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Subpicomolar sensing of hydrogen peroxide with ovalbumin-embedded chitosan/polystyrene sulfonate multilayer membrane

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ABSTRACT

The use of ovalbumin (OVA)-immobilized layer-by-layer-assembled chitosan/polystyrene sulfonate membranes for the detection of hydrogen peroxide (H_2O_2) at subpicomolar levels is reported. The detection of mercuric chloride ($HgCl_2$) and potassium iodide (KI) was also investigated. While the detection limits of $HgCl_2$ and KI remained in the micromolar concentration range, H_2O_2 could be sensed to a remarkably lower range (subpicomolar). Analysis of fluorescence quenching data of OVA by H_2O_2 using Stern–Volmer plots revealed a static quenching mechanism with high Stern–Volmer quenching constant (9.10×10^{12} L mol^{-1}) and k (5.82×10^{21} L mol^{-1} s^{-1}). The possibility of the conformational transition of OVA in the immobilized state is discussed using steady-state and time-resolved spectroscopic techniques. The resulting increased accessibility of tryptophan residues together with the reversibility of the bilayer for the sensing of H_2O_2 is also illustrated.

Polyelectrolyte multilayer buildup is used extensively for the surface functionalization. The surface structure and composition can be well tuned on a nanometer scale by controlling the construction parameters. The buildup is generally realized by sequential adsorption of alternately charged polyelectrolytes from aqueous solution [1]. Biomacromolecules, dyes, and other particles can be immobilized to the film using this versatile film fabrication tool [2,3]. Self-assembled polyelectrolyte multilayer membranes often provide a safe electrostatic cage for the embedded biomolecules wherein their bioactivity is preserved [4,5]. Polyelectrolytes stabilize protein molecules so that their secondary structure is preserved, leading to protein-resistant/adhesive surfaces, paving the way to drug delivery applications [6,7]. The embedded biomolecules can show enhanced properties, as the matrix provides a very high ratio of surface area to volume [8]. This is exactly the desired property in sensing applications, as it would lead to high accessibility to analyte molecules. Biosensing is another area in which the inherent fluorescence property of embedded protein molecules can be utilized [9–11]. The intrinsic fluorescence of proteins arises from the presence of tyrosine, tryptophan, and phenylalanine [12]. Tryptophan is the prominent fluorescent amino acid, with a quantum yield of 0.13 in solution. The fluorescence of proteins is sensitive to the tryptophan microenvironment and can provide information regarding protein structure, dynamics, and protein folding. The immobilization of proteins during physical adsorption is susceptible to defolding [6,13]. This might lead to the exposure of tryptophan residues. So, two main challenges to utilizing protein fluorescence for sensing are to optimize the experimental variables so as to provide access to buried tryptophan residues and to increase the surface area of the immobilized matrix. From this perspective membranes are superior to other solid supports [8]. In addition to this, based on our previous experience working with chitosan/polystyrene sulfonate (CHI/PSS) multilayer membranes, the adsorption/sorption pattern of the protein to the multilayer is entirely based on the experimental variables [14]. So we anticipated that through careful control of the morphology of the CHI/PSS multilayer it may be possible to provide good access of analytes to tryptophan residues.

The detection of hydrogen peroxide at trace levels is important for biodiagnostic applications. Hydrogen peroxide (H_2O_2) is generated enzymatically for the indirect evaluation of clinically relevant molecules such as glucose and cholesterol [15,16]. Although it is involved in many redox processes in the body, an excess percentage always suggests the presence of high-risk-category diseases. Therefore, its detection at trace levels has always been a topic of interest. Traditionally electrochemical detection is the most preferred method [17,18]. The shortcomings of platinum electrodes

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for the electrochemical oxidation of H_2O_2 have been discussed widely and alternate routes were also proposed [19]. Redox and chemical mediators are also being used for the electrochemical detection, though prone to electrode fouling [20,21]. Biosensors based on horseradish peroxide (HRP) are the most common route for the detection of hydrogen peroxide. Ali et al. [22] realized H_2O_2 detection using an HRP-attached single nanochannel redox reaction with ABTS as the substrate.

In this report the fluorescence of ovalbumin (OVA) embedded in CHI/PSS nanolayers is used for sensing H_2O_2. The polyelectrolyte nanolayers are self-assembled on a polyethersulfone porous support through a pressure-driven ultrafiltration (UF) process as described earlier [14,23]. The fluorescence of OVA arises from three tryptophan residues: Trp 148, Trp 184, and Trp 267 [24]. Hydrogen peroxide is a polar (2.26 D), uncharged compound, known to quench tryptophan fluorescence by collisional process [12]. This property is used here to fabricate a highly efficient system for the detection of hydrogen peroxide in the subpicomolar range in solution. Herein, we report the first system that allows us to localize tryptophanyl residues to the surface with a high surface-area-to-volume ratio that permits exposure of otherwise buried tryptophan residues.

Materials and methods

Materials and reagents

Supor 450 microfiltration membranes (0.45-μm pore size, polyethersulfone) used as the supporting membrane were procured from Pall Life Sciences. Chitosan (medium M_w, 75–85% deacetylated) and poly(styrene sulfonic acid) sodium salt (M_w 200,000, 30 wt% in water) were acquired from Aldrich. Other reagents included ovalbumin (M_w 45,000), glycine–NaOH, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride, from SRL-Mumbai, and citric acid–sodium citrate from Ranbaxy-India. Sodium chloride, H_2O_2 (30%, w/v), mercuric chloride ($HgCl_2$), and potassium iodide (KI) from Merck-India were used as received.

Film deposition

The polyether sulfone supporting membrane was kept in Millipore water (18.2 MΩ) for 24 h. The clean membrane was alternately exposed to polycationic (CHI) and polyanionic (PSS) solutions. The pH of the polyelectrolyte solutions was adjusted to 1.7 (1.72 for CHI and 1.74 for PSS) using HCl. The supporting membrane was first immersed in 0.01 M chitosan solution in water (molarities of polyelectrolytes were taken with respect to repeating unit). The immersion time was 15 min. After immersion, the membrane was rinsed with 50 ml distilled water for 1 min. Then the membrane was immersed in 0.01 M PSS solution prepared in 0.1 M NaCl. The membrane was then rinsed with 50 ml distilled water. This results in one bilayer. The above steps were repeated until the required number (6 to 9) of bilayers was formed. The bilayered membrane always had PSS as the surface layer. The bilayered membranes were kept in water until ultrafiltration started. An OVA solution (0.25 mg/ml) at pH 4.5 was prepared in citrate buffer. The protein was immobilized to CHI/PSS nanolayers through UF (Amicon 8050-ultrafiltration cell, 10 psi, 400 rpm; Millipore) at room temperature (28–30 °C). All filtration experiments were conducted in triplicate. The error limit was within 2%. It was observed that, for good reproducibility, the membranes must be prepared under identical conditions.

Characterization of membranes

The membranes were washed and dried (at room temperature) for recording Fourier Transform Infra Red (FTIR) spectra, scanning electron microscopy (SEM), and Atomic Force Microscopy (AFM). For the confirmation of development of multilayers, sulfonate peak (SO_3^-) at 1033 cm^{-1} in the Attenuated Total Reflectance (ATR)–FTIR spectrum was used as a reference peak. The spectra were recorded on a PerkinElmer Spectrum 400 instrument with PIKE Gladi ATR attachment and DTGS detector on a diamond crystal with 15 scans at 4 cm^{-1} resolution using Spectrum 400 software (version 6.3) in the region 650–4000 cm^{-1}. The FTIR spectra of OVA and OVA in the presence of H_2O_2 were also recorded using an ATR attachment with a control buffer spectrum for each sample. To evaluate the protein immobilization, the amide I band at 1650 cm^{-1} was used as an indicator peak. Spectral manipulations were done using Origin software (version 6). The first and second derivative spectra were generated for picking the peaks and the spectrum was fitted using a Gaussian function. Time-dependent FTIR–ATR measurements (immediately after ultrafiltration to 3 h) were also carried out on a PerkinElmer instrument.

A scanning electron microscope (JSM-6390) was used for the characterization of the multilayer deposited on the bare membrane. Samples were fractured in liquid nitrogen and sputter coated with platinum using a Jeol JFC-1600 autofine coater. Images of layer-by-layer-coated and protein-immobilized surfaces were taken.

UV/Vis absorption studies were carried out using a UV-1700 Shimadzu spectrophotometer equipped with a 1-cm quartz cell at a wavelength range of 200–900 nm. Ovalbumin (0.025, 0.1, 0.25 mg/ml) in an aqueous solution of citrate buffer (pH 4.5) was used for steady-state absorption measurements. H_2O_2 stock solutions were prepared in water at a concentration of 1×10^{-1} M and the desired concentrations were prepared by successive dilution.

Fluorescence measurements

Steady-state fluorescence measurements were carried out using an LS 55 spectrofluorimeter at an excitation wavelength of 290 nm with emission spectral data in the range 300–500 nm. Fluorescence excitation spectra were recorded in the wavelength range 200–350 nm at an emission wavelength 340 nm. For solution-state fluorescence measurements ovalbumin (0.1 mg/ml) in an aqueous solution of citrate buffer (pH 4.5) was used. Quencher (H_2O_2, $HgCl_2$, and KI) stock solutions were prepared in water at a concentration of 1×10^{-1} M and desired concentrations were achieved by successive dilution. Membranes were cut and placed (diagonal dimension) in a 1-cm quartz cuvette (Sigma) filled with Millipore water into which varying concentrations of quenchers (H_2O_2, $HgCl_2$, and KI) were injected with the help of a microsyringe to get a net concentration ranging from micromolar to subpicomolar levels. The experimental setup is shown in Supplementary Fig. 1. The synchronous fluorescence spectra were also measured at an excitation wavelength of 290 nm keeping a constant wavelength difference of $\Delta\lambda$ 15 nm and $\Delta\lambda$ 60 nm for tyrosine and tryptophan residues, respectively.

The fluorescence quenching data were analyzed using the Stern–Volmer equation given by

$$F_0/F = 1 + k\tau_0[Q] = 1 + K_{SV}[Q],$$

where F_0 and F are the steady-state fluorescence intensities in the absence and in the presence of quencher. The Stern–Volmer quenching constant is given by K_{SV}, which measures the efficiency of quenching; k is the bimolecular quenching rate constant; τ_0 is the average lifetime of fluorophore without the quencher; and [Q] is the concentration of quencher.

Time-resolved fluorescence measurements

Time-resolved intensity decays of the OVA and OVA–CHI/PSS samples were measured using a Fluorocube Life Time System from