

Characterization of enzyme–polymer interaction using fluorescence†

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The study of the interaction between free fluorophores or those attached to the enzyme (alcohol dehydrogenase) with polymer (Eastman AQ 55) using polarization and confocal microscopy has been performed. The potential of fluorescence to visualize the polymer structure as well as the distribution of incorporated species has been demonstrated in the case of Eastman AQ[®] 55 and Nafion[®] polymer films. Negatively and positively charged probes were found to exhibit different distribution patterns, suggesting an electrostatic contribution to the probe–polymer interaction. In the case of enzyme, however, the charge introduced by the probe tagging did not alter the interaction or distribution of the enzyme in the polymer, suggesting that the distribution obtained for the tagged enzyme correctly represents the distribution of non-tagged enzyme.

Polymer modified electrodes that entrap enzymes and redox mediators have been extensively developed as electrochemical sensors^{1,2} and biofuel cell anodes.^{3–5} Polysulfonic electrolytes such as Nafion[®] and Eastman AQ[®] have been used as immobilization films because of their known ability to incorporate large, hydrophobic cations, and to exclude negatively charged species.⁶ To characterize these enzyme–polymer films various methods such as scanning electron microscopy,⁷ infrared spectroscopy, X-ray diffraction,⁸ AFM,⁹ and small angle neutron scattering¹⁰ have been applied. However, these techniques have not been successful in visualization of the three-dimensional distribution of enzyme or in explaining enzyme interaction with the polymer matrix. Recent advances in fluorescence instrumentation, coupled with the large number of commercially available probes, suggest that fluorescence can now be applied in this regard. Despite its abundant use in biochemistry and some applications in biocatalysis,^{11,12} the full potential of fluorescence as a characterization technique for enzyme–polymer systems has not yet been reported.

Herein, we demonstrate the possibility of using fluorescence to study the interaction between the polymer (Eastman AQ[®] 55) and incorporated species (fluorophores, enzymes, *etc.*) as well as the spatial distribution of the latter in the polymer matrix.

The impact of electrostatic interactions between the polymer and the fluorescent probes of varied charge was investigated using fluorescence polarization¹³ as the measure of the rotational

diffusion of the probe. As can be seen in Fig. 1, the polarization of the dyes carrying positively charged groups (rhodamine, TAMRA[®], 7-nitrobenzofurazan (NBD)) increased substantially in the presence of the polymer, while no difference in polarization of the negatively charged probe (Alexa[®] 488) was observed.

Considering the presence of negatively charged sulfonic groups in the polymer, this result suggests that electrostatic forces significantly contribute to the probe–polymer interaction in the solution. The titration of the probes with polymer further revealed that the binding of the probe was proportional to the extent of positive charge it carried. Thus, Eastman AQ[®] bound the most positively charged rhodamine 44 times more effectively than TAMRA[®], which in its turn was bound better than NBD. Similar results were obtained by Guan *et al.* while studying the absorption of pentamethine indocarbocyanines by Nafion[®].¹⁴ These authors reported that no absorption of negatively charged species was observed unless inorganic salt (NaCl) was present to neutralize electrostatic interactions.¹⁴ In the present study the presence of 50 mM buffer led to slightly lower polarization of rhodamine in Eastman AQ[®] containing solution.

To see if the difference in charge affected the distribution of probe within the polymer, confocal laser scanning microscopy was used to visualize the distribution of probes in thin films cast from probe–polymer mixtures. The images revealed concentration of fluorescent material along the edges of the droplet. It was also found that circular wells were formed within the middle of each droplet. The images of the droplet formed by mixing negatively (fluorescein) and positively (rhodamine) charged probes with Eastman AQ[®] are presented in Fig. 2.

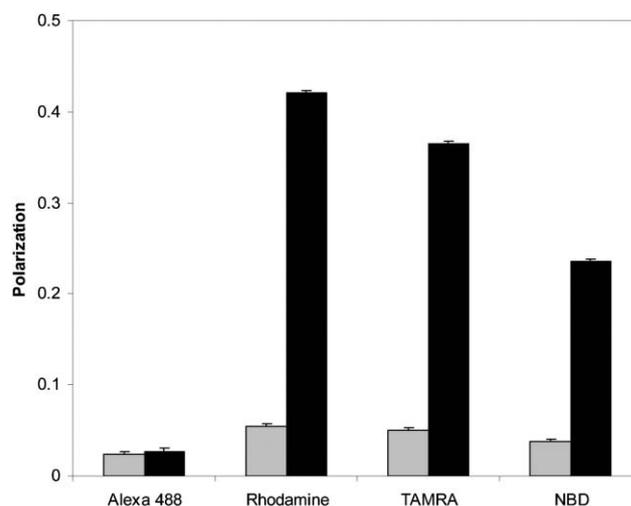


Fig. 1 Polarization of the free probes in water (grey) and Eastman AQ[®] 55 containing solutions (black).

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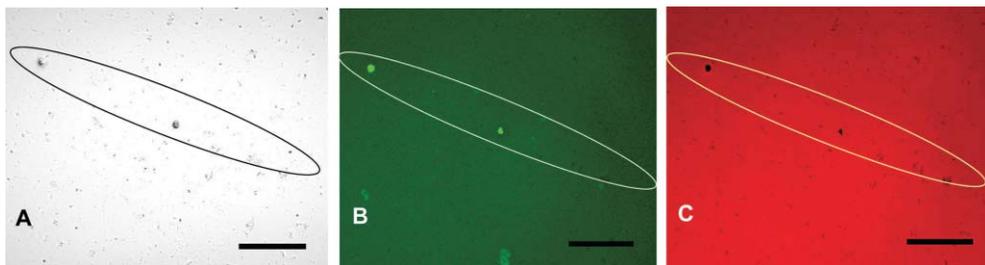


Fig. 2 Confocal images of the Eastman AQ[®] polymer containing mixtures of fluorescein and rhodamine. The images were obtained using the following excitation wavelengths: A—white light; B—488 nm; C—510 nm illumination. The bars on the images are 50 μm.

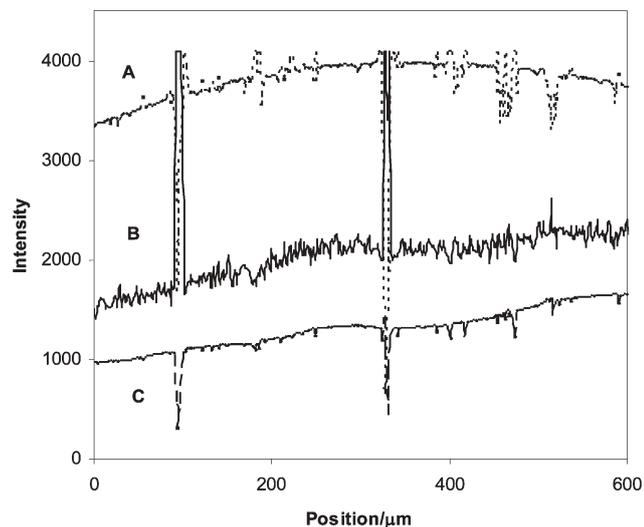


Fig. 3 Intensity profiles of the circled areas in Fig. 2. Line A—Fig. 2A, line B—Fig. 2B, line C—Fig. 2C.

Two wells can be seen on the surface of the polymer while using white light illumination (Fig. 2A). As can be seen (in the circled area), those wells appear as bright spots on the fluorescein image (Fig. 2B) and as dark spots in the rhodamine image (Fig. 2C). The intensity profiles of the line going through the wells (circled areas in Fig. 2) are presented in Fig. 3. Negative peaks corresponding to the white light image (Fig. 3, line A) indicate that the observed species were depressions or wells. The high positive peaks on the intensity graph (Fig. 3, line B) confirm concentration of fluorescein in the wells, while negative peaks corresponding to the rhodamine

picture (Fig. 3, line C) show that rhodamine was not concentrated in the wells.

To test whether these results applied to tagged enzyme, several probes (Alexa[®] 488, NBD, TAMRA) were covalently attached to the enzyme and the polarization measurements were conducted under the same conditions as with free probes. In this case, different probes were attached to alcohol dehydrogenase, which bears an overall negative charge at pH 6–7. Although not shown, we found that the polarization of the probe attached to the enzyme was not affected by the presence of the polymer in solution for all probes tested. This result suggests that the probe charge does not alter the interaction of the polymer with the enzyme and the distribution of the labeled enzyme coincides with that of the unlabeled enzyme.

To verify that the attachment of a probe to the enzyme did not effect its final distribution within the polymer film, the distribution of the tagged enzyme was also visualized using confocal laser scanning microscopy. A representative image of this film is shown in Fig. 4A along with a three-dimensional reconstruction of the film in Fig. 4B. As can be seen in Fig. 4 (A, B), Alexa[®] 488 labeled enzyme was concentrated in the channels in a similar manner to the fluorescein (Fig. 2B). The concentration of the tagged enzyme in small segregates was attributed to the repulsion between the negatively charged polymer sulfonic group and the enzyme, which bears an overall negative charge at neutral pH. Similar distributions were obtained for NBD- and TAMRA-labeled alcohol dehydrogenase (data not shown).

To investigate if the observed distribution of enzyme was dependent on the polymer type, Eastman AQ[®] 55 was substituted with Nafion[®]. A confocal image of alcohol dehydrogenase tagged with Alexa[®] 488 that is entrapped within a Nafion[®] polymer is

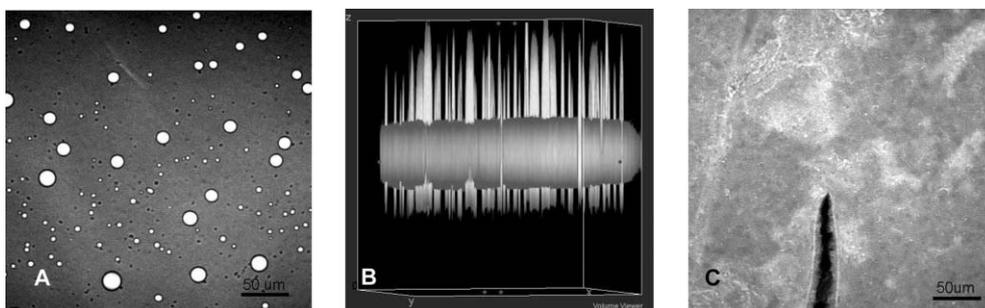


Fig. 4 Images of the distribution of alcohol dehydrogenase tagged with Alexa[®] 488 entrapped in Eastman AQ[®] 55 polymer film: (A) confocal image; (B) three-dimensional reconstruction of the stack of confocal images, (C) Alexa[®] 488 tagged enzyme in Nafion[®].

presented in Fig. 4C. As can be seen, there exists a substantial difference in the distribution of the tagged enzyme as compared to Eastman AQ[®] (Fig. 4A). The distribution within Nafion was found to be less homogeneous and suggestive of a more complex three-dimensional structure as compared to Eastman AQ[®] which had circular wells. It was also observed that the fabrication of the Nafion[®] film was sensitive to the processing conditions, implying that the film had less tolerance toward the nature and concentration of incorporated species, manifested in extensive cracking of the film.

In summary, the potential of fluorescence to visualize the polymer structure as well as the distribution of incorporated species has been demonstrated in the case of Eastman AQ[®] 55 polymer films. Moreover, the differences in the network structures between Eastman AQ[®] and Nafion[®] were also visualized. The negatively and positively charged probes exhibit different distribution patterns suggesting an electrostatic nature of the probe–polymer interaction. In the case of the tagged enzyme, the charge introduced by the probe tagging did not alter the interaction or distribution of the enzyme in the polymer. This suggests that the distribution obtained for the tagged enzyme correctly represents the distribution of non-tagged enzyme. The influence of the enzyme's charge on its distribution is currently under investigation. Considering the ease of preparation and high catalytic activity of the tagged enzyme (assuming that an appropriate probe-to-enzyme ratio is used), together with the vast number of different probes available, fluorescence coupled with polarization and confocal microscopy is a most useful characterization tool for investigating the interaction of enzymes with and incorporation into polymer matrices.

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