of PCHMs used in the test. MAIPA was performed according to previously reported standard procedures [5,26] and was used as the comparison.

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