Immunosensors

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Biocatalytic Evolution of a Biocatalyst Marker: Towards the Ultrasensitive Detection of Immunocomplexes and DNA Analysis**

*Bella Shlyahovsky, Valeri Pavlov, Lubov Kaganovsky, and Itamar Willner**

The amplification of biorecognition events is a fundamental topic in bioanalytical chemistry. The use of enzyme labels as catalysts that generate numerous readable product molecules as the result of a single recognition event is the basis of the enzyme-labeled immunosorbant assay (ELISA) technology.[1] Different enzyme-linked configurations of biosensors have been developed, in which color,[2] fluorescence,[3] or electroactive products[4] are used as readout signals. Also, enzyme labels have been used to catalyze the precipitation of insoluble products on electrode supports, and the resulting interfacial electron-transfer resistance,[5] or the microgravimetric changes on piezoelectric quartz-crystal microbalance crystals,[6] are used as readout signals of the precipitation processes. Different biosensing schemes that include enzyme labels as amplifying agents have been developed for different biorecognition events such as DNA hybridization,[7] antigen–antibody complexes,[8]and others.[9] Other amplifying labels that have been developed in recent years include nanoparticles,[10] beads that act as containers for redox-active units,[11] and liposomes.[12,13]

Herein, we describe a new concept to amplify biorecognition events based on the catalytic evolution of a biocatalytic label that leads to a readable signal of the biosensing process. Specifically, we applied ecarin (EC) conjugates that catalyze the transformation of prothrombin (PTh) to thrombin (Th).[14] The latter product acts as a biocatalyst for the hydrolysis of the nonfluorescent moiety bis(*p*-tosyl-Gly-Pro-Arg)rhodamine110 (**1**) to the fluorescent product **2** (Figure 1 A). That is, the surface concentration of the catalytic EC conjugate is low due to the low coverage by the recognition sites of the analyte units. The EC-mediated conversion of PTh into Th evolves the catalyst for the hydrolysis of **1** and leads to the formation of numerous fluorescent molecules of **2**. We applied this amplification method for the detection of antigen–antibody and DNA recognition complexes and demonstrate the use of the analytical procedure for the detection of telomerase in cancer cells.

[*] B. Shlyahovsky, Dr. V. Pavlov, L. Kaganovsky, Prof. I. Willner
Institute of Chemistry
Farkas Center for Light-Induced Processes
The Hebrew University of Jerusalem
Jerusalem 91904 (Israel)
Fax: (+972) 2-652-7715
E-mail: willnea@vms.huji.ac.il

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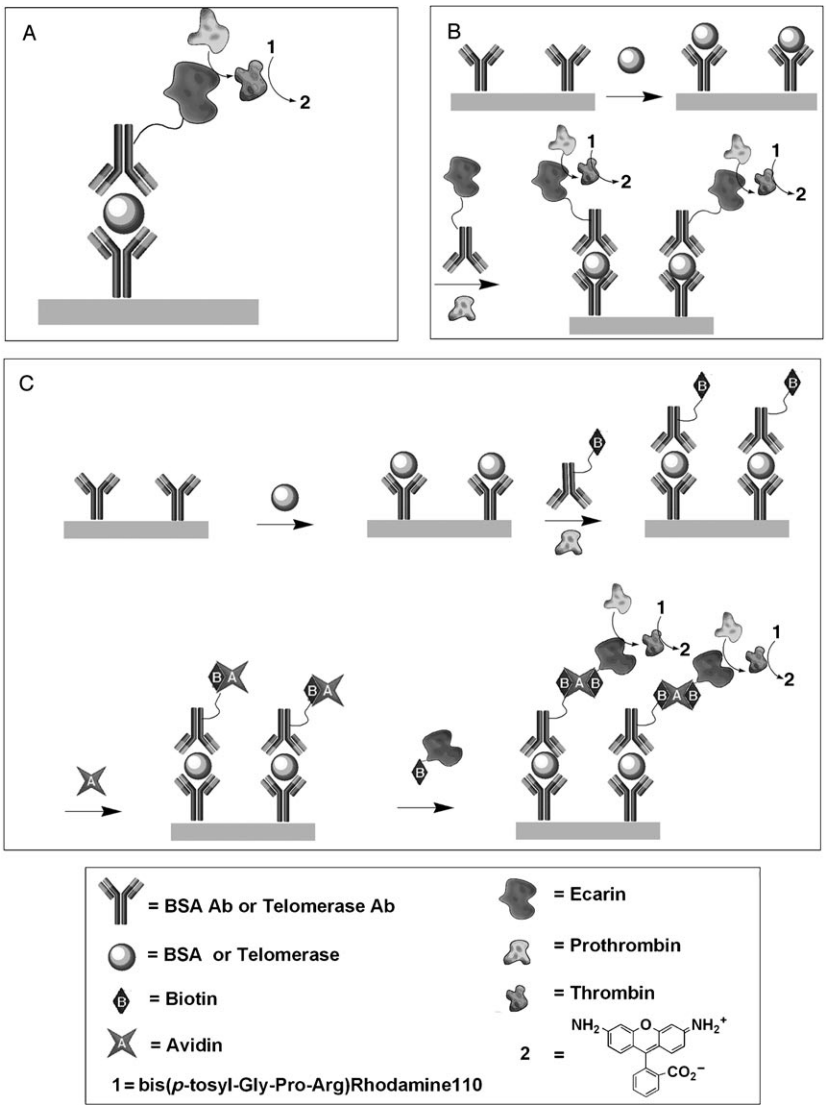

Figure 1. Amplified analysis of immunocomplexes by the ecarin-catalyzed evolution of the thrombin biocatalyst. A) Concept for ecarin-stimulated evolution of thrombin and the subsequent hydrolysis of nonfluorescent bis(*p*-tosyl-Gly-Pro-Arg)-R110 (**1**) to the fluorescent product **2** upon analysis of an antigen–antibody complex. B) Analysis of BSA. C) Analysis of telomerase originating from HeLa cancer cells.

Anti-bovine serum albumin (polyclonal BSA Ab) was immobilized on ELISA plates (Figure 1B). The resulting surfaces were blocked to minimize nonspecific adsorption and then interacted with different concentrations of bovine serum albumin (BSA). The rinsed surfaces were then treated with ecarin-labeled BSA Ab, then with PTh, and last with bis(*p*-tosyl-Gly-Pro-Arg)-R110 to yield the fluorescent product **2**.[15] Figure 2A shows the integrated fluorescence intensities generated in the solution of a single well upon analyzing different concentrations of the BSA antigen. The antibody was detected with a sensitivity limit that corresponds to 2×10^{-18} M, with a signal-to-background ratio of about 1.8, with respect to the background fluorescence originating from nonspecific adsorption. Figure 2B depicts the extracted calibration curve.

This amplification procedure was applied to detect telomerase, a ribonucleoprotein that catalyzes the elongation of telomer units on chromosomes. The shortening of telomers during the cell life cycle provides an intracellular trigger to terminate cell growth and proliferation.[16,17] The appearance of telomerase in malignant or cancer cells leads to the constant elongation of the telomers and to their transformation into immortal cells. Indeed, in over 95% of the different cancerous cells elevated amounts of telomerase were detected, thus providing a general marker for cancer cells.[18,19] The detection of telomerase is usually based on the analysis of the enzyme activity that originates from cell extracts. The TRAP test is based on the labeling of the resulting telomers with fluorescent dyes and their electrophoretic analysis.[20] Other methods to detect telomerase activity include electrochemiluminescent detection,[21] fluorescent analysis of the telomerase-generated telomers on semiconductor nanoparticles,[22] or optical detection of the telomerase-synthesized telomers.[23]

In contrast to previously reported procedures for the analysis of telomers, the telomerase itself was detected in the present study. The advantages are obvious, as we do not need to rely on the time-consuming telomerization process, the labeling of the telomers, or the secondary separation of the

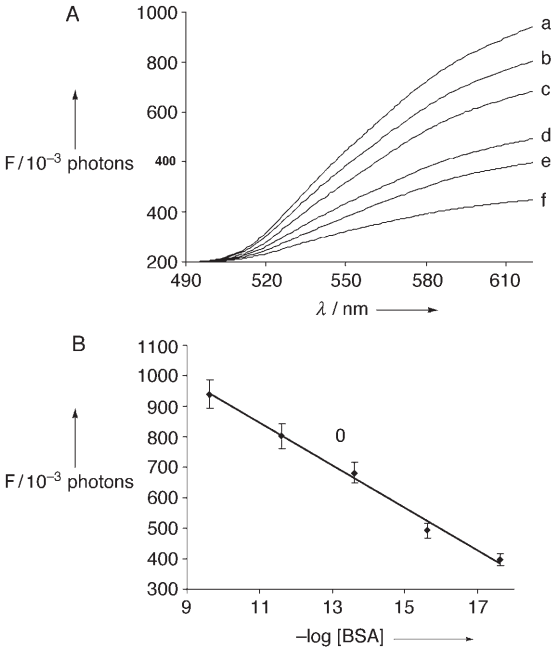

Figure 2. A) Integrated light intensities emitted upon the analysis of different concentrations of BSA according to Figure 1B: a) 2.43×10^{-10} M, b) 2.43×10^{-12} M, c) 2.43×10^{-14} M, d) 2.43×10^{-16} M, and e) 2.43×10^{-18} M; f) curve shows a control experiment in which the entire analytical protocol is applied in the absence of BSA (error bars were derived from five independent experiments). B) Calibration curve corresponding to the emitted light intensities at $\lambda = 620$ nm upon analyzing different concentrations of BSA.

telomers. Figure 1C outlines the method for detecting telomerase. The polyclonal rabbit anti-telomerase antibody was immobilized on the ELISA plate and treated with telomerase that originated from different numbers of HeLa cancer cells. The association of the telomerase was followed by the binding of the biotinylated polyclonal anti-telomerase antibody. Subsequently, the association of avidin and biotinylated ecarin resulted in the biocatalytic structure for the evolution of the thrombin biocatalyst. The latter product biocatalyzes the hydrolysis of **1** to **2**. Figure 3A shows the integrated light intensity emitted upon analyzing the telomerase originating from extracts of different number of HeLa cells. Telomerase originating from 1000 cells can be analyzed.

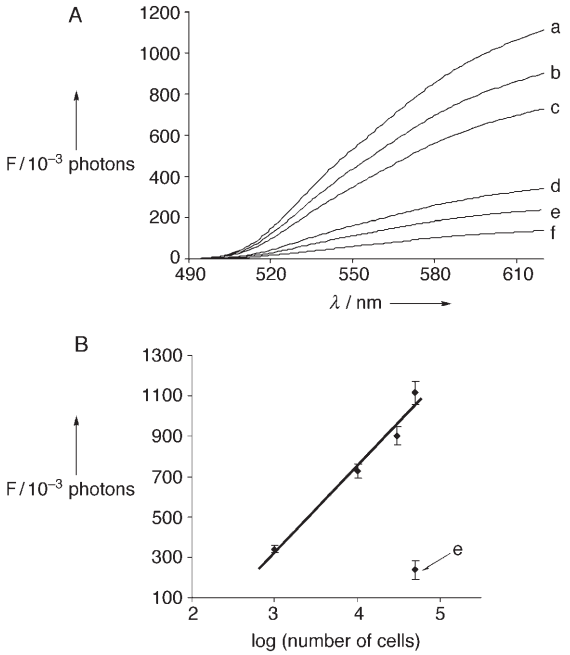

Figure 3. A) Integrated light intensities emitted upon the analysis of different numbers of HeLa cancer cells (or control system consisting of normal cells) according to Figure 1C: Curves a–d correspond to the analysis of 50000, 30000, 10000, and 1000 HeLa cells, respectively. Curve e corresponds to the analysis of 50000 HaCat normal cells. Curve f corresponds to a control experiment in which no HeLa cell extract (or telomerase) is added to the system. B) Calibration curve corresponding to the light intensity emitted by the system at $\lambda = 620$ nm upon the analysis of telomerase originating from different numbers of HeLa cancer cells. For comparison, the point marked (e) corresponds to the normal HaCat cells.

Figure 3A, curve e, depicts the application of the analyzing protocol on an extract of the cells that does not include telomerase (HaCat, normal skin). The resulting light intensity may be considered as the background level, as a result of nonspecific adsorption of the biocatalytic labels onto the sensing interface. Thus, the telomerase originating from 1000 HeLa cells is detected with a signal-to-background ratio of 2. Figure 3B shows the derived calibration curve. Note that we failed to analyze telomerase activity originating from 1000 HeLa cells by using the commercial TRAP assay.

The amplification of the biorecognition events by the ecarin/prothrombin system was also applied to analyze DNA (Figure 4). The thiolated nucleic acid **4** was assembled on an Au surface and hybridized with the analyzed DNA **3**. The

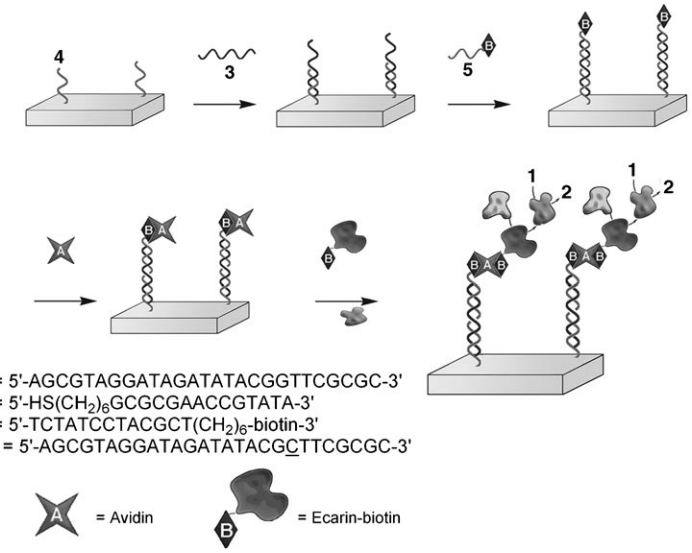

Figure 4. Amplified analysis of DNA by the ecarin-catalyzed evolution of the thrombin biocatalyst and generation of the fluorescent product **2**.

surface coverage of **4** was determined by Tarlov's method[24] and corresponded to approximately 5×10^{11} DNA strands per cm^2. The subsequent hybridization of the biotinylated nucleic acid **5**, followed by the association of avidin and biotinylated ecarin, led to the formation of the catalytic label on the gold surface. Figure 5A shows the integrated light intensity emitted by the system upon analyzing different concentrations of DNA. For comparison, curve e in Figure 5A shows the integrated light emitted by the system upon analysis of the nucleic acid **3a**, which includes one base-mismatch relative to **3**. The results indicate that the analyzed DNA **3** can be detected by the method with a sensitivity limit corresponding to 3×10^{-12} M. We also note that the DNA analyzed at a concentration of 3.4×10^{-8} M (Figure 5A, curve a) yields an integrated light intensity that is about threefold higher than the light intensity emitted by the system in which the DNA with one base-mismatch was analyzed at a concentration of 1×10^{-8} M (Figure 5A, curve e). These results imply that the analysis of DNA is selective and that the analyte DNA can be detected with a sensitivity limit of 3×10^{-12} (signal/background > 1.5). Figure 5B shows the derived calibration curve.

To conclude, the present study has introduced the catalytic evolution of a biocatalyst as a new concept to amplify biorecognition events. We employed ecarin and prothrombin as a catalytic evolution system for the generation of thrombin. The advantage of the reported system rests on the fact that the content of the enzyme (thrombin) increases in the system with time, and thus a nonlinear enhancement in the intensities of the evolved fluorescence is observed. Accordingly, by prolonging the time intervals for evolution of the thrombin the sensitivity limits might be further improved. It is difficult to perform a fair comparison of the proposed method with

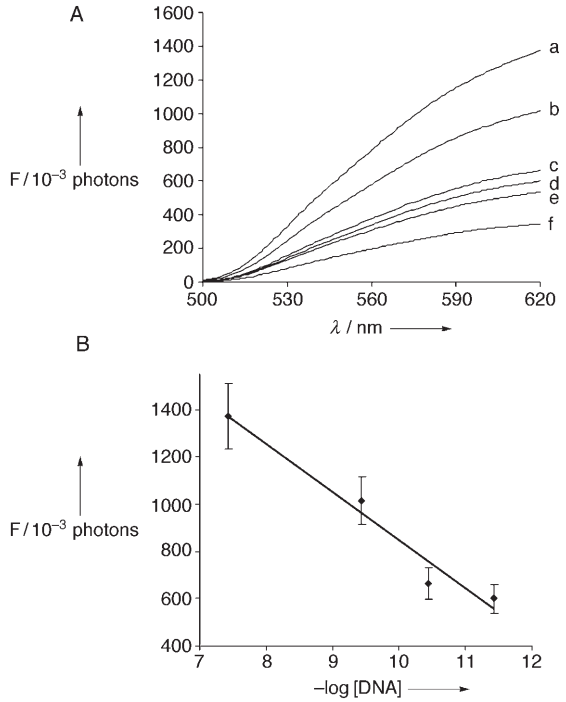

Figure 5. A) Integrated light intensities emitted by systems analyzing different concentrations of DNA **3** according to Figure 4. The concentrations of **3** correspond to a) 3.4×10^{-8} M, b) 3.4×10^{-10} M, c) 3.4×10^{-11} M, d) 3.4×10^{-12} M, and f) 0 M; curve e corresponds to the analysis of DNA **3a** with one base mismatch at a concentration of $1\xi10^{-8}$ M. B) Calibration curve corresponding to the emitted light intensity at $\lambda = 620$ nm upon analysis of different concentrations of DNA **3** (error bars were derived from four independent experiments).

available enzyme-amplified bioanalytical procedures because thrombin exhibits a substantially lower specific activity relative to other enzymes (e.g. peroxidase). Nonetheless, we note that for the analysis of BSA we find a 10^2–10^3-fold higher sensitivity in the ecarin–prothrombin system as compared to a system that employed the horseradish peroxidase–BSA Ab conjugate. For the analysis of DNA using biocatalysts as amplifying labels, we note that our system reveals an approximate 10^2-fold enhanced sensitivity as compared to a system that employed the horseradish peroxidase–nucleic acid label.[25] Also, our analytical procedure reveals comparable sensitivity to the reported bioelectrocatalytic detection of DNA using the polymerase-induced replication of redox-active DNA replica, which activates the electrocatalytic oxidation of glucose in the presence of glucose oxidase.[26] The simple and relatively rapid procedure described here for analyzing DNA is certainly an advantage. We thus anticipate that the method described here will find immense practical applications, as it can be adapted for any biorecognition process, and specifically, for systems that require high sensitivities.

Experimental Section

Preparation of ecarin–BSA Ab conjugate: Rabbit anti-bovine serum albumin IgG (Bethyl Laboratories; 1.64×10^{-7} M) was reduced with 2-mercaptoethylamine (1.3×10^{-5} M) in 500 μL of phosphate buffer (20 mM, pH 7.4, 0.15 M NaCl) at 37 °C for 90 min, and the product was purified using centrifugal filter devices (Centricon, 30000 MWCO, Millipore). Ecarin from *Echis carinatus* venom (55 kDa, Sigma-Aldrich; 6×10^{-7} M) was treated with *N*-(maleimidobutyroxy)sulfosuccinimide ester (1.25×10^{-4} M) in 450 μL of phosphate buffer (20 mM, pH 7.4, 0.15 M NaCl) for 30 min at room temperature and was then purified again using the Centricon filter (30000 MWCO). The resulting functionalized ecarin was reacted with the reduced BSA Ab for 30 min at room temperature.

Preparation of biotinylated anti-telomerase Ab: Anti-telomerase Ab (rabbit polyclonal antiserum, Oncogene Research Products) was reduced by a similar procedure as that described above for the reduction of BSA. The product was reacted with biotin-maleimide (Sigma-Aldrich; 3.3×10^{-4} M) in 1 mL of phosphate-buffered saline (PBS; pH 7.2).

Preparation of biotinylated ecarin: Ecarin (2×10^{-5} M) was treated with sulfo-NHS-LC-LC-biotin (Pierce Biotechnology; 1.5×10^{-5} M) in 1000 μL of phosphate buffer (20 mM, pH 7.4, 0.15 M NaCl) for 30 min at room temperature.

Analysis of BSA: For the analysis of BSA, an ELISA Nunc-Immuno Plate MaxiSorp Surface was treated with polyclonal BSA Ab for 60 min at 37 °C. The resulting plates were blocked with PBS solution that included 1 % goat serum. After rinsing, the plates were treated with different concentrations of BSA in borate saline buffer (BSB; pH 8.4) for 90 min at 37 °C. The plates were then rinsed and treated with the ecarin–BSA Ab conjugate in BSB (pH 8.4) for 60 min at 37 °C. The rinsed plates were then treated with a solution of prothrombin (Sigma-Aldrich; 1×10^{-8} M) in Tris buffer (20 mM, pH 8, 0.1 M NaCl) for 40 min, and then **1** (8×10^{-6} M) was added during 20 min to allow the hydrolysis to **2**. The fluorescence of the resulting solutions was monitored by using a photon-counting spectrometer (Edinburgh Instruments, FLS 920) connected to a computer (F900 v. 6.3 software).

Analysis of telomerase: HeLa cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) solution that included 2.5 % foetal bovine serum (Biological Industries, Beit Haemek, Israel) during 4 days. Trypsin (3 mL) was added to the resulting suspension (50 mL), and the cells were centrifuged and washed with the DMEM solution. Lysis of the HeLa cells was performed by treatment of a pellet of about 10^6 HeLa cells with 200 μL CHAPS (3,3-cholamidopropyl-dimethylammonio-1-propanesulfonate) buffer solution for 30 min at 4 °C. The resulting suspension was then centrifuged to yield the cell extract. The anti-telomerase Ab was deposited on the ELISA plates and blocked against nonspecific adsorption in a similar manner to that described for BSA Ab. The plates were then treated with solutions containing the extracts of variable numbers of HeLa cells. The resulting rinsed plates were interacted with biotinylated anti-telomerase Ab and then treated with avidin and biotinylated ecarin (the plates were rinsed between the reaction steps). Finally, the resulting plates were treated with prothrombin and **1** as described for the analysis of BSA.

Analysis of DNA: For the analysis of DNA, a 100-μL drop of solution of thiolated nucleic acid **4** (OD = 0.5) was placed on the plates and allowed to react overnight. Then, the surfaces were blocked with mercaptohexanol (1 mM) during 60 min. The modified plates were then hybridized with different concentrations of **3** in 100 μL of phosphate buffer (10 mM, 0.3 M NaCl) for 3 h. The double-stranded assemblies were then hybridized to the biotinylated nucleic acid **5** (1×10^{-6} M) at room temperature. Subsequently, the surface was treated with avidin and biotinylated ecarin (with rinsing between each of the steps). The resulting surfaces were then treated with prothrombin as described for the analysis of BSA, and the resulting fluorescence was monitored.

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