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Keywords: azides · glycans · glycoconjugates · ketones · oligosaccharides · sialic acid · Staudinger ligation

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Lighting Up Biochemiluminescence by the Surface Self-Assembly of DNA – Hemin Complexes

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The discovery of catalytic RNAs (ribozymes) has sparked scientific activities directed to the preparation of new biocatalysts and raised the suggestion that these biomolecules participated in the evolutionary process as preprotein catalysts.[1, 2] Analogously, deoxyribozymes, catalytic DNAzymes, are not found in nature but extensive research efforts have demonstrated the successful synthesis of catalytic deoxyribozymes for many chemical transformations.[3, 4] One interesting example of a catalytic DNA that reveals peroxidase-like activity includes a supramolecular complex between hemin and a single-stranded guanine-rich nucleic acid (aptamer).[5] This complex was reported to catalyze the oxidation of 2,2′-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) by H_2O_2, a common reaction used as an assay for peroxidase activity. It was suggested[6] that the supramolecular docking of the guanine-quadruplex layers facilitates the intercalation of hemin into the complex and the formation of the biocatalytically active hemin center.

Enzymes[7] and, specifically, horseradish peroxidase (HRP)[8, 9] are used as biocatalytic labels for the amplified detection of DNA-sensing events. The electrochemical amplified detection of DNA has been accomplished in the presence of different enzymes[7, 8] and the chemiluminescent analysis of DNA in the presence of HRP has been reported.[9] The integration of a DNA biocatalyst into DNA-detection schemes could provide a new method for the detection of nucleic acids that might reveal important advantages: 1) The catalytic DNA may substitute the protein-based biocatalysts, and thus eliminate nonspecific binding phenomena; 2) Tailoring of the DNA biocatalyst as part of the labeled nucleic acid might reduce the number of analytical steps for DNA detection. Here we report that two separated nucleic acids that include the segments A and B—constituting the single-stranded peroxidase deoxyribozyme, which forms a layered G-quadruplex structure (see Scheme 1)[10]—self-assemble in the presence of hemin to form a biocatalyst for the generation of chemiluminescence in the presence of H_2O_2 and luminol. The effect of hybridization with the DNAzyme compounds on the resulting biochemiluminescence is discussed. We also demonstrate the self-assembly of biocatalytic, supramolecular hemin – nucleic acid complexes on gold electrodes in monolayer configurations, and describe the biocatalytic and bioelectrocatalytic formation of chemiluminescence at the

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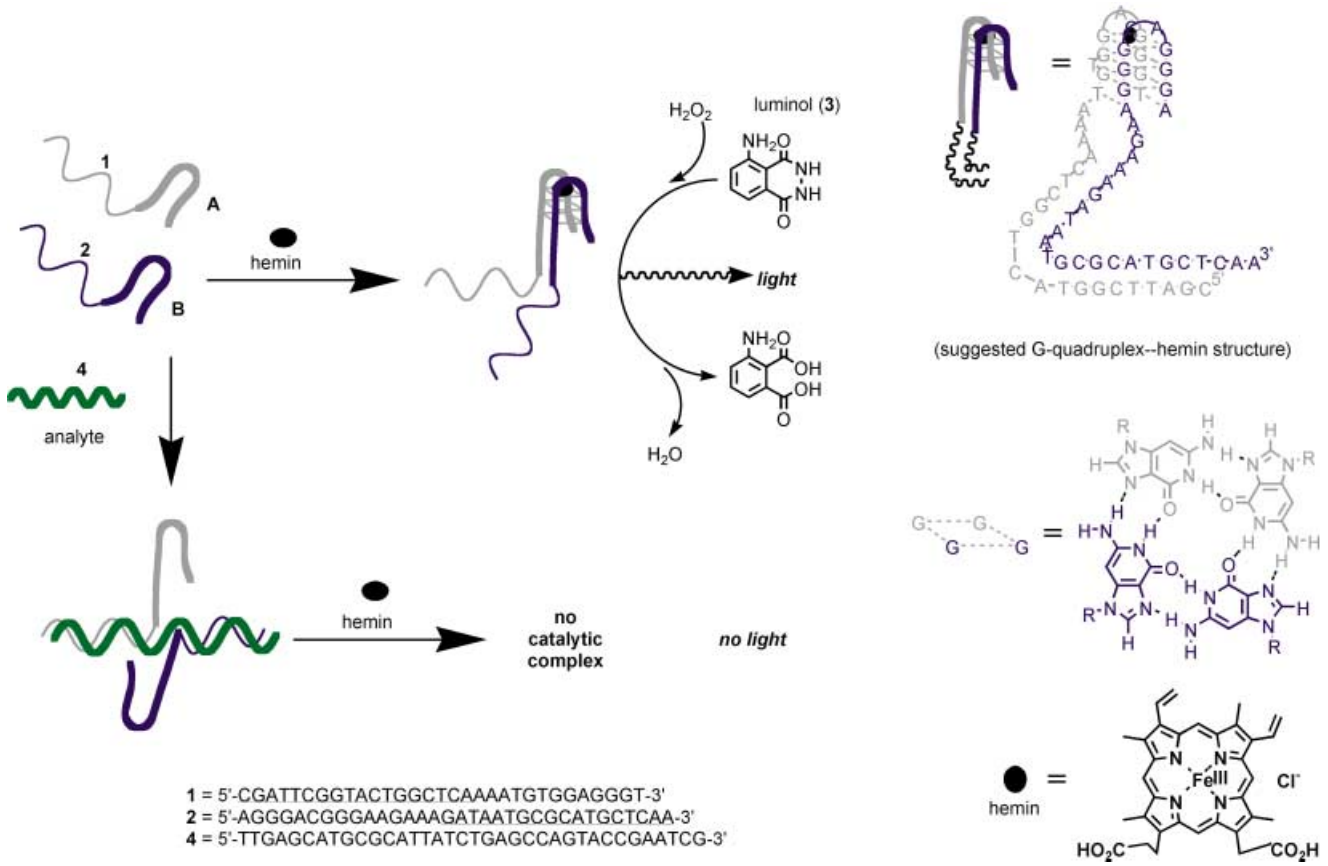


Scheme 1. *Chemiluminescence generated by a supramolecular nucleic acid – hemin complex and the inhibition of the DNAzyme activity by hybridization.*

interfaces as well as the inhibition or blocking of the light emission processes by hybridization.

The two nucleic acids **1** and **2** include the segments A and B that self-assemble on the hemin site. Treatment of hemin (12 μM) with **1** and **2** (12.5 μM each) results in the formation of a 1:1:1 supramolecular complex ($K_d = 130\ \mu M^2$, Scheme 1). This supramolecular complex reveals biocatalytic functions, and, in the presence of H_2O_2 and luminol (**3**), the system generates chemiluminescence. Curve a in Figure 1 shows the integrated light intensity emitted from the system. Control experiments reveal that the formation of the supramolecular complex is essential for light emission. Hemin itself yields very low chemiluminescence (curve b) and hemin in the presence of the separated nucleic acids **1** and **4** (the analyte) generates a very low light output (curve c). (The hybridization of **2** and **4** in the presence of hemin leads to negligible light emission.) Nucleic acid **4** does not emit any light in the absence of hemin (curve d). These results indicate that the self-assembly of **1** and **2** with hemin is essential to generate the biocatalyzed light. The nucleic acid chains linked to segments A and B of **1** and **2** are complementary to the 5′ and 3′ ends of nucleic acid **4**. Curves e – h in Figure 1 show the effect of hybridization of **4** with nucleic acids **1** and **2** in the presence of hemin on the emitted light intensity. Evidently, as the concentration of **4** increases the biocatalytic light emission decreases, and at a concentration of 12.5 μM of **4** 70 % of the original chemiluminescence is blocked. A

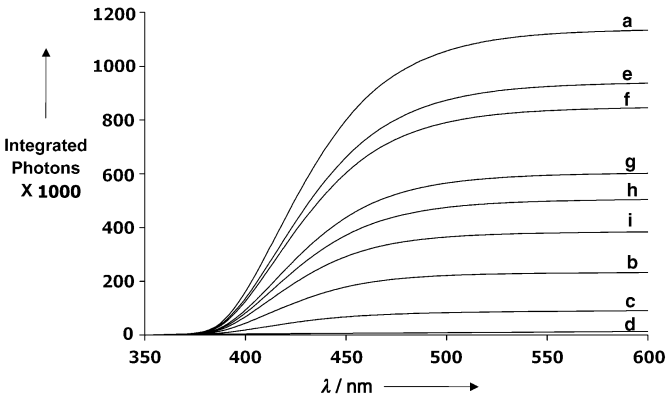


Figure 1. *Integrated photons recorded in the systems consisting of a) nucleic acids **1** and **2** (each 12.5 μM) and hemin (12 μM); b) hemin (12 μM); c) nucleic acids **1** and **4** (each 12.5 μM) and hemin (12 μM); d) nucleic acid **4** (12.5 μM) without hemin; e) – h) nucleic acids **1** and **2** (each 12.5 μM), hemin (12 μM), and competitive hybridizing nucleic acid **4** at concentrations that correspond to 3, 6, 9.5 and 12.5 μM, respectively; i) nucleic acid **4** (12.5 μM) and hemin (12 μM) without added **1** and **2**.*

control experiment that examined the light emission from nucleic acid **4** in the presence of hemin revealed low-level chemiluminescence (Figure 1, curve i). Thus, the decrease in chemiluminescence generated by the supramolecular hemin/**1**/**2** complex system upon addition of **4** is attributed to the

separation of the biocatalytically-active DNAzyme upon hybridization with **4**. Presumably, hybridization of **1** and **2** with **4** distorts the segments A and B to a configuration that cannot form the biocatalyst structure.

The biocatalytic generation of chemiluminescence on surfaces was also examined. Hemin was covalently linked to a gold surface (Scheme 2). The tethered hemin units were then used as sites for the reconstitution of the biocatalytic peroxidase-like supramolecular complex on the surface through the interaction of the functionalized surface with nucleic acids **1** and **2**. Coulometric assay of the redox wave of the heme units indicated a surface coverage of 3.5×10^{-11} mol cm^{-2}. Thus, about 18% of the hemin units are reconstituted with the nucleic acids **1** and **2**. Curve a in Figure 2 shows the integrated light intensity emitted by the DNAzyme interface in the presence of H_2O_2 and luminol (**3**). Control experiments confirm that very low light emission is stimulated by the hemin monolayer alone (curve b) and that hemin in the presence of **1** or **2** alone does not lead to any significant chemiluminescence (curve c). Curves d – g show the light emission in the presence of different concentrations of added **4**. As the concentration of **4** increases, the emitted light intensity decreases. Microgravimetric quartz-crystal microbalance experiments indicate that hybridization of the free nucleic acid parts of **1** and **2** with **4** leads to the dissociation of the hemin – nucleic acids complex from the surface, and at a concentration of **4** of 2.5 μM, the crystal frequency is almost similar to that of the hemin-monolayer-functionalized crystal prior to reconstitution with **1** and **2**. Thus, the hybridization of **1** and **2** with **4** presumably distorts the segments A and B leading to the dissociation of the surface-confined biocatalytic supramolecular complex.

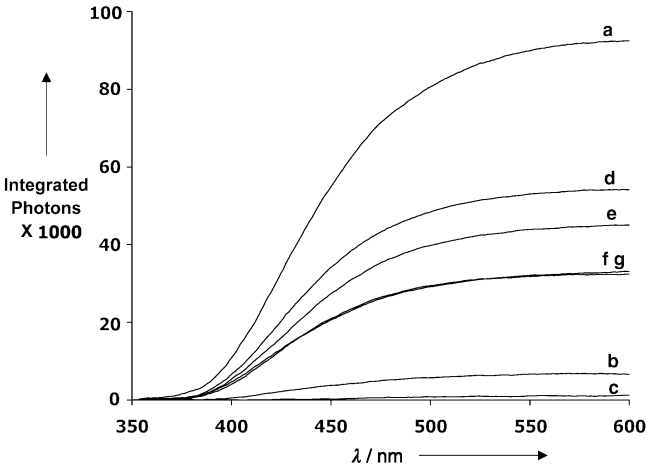
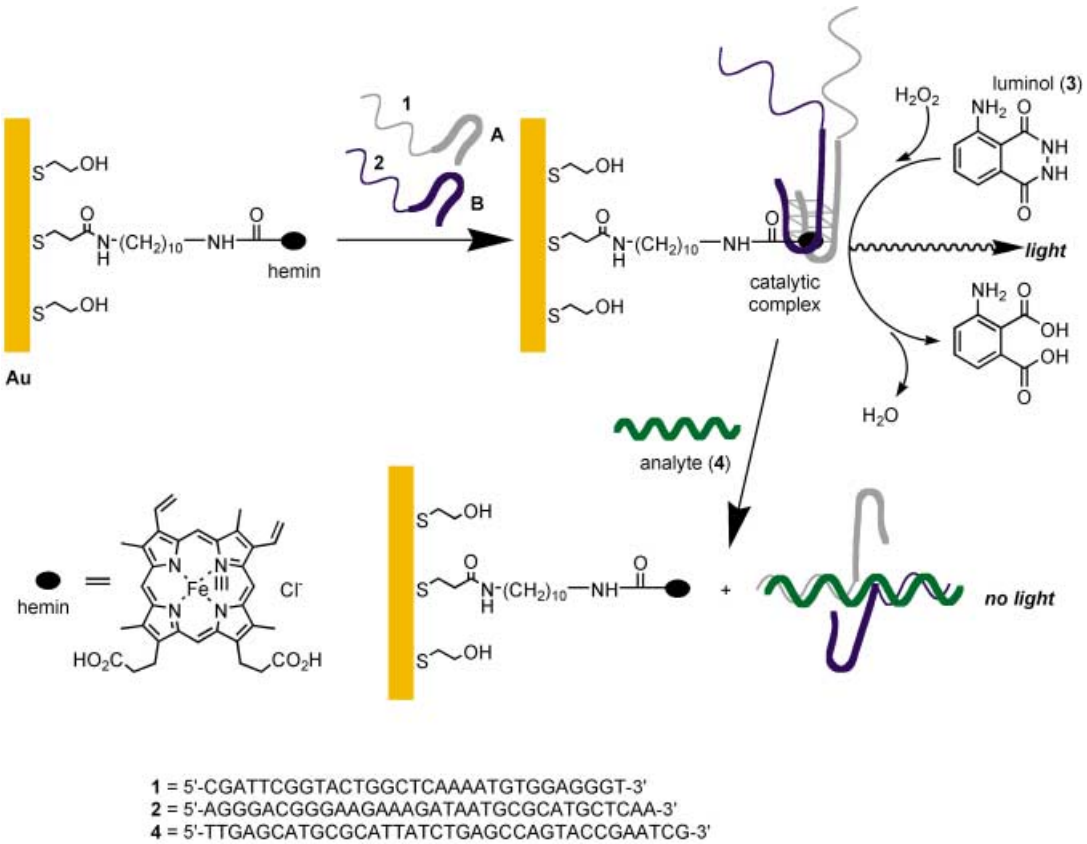
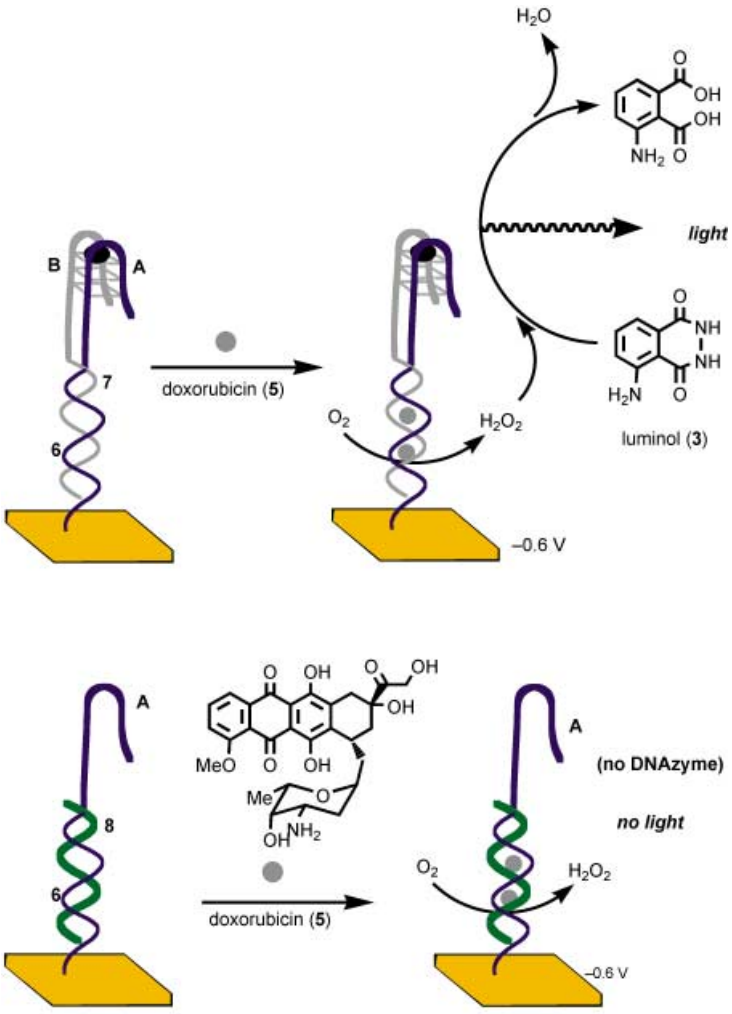


Figure 2. *Integrated photons emitted by a) the hemin-modified surface reconstituted with both **1** and **2** (each 2.5 μM); b) the hemin-modified surface without such reconstitution; c) the hemin-modified surface reconstituted with either **1** or **2** (each 2.5 μM); d) – g) the hemin-modified surface reconstituted with **1** and **2** (each 2.5 μM) in the presence of **4** at concentrations of 0.6, 1.2, 1.9, and 2.5 μM, respectively.*



Scheme 2. *Reconstitution of nucleic acids on a hemin-monolayer-modified surface, generation of a biochemiluminescence DNAzyme, and the inhibition of the process by hybridization.*



6 = 5'-HS$(CH_2)_6$CGATTCGGTACTGGCTCAAAATGTGGAGGGT-3'
7 = 5'-AGGGACGGGAAGATGAGCCAGTACCGAATCG-3'
8 = 5'-TGAGCCAGTACCGAATCG-3'

Scheme 3. *The assembly of a nucleic acid – hemin complex on an electrode for the electrochemical generation of chemiluminescence and the inhibition of the process by hybridization.*

A further surface-confined biocatalytic system for the generation of chemiluminescence in the presence of the DNAzyme was designed by the in situ generation of H_2O_2. Previous studies have demonstrated that the intercalation of doxorubicin (**5**) into double-stranded DNA immobilized on an electrode allows the electrocatalyzed reduction of O_2 to H_2O_2 by the intercalated quinone and the subsequent light emission in the presence of HRP and luminol (**3**).[9] Scheme 3 shows the assembly of the DNAzyme system on an electrode for the biocatalyzed generation of chemiluminscence and its application for nucleic acid analysis. The thiolated nucleic acid **6**, which includes the nucleic acid component "A" of the DNAzyme, is assembled on the electrode. The hybridization of the nonenzymatic part of **6** with the complementary part of the nucleic acid **7**, which includes the segment "B" of the DNAzyme, yields the interface that binds hemin and generates the peroxidase-mimicking DNAzyme. The intercalation of doxorubicin (**5**) into the double-stranded DNAzyme produces the bioelectrocatalytic interface for electrocatalyzed light emission. The electrocatalyzed reduction of **5** produces H_2O_2, and the DNAzyme catalyzes light emission in the presence of luminol (**3**). The surface coverage of the thiolated nucleic acid **6** and the double-stranded nucleic acid structure **6**/**7** was determined by microgravimetric quartz-crystal microbalance experiments to 9.5 and 4.6×10^{-12} mol cm^{-2}, respectively. The coulometric assay of the doxorubicin response indicated a surface coverage of about 2.8×10^{-11} mol cm^{-2}. Thus, approximately six doxorubicin units are intercalated into each double-stranded DNA. Curve a in Figure 3 shows the time-dependent light intensity emitted upon application of a potential of -0.6 V versus saturated calomel electrode (SCE) on the electrode. This potential reduces the amount of doxorubicin associated with the double-stranded DNA on the surface. Doxorubicin mediates the catalyzed generation of H_2O_2 during the reduction process, and the electrogenerated H_2O_2 leads to biochemiluminescence in the presence of luminol (**3**). Control experiments indicate that no light emission is observed upon application of the same sequence of reactions to the surface modified with **6** without hybridization with **7** (Figure 3, curve b). A further control experiment shows that light is emitted from the system consisting of the electrode functionalized with **6** upon interaction with nucleic acid **8** that lacks segment "B" of the DNAzyme when this is further treated with hemin and doxorubicin (**5**) and subjected to the potential of -0.6 V versus the SCE in the presence of luminol (Figure 3, curve c). Clearly, the emitted light intensity is negligible; this implies that hybridization of **8** with the interface inhibits the formation of the biocata-

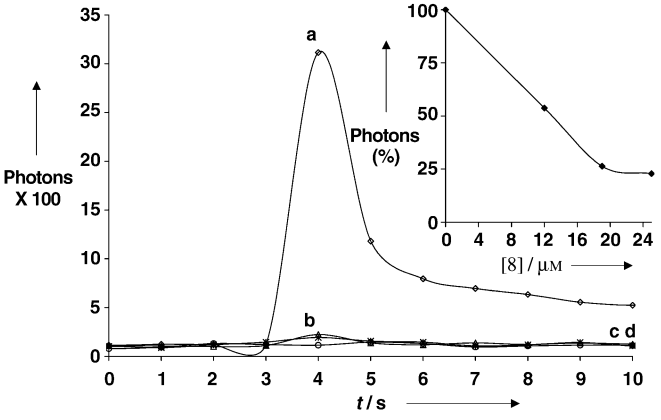


Figure 3. *Time-dependent photons counted in the system consisting of a) the electrode functionalized with **6**/**7** and treated with hemin (1.2 μM) and doxorubicin (**5**, 5 μM; ◇); b) the electrode functionalized with **6** (△); c) the electrode functionalized with **6** and hybridized with **8** (2.5 μM; ○); d) mercaptohexanol-functionalized electrode hybridized with **8** (2.5 μM) and treated with hemin (1.2 μM) and **5** (5 μM; ×). Inset: Calibration curve, percentage of photons as a function of the different concentrations of **8** (0, 1.2, 1.9, and 2.5 μM). Light emission was detected upon applying a potential of -0.6 V versus SCE.*

lytic interface for chemiluminescence. Also, the interaction of the mercaptohexanol-functionalized surface with **8** and then with hemin and doxorubicin followed by the application of the potential of −0.6 V versus SCE in the presence of luminol (**3**) did not yield any electrogenerated chemiluminescence (Figure 3, curve d). Thus, the control experiments reveal that the hybridization between **6** and **7** is essential to forming the complex with hemin and to intercalating doxorubicin (**5**) into the double-stranded assembly. The electrochemical reduction of the intercalator supplies the H_2O_2 for the DNAzyme, and this activates the light-emission process. Addition of nucleic acid **8** that is complementary to the surface-associated nucleic acid **6** competes with **7** in the hybridization process. Since **8** lacks the "B" part for self-assembly of the biocatalytic complex with hemin, light emission in the presence of hybridized **8** should be blocked. Figure 3 (inset) shows the calibration curve of light intensities emitted by the electrode modified with **6** upon hybridization with **7** in the presence of different concentrations of **8**, and upon treating the interface with hemin and **5** and applying the reductive potential in the presence of luminol, as described above.

The systems described in the present study reveal the possibility of detecting DNA at a limit of 0.6 μM by using the DNAzyme as a biocatalytic label. Although this sensitivity is adequate to analyze DNA in samples generated by PCR, it is far lower than the values reached recently by electrochemical means.[7–9] Furthermore, the systems described in this study result in a signal decrease upon analysis of the DNA; this is analytically unsatisfactory. The design of systems with a positive build-up of chemiluminescence upon DNA analysis is, however, technologically feasible, and experiments in this direction are underway in our laboratory. The advantages of using DNAzymes as catalytic labels for the DNA analysis rest, however, in the enhanced specificity of the analytical methods. While the use of enzymes and enzyme conjugates always involves nonspecific adsorption, the application of nucleic acid catalysts eliminates this phenomenon. One important aspect of the present study is the demonstration that self-assembly of two specific nucleic acids and hemin may yield a supramolecular biocatalytic entity.

In conclusion, the present study has revealed the novel functions of a supramolecular hemin–nucleic acid complex as a DNAzyme with peroxidase-like chemiluminescence activity. Besides the fundamental interest in their DNAzyme activities, the systems have important practical implications since the chemiluminescent DNAzyme may act as an internal nucleic acid biocatalytic label for DNA sensing. This means that one may design a protein-free amplified DNA-detection scheme by using chemiluminescence for transduction.

Experimental Section

Materials: Hemin was purchased from Porphyrin Products (Logan, Utah) and used without further purification. A hemin stock solution (4.6 mM) was prepared in DMSO and diluted with DMSO to the desired final concentrations. Solutions were frozen and stored in the dark at −20 °C. The concentrations of hemin solutions were determined by using standard spectroscopic methods.[11] 5-Amino-2,3-dihydro-1,4-phthalazinedione (luminol, **3**) and other chemicals were obtained from Sigma and used as supplied. All buffers used for analyzing the DNAzyme chemiluminescent activities contained the nonionic detergent Triton X-100 (0.05%, *w*/*v*) and 1% DMSO.

Nucleic acids synthesis: Nucleic acids were synthesized by Sigma Genosys and purified by using the PAGE method. The sequences of the oligomers are given below:

(**1**) 5′-CGATTCGGTACTGGCTCAAAATGRGGAGGGT-3′
(**2**) 5′-AGGGACGGGAAGAAAGATAATGCGCATGCTCAA-3′
(**4**) 5′-TTGAGCATGCGCATTATCTGAGCCAGTACCGAATCG-3′
(**6**) 5′-HS$(CH_2)_6$CGATTCGGTACTGGCTCAAAATGRGGAGGGT-3′
(**7**) 5′-AGGGACGGGAAGATGAGCCAGTACCGAATCG-3′
(**8**) 5′-TGAGCCAGTACCGAATCG-3′

Preparation of DNA–hemin complexes: Nucleic acids **1** and **2** (each 25 μM) and competitive hybridizing nucleic acid **4** (0, 6, 12, 19, and 25 μM) were heated to 95 °C for 9 min in Tris-HCl (10 mM, pH 7.4) to dissociate any intermolecular G-quadruplex, then allowed to cool to room temperature. An equal volume of the hybridization buffer (50 mM HEPES, 40 mM KCl, 400 mM NaCl, 0.1% Triton X-100, 2% DMSO; pH 7.4) was added to the nucleic acid mixtures, and the systems were allowed to hybridize and fold overnight at room temperature. Hemin (12 μM) was then added (final concentration of DMSO less than 2%) to form the G-quadruplex structures (12 h, room temperature).

Immobilization of hemin as a monolayer and reconstitution of DNAzyme on the gold surface: The Au-coated glass plate (50 nm gold layer, 22 × 11 mm) was immersed into a piranha solution (70% conc. sulfuric acid, 30% hydrogen peroxide) for 20 min and afterwards thoroughly rinsed with triple-distilled water. The plate was then soaked in concentrated nitric acid for 5 min and rinsed with water again. The plate was treated with a solution of 3-mercaptopropionic acid in ethanol (1×10^{-2} M) that contained 6-mercaptohexanol (1×10^{-3} M) for 12 h and afterwards rinsed with ethanol to remove any nonspecifically adsorbed material. The covalent coupling of 1,10-diaminodecane to the thiol monolayer-modified plate was performed by soaking the plate in HEPES buffer solution (0.01 M, pH 7.2) that included 1,10-diaminodecane (5×10^{-4} M) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC; 1×10^{-3} M) for 2 h at room temperature. The resulting plate was washed with HEPES buffer (0.01 M, pH 7.2) and incubated in HEPES buffer (0.01 M, pH 7.2) that contained hemin (5×10^{-4} M), Triton X-100 (0.05%), 1% DMSO, and EDC (1×10^{-3} M) for 4 h at room temperature. The resulting plates were then rinsed with the hybridization buffer. A mixture of nucleic acids **1** and **2** (each 25 μM) in Tris buffer (0.01 M, pH 7.4) was heated to 95 °C for 9 min, then allowed to cool to room temperature. An identical volume of the hybridization buffer was added to the nucleic acid mixture to allow proper folding (12 h, room temperature). The hemin-modified electrode was then immersed in the nucleic acid solution (2.5 μM) for surface reconstitution (12 h, room temperature). The resulting surface-reconstituted layer of the hemin–nucleotide complex was then treated with **4** (0, 0.6, 1.2, 1.9, or 2.5 μM) in phosphate buffer (0.1 M; containing 25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO; pH 7.4) for 12 h at room temperature.

Immobilization of 6 on the Au surface and its hybridization with 7 or 8: The Au plate was treated with a solution of **6** (6 μM) in phosphate buffer (0.4 M, pH 7.4) for 12 h, and the resulting surface was then treated with a solution of 1-mercaptohexanol (1 mM) in phosphate buffer (0.1 M) for 1 h. The resulting monolayer-functionalized surface was then treated with the complementary nucleic acids **7** (2.5 μM) and **8** (0, 1.2, 1.9, or 2.5 μM) in a solution composed of

phosphate buffer (0.1 M) and Perfect Hyb hybridization buffer (Sigma, 1:1 *v/v*) for 5 h to give double-stranded DNA assembly on the surface. The resulting surfaces were rinsed with the hybridization buffer and immersed in a solution of hemin (1.2 μM) in buffer (25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO; pH 7.4) for 12 h at room temperature. The resulting system was further treated with doxorubicin (**5**, 5 μM) in phosphate buffer (0.1 M, pH 7.4) for 1 h at room temperature.

Light-emission measurements: Light emission was performed by using a photon-counting spectrometer (Edinburgh Instruments, FLS 920) equipped with a cooled photomultiplier detection system, connected to a computer (F900 v. 6.3 software). Before the samples analyses, the background light was recorded and integrated. This background was subtracted from the recorded integrated spectra of the respective samples. Sample analyses were performed by adding the DNAzyme solution (15 μL) or the modified surface to luminol (0.5 mM) and H_2O_2 (30 mM) in buffer solution (3.3 mL; 25 mM HEPES, 20 mM KCl, and 200 mM NaCl; pH 9.0) in a cuvette.

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Keywords: biocatalysis · DNA · hemin · luminescence · monolayers · self-assembly

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Probing Protein – Carbohydrate Interactions with Microarrays of Synthetic Oligosaccharides

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Formerly a "neglected dimension" of biochemistry, recent years have seen growing interest in studying the biological function of carbohydrates and glycoconjugates. An emerging understanding of the physiological role of these biomolecules has uncovered their vital participation in a host of fundamental cellular processes. In the form of glycopeptides, glycolipids, glycosaminoglycans, and proteoglyans, glycoconjugates are known to be involved in inflammation,[1] cell – cell interactions,[2] signal transduction,[3] fertility, and development.[4, 5] Unfortunately, current methods for elucidating the biochemical roles of glycoconjugates are often cumbersome. This demonstrates the need to develop techniques that will satisfy this growing field of study by enabling rapid and facile exploration of biochemical events involving carbohydrates.

Inspired by the success of DNA and protein microarrays,[6, 7] the chip-based approach has been put forward as a useful tool in the emerging field of glycomics.[8–10] Nitrocellulose-coated slides have been employed for the noncovalent immobilization of microbial polysaccharides and neoglycolipid-modified oligosaccharides.[11, 12] Hydrophobic interactions have been utilized to anchor lipid-bearing carbohydrates on polystyrene microtiter plates.[13] Self-assembled monolayers presenting benzoquinone groups enabled the Diels – Alder-mediated immobilization of cyclopentadiene-derivatized monosaccharides on a gold surface.[14] Another covalent immobilization chemistry involved treating maleimide-functionalized mono- and disaccharide glycosylamines with a thiol-derivatized glass slide,[15] or, alternatively, thiol-functionalized carbohydrates with a self-assembled monolayer presenting maleimide groups.[16]

Our motivation for developing a system for arraying carbohydrates is based on the need to have microarrays that are fully

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