

iNOSoxy/polyethyleneimine layer-by-layer thin films:
effect of pH on iNOSoxy loading and implication on its
catalytic activity

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Abstract:

Layer-by-layer (LbL) immobilized alternating protein-polyelectrolyte films provide the opportunity to build nano-coatings with functional components such as enzymes. Active enzymes in thin films make such coatings biologically active, and can be used as functional coatings to play a variety of roles. In this regard, surface-bound nitric oxide synthase (NOS) can be used for targeted release of nitric oxide, which is known to drive antithrombotic activity on medical implant surfaces.

In this project, we investigate the layer-by-layer adsorption of inducible nitric oxide synthase oxygenase domain (iNOSoxy) as the functional component on polyethylenimine (PEI) as the matrix on graphite surfaces. Our research has previously established that these films, when exposed to the ingredients of the NOS reaction, release fluxes of nitric oxide (NO).

At pH 7, polyethylenimine carries a net positive charge in solution versus a net negative charge carried by the NOSoxy protein. In this work we examined if the pH of the protein solution, which affects the net surface charge on the protein in solution, can modulate the amount of iNOSoxy protein that can be adsorbed onto a PEI-coated surface. To this end, we used buffered iNOSoxy solutions and PEI solutions adjusted to desired pHs to investigate the charge-driven layer-by-layer adsorption process.

We prepared films of iNOSoxy/PEI from iNOSoxy solutions at 2 different pHs. First, we used atomic force microscopy (AFM) to study the morphologies of the two films. Evaluation of cluster densities in the two preparations qualitatively suggests higher levels of adsorbed iNOSoxy for films formed with protein solution at pH 8.6.

Next, we used the redox active Fe^{3+} -heme of iNOSoxy to closely monitor the overall amount of iNOSoxy adsorbed in the two sets of films. Integration of the voltammetric current provides the faradaic charge passed, which can be converted to give the accurate amount of iNOSoxy present in the film. Integrals show higher faradaic charge for electrodes modified with PEI/iNOSoxy films grown with iNOSoxy solution at pH 8.6 compared to those formed with iNOSoxy at pH 7.0 (Figure 1). In average, we find that films constructed at pH 8.6 adsorb at least 3x the amount of iNOSoxy compared to pH 7.0 films (0.6 pmole at pH 8.6 versus 0.2 pmole at pH 7.0).

The catalytic reduction of nitric oxide mediated by iNOSoxy in PEI films is another property that we used to study the effect of pH during the LbL preparation process. Figure 2 shows the peaks of nitric oxide reduction (~ -0.9 V vs. Ag/AgCl) catalyzed by iNOSoxy for films prepared at pH 8.6 and 7.0. Plots of normalized catalytic current against nitric oxide concentration exhibit

typical enzyme saturation kinetics. iNOSoxy/PEI films grown at pH 8.6 show higher turnover rates compared to films formed at pH 7.0. Non-linear fitting of observed traces using the Michaelis-Menton kinetic model yields the K_m values for the two films. The extracted K_m values and the overall turnover rates observed for the two sets of iNOSoxy/PEI films will be compared and discussed in light of FTIR and other spectroscopic characterization of the films.

Finally, we measured the iNOSoxy enzymatic function in PEI films. We used the NOS second enzymatic step, which converts N-hydroxy-L-arginine (NHA) to citrulline and the product NO. Films are dipped in a solution containing the NOS reaction cocktail, including NHA and the tetrahydrobiopterin cofactor. We measure NO release in the form of nitrite using the Griess assay on aliquots drawn over time for up to 72 hours. Results show higher levels of nitric oxide (2x to 3x) released from iNOSoxy/PEI films constructed with iNOSoxy solution at pH 8.6 compared to pH 7. While this is consistent with our earlier conclusion that the higher pH drives adsorption of higher amounts of iNOSoxy, other considerations may be at play and will be discussed in this paper.

Overall, our results show that the pH of the iNOSoxy protein solution modulates the overall amount of protein immobilized in iNOSoxy/PEI films during the LbL process. We will discuss the implication of this finding in the optimization of the general NOS-based nanofilms as potential antithrombotic coatings.

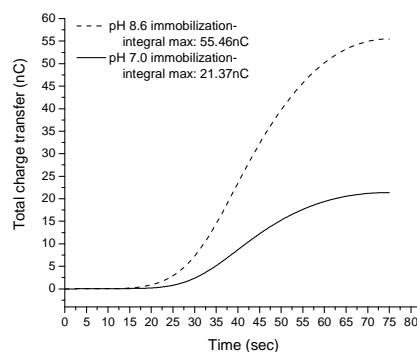


Figure 1: Integrals of cyclic voltammograms showing different amounts of immobilization at different pHs

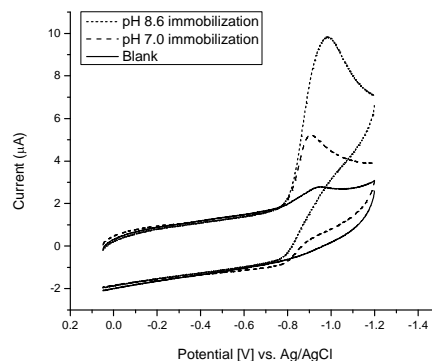


Figure 2: Catalytic NO reduction by different amounts of iNOSoxy immobilized at different pHs