JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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A Homogeneous Chemiluminescent Immunoassay Method

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Supporting Information

ABSTRACT: A new homogeneous chemiluminescent immunoassay method featuring the use of specific binding members separately labeled with an acridan-based chemiluminescent compound and a peroxidase is reported. Formation of an immunocomplex brings the chemiluminescent compound and the peroxidase into close proximity. Without any separation steps, a chemiluminescent signal is generated upon addition of a trigger solution, and the intensity is directly correlated to the quantity of the analyte.

A huge effort has been expended in the field of assay development, particularly immunoassay development, to simplify the assay process while preserving the essential benefits in sensitivity, robustness, broad applicability, and suitability to automation. One approach has been to devise so-called homogeneous assay formats where no separation of a detectably labeled specific binding member is needed.[1] This type of methodology relies on devising a detection principle that is modulated and either turned on or turned off as a result of the binding reaction. In contrast, heterogeneous assay formats rely on physical separation of bound and free detectably labeled specific binding members before quantification. Such physical separation, which consists of extensive washing steps, is not only laborious and time-consuming but also makes the performance of an assay more complex and susceptible to cumulative error. A large number of current homogeneous immunoassays are based on detection of fluorescence, using fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), or time-resolved FRET (TR-FRET).[2,3] These assays usually require proprietary or specialty fluorescent labels and in many cases tend to have background issues due to autofluorescence of biological samples and/or interfering compounds.[4] Compared with fluorescence detection, chemiluminescence offers enhancements of 10^1−10^2 in sensitivity and a broader dynamic range.[5] Several chemiluminescence-based homogeneous immunoassay technologies have recently emerged. These include singlet oxygen-induced luminescence proximity assays[6] and electrochemiluminescence assays based on the redox reaction of ruthenium ions proximal to an electrode.[7] Another technology utilizes enzyme fragment complementation of β-galactosidase to generate a chemiluminescence signal when a binding event occurs.[8] It should be noted here that in the field of immunoassays, the term "homogeneous" just means that no separation step is needed; it does not have the same definition as in physical chemistry, since some homogeneous assay methods, for instance Ru-based electrochemiluminescence assays, involve the use of a solid phase. While each of these technologies has its own uniqueness,[9] the quest for new homogeneous immunoassay technologies continues as both the clinical diagnostic and life science research communities constantly look for assay technologies that are sensitive, robust, and easy to implement.

We recently reported a new class of robust chemiluminescent compounds for peroxidase detection.[10] These acridan-based compounds, in formulations containing a phenolic enhancer, generate instantaneous chemiluminescence of superior intensity when in contact with a peroxidase (Figure 1). Such formulated solutions have found immediate applications as substrate reagents for ultrasensitive detection of horseradish peroxidase (HRP). Recently their superb chemiluminescence intensity as well as extraordinary stability prompted us to explore the use of

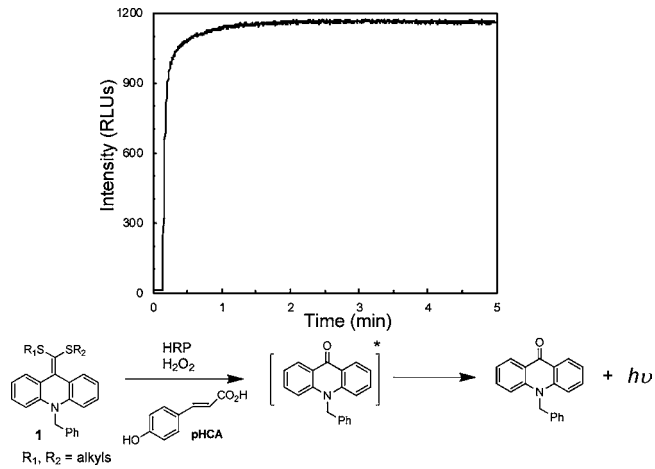

Figure 1. New acridan-based chemiluminescent compounds.

Received: December 10, 2012
Published: March 11, 2013

   dx.doi.org/10.1021/ja312039k | J. Am. Chem. Soc. 2013, 135, 4191−4194

these compounds as chemiluminescent labels. We report here our recent study leading to the development of a nonseparation immunoassay method featuring the use of such a chemiluminescent label and an HRP-conjugated specific binding member for inducing a chemiluminescent reaction.

The initial idea was that in an immunoassay using binding members separately labeled with compound **1** and HRP, the immunological binding event would bring **1** and HRP into close proximity, and as a result, the effective concentration of HRP relative to **1** in the microenvironment of each immunocomplex would be much higher than in the rest of the bulk solution, where no binding event would take place. We reasoned that at given concentrations of both **1** and HRP during an immunoassay, the formation of a greater number of immunocomplexes would produce a more intense chemiluminescence signal, even without separation of unbound binding partners. This would be especially true if the measurements were to be taken during the initial stage of the chemiluminescent reaction, since the chemiluminescence generation is exceptionally rapid (Figure 1) and, as observed in our previous experiments, when the concentration of HRP increased significantly with a given amount of **1**, the chemiluminescence from the system started to change from a glow profile to a flash profile because of fast depletion of **1**.

For proof-of-concept experiments, amino-reactive labeling compounds **2** with the structures shown in Figure 2 were

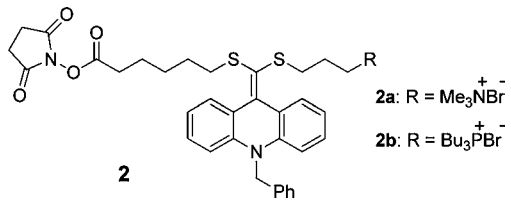

Figure 2. Acridan-labeled chemiluminescent compounds **2**.

synthesized and used to label binding members. First, a microtiter-plate-based mouse immunoglobulin G (IgG) assay was examined as a quick test. In this case, a white microtiter plate was coated with **2**-labeled sheep anti-mouse IgG antibody. To the plate were added mouse IgG standard solution and goat anti-mouse IgG F(ab)2-HRP. Without any washing steps to remove unbound HRP-conjugated antibodies, a trigger solution containing hydrogen peroxide and *p*-hydroxycinnamic acid (pHCA) was added to generate the chemiluminescence signal. This signal was measured for 5 s upon addition of the trigger solution and showed clear dose–response behavior as the concentration of mouse IgG changed. It was also interesting to note that in a similar experiment where the plate was coated with plain unconjugated anti-mouse IgG antibody and then blocked with **2**-labeled bovine serum albumin (BSA) (instead of native BSA as in a normal blocking procedure), clear dose–response behavior was once again observed. Here the chemiluminescent compound **2** was not directly conjugated to the antibody but sat in close vicinity. This indicated flexibility in terms of how the labeling could be done.

Encouraged by these initial experiments, we set out to examine various assay formats in more detail, including microparticle-based assays. In such cases, the microparticle carried not only a capture antibody (as in a normal washed assay) but also compound **2**. With a protocol that required no washing step as described above, calibration curves were easily established in all of the cases we investigated. More details can be found in a recently published patent.[11]

As our investigation continued, the new no-wash assay method enjoyed success in the case where a capture antibody and compound **2** were both immobilized on a solid phase, either a microtiter plate well or a microparticle. However, in the cases where **2**-labeled antibody was used directly in conjunction with an HRP-conjugated complementary antibody or antigen in homogeneous assays without any solid phase involved, we saw less impressive signal-to-noise ratios because of high background due to the chemiluminescence observed when no target analyte was present. This remained a challenge until recently, when another aspect of this no-wash assay method was discovered.

We recently noted that the performance of no-wash assays involving the use of solid phases could be further improved by addition of certain compounds. Among the most effective were ascorbic acid, 2-aminophenol, hydroxylamines, and certain other compounds with antioxidant properties. Shown in Table 1 are the results of a no-wash prostate-specific antigen (PSA)

Table 1. Effect of Selective Signal Inhibiting Agents

		no SSIA		Et_2NOH added		
signal	PSA (ng/mL)	RLU[a]	S/S_0	RLU[a]	S/S_0	signal reduction
S_0	0	15368	–	6694	–	56%
S_1	0.46	680002	44	446120	67	34%
S_5	129	54298678	3533	43144054	6445	20%

[a]Signal intensity measured in relative luminescence units (RLU).

assay using silica microparticles immobilized with both a monoclonal anti-PSA antibody and compound **2a**. Significant improvements in S/S_0 were observed when Et_2NOH was added before signal generation with a trigger solution. While these so-called selective signal inhibiting agents (SSIAs) caused signal suppression at all PSA levels, they induced a much more significant reduction in the background chemiluminescence.

More profoundly, when incorporated into our homogeneous assays using directly labeled antibodies without any solid phase, which had previously given less satisfactory results, SSIAs made such a drastic difference that these assays started to work and displayed similar or better performance than the corresponding washed assays. This is considered a significant advancement because it makes the assay truly homogeneous, a feature desired for faster immunoreaction kinetics. More importantly, in comparison with current homogeneous assay technologies, this new homogeneous immunoassay method has the advantage that the use of directly labeled antibodies in conjunction with readily available and widely used HRP conjugates makes it an easy task to convert any existing heterogeneous immunoassay to a homogeneous chemiluminescent assay (Figure 3).

Figure 4 shows the results of such a homogeneous chemiluminescent assay for IL-8, a widely studied cytokine. Herein, a capture antibody (mouse monoclonal anti-human IL-8) was labeled with compound **2a** following standard procedures. A solution containing this **2a**-labeled capture antibody and a commercially available HRP-conjugated detection antibody was incubated with the sample solution in a microtiter plate well. Without any further steps after the incubation, a solution of ascorbic acid was added. Luminescence was then generated by injection of the trigger solution

dx.doi.org/10.1021/ja312039k | J. Am. Chem. Soc. 2013, 135, 4191–4194

Figure 3. A new homogeneous chemiluminescent immunoassay method.

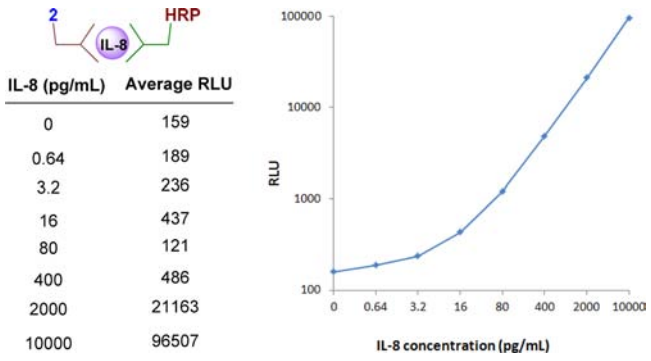

Figure 4. Results of a homogeneous sandwich immunoassay for IL-8. Assay conditions: 96-well plate; 20 μL of **2a**-labeled mouse anti-human IL-8 antibody (2.0 μg/mL), 20 μL of HRP-conjugated mouse anti-human IL-8 antibody (1:500 dilution from a commercial stock solution), and 30 μL of sample solution; 1 h incubation at room temperature; addition of 10 μL of ascorbic acid solution (11 mM) before injection of 100 μL of trigger solution and 2 s read.

and measured on a luminometer. While the simple protocol consisted of just a one-step incubation followed by injection and reading, the assay exhibited excellent sensitivity with a limit of detection as low as 0.64 pg/mL, which is comparable to or better than those of current washed enzyme-linked immunosorbent assay (ELISA) methods.[12]

Another surprising yet attractive feature of the homogeneous assay method is that with SSIAs, one can use an unpurified antibody directly from the labeling reaction mixture without a desalting step to remove unconjugated compound **2**. This further simplifies the process of assay development. Additionally, conditions to reduce the assay volume without compromising the assay performance can be easily worked out, a benefit when considering reduced reagent consumption and miniaturization. An IL-8 assay using an unpurified **2a**-labeled antibody and reduced reagent/sample volumes gave performance similar to that described above.

Our homogeneous immunoassay method works equally well with competitive assays. Figure 5 shows the results of an assay for cyclic adenosine monophosphate (cAMP) configured using this method. In this assay, cAMP–HRP competed with cAMP present in the sample to bind to an anti-cAMP antibody labeled with compound **2b**. The quantity of immunocomplexes containing both compound **2b** and HRP was inversely correlated to the quantity of cAMP present in the test sample. Once again, the assay protocol was simple and straightforward, consisting of a 30 min incubation followed by trigger injection and reading on a luminometer. Under the conditions indicated in Figure 5 without optimization, the assay yielded an IC_{50}

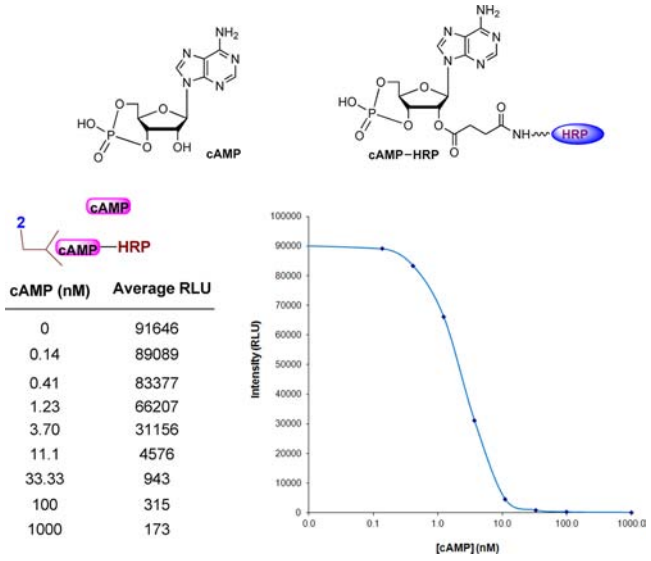

Figure 5. Results of a homogeneous competitive assay for cAMP. Assay conditions: 384-well plate; 3 μL of **2b**-labeled rat anti-cAMP antibody (0.275 μg/mL, diluted directly from the labeling reaction mixture), 3 μL of HRP-cAMP (0.094 μg/mL), and 4 μL of sample solution; 30 min incubation at room temperature; addition of 1 μL of 2-aminophenol solution (0.69 mM) before injection of 10 μL of trigger solution and 1 s read.

value of 2.6 nM, equivalent to 10.4 fmol/well, a sensitivity in line with or better than those of current methods.[4]

To evaluate the practical utility of this assay technology, a method comparison experiment was performed with PSA against the Beckman Coulter Hybritech PSA assay on a modified DxI using 69 previously frozen patient samples (normal and positive). The homogeneous PSA assay with a 4.5 min incubation time achieved a calculated (2 standard deviations from S_0) analytical sensitivity of 0.0065 ng/mL, similar to that of the Hybritech PSA assay, which includes washing steps in the protocol. The correlation coefficient with respect to the Hybritech PSA assay was $R = 0.979$ with a slope of 1.021. Figure 6 shows the plot for the clinically significant range.

We performed some studies to investigate the chemical mechanism of chemiluminescence signal generation during our assay. Multiple intermediates were identified, indicating the presence of more than one reactive species during the process

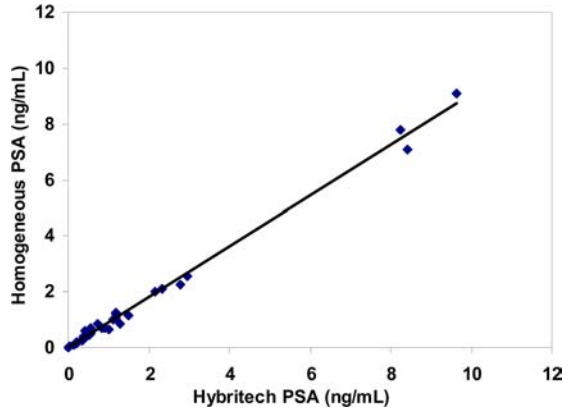

Figure 6. Correlation between the homogeneous and Hydritech PSA assays.

dx.doi.org/10.1021/ja312039k | J. Am. Chem. Soc. 2013, 135, 4191–4194

after addition of the trigger solution. These results will be published separately in due course.

In summary, we have developed a novel homogeneous chemiluminescent immunoassay method using specific binding members labeled separately with a chemiluminescent acridan-based compound and HRP. This new assay methodology provides a truly homogeneous nonseparation assay technology with wide applicability, realizing a long-sought goal in the field. It does not involve complicated reaction schemes or special constructs. Compared with current homogeneous assay technologies, the new method is further characterized by its simplicity in terms of assay format and great ease in implementing new assays or converting existing heterogeneous assays.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods and details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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