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Effect of architecture on the activity of glucose oxidase/horseradish peroxidase/carbon nanoparticle conjugates

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ABSTRACT

We investigate the activity of glucose oxidase (GOx) together with horseradish peroxidase (HRP) on carbon nanoparticles (CNPs). Because GOx activity relies on HRP, we probe how the arrangement of the enzymes on the CNPs affects enzymatic behavior. Colorimetric assays to probe activity found that the coupling strategy affects activity of the bienzyme–nanoparticle complex. GOx is more prone than HRP to denaturation on the CNP surface, where its activity is compromised, while HRP activity is enhanced when interfaced to the CNP. Thus, arrangements where HRP is directly on the surface of the CNP and GOx is not are more favorable for overall activity. Coverage also influenced activity of the bienzyme complex, but performing the conjugation in the presence of glucose did not improve GOx activity. These results show that the architecture of the assembly is an important factor in optimization of nanoparticle–protein interfaces.

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1. Introduction

Glucose oxidase (GOx) has been exploited for a wide range of industrial and medical applications, including electrochemical blood glucose sensors, food preservation, and biofuel cells. One challenge that all these applications face is that they require GOx immobilization on a solid substrate for electrical contact, enable separation, or improve thermal stability for storage [1,2], where a suitable enzyme–surface interface is critical [3,4]. Immobilization must be done in a way that maintains not only the secondary structure of the protein, but also the protein microenvironment, orientation, and the ability of substrate and product to diffuse to/from the binding pocket and active site [5]. Nanoparticle (NP) or nanostructured surfaces are desirable for supports due to their high surface area and consequently high capacity for enzyme, as well as their ability to be solubilized [6]. Unfortunately, many enzymes including GOx denature when immobilized on flat and nanostructured surfaces [7,8], suffering from lower activity and weaker substrate binding [7,9–12].

Furthermore, observation of GOx activity often relies on the presence of a second enzyme, horseradish peroxidase (HRP). Both enzymes act in cascade – in oxidizing glucose, GOx produces H_2O_2, which HRP uses as a substrate. HRP presence is key for sensing GOx activity as it generates either a colorimetric change in a molecule or a current in an electrode, amplifying the signal. However, HRP is often assumed to be fully folded and functional, where interface issues are non-existent. Consequently, to construct a functional GOx–HRP complex, one needs to consider not only the interface of the GOx to the NP, but also account for HRP and its role in the chain of activity [13,14]. Co-immobilization of HRP and GOx can increase activity, as the proximity of the enzymes aids transport of H_2O_2 from GOx to HRP [15]. However, how to build the bienzyme complex such that detrimental interface effects are minimized is not immediately clear, especially since the enzymes work in concert and not independently. Previous studies of multi-enzyme–NP complexes show that enzyme arrangement on the NP surface influences overall activity [16,17]. Furthermore, HRP and GOx have different surface charges and sizes, and may have different affinities for the NP surface or propensities towards denaturation. Because the product of GOx is the substrate for HRP, diffusion of both substrate/product within the complex needs to be taken into account. Thus, constructing an optimal multi-enzyme-NP conjugate involves multiple issues that become more complicated due to the intricacies of the nano-bio interface.

Even though the NP surface is often problematic for immobilized enzymes, its properties can be advantageous [18]. Carbon structures have attracted considerable interest due to their

Abbreviations: CNP, carbon nanoparticle; GOx, glucose oxidase; HRP, horse radish peroxidase; DLS, Dynamic Light Scattering.

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versatility of presenting crystalline and amorphous phases, owing to their unique electronic structure that allows both sp^2 and sp^3 bonding. Carbon NPs (CNPs) possess size-dependent properties due to delocalization of sp^2-π electrons and exhibit field emission properties comparable to carbon nanotubes due to their degree of graphitization [19]. Like other carbon structures, CNPs are low density, have high surface area, and a relatively high chemical and thermal stability [20]. Moreover, production of CNPs is more affordable than production of NPs made of semiconductors, noble metals, metal oxides, and other crystalline or specialty materials. While the majority of studies of GOx has focused on NPs made of gold, platinum, or silver, CNPs are particularly attractive as solid supports due to their electrical conductivity [21] which can aid electrochemical detection. Furthermore, the carbon substrate is not inert, and depending on its form, can play a role in redox activity. Others have found that graphene oxide can exhibit peroxidase activity [5] and HRP activity on carbon surfaces is enhanced [22]. Therefore, constructing the interface can be done in a way that takes advantage of the material properties of the CNP.

Here we study how the assembly approach and conjugate configuration affects GOx and HRP function on CNPs (Scheme 1). GOx and HRP activity is measured for different NP–bienzyme architectures showing that how the complex is assembled affects overall activity. The NP support is not an inert substrate, and GOx is more negatively impacted by the CNP surface than HRP. Moreover, HRP activity is actually enhanced by the CNP, showing that interfaces are not always detrimental for enzymes. These results highlight the importance and complexity of the enzyme–nanoparticle interface, and that the CNP is not necessarily passive. These issues are critical for glucose sensors, biofuel cells, or any complex which requires multiple immobilized enzymes that work in concert.

2. Results and discussion

2.1. CNP characterization

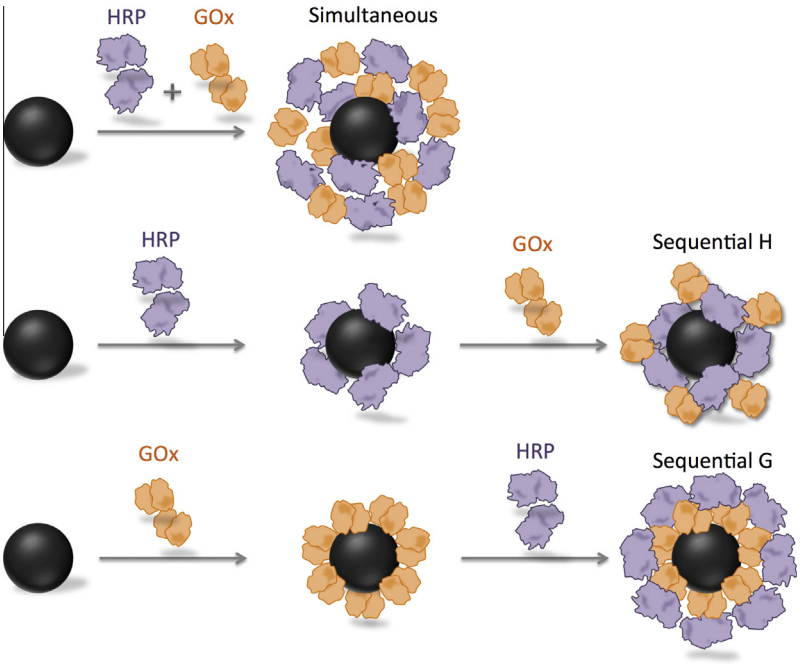
CNPs from soot collection are water-insoluble due to the intrinsic hydrophobicity of carbon. HNO_3 treatment oxidizes the CNP and introduces surface –OH and –COOH groups [23], resulting in negatively charged CNPs which are water-soluble and amenable for protein conjugation [24,25]. TEM images of the CNPs showed mostly spherical particles (Fig. 1a) with an average diameter of 43 ± 6 nm. DLS measurements revealed an average hydrodynamic diameter of D_H = 165 ± 30 nm (peakwidth) (Fig. 1b). The difference between the DLS and TEM indicates that the CNPs form stable agglomerates, which could be due to incomplete oxidation of the CNP surface. Quantitative analysis of TEM images was hindered by the overlapping of the particles and the low contrast of carbon, therefore estimation by DLS was used for further calculations.

2.2. Quantifying enzyme coverage on the CNPs

GOx is a homodimeric glycoprotein, where each monomer has 583 amino acid residues, 17 α-helices, and 30 β-strand structures. The two monomers are linked via disulfide bonds and each subunit possesses one tightly bound FAD cofactor [26]. The overall size of the monomer is ~6.0 × 5.2 × 3.7 nm [27] (UnitprotKB accession number P13006). HRP is a 44 kDa protein with 309 amino acids with a size of ~4.0 × 4.4 × 6.0 nm [28]. It is largely α-helical and contains a heme cofactor (UnitprotKB accession number P00433) [29]. All assays were performed at the pH optimal for GOx activity (pH 5.5), at which GOx is negatively charged (pI 4.2) and HRP (pI 7.2) positively charged.

GOx and HRP were conjugated to CNPs by simultaneous or sequential incubation with CNPs (Scheme 1). Spectroscopic properties of HRP and GOx were used to quantify coverage on the CNPs, as their absorption and fluorescence spectra are distinct [30–32]. The HRP heme gives rise to a Soret peak at ~403 nm (Fig. 2a, orange). GOx, however, lacks a heme and thus has minimal absorption in this region (Fig. 2a, purple), so the Soret peak can be used to quantify HRP. CNPs in this spectral region have only a featureless absorption (Fig. 2c) which can be subtracted from the spectrum of the CNP–enzyme conjugates.

To quantify GOx concentration, we used its tryptophan (W) fluorescence peak at 340 nm (Fig. 2b, purple). GOx possesses 10 W, 18 F, and 24 Y, resulting in strong fluorescence. HRP exhibit much weaker W fluorescence in comparison (Fig. 2b, orange), as



Scheme 1. Schematic of the different assembly approaches for GOx–HRP–CNP complexes. Simultaneous (HRP and GOx together), HRP first and then GOx ("Sequential H"), and GOx first and then HRP ("Sequential G").