Biomaterials 29 (2008) 4447–4454

Contents lists available at ScienceDirect

Biomaterials

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

Application of highly sensitive, modified glass substrate-based immuno-PCR on the early detection of nasopharyngeal carcinoma

Tzu-Wei Wang [a,b,c], Hsiang-Yin Lu [b,c], Pei-Jen Lou [d,**], Feng-Huei Lin [b,c,*]

[a] Tissue Engineering Laboratories, Brigham and Women's Hospital, Harvard Medical School and VA Boston Healthcare System, MA, USA
[b] Institute of Biomedical Engineering, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC
[c] College of Engineering, National Taiwan University, Taipei, Taiwan, ROC
[d] Department of Otolaryngology, National Taiwan University Hospital, Taipei, Taiwan, ROC

ARTICLE INFO

Article history:
Received 2 June 2008
Accepted 10 July 2008
Available online 26 August 2008

Keywords:
Surface modification
Silane
Immunoassay
Immuno-PCR
Cancer

ABSTRACT

In this study, we investigated the utilization of highly sensitive immuno-PCR (IPCR) method as a powerful tool to detect NPC in early disease stage. We established a substrate-ELISA platform as a model system for evaluation of the feasibility of our idea after surface modification process on glass beads. Therein the DNA–antibody conjugation was added to sensitize prior enzyme substrate–antibody complex. In the study, the detection efficiency of two different systems regarding sensitivity, affinity, and specificity was evaluated. Moreover, to show the efficacy of our IPCR system, commercialized ELISA kit was also included for comparison with our IPCR glass substrate-based capture system. The surface physical properties of the modified substrates were also tested with atomic force microscopy and X-ray photoelectron spectroscopy, together with the measurement of the water contact angle. In the results, various factors in the production of IPCR detection system were determined to maximize the effect on assay performance, including the modification of the glass surface properties, primary and secondary antibody optimal concentrations, and biotinylated reporter DNA concentration. We found that the sensitivity of IPCR was approximately over two order magnitude higher than that of conventional ELISA method. The result suggests that our IPCR system could be an applicable and reliable tool for early detection of NPC.

1. Introduction

Incidence of nasopharyngeal carcinoma (NPC) has remained high in endemic regions. Poor prognosis often resulted from delayed clinical diagnostics because of the occurrence of NPC usually in deep anatomical site and along with vague symptoms. Epstein–Barr virus nuclear antigen (EBNA1) is a DNA-binding nuclear phosphoprotein, which is required for the replication and maintenance of the episomal Epstein–Barr virus (EBV) genome. Directing EBNA1 expression to lymphocytic cells in transgenic mice has been shown to result in B-cell lymphomas suggesting that EBNA1 may have a direct role in oncogenesis [1]. EBV-encoded RNA signal is present in all nasopharyngeal carcinoma cells, and diagnosis of the disease is possible through the detection of raised antibodies against EBV [2]. However, so far the analysis of the pathophysiological role of EBNA1 in human plasma is difficult to achieve using conventional assays because of the low concentrations of EBNA1.

An immunoassay is defined as an analytical method that uses antibodies or antibody-related reagents for the determination of sample components [3]. The selective nature of antibody binding allows these reagents to be employed in the development of methods that are highly specific and that can often be used directly with even complex biological matrixes such as blood, plasma, or urine. To extend the scope of PCR to the high-sensitivity detection of proteins, we here established an immuno-PCR (IPCR) system to take advantages of specific conjugates comprising an antibody and a DNA marker fragment. Combining the versatility of enzyme-linked immunosorbent assays (ELISAs) with the amplification power and sensitivity of the PCR, IPCR is based on chimeric conjugates of specific antibodies and nucleic acid molecules, the latter of which are used as markers to be amplified by PCR for signal generation. The enormous efficiency of nucleic acid amplification typically leads to a 100–10,000-fold increase in sensitivity, as compared with the analogous enzyme-amplified immunoassay [4,5]. In addition to the great increase in sensitivity, IPCR offers the opportunity to freely choose the sequence of the DNA marker and hence opens up access to an almost unlimited range of specifically labeled antibodies,

* Corresponding author. Institute of Biomedical Engineering, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC. Tel.: +886 2 23912641; fax: +886 2 23940049.
** Corresponding author.
E-mail address: double@ha.mc.ntu.edu.tw (F.-H. Lin).

0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.07.015

which even enable multiplex detection of various different antigens [6]. The specific and sensitive detection of these markers is considered to be one of the most important challenges in the monitoring of many diseases, such as cancer and autoimmune diseases [7].

Owing to the versatility and high affinity of the biotin–streptavidin binding, we have explored and utilized this coupling system to generate IPCR reagents. In principle, the glass substrates were used to conjugate with epoxy-terminated silane group for binding with anti-EBNA1. After conjugating with the anti-EBNA1 IgA antibody in human plasma, biotinylated secondary antibody was then applied on it. The free streptavidin binding sites were linked to the biotinylated reporter plasmid DNA. The signal was amplified by conventional PCR and the products were analyzed by gel electrophoresis. In this study, we were interested in further enhancing the sensitivity of antigen–antibody detection systems. This should facilitate the specific detection of rare antigens, such as EBNA1, which are present only in very small numbers, and thus could expand the application of this technique in early detection of nasopharyngeal carcinoma.

2. Materials and methods

2.1. Surface modification and activation of glass substrates

The surface activation and modification process were carried out as previously described by Wong and Krull [8]. In brief, untreated slides were washed with ethanol and then etched by immersion in piranha solution (70% H_2SO_4 + 30% H_2O_2) at room temperature for 1 h. Subsequently, the slides were cleaned by sonification for 15 min. All slides were dried with nitrogen. They were then rinsed several times in water, washed in ethanol and derivatised in the 2.5% 3-glycidoxypropyltrimethoxysilane (GPTS) solution at 60 °C for 4 h followed by a sonification step. After silanisation, GPTS-treated slides were washed thoroughly with 95% ethanol. The slides were finally baked in oven for cross-linking and well preserved for the further experiments.

2.2. Characterization

X-ray photoelectron spectroscopy (XPS) analysis was performed with a VG MICROTECH, MT-500, UK spectrometer. The X-ray source was unmonochromated Mg Kα and the sample size was 1×1 cm^2. The excitation voltage was 1253.6 eV. The pass energy of 192 eV was used for low resolution survey and elemental composition analysis, whereas a pass energy of 48 eV was used for high resolution scans.

The atomic force microscope (AFM) images of the glass surfaces were obtained from a Digital Instruments Nanoscope Atomic Force Microscope (Asylum Research, Santa Barbara, CA, USA). The imaging was done in air in tapping mode. The AFM tip was made of silicon nitride with a spring constant of 0.12 N m^{-1} and a nominal radius of 20–60 nm. The software used to process the images was Nanoscope IIIa (Santa Barbara, CA, USA).

2.3. Wettability measurements

A 5 μL droplet of double-distilled water was deposited near the edge of an untreated or GPTS-modified glass slide. A light microscope that was equipped with a protractor was placed at its side as well as the illumination source so that the light would travel parallel to the bench top. A sample stage supported the slide enabling the shadow of the water droplet to be observed. Each measurement was made within 1 min of water deposition and was repeated for five other spots along the edge of the slide.

2.4. Immunochemistry analysis

The commercialized Super Sensitive™ Non-Biotin HRP Detection System (Invitrogen, CA, USA) was purchased to evaluate the capability of protein immobilization on glass substrate. In brief, silanized slide was grafted with mouse IgG for reaction overnight at 4 °C, and then washed with PBS buffer. Supper enhencer™ reagent was dropped onto the slide, incubated for 20 min at room temperature followed by washing with PBS. Poly-HRP reagent was finally added to react for 30 min and colored by DAB solution.

2.5. ELISA

Hundred microliter of diluted serum specimens, positive control, and negative control was added into microplate wells, respectively. Incubate at 37 °C for 1 h. The serial dilution of enzyme–antibody conjugates was then added and incubated at 37 °C for 30 min. Tetramethylbenzidine (TEB) solution was used for staining. The reaction was stopped by 1 N HCl addition. The results were measured at the wavelength of 450 nm by spectrophotometer.

2.6. Modeling of immuno-PCR

The detection protocol used is modified according to the conventional ELISA methods [9]. In brief, 50 μl of EBNA1 protein (10 μg/ml) was added onto glass substrate for conjugation with immobilized silane groups for 4 h at 37 °C. Unbound antigens were then aspirated and washed three times with washing buffer containing 10 mM Tris pH 7.3, 150 mM NaCl. Substrate surface was blocked by incubation with 10 mM Tris/HCl for 1 h at 37 °C, pH 7.6, containing 6% (w/v) non-fat milk powder, 0.2% (w/v) NaN_3, 0.05% Tween-20, and 5 mM EDTA. NPC patient's serum with anti-EBNA1 IgA antibody was applied and then incubated for 1 h at RT. After washing, samples were further incubated for 1 h at RT with biotinylated goat anti-human IgA secondary antibody (KPL Europe). After that, the specimens were washed and incubated for 30 min at RT with 100 ng/ml streptavidin (KPL Europe). 1 ng/ml biotinylated PCR target DNA (Sigma) was incubated with the specimens for 30 min at RT. Unbound target DNA was rigorously removed by washing several times with PBS/Tween solution (Zymed Laboratories Inc.). The brief scheme is illustrated in Fig. 1.

After addition of commercialized PCR kit reagents, contents were subjected to 30 cycles of PCR amplification (PCR Thermocycler, Corbett Life Science, Australia). PCR was performed in 50 μl of 50 mM KCl in 10 mM Tris/HCl, pH 8.3, containing 1 mM Mg^{2+}, 50 μM of dNTP (PCR Master Mix, GeneMark), 50 pmole of each primer. A primer set nested to those used above for production of biotinylated PCR target DNA was used, and gave a 235 base-pair PCR product (sense primer sequence corresponding to pHSP-70: 5′-CTCCAGCCGACAAGAAGC-3′; antisense primer sequence: 5′-ACGGTGTTGTGGGGGTTCAGG-3′). Tests were initially heated to 95 °C and held at this temperature for 5 min, then subjected to 30 cycles of 55 °C, 30 s, 72 °C, 30 s, 94 °C, 30 s, followed by 72 °C for 15 min, then cooling to 4 °C. Resulting biotinylated DNA was purified using a QIAGEN Gel/PCR DNA Fragments Extraction Kit (Qiagen, USA.) and stored frozen in batches at −20 °C. The resulting PCR products were analyzed by 1.5% agarose gel electrophoresis after staining with ethidium bromide.

3. Results

3.1. Characteristics of glass substrate surface properties

3.1.1. Water contact angle

The water contact angle was measured to confirm the glass surface modification process. In Fig. 2, the water contact angle on the surface of unmodified glass substrate was 40.75° ± 3.77. After piranha solution treatment, a layer of hydroxyl groups was formed

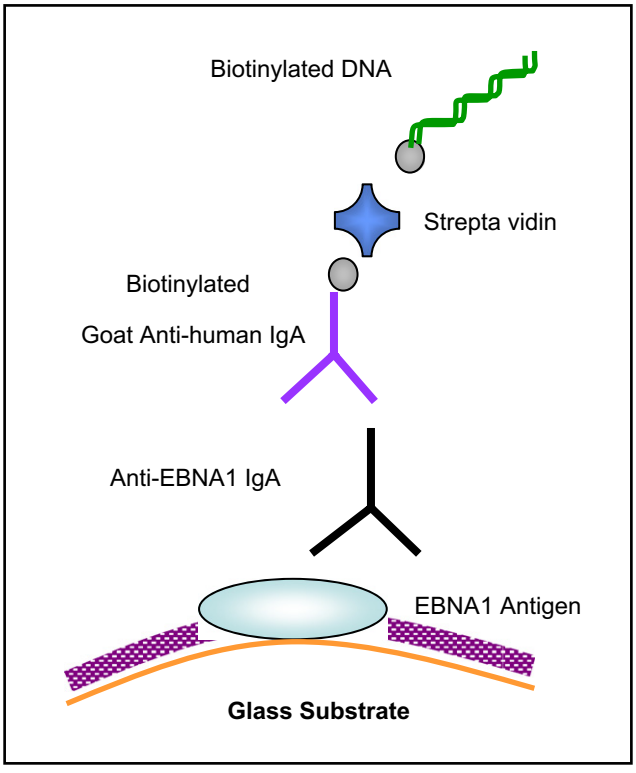

Fig. 1. The scheme of modeling process of immuno-PCR.