0273-2289/01/9601-3/0287/$13.75

Biomaterials for Molecular Electronics Development of Optical Biosensor for Retinol

Kumaran Ramanathan,*[,1] Juraj Svitel,[1] Anatoli Dzgoev,[1] P. V. Sundaram,[2] and Bengt Danielsson[1]

[1]Department of Pure and Applied Biochemistry, Box 124, Center for Chemistry and Chemical Engineering, Lund University, Lund 221 00, Sweden, E-mail: alamelu6703@hotmail.com; and
[2]Centre for Protein Engineering and Biomedical Research, The Voluntary Health Services, Adyar, Madras 600 113, India

Abstract

Molecular electronics involves expertise from several branches of science. Various biomaterials and electronics are involved in the fabrication of such devices. While passive biomaterials are involved in anchoring the active biomolecules, the latter are involved in switching and/or signal transduction. In the present investigation we have used a glass-capillary-based approach to design a biosensor for retinol. The sensing element is retinol-binding protein (RBP). The affinity of retinoic-acid–horseradish peroxidase (conjugate) to RBP is tested using a surface plasmon resonance technique. A simple photomultiplier-tube-based system is exploited to monitor the chemiluminescent signal generated upon reaction of hydrogen peroxide and luminol with the conjugate bound to RBP. The photomultiplier tube is directly coupled to a computer for data logging.

Index Entries: Retinol; retinol-binding protein; molecular electronics; biosensor; chemiluminescence; capillary.

Introduction

Molecular electronics is a multidisciplinary area in which molecular systems are employed for coding, manipulation, and retrieval of information *(1–8)*. In particular, biomolecular electronics involves application of biologic materials for the design of newer devices at molecular dimensions *(9)*. A few decades ago, the idea of using biomolecules for electronics was conceived, and this has resulted in intense research activities on several

*Author to whom all correspondence and reprint requests should be addressed.

molecules with potential use in biomolecular electronics *(10)*. The interdisciplinary nature of this field requires expertise from biologists *(11)*, chemists *(12,13)*, physicists *(14)*, instrumentation specialists, microscopists *(15,16)*, and more so from material scientists *(17,18)*.

The biomaterials employed for the design and fabrication of biomolecular electronics devices are classified into two types: passive and active biomaterials. Passive biomaterials are involved in anchoring an active biomaterial and providing a suitable environment for maintaining its activity *(19)*. Examples of passive biomaterials include sol-gel/hydrogel *(20–22)* and their composites *(23)*, receptor elements such as retinol-binding protein *(24)*, inorganic-organic composites *(25)*, Langmuir-Blodgett films *(26)*, and self-assembled structures *(27–31)*.

Active biomaterials are involved in the switching and transduction of the signal. The nature of the signal may be in the form of electron transfer or energy transfer. Examples of active biomaterials include rhodopsin *(32)*, bacteriorhodopsin *(33)*, DNA *(34–39)*, photosynthetic electron transfer agents *(40)*, porphyrin-based structures *(41–43)*, riboflavin/flavine adenine dinucleotides *(44)*, dyes *(45–49)*, phthalocyanine *(50,51)*, β-carotene *(52)*, polymers *(53)*, and conducting polymers *(54,55)*.

Among the active biomaterials, bacteriorhodopsin is being extensively investigated as a photoactive and fast-switching molecule *(56,57)*. Bacteriorhodopsin from purple membrane of *Halobacterium halobium* is sufficiently stable, and its photochromic and photoelectric properties *(58)* are used for constructing biophotonic and bioelectronic devices. The photosignal has been well characterized for this single polypeptide (248 amino acids) transmembrane protein *(59)*.

The excitability of bacteriorhodopsin is based on the attachment of the photoactive chromophore "retinal" (vitamin A aldehyde) to Lys-216 of apoprotein bacterio-opsin, which confers an intense purple color to bacteriorhodopsin in the ground state *(60)*. Illumination at 590 nm for 1 μs stimulates the isomerization of retinal leading to a conformational change in bacteriorhodopsin. This results in translocation of protons from the intra- to extraaqueous phase across the membrane. As bacteriorhodopsin maintains a preferential orientation, the light-induced proton movement is manifested as a photocurrent, followed by an exponential relaxation *(61)*. An interesting feature is that the time interval between onset and reversal of photocurrent is 9 μs, while the switch between all-*trans* retinal to 13-*cis* retinal occurs in 3 ps. These properties are exploited in several investigations with both *in situ* and immobilized bacteriorhodopsin for prospective biomolecular electronics applications.

The well-established techniques that are useful in the design of biomolecular electronics devices include immuno/receptor assay as a cost-effective and sensitive method for screening clinical as well as environmental samples. However, the end use of enzyme-linked immunosorbent assay (ELISA) is limited owing to the requirement of trained personnel and longer assay time. These disadvantages have initiated the development of

immuno/receptor-based sensors using sandwich and competitive assay formats at the surfaces of acoustic waveguides *(62)*, optical fibers *(63,64)*, planar waveguides *(65)*, plasmon resonance surfaces *(66)*, and piezoelectric devices *(67)*. In this regard, flow displacement assays have also been reported *(68)*. The nature of the signal generated could be either optical, electrochemical, or electronic in nature. Accordingly, several forms of detection principles and instrument design have been employed in such investigations. These include charge-coupled devices (CCDs) *(24)*, chemiluminescent detection using photomultipliers, scanning array of DNA/biomolecules *(69)*, and neural network analysis *(70,71)*.

In the case of optical measurements, the chemistry within glass capillaries is advantageous because of the high surface-to-volume ratio, laminar flow, and reduced diffusion effects *(72)*. Although capillaries have proved to be very successful in immunoassays, their use in receptor-based assays is as yet less explored. Several recent reports do describe other capillary-based sensors *(73,74)*.

In our investigations, we chose a capillary-based measurement for retinol and retinoic acid to understand their binding to retinol-binding protein (RBP). This system could be useful in anchoring retinol or retinal at desired locations in an immobilization matrix for future biomolecular electronics devices. We demonstrate a scheme wherein the binding of retinoic acid–horseradish peroxidase (HRP) (conjugate) to RBP could be exploited for developing a retinol biosensor based on an optical detection scheme.

Materials and Methods

Materials

HRP (EC 1.11.1.7) type VI, RBP, bovine serum albumin (BSA), *N*-hydroxysuccinimide, dicyclohexyl carbodiimide (DCC), luminol (8-amino-2,3-dihydro 1,4-phthalazinedione), and triethylamine were from Sigma (St. Louis, MO). Anti-RBP antibodies were obtained from DAKO (Glostrup, Denmark). Glass (Kwik-fil borosilicate) capillaries were from World Precision. Toluene, tetrahydrofuran, acetone, hydrogen peroxide (30%), sodium bicarbonate, and sodium phosphate buffer salts were from Merck (Germany). Water (Maxima Ultrapure) was from ELGA (Sweden), and 3-glycidoxy propyltrimethoxysilane (GPTES) was from Aldrich. Sephadex G-25 was from Pharmacia (Uppsala, Sweden), and blocking reagent for ELISA was from Boehringer Mannheim. Dialysis membrane (mol wt cutoff of 12,000–14,000) was from Spectrum.

Instrumentation

The photomultiplier tube (PMT) HC135-02 module was from Hamamatsu (Kista, Sweden). The casing for the PMT was fabricated at the local workshop. The design is referred elsewhere *(75)* and was interfaced to a computer with a custom-made software for data logging. Results were analyzed on a PC running MS-excel 97. The fraction collector was from LKB

Bromma (Sweden), and spectrophotometer model 8453 was from Hewlett Packard. The SPR chips CM-5 coated with carboxymethylated dextran and amine coupling kit were purchased from Biacore (Sweden), and the SPR analysis was carried out on BIAcore (Pharmacia).

Preparation of Retinoic Acid–HRP Conjugate

The method of synthesis is described elsewhere *(76)*. In brief, 0.1 mmol of retinoic acid was dissolved in 2.4 mL of dimethylformamide (DMF), while, 0.1 mmol of NHS and 0.1 mmol of DCC were dissolved in 0.1 mL of DMF, respectively. The three solutions were mixed in a round-bottomed flask, and the reaction was allowed to proceed for 20 h under a nitrogen atmosphere. The precipitate was sedimented by centrifugation at 1500 rpm for 5 min. The supernatant was mixed with 500 U of HRP dissolved in 1 mL of 0.1 *M* sodium borate buffer (pH 8.9). The reaction mixture was stirred for 20 h.

Purification of Conjugate

The conjugate obtained was purified initially by dialysis against 0.1 *M* phosphate buffer followed by G-25 Sephadex column (40 × 1 cm) chromatography. The purity was tested spectrophotometrically for the enzyme peak (280 nm). The enzyme activity was also estimated based on a colorimetric procedure *(77)*.

Silanization of Glass Capillaries

Initially, the glass capillaries were washed twice (5 min at 80°C) with water followed by washings with anhydrous acetone, tetrahydrofuran, and toluene. The capillaries were then filled with a solution of 300 µL of GPTES and 30 µL of triethylamine in 15 mL of toluene. The silanization was allowed to proceed at room temperature (22°C) for 18 h. After silanization the capillaries were washed twice with anhydrous toluene, tetrahydrofuran, and acetone. The capillaries were stored under dry condition prior to use.

Surface Plasmon Resonance Measurements

The retinoic acid–HRP conjugate was dialyzed against HEPES buffer (0.01 *M*, pH 7.4) with several buffer changes for 24 h with continuous stirring and at 4°C. The volume of the conjugate increased during dialysis from 100 to 300 µL. The excess of retinol precipitated during dialysis and was centrifuged. The dialysate was filtered through a 0.22-µm Millex-GV filter (Millipore) and used for immobilization on the chip surface. The surface plasmon resonance (SPR) measurements were performed with a Biacore 2000 (Biacore AB) operated with BIAcore control software 1.2. All measurements were performed at 25°C and a flow rate of 5 µL/min. The pH 7.4 buffer consisted of 0.01 *M* HEPES and 0.15 *M* NaCl. The chip used for the experimental work was modified by covalently bound HRP-retinol conju-

gate or with RBP. A standard amine coupling kit was used for the modification that was performed by injecting 50 µL of HRP-retinol conjugate or 0.5 mg/mL of RBP into the flow stream.

Coating RBP onto Capillaries

The capillaries were filled with an 800-fold dilution of anti-RBP antibodies prepared in 50 m*M* carbonate buffer (pH 9.6) and incubated overnight at 4°C, followed by coating with a 0.5 mg/mL solution of RBP. Each capillary could hold a volume of approx 50 µL. The capillaries were incubated at 4°C for 24 h. The capillaries were washed three times with 0.1 *M* phosphate buffer (pH 7.4) containing 0.139 *M* NaCl and 0.1% (v/v) Tween-20 (PBST). The capillaries were filled with the blocking reagent (27 mg/mL) and left for 30 min at room temperature (22°C) followed by washing with PBST (five times). After washing, the capillaries were filled with the conjugate and incubated at 4°C for 24 h. After incubation, the capillaries were washed with PBST (containing 0.05% Tween-20) and filled with the substrate solution consisting of 1 m*M* luminol and 3.4 µL of H_2O_2 (30%) prepared in 10 mL of 0.1 *M* Tris-HCl, pH 8.6. Immediately thereafter the capillaries with the substrate solution were placed in the holder of the PMT casing for measuring the chemiluminescent signal.

Results and Discussion

Within the purview of biomolecular electronics, it was very essential to understand the interactions of molecules in general and specifically the nature of the active biomaterials that are employed for the fabrication of biomolecular electronics devices. In this regard, we thought it interesting to investigate the binding chemistry of retinol, a closely related analog of retinal, to understand the mechanisms involved in the binding of retinol to its natural receptor RBP. The understanding of such receptor-based anchoring of chromophore would enable the design of biomolecular electronics devices wherein the anchoring of the biomolecules to specific locations on an immobilization matrix would be the crux of the technology. The binding studies were carried out using the SPR technique, and we further exploited this understanding to design a possible scheme for the optical sensing of retinol.

In an earlier investigation, we had demonstrated the quantification of RBP in standard samples using a CCD camera–based approach *(24)*. Herein, both individual capillary and an array of capillaries could be simultaneously detected and quantified for calibrating the system. In this approach, anti-RBP antibodies were immobilized on glass capillaries followed by the binding of the RBP and an anti-RBP-HRP conjugate for generating the chemiluminescent response. The scheme for generation of the chemiluminescence was described in an earlier work *(75)*.

Following the understanding of the RBP biosensor, we designed a conjugate of retinoic acid with HRP and employed the same for screening

a variety of organic solvents for their ability to break the RBP–retinoic acid–HRP complex using a thermometric approach *(76)*. Herein, HRP was used as a label because of its stability in a variety of organic solvents and also for its high free energy change on reaction with H_2O_2 or butanone peroxide.

In the present investigations, a similar approach of retinoic acid–HRP conjugate was employed for testing the binding of retinoic acid with RBP and also to act as a competitor to retinol in the design of a retinol biosensor. Note that retinoic acid, retinol, and retinal are structurally similar except that the terminal carbon could be bonded to either COOH (carboxylic acid), OH (alcoholic), or CHO (aldehydic) groups, respectively. The remaining parts of the structure in all three instances are identical.

Initially, the binding of the retinoic acid–HRP (conjugate) to RBP was investigated using the SPR-based analysis, using a Biacore instrument, followed by the measurement of the conjugate on immobilized RBP using a PMT detector. An overview of the assay procedure is shown in Fig. 1. Briefly, RBP was incubated within the capillary precoated with an anti-RBP antibody. After washing out the unreacted RBP, the excessive reactive sites within the capillary were blocked with a blocking reagent (see Materials and Methods). Varying dilution of the conjugate was added to the capillary after washing the capillary with a detergent containing buffer. The conjugate was incubated overnight followed by washing to remove the unreacted conjugate. Finally, a chemiluminescent substrate was added to each capillary, and the chemiluminescence produced was measured using a PMT interfaced to a PC for data logging (see Materials and Methods).

Prior to initiating investigations on the RBP-conjugate system, the conjugate was purified using the G-25 column chromatography. Figure 2 shows the elution peak of the conjugate around fraction number 15. The observed peak corresponds to the absorption, by both the HRP and the all-*trans* retinoic acid in the conjugate. The major contribution was from HRP, which was verified independently by running HRP as a control. Although all-*trans* retinol is known to emit fluorescence following excitation at 280 nm, retinoic acid does not exhibit this effect. The peaks observed around fraction 30 may be attributed to the elution of unreacted retinoic acid during the synthesis of the conjugate.

Figure 3 shows a typical SPR recording of the binding of RBP to the surface of retinoic acid–HRP conjugate. In this case, the conjugate was immobilized on the surface of the chip, and the RBP (0.5 mg/mL) was injected into the flow stream. The change in the refractive index of the SPR signal, represented as response units (RU), between the point of injection and 10 min clearly indicates the binding of the RBP to the surface of the conjugate via the retinoic acid moiety. However, the initial change in the baseline (the vertical increase at 0 min) could be attributed to the bulk contribution from the buffer (~995 refractive index [RI]) and the nature of the buffer (i.e., ionic strength, dissolution of the solutes in the buffer, and RI of the buffer). The buffer in the flow stream was HEPES while the RBP was prepared in phosphate buffer.

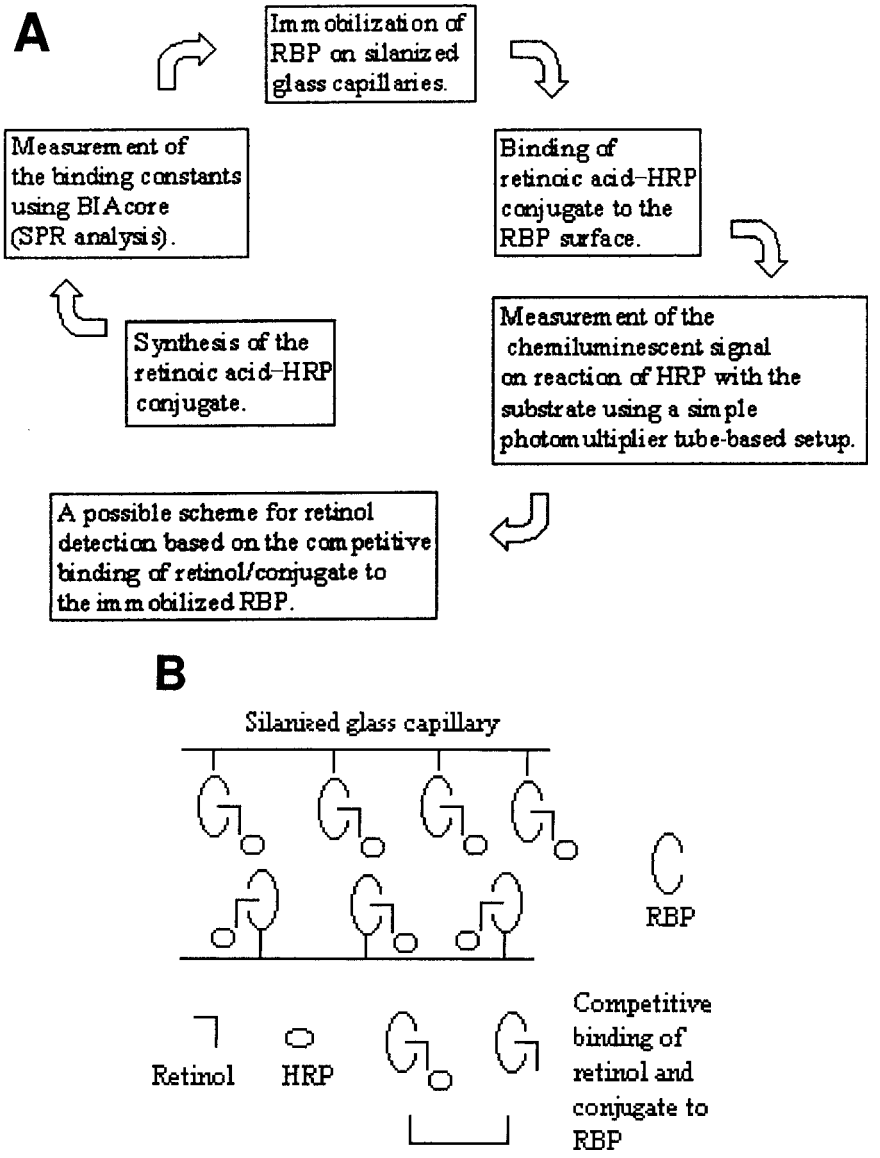

Fig. 1. **(A)** Overview of the procedure followed in the present investigations; **(B)** Schematic illustrating the steps.

The almost linear increase in the response is an indication of the binding of the RBP to the retinoic acid moiety within the conjugate. The binding of the β-barrel structure of RBP to the conjugate was through the β-ionone ring of the all-*trans* retinol, and the functional group (–COOH group) was outside this complex *(78)*. The almost flat region of the curve after 600 s was fitted to a simultaneous k_a/k_d kinetics model for calculating the binding constants, as shown in Table 1. The calculated dissociation constant k_d (2.46×10^{-7}) was in close agreement with those calculated earlier using fluorimet-

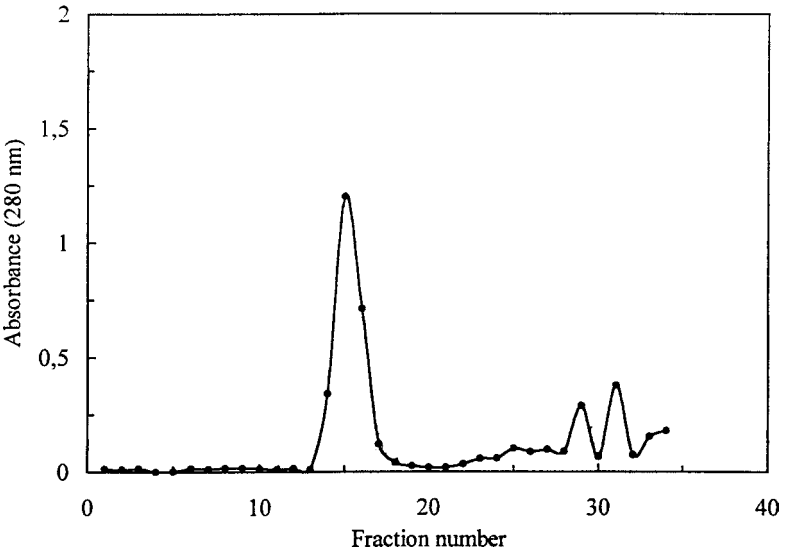

Fig. 2. Purification of the retinoic acid–HRP conjugate using a Sephadex G-25 packed column (40 × 1 cm). A 0.1 *M* $NaHCO_3$ buffer (pH 4.5) was used for the elution at a flow speed of 1 mL/min. The absorbance of the collected fractions was recorded at 280 nm on a Hewlett Packard spectrophotometer.

ric titration technique (i.e., 2.2×10^{-7}) *(79)*. This calculated value of the k_d was used to fit the linear rising portion of the curve (Fig. 3) using the kinetics separate k_a model, and the k_a value is shown in Table 1. The close agreement of our data with those reported earlier point to the correctness of the model we have employed. This motivated us to continue further to design a retinol biosensor based on its affinity for RBP.

It is important to mention that the reverse effect—the binding of the conjugate to RBP—was not observed (Fig. 4) on immobilization of the RBP on the chip and injection of the conjugate. This could be attributed to several reasons: the orientation of the RBP on the surface of the sensor chip (as the β-barrel of RBP structure is only accessible at one end *(78)*, the steric hindrance caused by the proximity of the HRP in the conjugate and the immobilized RBP, or insufficient time of contact (between the conjugate and the immobilized RBP) for a successful binding at a flow rate of 5 µL/min. Some of these possibilities will be addressed in future investigations.

The binding studies were followed by a scheme for designing a retinol biosensor. Thus, it was essential to immobilize the RBP and optimize the binding of the conjugate to the surface of the RBP. Figure 5 depicts the successful binding of the conjugate to the surface of the RBP and the consequent chemiluminescent signal generated on addition of the substrate (1 m*M* luminol and 0.34 µL/mL of 30% H_2O_2). As observed in the plot of chemiluminescent signal vs time (Fig. 4), there was an immediate rise in the signal owing to the reaction of the HRP with the H_2O_2. The initial rise (up to 72 s) was followed by decay owing to the depletion of the substrate

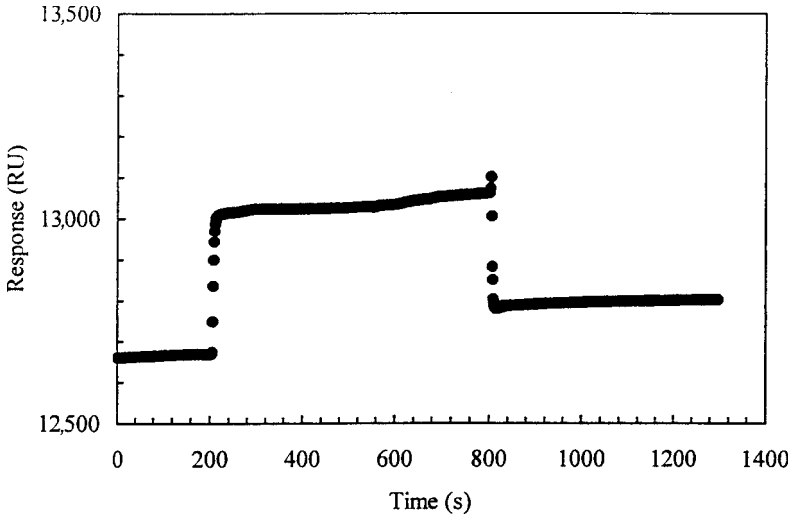

Fig. 3. Interaction of RBP with retinoic acid–HRP conjugate immobilized on the SPR chip surface. The signal was generated on injecting 50 µL of 0.5 mg/mL RBP (2.34×10^{-5} mol/L) in HEPES buffer. The binding was fitted to k_a/k_d kinetics using the BIAcore analysis software. a.u., arbitrary units.

Table 1
Various Parameters Reported by BIAcore Analysis Software for Binding of RBP to Immobilized Conjugate on SPR Chip Surface

Kinetic model	Concentration of analyte (M)	R_{max}	RI	Tk_d/Tk_a	k_d/k_a
k_a/k_d	2.34×10^{-5}	4.43×10^3	517	0.501	2.46×10^{-7}
k_a	2.34×10^{-5}	4.60×10^3	975	177	6.70×10^1

within the capillary. The initial rise followed a logarithmic relationship: $y = 683.87\ln(x) + 9170.7$ with a correlation coefficient of $R = 0.95$.

The signal decay initially between 72 and 400 s was rapid and then declined slowly up to 800 s. This decaying region of the chemiluminescent signal could be successfully employed for generating a calibration graph for sensing varying dilution of the conjugate. The decay was typically exponential in nature and followed the relationship $y = 10518e^{-0.0005x}$ with a correlation coefficient of $R = 0.9255$. The exponent $-0.0005x$ could be correlated to $-\Delta G/kT$ wherein ΔG is the change in free energy of the system, k is the Boltzmann constant, and T is the temperature (in Kelvin) during measurements. Several useful parameters may be obtained using this relationship for design of a chemiluminescent device from the biomolecular electronics point of view.

The kinetics of the stability of the chemiluminescent emission from the HRP/luminol reaction were analyzed by detecting the photons continuously for a period of about 18 min and plotting this as a function of

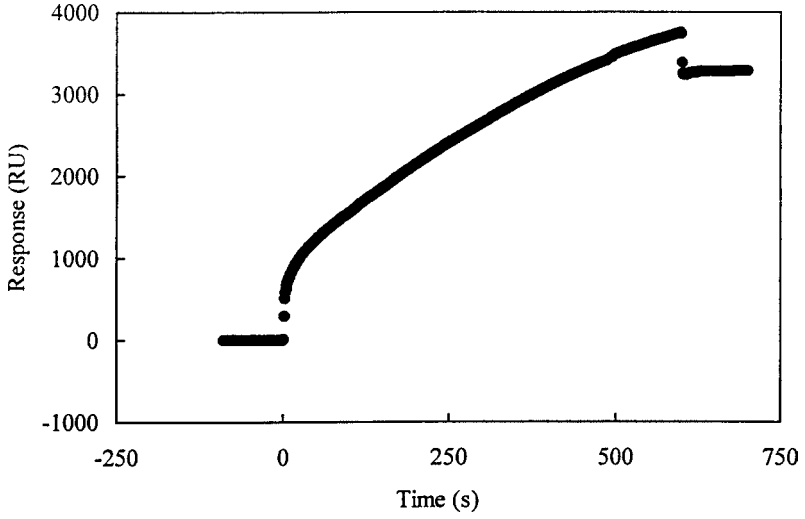

Fig. 4. Interaction of the conjugate with the immobilized RBP on the Biacore chip surface. Other details are similar to those in Fig. 3.

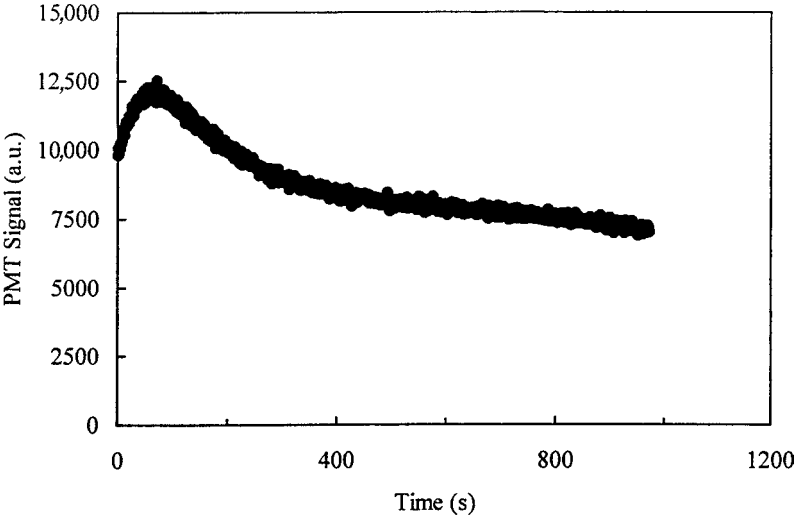

Fig. 5. Stability of the chemiluminescent signal recorded using a 10^{-5} dilution of the conjugate for a period of 1000 s. The signal was detected using a PMT at a bias voltage of 800 V.

PMT signal intensity (Fig. 5). The chemiluminescent signal from the HRP/luminol reaction rises and decays rapidly over this time course. The substrate becomes limiting only at the very high HRP concentrations. For practical purposes, a relatively short exposure time of 60 s was employed throughout these studies. However, the long-term stability of the signal indicates that it was possible to improve the sensitivity by a factor of 10 or more by simply changing the time of exposure (data not shown).

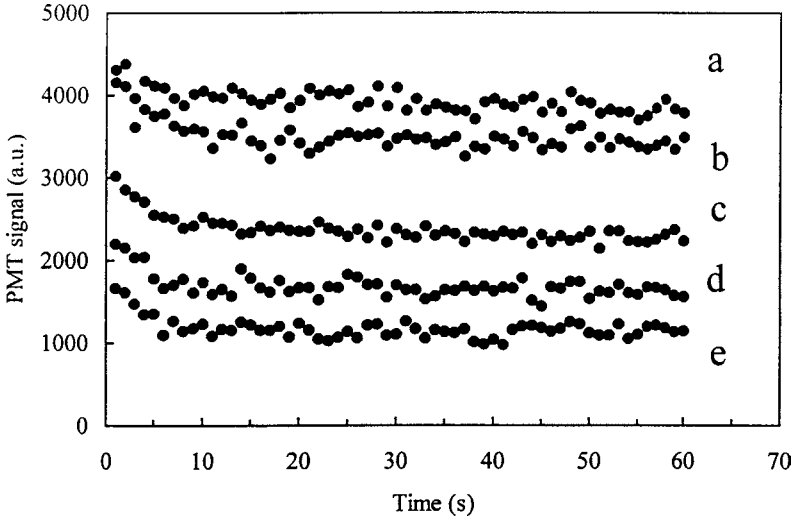

Fig. 6. Chemiluminescent signal recorded on a PMT detector for five different dilutions of the conjugate: (a) 10^{-1}, (b) 10^{0}, (c) 10^{-2}, (d) 10^{-3}, and (e) 10^{-4}. a.u., arbitrary units.

Figure 6 depicts the decay in the chemiluminescent signal obtained by varying dilutions of the conjugate from 1:1 to $1:10^{-4}$. In all instances, the signal followed a similar decay pattern. Note that in all these instances it was not possible to obtain the peak response, because the decay in the signal was quite rapid. Some of the signal was lost between filling the capillaries with the substrate solution and placing them in the capillary holder facing the detector (PMT). This time was on average about 10 s. Therefore, the 20-s value was chosen to plot the effect of conjugate dilution vs the chemiluminescent response.

Several variables were optimized prior to recording the signal. The distance between the capillary and the PMT detector was fixed to avoid inconsistency in the cone of light (issuing out of the capillary) that was incident on the detector. The presence of air bubbles in the capillary contributed to a noisy signal and was prevented during the measurements. The light produced by the chemiluminescent reaction had the freedom to travel along the capillary in either direction (i.e., toward the PMT detector or to the opposite end). Hence, the signal reported was the photons incident on the PMT after multiple reflections from the capillary walls. Last, the voltage set on the PMT during the measurement (800 V) had a major influence on the intensity of the signal. A decrease in the applied voltage resulted in a very weak signal. At this voltage (800 V), an exposure time of 60 s was sufficient for the measurements. The background signal was always corrected prior to signal measurement.

The reproducibility of the signal was also an important variable, and in order to verify the same, two capillaries with similar concentration of the conjugate (10^{-1} and 10^{-3}) were chosen. Figure 7 demonstrates the reproducibility of the signal for the same concentration of the conjugate (10^{-3}). The

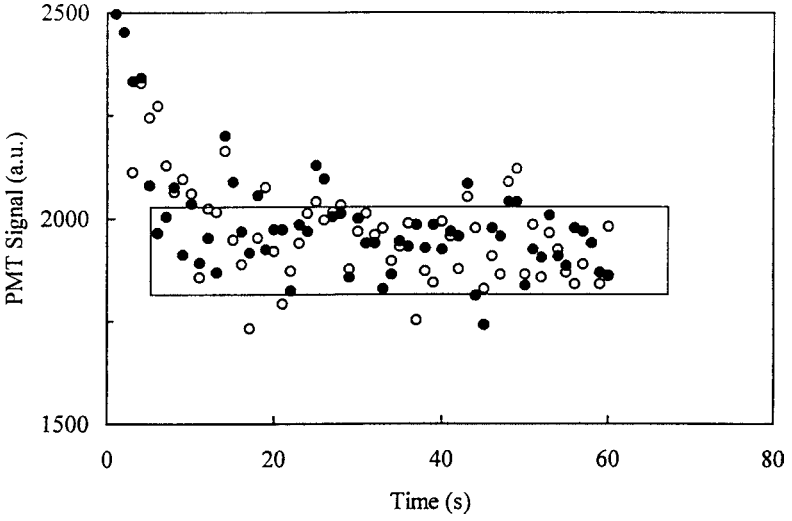

Fig. 7. Reproducibility of the chemiluminescent signal recorded on a PMT using a 10^{-3} dilution of the conjugate in a glass capillary with immobilized RBP. The area demarcated by the rectangle represents the dispersion in the signal in the two instances, calculated to be between 10 and 15%. a.u., arbitrary units.

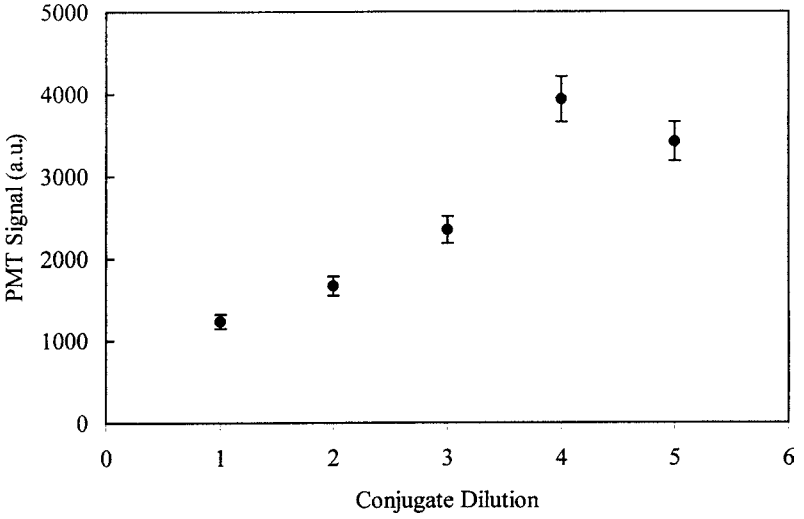

Fig. 8. Plot of the conjugate dilution vs the PMT reading at 20 s for 10^{0} (5), 10^{-1} (4), 10^{-2} (3), 10^{-3} (2), and 10^{-4} (1) dilution, respectively. a.u., arbitrary units.

points within the rectangular area show the scatter in the points for the two measurements. The reproducibility was found to be within 10–15%.

The conjugate concentration at which the specific binding of the conjugate to RBP was greatest with respect to the nonspecific binding of conjugate was sought (Fig. 8). A dilution series of conjugate was prepared in 0.1 *M* phosphate buffer (pH 7.4) and incubated with the immobilized RBP

(0.5 mg/mL, 50 µL per capillary). Dilution of conjugate stock solution was between 1:1 and $1:10^{-4}$. Results indicate that nonspecific conjugate binding was observed at all conjugate dilutions. The greatest ratio between the specific and nonspecific binding was observed at 1:10 conjugate dilution (equivalent to 2 µg of protein). The 2000-unit response difference was considered sufficient for sensor development. The overall response of 4000 units obtained at this conjugate dilution could be employed for measuring retinol concentration in real samples.

The 1:10 conjugate dilution gave the highest response and would be employed for subsequent experimentation. The decrease in response at higher conjugate concentrations was believed to be owing to "overloading" of the capillaries. Washing the capillaries after the binding step may result in removal of insufficiently adsorbed conjugate. High loading may also result in the creation of a diffusion barrier. In this regard, the commercial blocking reagent had a maximum effect on nonspecific binding sites, compared to BSA.

Conclusion

The present investigations suggest that the retinoic acid–HRP conjugate is stable and shows good binding with the immobilized RBP. The optical signal generated as compared to the blank was detectable on a simple PMT-based detection system. The chemiluminescent signal was stable at least for 18 min and provided a sufficient time interval for the assay to be optimized for fixed-time measurement of real samples. The method is sensitive and could be employed for designing a competitive receptor-binding assay for all-*trans* retinol based on optical biosensing. The ideas presented in these investigations could be successfully employed to understand and design specific biomolecular electronics devices based on all-*trans* retinol/retinal. The latter is an integral part of bacteriorhodopsin and is the most likely candidate for a workable light-addressable biomolecular electronics device in the coming century.

Acknowledgment

We gratefully acknowledge financial support from Swedish International Development Cooperation Agency (SIDA)/Sidas Department for Research Cooperation (SAREC) and The Swedish Research Council for Engineering Services (TFR).

References

1. Hong, F. T., ed. (1989), *Molecular Electronics: Biosensors and Biocomputers*, Plenum, NY.
2. Aviram, A. (1992), *Adv. Mater.* **4**, 441,442.
3. Mirkin, C. A. and Ratner, M. A. (1992), *Annu. Rev. Phys. Chem.* **43**, 719–754.
4. Friend, R. (1991), *Nature* **352**, 377–378.
5. Bryce, M. (1991), *Chem. Br.* **27,** 707–710.
6. Göpel, W. (1991), *Sens. Act. B* **4**, 7–21.

7. Bloor, D. (1991), *Physica Scripta* **T39**, 380–385.
8. Thomas, J. M. (1988), *Angew Chem. Int. Ed. Engl.* **27**, 1751–1754.
9. Strong, A. E. and Moore, B. D. (1999), *J. Mater. Chem.* **9**, 1097–1105.
10. Birge, R. (1995), *Sci. Am.* **3**, 90–95.
11. Ashkenazi, G., Ripoll, D. R., Lotan, N., and Scheraga, N. A. (1997), *Biosens. Bioelectron.* **12**, 85–95.
12. Lindsey, J. S. (1991), *N. J. Chem.* **15**, 153–180.
13. Kiselev, V. F. (1990), *Kinetika I Kataliz* **31**, 273–283.
14. Swalen, J. D. (1991), *Annu. Rev. Mater. Sci.* 21, 373–408.
15. Kelley, T. W., Granstrom, E. L., and Frisbie, C. D. (1999), *Adv. Mater.* **11**, 261–264.
16. McCarty, G. S. and Weiss, P. S. (1999), *Chem Rev.* **99**, 1983–1990.
17. Englebienne, P. (1999), *J. Mater. Chem.* **9**, 1043–1054.
18. Martin, C. R. and Mitchell, D. T. (1998), *Anal. Chem.* **70(9),** 322A–327A.
19. Dagani, R. (1999), *C EN* **77**, 6.
20. Weetall, H. H. (1996), *Biosens. Bioelectron.* **11**, 327–333.
21. Barr, J. (2000), Paper presented at: *The Fourteenth Annual Morton D. Sarver Lecture Series on Contact Lenses and the Cornea*, Morton D. Sarver Laboratory, Berkeley, CA.
22. Weetall, H. H., Robertson, B., Cullin, D., Brown, J., and Walch, M. (1993), *Biochim. Biophys. Acta* **1142**, 211–213.
23. Stupp, S. I. and Braun, P. V. (1997), *Science* **277**, 1242–1248.
24. Momeni, N., Ramanathan, K., Larsson, P. O., Danielsson, B., Bengmark, S., and Khayyami, M. (1999), *Anal. Chim. Acta* **387**, 21–27.
25. Hradsky, A., Bildstein, B., Schuler, N., Schottenberger, H., Jaitner, P., Ongania, K. H., Wurst, K., and Launay, J. P. (1997), *Organometallics* **16**, 392–402.
26. Weetall, H. H., Druzhko, A. B., Samuelson, L. A., deLera, A. R., and Alvarez, R. (1997), *Bioelectrochem. Bioenerg.* **44**, 37–43.
27. Sampath, S. and Lev, O. (1997), *Adv. Mater.* **9**, 410–413.
28. Wittstock, G., Hesse, R., and Schumann, W. (1997), *Electroanalysis* **9**, 746–750.
29. Silin, V., Weetall, H., and Vanderah, D. J. (1997), *J. Coll. Interf. Sci.* **185**, 94–103.
30. Doron, A., Portnoy, M., Dagan, M. L., Katz, E., and Willner, I. (1996), *J. Am. Chem. Soc.* **118**, 8937–8944.
31. Finklea, H. O. and Hanshew. D. D. (1992), *J. Am. Chem. Soc.* **114**, 3173–3181.
32. Ostrovsky, M. A. and Weetall, H. H. (1998), *Biosens. Bioelectron.* **13**, 61–65.
33. Stoeckenius, W. (1976), *Sci. Am.* **234**, 38–46.
34. Vo-Dinh, T. (1998), *Sens. Act. B* **51**, 52–59.
35. Yershov, G., Barsky, V., Belgovskiy, A., Kirillov, E., Kreindlin, E., Ivanov, I., Parinov, S., Guschin, D., Drobishev, A., Dubiley, S., and Mirzabekov, A. (1996), *Proc. Natl. Acad. Sci. USA* **93**, 4913–4918.
36. Rogers, K. R., Apostol, A., Madsen, S. J., and Spencer, C. W. (1999), *Anal. Chem.* **71**, 4423–4426.
37. Service, R. F. (1997), *Science* **277**, 1036,1037.
38. Hoheisel, J. D. (1997), *Tibtech* **15**, 465–469.
39. Storhoff, J. J. and Mirkin, C. A. (1999), *Chem. Rev.* **99**, 1849–1862.
40. Lee, I., Lee, J. W., and Greenbaum, E. (1997), *Phy. Rev. Lett.* **79**, 3294–3297.
41. Sigmund, E., Gribi, P., and Isenmann, G. (1993), *Appl. Surf. Sci.* **65/66**, 342–348.
42. Tsionsky, M. and Lev, O. (1995), *J. Electrochem. Soc.* **142**, 2132–2138.
43. Porteu, F., Palacin, S., Teixier, A. R., and Barraud, A. (1991), *Makromol. Chem. Macromol. Symp.* **46**, 37–45.
44. Alva, S., Phadke, R. S., and Govil, G. (1883), *Mol. Cryst. Liq. Cryst. Sci. Tech. Sect. A* **234**, 377–383.
45. He, G. S., Signorini, R., and Prasad, P. N. (1998), *Appl. Optics* **37**, 5720–5726.
46. Wang, X., Chen, J. I., Marturunkakul, S., Li, L., Kumar, J., and Tripathy, S. K. (1997), *Chem. Mater.* **9**, 45–50.
47. Chen, Z., Kaplan, D. L., Yang, K., Kumar, J., Marx, K. A., and Tripathy, S. K. (1997), *Appl. Optics* **36**, 1655–1659.
48. Daub, J., Beck, M., Knorr, A., and Spreitzer, H. (1996), *Pure Appl. Chem.* **68**, 1399–1404.

49. Samuelson, L. A., Miller, M., Galotti, D. M., Marx, K. A., Kumar, J., Tripathy, S. K., and Kaplan, D. L. (1992), *Langmuir* **8**, 604–608.
50. Casstevens, M. K., Samoc, M., Pfleger, J., and Prasad, P. N. (1990), *J. Chem. Phys.* **92**, 2019–2024.
51. Wilson, E. G. (1995), *Jpn. J. Appl. Phys.* **34**, 3775–3781.
52. Orczyk, M. E., Samoc, M., Swiatkiewicz, J., and Prasad, P. N. (1993), *J. Chem. Phys.* **98**, 2524–2533.
53. He, J. A., Samuelson, L., Li, L., Kumar, J., and Tripathy, S. K. (1998), *Langmuir* **14**, 1674–1679.
54. Bradley, D. C. (1991), *Chem. Br.* **27(8),** 719–723.
55. Pal, A. J., Östergård, T. P., Österbacka, R. M., Paloheimo, J., and Stubb, H. (1998), *IEEE J. Select Top. Quant. Electron.* **4**, 137–143.
56. Pandey, P. C., Upadhyay, B. C., Pandey, C. M. D., and Pathak, H. C. (1999), *Sens. Act. B* **56**, 112–120.
57. Xu, D., Martin, C., and Schulten, K. (1996), *Biophys. J.* **70**, 453–460.
58. Petrak, M. R. and Hong, F. T. (1998), *Bioelectron. Bioenerg.* **45**, 193–201.
59. Fuller, B. E., Okajima, T. L., and Hong, F. T. (1995), *Bioelectrochem. Bioenerg.* **37**, 109–124.
60. Lewis, A. and Del Priore, L. V. (1988), *Phys. Today* **41,** 38–46.
61. Drachev, et al. (1974), *Nature* **249**, 321.
62. Welsch, W., Klein, C., von Schickfus, M., and Hunklinger, S. (1996), *Anal. Chem.* **68**, 2000–2004.
63. Wolfbeis, O. S. (1991), in *Fiber Optic Chemical Sensors and Biosensors*, vol. 1, Wolfbeis, O. S., ed., CRC, Boca Raton, FL, pp. 1–23.
64. Walt, D. R. (1998), *Acc. Chem. Res.* **31**, 267–278.
65. Wolfbeis, O. S. (1996), *Tr. Anal. Chem.* **15**, 225–232.
66. Svitel, J., Dzgoev, A., Ramanathan, K., and Danielsson, B. (2000), *Biosens. Bioelectron.*, **15,** 411–415.
67. Guilbault, G. G. and Schmid, R. D. (1991), in *Electrochemical, Piezoelectric and Fiber-Optic Biosensors*, vol. 1, JAI, Greenwich, CT, pp. 257–289.
68. Narang, U., Gauger, P. R., and Ligler, F. S. (1997), *Anal. Chem.* **69**, 1961–1964.
69. Vo-Dinh, T. (1988), *Sens. Act. B* **51**, 52–59.
70. Lehn, J.-M. (1988), *Angew. Chem. Int. Ed. Engl.* **27**, 89.
71. Chen, J. C. and Conrad, M. (1997), *Neural Networks* **10**, 111–123.
72. Ayyagari, M. S., Pande, R., Kamtekar, S., Gao, H., Marx, K. A., Kumar, J., Tripathy, S. K., Akkara, J. A., and Kaplan, D. L. (1995), *Biotech. Bioeng.* **45**, 116–121.
73. Ben-David, O., Shafir, E., Gilath, I., Prior, Y., and Avnir, D. (1997), *Chem. Mater.* **9**, 2255–2257.
74. Dzgoev, A., Mecklenburg, M., Xie, B., Miyabayashi, A., Larsson, P.-O., and Danielsson, B. (1997), *Anal. Chim. Acta* **347**, 87–93.
75. Dzgoev, A., Gazaryan, I., Ramanathan, K., and Danielsson, B. (1999), *Anal. Chem.* **71**, 5258–5261.
76. Ramanathan, K., Jönsson, B. R., and Danielsson, B. (2000), *Anal. Chem.* **72,** 3443–3448.
77. Bovaird, J. H., Ngo, T. T., and Lenoff, H. M. (1982), *Clin. Chem.* **28**, 2423–2426.
78. Newcomer, M. E., Jones, T. A., Åqvist, J., Sundelin, J., Eriksson, U., Rask, L., and Peterson, P. A. (1984), *EMBO J.* **3**, 1451–1454.
79. Cogan, U., Kopelman, M., Mokady, S., and Shinitzky, M. (1976), *Eur. J. Biochem.* **65**, 71–78.