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Probing phosphatase activity using redox active nanoparticles: A novel colorimetric approach for the detection of enzyme activity

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ABSTRACT

A new colorimetric assay for the detection of alkaline phosphatase (ALP) activity is reported based on the surface reactivity and optical properties of redox active nanoparticles of cerium oxide, or nanoceria. The method takes advantage of nanoceria color changes after interaction with products of the ALP catalyzed reaction, resulting in charge transfer complexes with very strong absorption characteristics. The developed assay is easy-to-use, robust and cost effective and does not involve labeled reagents, secondary enzymes or soluble dyes. Hydrolytic products of more stable substrates (catechol monophosphate, ascorbic 2-phosphate and hydroquinone diphosphate) that could previously not be used in ALP assays can be conveniently colorimetrically detected with this assay. A detection limit of 0.04 U/L ALP with a linear range up to 2 U/L was obtained with ascorbic 2-phosphate substrate. The proposed assay can eliminate multistep procedures and minimize problems associated with the poor stability of substrates and enzyme labels of conventional ALP assays. The assay has been adapted to a paper platform and has demonstrated functionality for ALP detection in human serum. This sensing concept can find wide applications as a general approach for improving sensitivity and simplifying detection schemes of colorimetric bioassays, e.g. enzyme, gene, immuno and aptamer assays and related affinity sensing methods.

1. Introduction

Alkaline phosphatase (ALP) activity assays are one of the most widely used assays with broad applicability in many fields. In clinical diagnosis, ALP is used as a biomarker of disease. In bioanalysis, ALP is used as an enzyme label and indicator reaction to quantify affinity binding processes in immunoassays, gene assays and other affinity methods. Although a variety of ALP assays with colorimetric, fluorescent, chemiluminescent and surface enhanced Raman scattering (SERS) detection have been developed (Blum et al., 2001; Jia et al., 2010; Liu and Schanze, 2008; Ruan et al., 2006), many of these assays require laborious sequential step reactions to detect the synthetic substrates, and some have low sensitivity and selectivity that limits broad applicability. In a classical assay the ALP activity is generally quantified by monitoring the enzymatic conversion of *p*-nitrophenyl phosphate substrate to *p*-nitrophenol which has an absorption peak at 405 nm (Iqbal, 2011). While widely used, *p*-nitrophenyl phosphate is very unstable posing significant challenges in bioaffinity assays. Furthermore, ALP is used in conjunction with other enzymes in order to achieve detectability levels (Szydlowska et al., 2006), increasing the complexity of the system. More robust sensing strategies that use more stable substrates and single-step procedures to probe ALP activity are highly desired.

Recent advancements in the development of enzyme activity assays include integration of nanoparticle based colorimetric schemes, in particular gold (Daniel and Astruc, 2004; Ghosh and Pal, 2007; Zhao et al., 2007) and silver (Wei et al., 2008) to indirectly detect ALP activity based on nanoparticle aggregation. Choi et al. used a short phosphorylated tripeptide with a positively charged guandine group on the argnine side chain, next to the negatively charged phosphate group on the tyrosine chain to prevent the aggregation of gold nanoparticles. The addition of ALP removed the negatively charged phosphate group from the peptide and triggered aggregation and color change of the nanoparticles (Choi et al., 2007). Li et al. used conjugated gold nanoparticles-adenotriphosphate (ATP) in which ATP induces aggregation of cetyltrimethylammonium bromide caped gold nanoparticles. The dephosphorylation reaction by ALP leaves the gold nanoparticles dispersed (Li et al., 2013). Most methods based on nanoparticle aggregation involve elaborate procedures, labeled reagents and specific substrates and in some cases their detection limits are higher than the normal values required for practical applications. Moreover, the presence of any charged species in the

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sample matrix could result in the aggregation of the nanoparticles, and subsequently may change the colorimetric results. Furthermore, the development of an easy-to-use and cost effective method for rapid detection of ALP activity is still of critical importance in many fields.

Herein we report a facile and robust method using redox active nanoparticles of cerium oxide as colorimetric agents to quantify ALP activity in a single step procedure. As opposed to detection schemes based on nanoparticle aggregation, the proposed design takes advantage of spectral and functionality changes of the nanoparticle surface in the presence of the ALP hydrolyzed products. Nanoceria particles have recently gained increasing interest due to their catalytic and free radical scavenging properties (Ispas et al., 2008; Karakoti et al., 2008) and their ability to participate in redox reactions due to the dual oxidation state (Ispas et al., 2008). Nanoceria have been shown to exhibit superoxide dismutase, catalase and oxidase like activity (Korsvik et al., 2007). Our previous studies have established that nanoceria can oxidize phenolic compounds to quinone derivatives that further attach to the nanoparticle surface, generating strongly absorbing charge transfer compounds, with unique surface and spectral properties (Sharpe et al., 2013). We envision that the enhanced optical properties of the nanoceria-phenol complex could be integrated in bioassays that involve phenolic type compounds to develop sensitive and robust sensing platforms. However, the development of an ALP activity assay based on the optical properties of nanoceria has not been reported.

Toward this end, we explore herein the use of nanoceria as a redox active colorimetric probe to measure the formation of the ALP reaction products. We then adapt this concept to fabricate a paper based ALP assay with immobilized nanoceria particles and demonstrate functionality in human serum. Colorimetric bioassays based on patterned paper are the least expensive, user-friendly alternative to conventional analytical assays. We demonstrate the usefulness and the advantages of this method for the detection of ALP activity. ALP is known to hydrolyze the phosphate ester functional group of a variety of phosphate compounds to the respective alcohol products (Preechaworapun et al., 2008), many of which are phenolic type generated from phenylphosphates. We hypothesize that phenolic products of the ALP reaction are rapidly oxidized by nanoceria to reactive radical intermediates that will bind to the particles and generate highly conjugated charge transfer complexes through the hydroxyl functionalities with enhanced optical properties as illustrated in Scheme 1. The intensity of the color formed is related to ALP activity while the high absorbance characteristics of the new nanoparticle complex provide enhanced sensitivity. In this configuration there is no need for secondary enzymes to amplify the signal and no need for soluble redox dyes for signal quantification, greatly simplifying the complexity and detection scheme of the assay.

2. Experimental

2.1. Chemicals and biochemicals

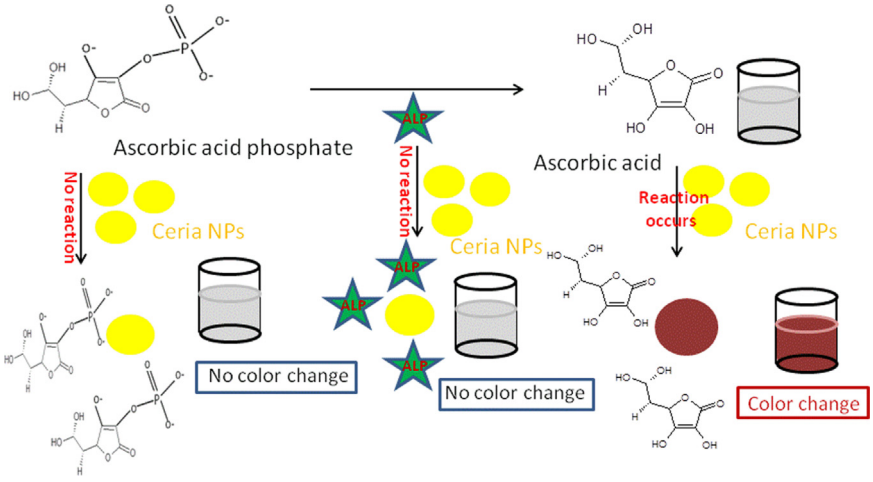
Alkaline phosphatase (activity 31130 U/mL; protein concentration 19.1 mg/mL with aspecific activity 1630 U/mg) from Calf intestine was purchased from CALBIOCHEM, prepared in ammonium chloride/hydroxide buffer (pH 9.0). Catechol (99%) and ascorbic acid were from Acros. Hydroquinone (1,4-benzenediol, 99%) was purchased from Sigma Aldrich. L-ascorbic acid-2-phosphate sesquimagnesium salt hydrate was from Santa Cruz Biotechnology. Buffer components for ammonium chloride/hydroxide buffer including ammonium chloride and ammonium hydroxide were from J–T. Baker.

2.2. Solutions and buffers

Ammonium chloride/hydroxide buffer was prepared by mixing 0.1 M ammonium chloride and 30% ammonium hydroxide solution. The pH of the buffer solutions was adjusted using diluted HCl. The enzyme stock solution (1313 U/L) and the solutions of catechol (4–20 μM), ascorbic acid (12–108 μM), and hydroquinone (30–270 μM) were prepared in ammonium buffer at pH 9. Distilled water (Millipore, Direct-Q-system) was used for the preparation of buffer solutions. Cerium oxide(IV) or nanoceria particles, 20 wt% colloidal dispersion in 2.5% acetic acid with an average size of 20–25 nm were purchased from Sigma Aldrich. The nanoceria dispersion was prepared in ammonium buffer and sonicated for 5 min prior experiments.

2.3. Instrumentation

UV–vis spectrophotometric measurements were performed with a Schimadzu P2041 spectrophotometer equipped with a 1-cm path length cell. Thermogravimetric analyses (TGA) were carried out by using a TG/DTA Seiko Exstar 6200 thermogravimetric analyzer. Quantitative colorimetric measurements were performed with an ELx800 absorbance microplate reader (Bio-Tek). Untreated, round "U" bottom, no lids, polystyrene 96-well microplates were from



Scheme 1. Proposed mechanism of the nanoceria based ALP sensing.