Immunooassay for Determining Low- and High-M\(_2\) Antigens with a Dry Multilayer Film, Yoshihiro Ashihara, Yoshikage Hirooka, Yoshikiko Makino, Hiroshi Shinoki, Naofumi Hora, Yuko Sudo, Masashi Ogawa, Tetsuji Tanimoto, Tadashi Ninomiya, Isao Nishizono, and Yasushi Kasahara (1 Fujiirebio Inc., Research Labs., 51 Komiya-cho Hachioji-shi, Tokyo 192, 2 Fuji Photo Film Co., Ltd., Research Labs., Asaka-shi, Saitamaken 351, Japan)

Dry chemistry assays have been applied to detection of serum glucose, cholesterol, and creatinine for many clinical diagnoses because of rapid and simple methodology without the use of solution chemistry. This technology is accepted for biochemical tests but not for immunooassays, because the latter require a separation between bound and free labels with a washing procedure. Because the washing procedure is eliminated in enzyme immunooassay, homogeneous enzyme immunooassay is well suited for dry chemistry, and makes it possible to apply the dry chemistry system to immunooassay.

We have developed a simple, dry immunooassay for determination of a hapten and a macromolecular antigen—*theophylline* and C-reactive protein (CRP), respectively—by a homogeneous enzyme immunooassay (1, 2). Specific antibody linked with \(\alpha\)-amylase (EC 3.2.1.1) is used as the labeling conjugate and insoluble starch is the substrate. Because \(\alpha\)-amylase in serum may interfere with this method, we include a specific inhibitor to human \(\alpha\)-amylase, termed AIC, isolated from bacteria. The AIC does not inhibit \(\alpha\)-amylase of the conjugate because the conjugate enzyme is derived from bacteria.

In this method for measurement of high-molecular-mass (HMM) antigen in a sample, because of the bulky structure of the complex between conjugate and HMM antigen, the insoluble starch cannot be digested efficiently by the enzyme of the conjugate, which is complexed with antigen immunologically in excess of HMM antigen. The activity of the enzyme is inversely proportional to amounts of HMM antigen in sample. On the other hand, for detection of low-molecular-mass (LMM) antigen in a sample, we used a competitive assay, and prepared polymerized antigen as a competitive antigen included in the slide, which is able to behave like HMM antigen in the absence of LMM antigen in the sample as mentioned above. In excess of LMM antigen, enzyme conjugate complexed with LMM antigen still has enzyme activity. For LMM antigen, enzyme activity is directly related to antigen concentration.

As indicated schematically in Figure 1, the dry slide for the detection of HMM antigen has three major layers: developing zone for immunological and enzymatic reactions, a barrier zone, and a color-developing zone. The 2.8 × 2.4 cm slide is a molded plastic case. The antibody conjugated with amylase and insoluble starch is contained in the developing zone, and when a diluted sample is applied to the slide, reagents are reconstituted and HMM antigen can complex with the conjugate. The free conjugate from the HMM antigen can react with the substrate in the developing zone, from which the digested product from starch moves to the color-developing zone through the barrier zone and produces a green signal by reaction with glucose oxidase/horseradish peroxidase.

The serum sample is diluted 20- to 220-fold with diluent buffer containing the amylase inhibitor. In the test procedure, 10 \(\mu\)L of serum is pipetted onto the slide, while in the Fuji Dri-Chem 5500 analyzer (Fuji). During incubation at 37 °C, the difference of reflected absorbance \((\Delta A)\) at 4 and 6 min is measured with a reflectance photometer during the color developing. The result is printed out automatically after the data have been processed by the analyzer.

We examined the time course of the color development to increase the assay sensitivity. Although the \(A_s\) of CRP at 0 mg/L slightly increased after incubation for 5 min, \(A_s\) at 20 mg/L increased substantially. The greater sensitivity was obtained by reading the \(A_s\) at 4 and 6 min and determining the difference between the two. The dynamic range of this

![Fig. 1. Schematic model of the dry slide and whole reaction for CRP assay in each zone](image)
assay system for CRP is 5–100 mg/L and for theophylline is 1–50 mg/L.

We compared this method for CRP with the Hoechst turbidimetric method and compared the method for theophylline with the EMT assay (Syva Co., Palo Alto, CA). The correlation of CRP between the present method (y) and the turbidimetric method (x) is \( y = 1.0045x - 0.399 \text{ mg/mL} \) \((r = 0.985, n = 31)\), with no discrepant results. Correlation of theophylline concentration between our method (y) and EMT (x) was also excellent: \( y = 0.979x + 0.237 \text{ mg/mL} \) \((r = 0.987, n = 25)\).

We also evaluated interference by hemoglobin, glucose, α-amylase, and bilirubin in the assay for CRP. No interference was observed by hemoglobin and bilirubin. Even in serum with high glucose (2 g/L), CRP exhibited the same results as in serum with no glucose. Furthermore, no interference from α-amylase in serum was observed up to 1000 kU/L, because the AIC included in the test slide inhibits strongly amylase derived not only from the pancreas but also from saliva.

The present dry chemistry assay is sensitive and rapid for both HMM protein and LMM drugs without any tedious procedures or complicated instrumentation. In addition, this method should be useful for routine assays in doctors' offices and for bedside tests.

References


Several years ago, we developed a direct chemiluminescent substrate, disodium 3-(4-methoxySpiro1,2-dioxetane-3,2-dioxacyclo-[3.3.1.1^6,7]-decan)-4-yl)phenyl phosphate (AMPPD; CAS no. 124951-96-8), for the enzyme alkaline phosphatase (EC 3.1.3.1) (1, 2). This substrate has now been widely used in ligand binding assays as a very sensitive, nonradioactive detection system (3, 4). AMPPD is a stable 1,2-dioxetane with a half-life of one year in aqueous solution at room temperature.

Dephosphorylation of AMPPD generates the metastable phenolate anion (AMPD), which chemiluminesces upon decomposition. AMPD, as a substrate for alkaline phosphatase, is superior to the colorimetric, bioluminescent, and fluorometric substrates. Its advantages have been demonstrated in the detection of proteins and nucleic acids in solution and on membrane supports (3–5).

We have observed that aqueous buffered solutions of AMPPD in the presence of alkaline phosphatase and its conjugates, with and without polymeric enhancers, exhibit a relatively long delay before reaching steady-state emission. AMPPD also generates nonenzymatically activated background chemiluminescence. The background or “noise” can be attributed to the emission from both 2-adamantanone and methyl m-oxybenzoate anion. The background signals and the long delay leading to constant light emission are due to the amphiphilic nature of AMPPD, which leads to the aggregation of AMPPD and its dephosphorylated anion.

To eliminate the problems described above, we designed a new class of dioxetane substrates for alkaline phosphatase. The new dioxetanes contain a modified hydrophobic adamantyl group to prevent aggregation (7). We have found that derivatization of the adamantyl portion of the dioxetane results in more rapid decomposition (<50% shorter half-lives for the Cl- and Br-substituted dioxetanes) of the intermediate dioxetane anion produced by alkaline phosphatase-catalyzed dephosphorylation (7). Because of the shorter half-lives, steady-state chemiluminescence is attained more rapidly. Furthermore, this molecular modification strongly affects the thermal or nonenzymatic background signal of the dioxetane. The background chemiluminescence observed with the Cl- and Br-substituted dioxetane was 50% lower than that of AMPPD.

Previously, we demonstrated the superior performance of AMPPD over the colorimetric substrate p-nitrophenyl phosphate as a substrate of alkaline phosphatase in a modified highly sensitive assay of thyrotropin (3). Here we compare Cl- and Br-substituted adamantyl dioxetane phosphates with AMPPD as substrates for alkaline phosphatase-labeled binders in the same assay. The new generation of 5-substituted adamantyl dioxetane phosphates exhibits improved performance in a wide range of applications as substrates for alkaline phosphatase conjugates. Constant light emission is reached more rapidly, and nonspecific backgrounds are lower than that achieved with the parent AMPPD.

AMPPD, CI-AMPPD (CSPD”), and the luminescence enhancers Emerald™ and Sapphire II™ are available from Tropix, Inc. (Bedford, MA 01730). Diethanolamine was from Aldrich (Milwaukee, WI 53233). Milli-Q “HPLC-grade” water (Millipore, Bedford, MA 01730) was used in the preparation of all buffers. The Tandem-E High Sensitivity TSH assays were purchased from Hybritech (San Diego, CA 92126). In addition to the standards supplied in the kit, we prepared 0.005 and 0.05 milli-int. unit/L thyrotropin samples by diluting the 0.5 milli-int. unit/L standard with zero control solution. The thyrotropin assays were performed according to the protocol supplied with the kits, up to the detection step. Before adding the chemiluminescent substrate, we washed the beads once with substrate buffer (0.1 mol of diethanolamine and 1 mmol of MgCl₂ per liter, pH 10.0). Substrate buffer (0.2 mL, with or without Sapphire II or Emerald) was then added to each tube to keep the beads from drying. We next added 0.3 mL of dioxetane (0.67 mmol/L, with or without Sapphire II or Emerald) to each tube. Serum samples previously analyzed for thyrotropin with a CoTube™ assay (Bio-Rad Labs., Hercules, CA 94547) were used for the intermethod comparison experiments. A Model LB 952T luminometer (Berthold, Nashua, NH 30363) was used to inject substrate solution and measure the chemiluminescence intensity (1-s integrals at 5.5, 20, 40, and 60 min).

The kinetics of light emission for the standards used in the Hybritech Tandem-E High Sensitivity TSH assay indicated a much faster increase to maximum light emission with CI-AMPPD in Sapphire II than with AMPPD in Sapphire II (data not shown; available upon request). A comparison of the standard curves obtained for an enzyme immunoas-