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'All-solid-state' electrochemistry of a protein-confined polymer electrolyte film

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Abstract

Interfacial redox behavior of a heme protein (hemoglobin) confined in a solid polymer electrolyte membrane, Nafion (a perfluoro sulfonic acid ionomer) is investigated using a unique 'all-solid-state' electrochemical methodology. The supple phase-separated structure of the polymer electrolyte membrane, with hydrophilic pools containing solvated protons and water molecules, is found to preserve the incorporated protein in its active form even in the solid-state, using UV–visible, Fluorescence (of Tryptophan and Tyrosine residues) and DRIFT (diffuse reflectance infrared Fourier transform) spectroscopy. More specifically, solid-state cyclic voltammetry and electrochemical impedance of the protein-incorporated polymer films reveal that the Fe^{2+}-form of the entrapped protein is found to bind molecular oxygen more strongly than the native protein. In the 'all-solid-state' methodology, as there is no need to dip the protein-modified electrode in a liquid electrolyte (like the conventional electrochemical methods), it offers an easier means to study a number of proteins in a variety of polymer matrices (even biomimetic assemblies). In addition, the results of the present investigation could find interesting application in a variety of research disciplines, in addition to its fundamental scientific interest, including protein biotechnology, pharmaceutical and biomimetic chemistry.

Keywords: Nafion; Hemoglobin; Redox behavior; All-solid-state; Cyclic voltammetry; Impedance

Metalloproteins constitute one of the most indispensable functional components of biological machinery, which span a wide range of utility depending on the nature of the metal center and the architecture of the encapsulating protein cage. The location (extracellular/intracellular), distribution and quarternary disposition of metalloproteins in biological systems are mainly decided based on the redox activity of the metal center [1]. Consequently, since the past few decades, understanding the redox properties and redox-induced conformational changes of metalloproteins has been a major focus of research. In this context, electrochemical techniques, owing to their versatility and simplicity, have often played a central role in delineating the intricacies of biological electron transfer processes [2].

However, achieving direct electron transfer between the electrode and the metalloprotein is often plagued by difficulties arising from the inaccessibility of the metal-containing prosthetic group deeply buried inside the protein cage. This problem is normally tackled either by electrochemical investigations of synthetic complexes mimicking the active site of the protein [3] or by modifying the electrode surface to anchor the native protein in addition to spectro-electrochemical methods [4]. Immobilizing the native metalloprotein on the electrode surface using a thin polymer film is the most common, mainly due to the ease of experimentation and interpretation [5], among the various protocols like interfacing the protein to the electrode surface using a self-assembled monolayer [6] and adsorbing the protein in

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a favorable configuration directly on a pyrolytic graphite electrode—popularly known as protein film voltammetry [7]. The polymer used for immobilization could be a redox active hydrogel [8], polymer electrolyte [9] or a multi-component surfactant. The polymer electrolyte, Nafion (a perfluoro sulfonic acid polyelectrolyte [10]) is proved to be one of the best polymers of choice for entrapping biomolecules due to its phase-separated structure with hydrophilic pools marked by hydrophobic domains, to preserve the biophysics of the protein molecules confining them in an environment similar to that in living cells in addition to its excellent proton conductivity ($\sim$0.1 S cm^{-1}). Nafion is also recognized well for its biocompatibility [11], especially due to the presence of sulfonate groups, known to be a good protein-interactive functionality [12]. Although the latter methodology offers a convenient and reproducible means for achieving direct electrochemistry of proteins, the interference of contributions from the film/solution interface (formed when the film-modified electrode is dipped in a liquid electrolyte) could not be neglected. For example, restrictions imposed by electro-neutrality requirements, often leads to ion ingress/egress at the polymer film/solution interface [13]. As a result, the effect of the polymer (Nafion) matrix on the stability and dynamics of the incorporated biomolecule is often neglected.

In this context, electrochemical investigations in an 'all-solid-state' configuration, (achieved by introducing the metalloprotein into the hydrophilic pools of a polymer 'electrolyte' membrane cast over the respective electrodes, without dipping the film further in a liquid electrolyte), offers a number of advantages. For example, the 'all-solid-state' methodology is a unique way to understand the interactions between the protein and the polymer matrix, in addition to advantages like the ease of sampling, experimentation under different gas atmospheres and more specifically, characterization of the 'nature' of protein/polymer interfaces. For instance, in our earlier report, we demonstrated the efficacy of the methodology in understanding the redox compatibility of polymer electrolytes, wherein we have identified self-electron exchange of cyanoferrate (III) complexes entrapped in a Nafion matrix [14]. The importance of such solid-state techniques could also be realized from a recent report by Navati et al., where they have incorporated heme proteins in sugar-derived glasses for tuning the protein function towards applications in protein-based solid-state devices [15].

In this paper, a detailed study of the redox behavior of the respiratory protein, hemoglobin confined in a solid film of Nafion (a polymer electrolyte) has been carried out using a unique 'all-solid-state electrochemistry' supplemented by spectroscopic characterization. Nafion and hemoglobin are chosen for the study because of their well-characterized electrochemical behavior, so that the significance of the methodology could be easily appreciated. However, the basic principles could be easily extended to a variety of systems addressing more complicated research problems related to diverse disciplines including biotechnology and polymer science.

Materials and methods

Chemicals

A 5 wt% solution of Nafion (eq. wt. 1000) in a mixture of water and lower aliphatic alcohols was procured from Aldrich chemicals. Hemoglobin from bovine blood (in Methemoglobin form) (M.Wt. 64,500) was purchased from Sigma chemicals. Sodium hydroxide (Analytical Reagent grade) was obtained from Ranbaxy Fine Chemicals Ltd., New Delhi, India and Sodium dihydrogen phosphate ('Excelar' grade) from Qualigens Fine Chemicals Ltd., Mumbai, India. All solutions were prepared using deionized water (Millipore 18 MΩ). N_2, O_2 gases were of INOX grade (99.9% purity) and SO_2 (1% in N_2) was obtained from Deluxe trading company, Pune, India.

Methods

Electrochemistry. All-solid-state voltammetry was carried out with a specially designed, homemade three-electrode cell made of Teflon, described in our earlier report [22] with a Pt wire of diameter 140 μm as working electrode, another Pt wire of diameter 0.5 mm as counter electrode and an Ag/AgCl reference electrode obtained by chloridation of a 0.5 mm dia. Ag wire. A thin film of Nafion (thickness 0.2 mm) cast over the electrodes by using a 5 wt% commercial dispersion, acted as the electrolyte. Then 70 μl of Methemoglobin (MetHb) solution (1 mg/ml) in 50 mM phosphate buffer (made using Sodium dihydrogen phosphate and Sodium hydroxide; pH 7), purified by filtering through a Whatmann 40 filter paper, was added to the film and enough time was given for the species to be absorbed in the membrane, confirmed by open circuit potential–time transients and the appearance of redox peaks in cyclic voltammograms (ESI Fig. 1). Electrochemical experiments were performed using a Solartron SI 1287 Electrochemical interface equipped with a 1255B Frequency Response Analyzer at room temperature (25 ± 0.5 °C) in a N_2 atmosphere (humidified to retain the water content of Nafion, relative humidity 30%) unless otherwise specified and the Nafion–Hb films were stored at 4 °C when not under study. The cell resistance was 70–75 Ω and was electronically compensated to 10–15 Ω by a positive feedback circuitry available with the instrument. Relative humidity was measured using a Metravi HT-3005 humidity sensor.

Spectroscopy. UV–visible spectra were recorded using a Varian Cary 50 double beam spectrophotometer with 1 nm resolution, with a MetHb concentration of 0.25 mg/ml in 50 mM phosphate buffer (pH 7) at a scan speed of 288 nm/min. Samples for solid-state UV–vis spectroscopy were prepared by casting a thin film (thickness 0.1 mm approx.) of 2 wt% Nafion on a quartz slide followed by the addition of 20 μl of Methemoglobin (MetHb) solution [1 mg/ml in 50 mM phosphate buffer, pH 7]. Fluorescence emission scans and lifetime analysis were recorded using a FLS 900 (Edinburgh) instrument operated at a resolution of 7 nm at 18 °C, with a MetHb concentration of 0.25 mg/ml in 50 mM phosphate buffer (pH 7) before and after the addition of 10 μl of 5 wt% Nafion. IR spectra of the solid Nafion–Hb films were recorded using Perkin–Elmer FTIR Spectrum One Spectrophotometer operated in the Diffuse Reflectance mode at a resolution of 4 cm^{-1}.

Results and discussion

Cyclic voltammetry

A schematic representation of hemoglobin (Hb) molecules entrapped inside the hydrophilic pools of water clusters marked by the pendent sulfonate groups of the polymer electrolyte is shown in the supplementary material (ESI Fig. 2).

Fig. 1 shows the cyclic voltammograms of MetHb-entrapped Nafion film ($C_{Hb} = 5.6 \times 10^{-7}$ mol cm^{-3}) under N_2 atmosphere (relative humidity: 30%) at room temperature with a set of redox peaks positioned at $E_{1/2} = -0.057$ V vs Ag/AgCl ($E_{1/2} = (E_{pa} + E_{pc})/2$, where E_{pa} and E_{pc} are the anodic and cathodic peak potentials, respectively) due to the $Fe^{2+/3+}$–heme couple [16–18]. The appearance of redox peaks for the heme couple in the solid polymer electrolyte indicates that the heme protein is present in an environment with sufficient hydration to preserve its biological function, provided by water molecules associated with the hydrophilic pools of the polymer. In fact, the presence of enough amount of water in the matrix (i.e., humidification) is crucial for the functioning of the polymer as 'electrolyte', which could be ensured by a qualitative comparison of the cyclic voltammograms of completely dry and humidified Nafion films (ESI Fig. 3). In addition, the difference in redox potential observed in the present case from that reported for hemin [19,20] (which is closely related to heme except for a few differences in substituents) indicates that the prosthetic group remains in tact in its protein cage and MetHb is considerably stable in the solid polymer electrolyte film.

Further, the linear nature of the plot of anodic peak current (I_{pa}) against the square root of potential scan rate indicates that diffusion is the principal mode for transporting the Hb molecules from the bulk electrolyte to the electrode surface. Accordingly, the diffusion coefficient of Hb in the solid polymer electrolyte matrix is calculated to be 3.04×10^{-9} cm^2/s using the expression for quasi-reversible systems [21],

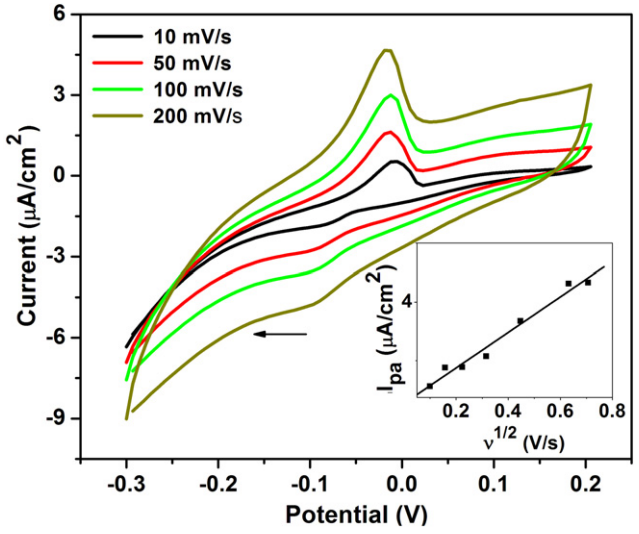

Fig. 1. Cyclic voltammograms of Nafion/Hb films in the solid-state at various scan rates ranging from 0.01 to 0.5 V/s with 150 μm Pt wire as the working electrode, a Pt counter electrode and AgCl coated Ag wire as reference electrode. The cell briefly consists of a cylindrical Teflon block with a shallow trough housing the working, counter and the reference electrodes over which the polymer film is cast and electrochemical experiments are carried out in a humidified N_2 atmosphere using a special provision available in the cell (cell design is available in Ref. [14]). The voltammograms correspond to the second potential cycle and no changes were observed in subsequent cycling, indicating the absence of any coupled chemical reaction.

$$i_p = (2.69 \times 10^5) n^{3/2} A D_o^{1/2} C_o^* v^{1/2} \quad (1)$$

where i_p is the peak current (after subtracting the non-faradaic contribution); 'n' is the number of electrons involved in the redox process (taken as 4 in the present case—one electron from each of the four heme groups in hemoglobin assuming that there is no intramolecular interaction between the heme groups); A is the area of the electrode; D_o is the diffusion coefficient of the electroactive species (cm^2/s); C_o^* is the bulk concentration and v is the potential scan rate. The diffusion coefficient of hemoglobin in the polymer matrix is found to be of the same order as that reported for hemoglobin solutions [22]. More significantly, the solid-state CVs show a quasi-reversible behavior (peak separation varies with scan rate; $I_{p,anodic} \neq I_{p,cathodic}$) in contrast to the reversible CVs often observed in the case of Electrode/Nafion film/liquid electrolyte systems [16]. In addition, the cathodic current is found to be lower than the anodic current despite the initial addition of the oxidized form, i.e., MetHb. Though in our earlier report, ferricyanide ($[Fe(CN)_6]^{3-}$) species incorporated in Nafion films was found to undergo spontaneous reduction due to specific chemical interactions with the polymer matrix [14], such a situation is chemically unlikely in the case of hemoglobin. Hence the above voltammetric features could be attributed to the differences in transport rates between oxidized (MetHb) and native (Hb) forms of the protein, in the solid polymer matrix, as further clarified by electrochemical impedance studies discussed in "Electrochemical impedance" section.

Oxygen uptake/release studies

Having analyzed the redox behavior of hemoglobin entrapped inside the polymer electrolyte membrane, it would be more interesting to understand its oxygen-binding characteristics, which is the biological role of the protein. Accordingly, the oxygen uptake/release characteristics of the polymer-entrapped protein are shown in Fig. 2. The anodic

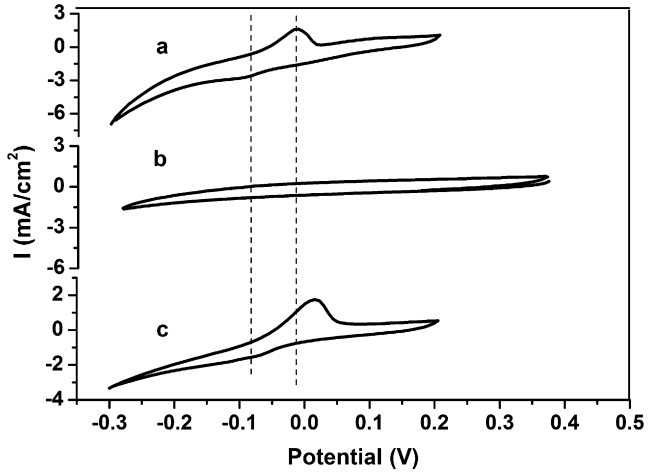

Fig. 2. Cyclic voltammograms of Nafion/Hb films in the solid-state in (a) N_2; (b) after passing O_2; (c) rejuvenation of the anodic peak after 2 days in N_2 at a scan rate of 25 mV/s with 150 μm Pt wire as the working electrode, a Pt counter electrode and AgCl coated Ag wire as reference electrode.

peak in the CV is found to vanish in O_2 atmosphere, which could be attributed to the formation of oxyhemoglobin, difficult to be oxidized electrochemically to the Fe^{3+} form. Another possibility could be that the anodic peak shifts beyond the experimental potential window accessible with the present system, because of differences in redox potentials arising from the chemical transformation of Fe (II) to the Fe (II)–O_2 state [23]. On the other hand, the cathodic peak could hardly be judged as vanishing owing to its insignificant shape. Surprisingly, in contradiction to earlier reports in which hemoglobin is found to release bound-oxygen instantaneously when the partial pressure of oxygen comes down in the atmosphere, the heme groups are found to bind oxygen very strongly in Nafion matrix, as evident from a slow rejuvenation of the anodic peak after 2 days (Fig. 2). This could be possible because oxygen binding, unlike electron exchange process, is known to be sensitive to steric constraints [24] that vary considerably when the protein is entrapped in a solid polymer membrane. In addition, the anodic peak is found to shift to a more positive value by 30 mV, indicating only a partial recovery of the deoxy state even after 2 days.

Further, we tried to trigger the release of bound oxygen by performing CV under SO_2 atmosphere, which is expected to bind to the metal center. However, the anodic peak did not reappear even after holding the potential at −0.4 V for 30 min. On the contrary, an instantaneous release of oxygen shown by a rejuvenation of the anodic peak, again with a shift to a more positive potential by 43 mV, is observed when a small amount (5 μl) of 0.2 M NaOH solution is added to the membrane (ESI Fig. 4).

Electrochemical impedance

A further examination of the system in different gas atmospheres using the electrochemical impedance technique is shown in Fig. 3. The Nyquist plots (imaginary vs real part of impedance) illustrate a semicircle in the high frequency region indicating the presence of a charge-transfer limited process followed by a linear behavior in the low frequency region corresponding to the diffusion of the species to the electrode surface. The high frequency part (100 kHz to 100 Hz) was fitted with a simple Randles equivalent circuit with a constant phase element considering the shift in the center of the semicircle found in the high frequency part (ESI Fig. 5) to yield the following trend in electron transfer resistance (indicative of the feasibility of hemoglobin oxidation).

$$R_{alkaline} < R_{N_2} < R_{O_2} < R_{SO_2}$$

Though this is in agreement with the appearance and disappearance of heme oxidation peaks in CV under respective conditions, the higher R_{ct} observed in SO_2 atmosphere and a more negative open circuit voltage shown in the OCV–time profiles (ESI Fig. 6) reveal a striking evidence for the involvement of sulfonate groups of the polymer in the redox equilibrium of hemoglobin. A subsequent

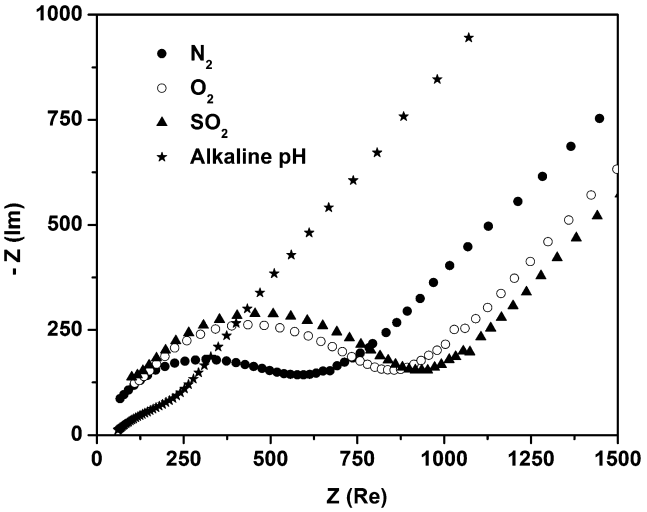

Fig. 3. Electrochemical impedance Nyquist plots ($Impedance_{real}$, Z(Re) vs $Impedance_{imaginary}$ Z(Im)) for Nafion/Hb films successively under N_2, O_2, SO_2 atmospheres and after addition of 10 μl of 0.2 M NaOH solution to the film with 150 μm Pt wire as the working electrode, a Pt counter electrode and AgCl coated Ag wire as reference electrode.

calculation based on the electron-transfer resistance values in N_2 atmosphere yields an apparent heterogenous rate constant of 0.52 cm/s, which is closely related to the reported values [25]. Surprisingly, a further analysis of the low frequency part of the impedance plots (ESI Fig. 7), results in a diffusion coefficient value of 4.82×10^{-6} cm^2/s, which is about three orders of magnitude higher than that estimated using cyclic voltammetry. This discrepancy could be arising from the assumption made while simplifying the expressions for Warburg impedance, that the diffusion coefficients of Hb and MetHb are equal. This clearly indicates that the transport rates of Hb and MetHb in the polymer electrolyte film are entirely different which could also form the reason behind the quasi-reversible nature of the cyclic voltammograms.

Spectroscopic investigations

The stability of the heme protein in the polymer matrix is verified using complementary spectroscopic tools viz., UV–visible, Fluorescence and FTIR spectroscopy. In Fig. 4 the UV–vis spectrum of MetHb-entrapped Nafion film cast on a quartz slide is compared with that of the MetHb solution in a pH 7, 50 mM phosphate buffer in Fig. 4. The former shows a Soret band at 406 nm similar to that of the MetHb solution indicating that the heme environment in the polymeric matrix is not very much different from that in the solution. However, the differences in peak shapes and lower signal-to-noise ratio between the solid-state and liquid-state spectra could be attributed to restricted degrees of freedom and slower relaxation processes in the 100 μm thick polymer film used for the spectral studies. Further, the fluorescence emission scans of MetHb solutions (1 mg/ml in pH 7, 50 mM phosphate buffer) after the addition of Nafion (10 μl of 1 wt% resin in

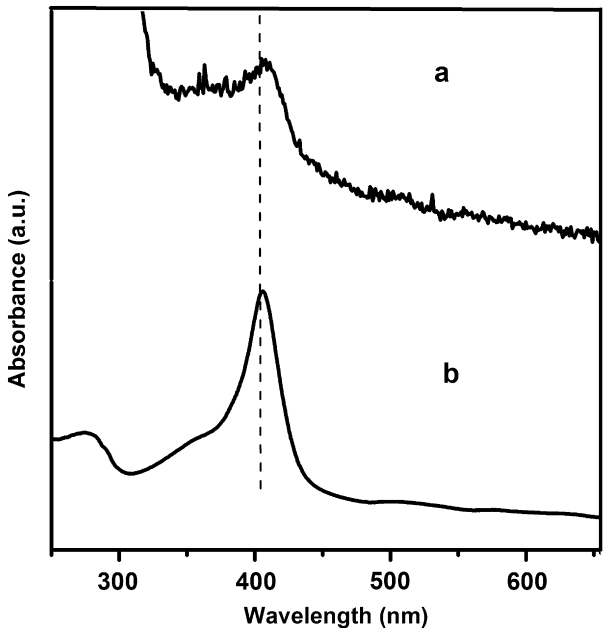

Fig. 4. UV–visible spectra of Nafion/Hb system (a) in the solid-state with a film recast on a quartz slide and (b) in the solution state with Hb dissolved in a 50 mM phosphate buffer (pH 7) containing 10 μl of 5 wt% Nafion resin.

1 ml of MetHb solution) at an excitation wavelength of 280 nm (which corresponds to Tyr fluorophores) and 295 nm (corresponding to that of Tryptophan [Trp] residues) result in emission maxima at 326 and 327 nm, respectively (ESI Fig. 8), indicating the retention of protein secondary structure in the polymer matrix. Also the amide I and amide II bands (1680 and $1564 \pm 4\ cm^{-1}$, respectively) [18], characteristic of the protein's conformation, are clearly visible in the FTIR spectra of the Nafion–Hb film compared to that of a pristine recast Nafion film (ESI Fig. 9) signifying that there is no major structural change/denaturation of the protein chains after incorporating in the polymer matrix.

Thus the efficacy of the 'all-solid-state' methodology in understanding protein electrochemistry is demonstrated in this communication with the help of hemoglobin and Nafion as the protein and polymer matrix, respectively. However, the methodology could be easily extended to a wide range of systems and the results of this investigation would have interesting implications in diverse disciplines of research including protein biotechnology and redox protein electrochemistry.

Summary and conclusions

Interfacial redox behavior of the respiratory redox protein hemoglobin confined in a solid polymer electrolyte membrane of Nafion has been investigated using an 'all-solid-state' methodology. The stability of the protein in the solid films has been confirmed using UV–visible, Fluorescence and FTIR spectroscopy. Interestingly, the Fe^{2+}-form of the polymer-confined protein is found to bind molecular oxygen more strongly than the native protein, which could be due to some specific interactions offered by the phase-separated structure of the polymer electrolyte. Hemoglobin and Nafion have been chosen for the present studies to demonstrate the all-solid-state methodology, as they are well-characterized by other electrochemical techniques, thus allowing easier comparison of the present results with the reported ones. However, this could be readily extended to any combination of redox protein–polymer electrolytes thereby providing a broader application in diverse fields like protein-based biotechnology and pharmaceutical industry.

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Appendix A. Supplementary data

Open circuit voltage vs time plots for Nafion–Hb and pristine Nafion films, effect of water content on CV, electrochemical impedance-equivalent circuit, Warburg impedance and diffusion coefficient calculation, fluorescence emission spectra of Hb solution with and without Nafion.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.09.118.

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