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Tunneling of redox enzymes to design nano-probes for monitoring NAD^+ dependent bio-catalytic activity

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ABSTRACT

Monitoring of bio-catalytic events by using nano-probes is of immense interest due to unique optical properties of metal nanoparticles. In the present study, tunneling of enzyme activity was achieved using redox cofactors namely oxidized cytochrome-c (Cyt-c) and Co-enzyme-Q (Co-Q) immobilized on Quantum dots (QDs) which acted as a bio-probe for NAD^+ dependent dehydrogenase catalyzed reaction. We studied how electron transfer from substrate to non-native electron acceptors can differentially modify photoluminescence properties of CdTe QDs. Two probes were designed, QD-Ox-Cyt-c and QD-Ox-Co-Q, which were found to quench the fluorescence of QDs. However, formaldehyde dehydrogenase (FDH) catalyzed reduction of Cyt-c and Co-Q on the surface of QDs lead to fluorescence turn-on of CdTe QDs. This phenomenon was successfully used for the detection of HCHO in the range of 0.01–100,000 ng/mL (LOD of 0.01 ng/mL) using both QD-Ox-Cyt-c (R^2=0.93) and QD-Ox-Co-Q (R^2=0.96). Further probe performance and stability in samples like milk, wine and fruit juice matrix were studied and we could detect HCHO in range of 0.001 – 100,000 ng/mL (LOD of 0.001 ng/mL) with good stability and sensitivity of probe in real samples (R^2=0.97). Appreciable recovery and detection sensitivity in the presence of metal ions suggests that the developed nano-probes can be used successfully for monitoring dehydrogenase based bio-catalytic events even in the absence of NAD^+. Proposed method is advantageous over classical methods as clean up/ derivatization of samples is not required for formaldehyde detection.

1. Introduction

Nano-particle based monitoring of bio-catalytic events has been a major focus in the past decade. There are many reports where optical monitoring of enzyme activity has been achieved using properties such as fluorescence resonance energy transfer (FRET) (Akshath et al., 2012; Chen et al., 2013; Choi et al., 2012; Freeman et al., 2010; Huang et al., 2008, 2012; Willner et al., 2007) and electron transfer (ET) (Akshath et al., 2014; Freeman et al., 2010; Yang et al., 2011) phenomenon. Gold nanoparticles (GNPs) and Quantum dots (QDs) are of immense interest in the construction of enzyme based biosensors due to their unique optical properties. Considering the fact that quantum dots (QDs) are colloidal nano-crystalline semiconductors possessing unique spectral properties due to quantum confinement effects, it makes them superior tool for QD-based biosensors (Freeman and Willner, 2012). Increased attention is being given to designing optical biosensing methods for monitoring enzymatic activities both *in-vitro* and *in-vivo* (Costa-Fernández et al., 2006). Also, studies on interaction of metal nanoparticles with enzymes have led to opening of new opportunities in electrochemical (Jena and Raj, 2006; Scott et al., 2008; Shlyahovsky et al., 2005), optical (Algar et al., 2010; Quek and Leong, 2012; Xiao et al., 2004; Yang et al., 2011; Zhang et al., 2011) and colorimetric (Beqa et al., 2011; Jv et al., 2010; Qi and Li, 2011) biosensing. Redox enzymes have been in the center of many of these studies since their activity is tunable to variety of substrates. However, since redox enzymes cannot have a 'direct-talk' with electrodes, they are always built-in by 'mediator-talk' based systems. Majority of studies are focused on direct electron transfer (DET) based systems or mediators through nano-wiring (Dhand et al., 2011; Diaconu et al., 2010; Jia et al., 2010; Li et al., 2012; Xiao et al., 2004). But, since amperometric based systems suffer from wide variety of drawbacks, optical systems using redox enzymes have emerged as an alternate tool. While cofactors such as NADH have been successfully used by

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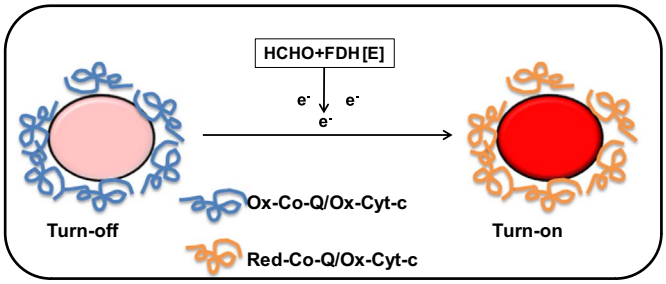

Scheme 1. Proposed turn-on biosensing using Ox-Cyt-c/Co-Q-QD based nano-probe.

several authors for nanoparticle coupled biosensing, literature on use of 'non-classical' electron carriers for tracking and monitoring enzyme reactions is extremely less explored. In this direction, only recently, non-classical co-factors like oxidized cytochrome-c (Ox-Cyt-c) and co-enzyme Q (Co-Q) are being used as sensing probes after their differential interaction with QDs have been understood (Gerhards et al., 2008). Li et al. (2011), used Cyt-c based interaction studies for *in-vitro* detection and *in-vivo* imaging of reactive oxygen species. A ubiquinone based biosensing and bio-imaging probe was developed by Ma et al. (2013).

In the present investigation, we attempted to design QD-Cyt-c/Co-Q based probe for monitoring the activity of a dehydrogenase enzyme. The differential and discriminative response of these probes was used in the detection of formaldehyde (HCHO), a Group I carcinogen, using the enzyme formaldehyde dehydrogenase (FDH). As well known, FDH catalyzes breakdown of HCHO to formic acid in the presence of NAD^+. However, as proposed in Scheme 1, we hypothesized that Oxidized Cyt-c/Co-Q immobilized on QDs can act as electron acceptors, in the absence of NAD^+, and reduction of Cyt-c/Co-Q on surface of QDs leads to change in fluorescence properties.

Formaldehyde is a carcinogenic (Group 1) and potent neurotoxic agent frequently added to consumable items (IARC Monograph, 2006). Reports on HCHO addition in wines, fruit juice, beers, noodles, milk, water and many other consumable items has created a need of developing 'non-classical' techniques for rapid and sensitive detection (Akshath et al., 2012). Classically, HCHO is detected by high-performance liquid chromatography (HPLC) (Wu et al., 2003), colorimetric methods/spectrophotometric methods (OSHA Mannual, 1989), gas sensors (Bianchi et al., 2007), and potentiometric methods based on immobilized FDH or alcohol oxidase (Ben Ali et al., 2007; Korpan et al., 2000). In our earlier study, we reported a FRET based biosensing of HCHO using CdTe QDs (Akshath et al., 2012). In an attempt to increase the specificity and sensitivity of detection, in the present study, we designed two probes QD-Cyt-c (Probe 1) and QD-Co-Q (Probe 2) for tunneling enzyme activity and monitor the enzyme reaction. To the best of our knowledge, this is the first attempt to design a non-classical co-factor based QD-Cyt-c and QD-Co-Q nano-probe for monitoring dehydrogenase activity and for the sensitive detection of HCHO.

2. Materials and methods

Cadmium acetate, mercaptopropionic acid, sodium borohydride, tellurium, cytochrome-c (Oxidized), co-enzyme-Q (Oxidized), NAD^+, HCHO and FDH were procured from Sigma Chemicals, St. Louis, USA. Aluminium chloride, zinc chloride, ferrous sulphate, magnesium chloride, manganese chloride, sodium chloride, potassium chloride were obtained from Sigma chemicals. All reagents used were of analytical grade and acquired from standard suppliers and used without any further purification. For analysis in real samples, water, milk, wine and fruit juice were procured from market and used directly without any pre-treatment. The instruments used were UV–Vis spectrophotometer (UV-1601, Shimadzu, Japan) for monitoring spectral changes during synthesis of CdTe QDs, Spectrofluorimeter (RF-5301 PC, Shimadzu, Japan) for photoluminescence measurements.

2.1. Synthesis and characterization of CdTe QDs

CdTe QDs with emission at 530–550 nm were synthesized as previously reported (Li et al., 2007), with minor modification. In brief, 0.02 M Cd-$(CH_3COO)_2$ salt was dissolved in 25 mL of milli-Q water and mixed with 0.05 M of MPA. The prepared solution was degassed followed by pH adjustment to 9.2 ± 0.2. In a separate reaction, sodium hydrogen telluride (NaHTe) was synthesized by reacting 0.03 M of $NaBH_4$ and 0.01 M of Te in ice-cooled water. Further, NaHTe was added drop wise to the above solution until the solution turned orange. The solution was refluxed at 100 ± 2 °C for 180 min Both absorption and emission spectra were recorded. TEM study was carried out using a drop of colloidal solution dried on a 400-mesh carbon copper grid at an acceleration voltage of 200 kV.

2.2. Enzyme assay

2.2.1. Immobilization of Oxidized Cytochrome-c/Co-Q onto CdTe QDs

Varying concentrations of Ox-Cyt-c (0.04–0.8 μM) and Co-Q (0.005–0.03 mM, stock solubilized in acetone) were prepared in phosphate buffer (PB; pH 7.5). 100 μl of individual concentrations of Ox-Cyt-c and Co-Q were added to 10 μl of synthesized CdTe QDs (0.1 absorbance units) and incubated for a period of 10 min After 10 min of incubation, absorbance spectra and emission spectra was recorded by exciting CdTe QDs at 450 nm. The designed probes were coded as QD-Cyt-c (Probe 1) and QD-Co-Q (Probe 2). Fluorescence emission was monitored as a function of Cyt-c/Co-Q concentration to study the effect of co-factors on QDs fluorescence and stability.

2.2.2. Enzyme tunneling in presence and absence of NAD^+

Experiments were carried out, both in presence and absence of NAD^+, to study the fluorescence behavior of probe 1 and 2 using FDH. A set of experiments were carried out by adding NAD^+ (2.5 mM) to both the probes and a set without NAD^+. Formaldehyde concentration [S] of 100 and 10,000 ng/mL followed by NAD^+ (2.5 mM) was added to probe 1 and probe 2. The mixture was incubated for 10 min This step was followed by addition of FDH (prepared in PB, pH 7.5) of 0.12 U for probe 1 and 0.52 U for probe 2. Fluorescence spectra was monitored after 15 min of incubation at 37 °C.

2.2.3. Designing QD-Cyt-c (probe 1) and QD-Co-Q (probe 2) nano-probe

For designing non-classical co-factor based fluorescent probe, CdTe QD having 0.1 absorbance unit was incubated separately with 0.04 μM Ox-Cyt-c and 0.03 mM of Co-Q for 10 min Further HCHO concentration [S] of 100 ng/mL and 1 ng/mL was added to probe 1 and 2 followed by addition of FDH at varying concentrations (0.02 – 0.6 U) in separate reactions. Absorbance and fluorescence spectra were monitored after 15 min of incubation at 37 °C.

3. Detection of HCHO using proposed system

3.1. Fluorescence spectra with designed probes, metal-ion