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CLCHAW 46 (9) 1291-1522 (2000)

International Journal of Laboratory Medicine and Molecular Diagnostics

in this issue

- Cardiac Troponins T and I
 - End-Stage Renal Disease
 - Heparin-Plasma vs Serum
- Bidirectional Fetomaternal Transfer of DNA
- Reference Preparations of PSA
- Leonard Skeggs on the AutoAnalyzer
- Oak Ridge Conference

Clinical Chemistry

CLCHAU 46 (9) 1291-1522 (2000)

International Journal of Laboratory Medicine and Molecular Diagnostics



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Reflectometry Interference Spectroscopy in Detection of Hepatitis B Surface Antigen, Fang Yu, Danfeng Yao, and Weiping Qian* (National Laboratory of Molecular and Biomolecular Electronics, Southeast University, Nanjing 210096, Peoples Republic of China; * author for correspondence; fax 86-25-7712719, e-mail wqian@seu.edu.cn)

Optical biosensors are of increasing interest for real-time detection of analytes without the use of additional labeled reagents. The sensing system is based on different interfacial physical properties that change upon the binding reaction. Combining high sensitivity and selectivity, label-free biosensors have the potential to provide low-cost detection and measurement technology for accurate and highly specific quantification of low-concentration analytes. Reflectometry interference spectroscopy (RIFS) is such a typical optical system that has been systematically developed and investigated by Brecht et al. (1). Based on the white-light interference effects occurring at thin transparent films, RIFS has shown high precision and high stability in monitoring the change of optical thickness attributable to interfacial molecular interaction and has been shown to be a useful approach for the detection of herbicides in river water and hydrocarbons in air (1,2).

At present, we are trying to find possible applications of RIFS in clinical diagnosis. ELISAs, the widely used label immunoassays in clinical laboratories, have inherent drawbacks, such as numerous pipetting and incubation steps. Additionally, the technique does not always show dose dependence in an extensive concentration range of analytes, exemplified by the so called high-dose hook effect (3). One of important factors that induces the high-dose hook effect is the introduction of labeled antibody, so it is expected that improved performance can be gained with RIFS technology, which is a label-free measurement.

We describe here a strategy based on RIFS and spin-coating, which can in principle eliminate several steps of the traditional ELISA. The molecule-substrate and intermolecule interactions can be recorded in real time with relative high sensitivity and reproducibility. The optical principle of RIFS measurement has been described in detail by Brecht et al. (4). The change of optical thickness caused by interfacial binding can be probed with a spectrometer, using white-light interference. In our apparatus, a CCD spectrometer (PC-1000; Ocean Optics Inc.) is used instead of a diode array spectrometer to decrease size. The optics use Y-shaped reflective optical fibers to illuminate and collect the reflected light of the transducer chip on a windowless Teflon cell (flow cell) with volume of $\sim 30 \mu\text{L}$. Self-developed software is used for the real-time acquisition of full spectra and online analysis. Solution sample handling was carried out using a peristaltic pump with a minimal flow rate of $100 \mu\text{L}/\text{min}$.

A schematic of the transducer is shown in Fig. 1A. A 10-nm Ta_2O_5 film was deposited on glass substrate to enhance the interference contrast (5). After the above treatment, polystyrene was spin-coated on the chip at 2000 rpm and dried in 60°C for 1 h. A transparent

polystyrene layer with thickness of $\sim 650 \text{ nm}$ was then generated and served as both the interference film and the hydrophobic surface for immobilization of antibodies.

In our experiments, yeast hepatitis B surface antigen (HBsAg) and its monoclonal antibodies (anti-HBs) were chosen as a model system. The adsorption of antibody occurred in self-designed Teflon cells mounted on the polystyrene-coated chips. Anti-HBs was dissolved in 50 mmol/L carbonate buffer, pH 9.6, at a concentration of 5 mg/L , and $200 \mu\text{L}$ of this solution was pipetted into each cell. After overnight incubation at 4°C , the chips were subsequently blocked for 1 h at 37°C with carbonate buffer supplemented with 10 g/L bovine serum albumin (BSA) and then washed five times with carbonate buffer before use.

With a "blank" chip, the coating and blocking process can be monitored by RIFS in real time at room temperature ($20 \pm 2^\circ\text{C}$). When the anti-HBs molecules were physically adsorbed onto a polystyrene film, an increase of $\sim 3.4 \text{ nm}$ in total optical thickness was detected. The following blocking process with BSA increased thickness only $\sim 0.3 \text{ nm}$ because of the small molecular weight of BSA and the dense distribution of antibody molecules previously adsorbed on the polystyrene surface.

Immunoreaction was conducted at room temperature. The chips were first rinsed with 50 mmol/L phosphate-buffered saline (pH 7.4) until a stable prerun baseline was recorded. The standard deviation of the baseline fluctuation was $\sim 17.8 \text{ pm}$. A series of antigen solutions ($0.1, 1, 10,$

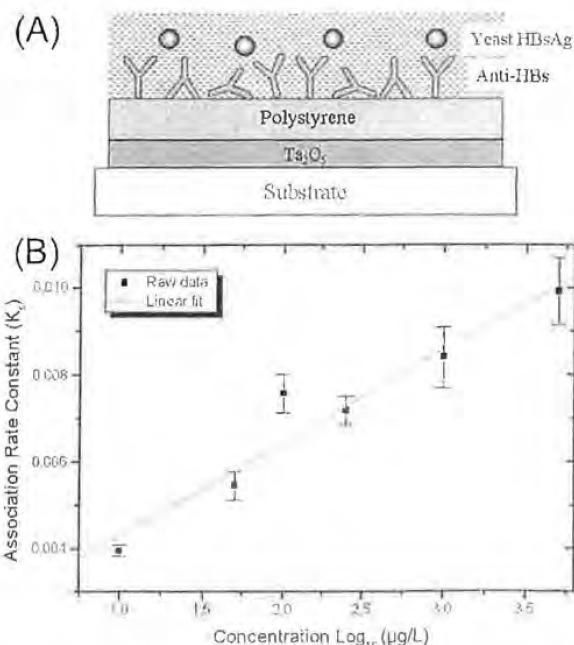


Fig. 1. Schematic of the RIFS transducer (A) and correlation between association rate constant (K_a) and HBsAg concentrations (B). Bars, SD.

100, 250, 1000, and 5000 $\mu\text{g/L}$ in phosphate-buffered saline with 500 mg/L BSA added) were then injected at 400 $\mu\text{L/min}$, and each binding curve was recorded. The curves were analyzed with the "CurveExpert" system (Ver. 1.34), using a pseudo-first-order exponential association model in an effort to determine each association rate (K_a) and hence the calibration curve. As negative control, BSA solution at a concentration of 1000 mg/L was injected and had only a negligible effect, indicating the specificity of the RIFS detection of immunoreaction. The RIFS signal was totally obscured by noise when the concentration of HBsAg sample was 1 $\mu\text{g/L}$.

The calibration curves extended into the $\mu\text{g/L}$ range (Fig. 1B). The interaction between HBsAg and immobilized Anti-HBs in BSA buffer provided a good signal at 10 $\mu\text{g/L}$ and a linear window range of 20–5000 $\mu\text{g/L}$ (Fig. 1B). By contrast, conventional ELISA has a lower linear range, usually 1–100 $\mu\text{g/L}$ for HBsAg quantitative analysis. However, the present RIFS is by no means optimized. It is expected that substantial improvement can be obtained by the use of a compensator to carefully control the system noise. Application of RIFS in the clinical detection of HBsAg is possible with the advantages of direct detection of analytes, shorter measurement time (a few minutes), no interference from the mobile phase, a favorable linear range, and reusability of the transducer.

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Purification of Prostate-specific Antigen from Serum by Indirect Immunosorption and Elution with a Hapten, Wolfgang Hoesel,^{1*} Jochen Peter,^{2†} Helmut Lenz,¹ and Carlo Untch,^{2‡} ¹Roche Diagnostics GmbH, Nonnenwaldstrasse 2, 82372 Penzberg, Germany; ²Technical University Munich, 85748 Garching, Germany; *author for correspondence: fax 49-8856-603341, e-mail wolfgang.hoesel@roche.com; †present address: National Institute of Environmental Health Sciences (NIH/NIEHS), Bldg. 101, Room F011, Research Triangle Park, NC 27709; ‡present address: Lehrstuhl für Bioorganische Chemie, Universität Bayreuth, Gebäude NW 1, 95440 Bayreuth, Germany)

Preparative purification of macromolecules, e.g., proteins, has reached a high standard because of efficient separa-

tion materials that are available for this task. However, this is limited to samples containing substantial amounts of the desired protein. When proteins that are present only in minute amounts (e.g., in the $\mu\text{g/L}$ range) must be isolated from a limited supply of complex biological material (e.g., human serum), a one-step method is recommended to obtain the protein in sufficient yield. When suitable antibodies are available, immunopurification often is the method of choice (1), but nonspecific binding of proteins to affinity matrices is a substantial problem because elution with acid or chaotropic agents very often washes off the impurities as well as the analyte (2). Thus, there is a need for methods that allow the specific elution of the desired proteins without eluting the impurities. Specific elution by competing with a low-molecular weight analog is appealing, but suitable analogs may not be available.

We wanted to isolate prostate-specific antigen (PSA) from serum and analyze its structure by mass spectrometry. The concentration of PSA in serum from patients with prostate cancer (PCa) ranges from ~ 3 to >3000 $\mu\text{g/L}$, $\sim 10^5$ – 10^7 times less than that of other serum proteins, e.g., albumin. We have developed a general indirect immunosorption method that follows to a certain extent an immunoassay principle developed by Hashida et al. (3) and Ishikawa et al. (4) and makes use of a digoxigenylated anti-analyte antibody. Initially, the PSA from the biological sample is bound to magnetic beads by an array of antibodies. The key step is the competitive release of the PSA-antibody pair by digoxigenin-lysine under neutral conditions. These conditions leave the impurities almost entirely bound to the matrix and yield the antibody-PSA complex in a pure form (5).

When we began isolating PSA from serum, we first tried a standard immunosorption method using magnetic beads combined with acidic elution. To this end, streptavidin-coated magnetic beads were loaded with a biotinylated anti-PSA monoclonal antibody (Mab) and incubated with serum from a PCa patient. After magnetic collection of the beads and several washing steps, the bound protein was released by a small amount of a mixture of either formic acid-water-acetonitrile (1:3:2, by volume) or 1 mol/L propionic acid, and the eluted material was analyzed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). It was found that mainly nonspecifically bound serum proteins were liberated from the beads (see lanes A1–A4 in Fig. 1). The high degree of impurities did not allow the unambiguous detection of free PSA and the PSA/ α_1 -antichymotrypsin (ACT) complex. This applied especially to the elution with formic acid-water-acetonitrile, a solvent used for preparing the matrix solution for MALDI-TOF MS and which would therefore be very suitable for that type of analysis. But when the elution mixture with this solvent was applied to the MALDI-TOF MS, almost all of the proteins detected in the mass spectrum were impurities. Free PSA was barely detectable, and the PSA/ACT complex could