Rapid naked eye detection of alkaline phosphatase using α-MoO$_{3-x}$ nano-flakes

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ABSTRACT

We present a novel platform enabled for rapid naked eye detection of alkaline phosphatase using two-dimensional α-MoO$_{3-x}$ nano-flakes as reporter molecule as first of its kind report. The detection system involved α-MoO$_{3-x}$ nano-flakes and zinc oxide as the primary constituents followed by the introduction of alkaline phosphatase. The rapid transformation of α-MoO$_{3-x}$ (blue) to α-MoO$_3$ (colorless) in presence of alkaline phosphatase with zinc as a cofactor is an indication for the presence of the enzyme. The sensitivity of the current biosensor was found to reach 0.1 μM of enzyme concentration. The stability and selectivity of the novel biosensor has been tested at different pH and with a wide range of analytes in the serum.

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1. Introduction

With the discovery of two-dimensional (2D) nanomaterials such as Graphene [1]; transition metal dichalcogenides (TMDs) [2,3] and transition metal oxides (TMOs) [4] have been studied extensively owing to their fluorescent and absorption characteristics respectively [5]. Among the TMOs, 2D Molybdenum trioxide (MoO$_3$) has been widely applied in environmental applications [6,7] and electrochemical storage devices [8] pertaining to it's photochromic [9], electrochromic [10] and thermochromic [11] properties. The surface defects on MoO$_3$ has been controlled to result in α-MoO$_{3-x}$ nano-flakes [6,12] with unique surface plasmon resonance (SPR). Following the discovery, it has never been applied for detection of biological analytes in solution, except some rudimentary speculations [13]. The most cardinal issues which prevent the application of α-MoO$_{3-x}$ nano-flakes in aqueous biosensors are attributed to the following reasons: 1. Relatively less stability/solubility in water [14], 2. The absence of inherent fluorescence. 3. Lack of size-

dependent surface plasmon resonance in the flakes [13]. To address the above issues, we have successfully transferred nano-flakes in water without compromising its stability and optical properties (see Supporting Information for Materials and Methods). This was mainly performed to remove ethanol from the mixture, which may interfere with the structural integrity of biological molecules. The SPR is an interesting phenomenon exhibited by α-MoO$_{3-x}$ nano-flakes in solution. We have exploited the sensitivity of SPR to negatively charged alkaline phosphatase (ALP) for its sensing in the physiological regime. A similar observation was recorded in the case of bovine serum albumin [13]. However, there are few issues linked with detection of key biological analytes in serum due to an overabundance of albumin along with other components. This serious issue compromises the selectivity of the method. ALP, on the other hand, is present inadequately high concentration in serum [15]. Higher levels of ALP in serum is a biological signature of a variety of ailments such as bone diseases, liver malfunction, breast and prostate cancers and diabetes [16–18]. The intricate participation of ALP is profuse in biochemical mechanisms demanding a sensitive and selective detection tactic. To address this issue, we have modified a selective protocol for detecting ALP in biological fluids. In contrast to the variety of systems designed for sensing ALP based on chromatography [19,20], fluorimeter [21], electrochemistry [22,23], chemiluminescent [24] and SERS [25], our method is
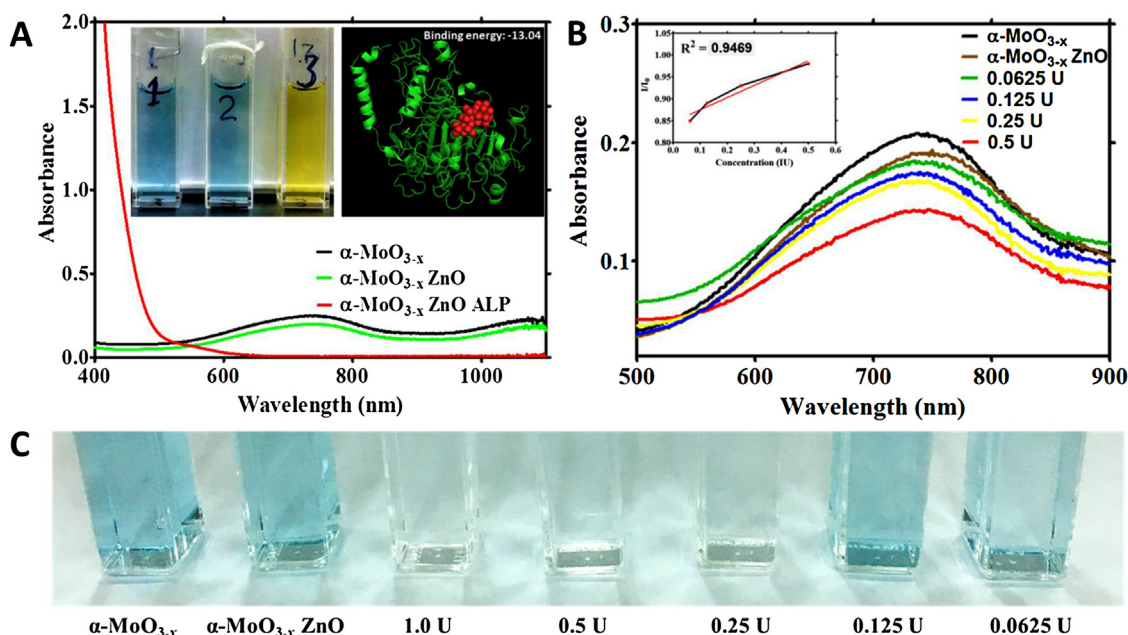
Fig. 1. (A) UV–vis spectra of α-MoO$_{3-x}$ and other conjugates. Inset (left) shows ambient light photograph of (1) α-MoO$_{3-x}$ + KI (2) α-MoO$_{3-x}$ − ZnO + KI (3) α-MoO$_{3-x}$ − ZnO-ALP + KI complex (KI added to photo-capture the generation of H$_2$O$_2$). Inset (right) shows molecular docking results of ALP and α-MoO$_{3-x}$ (Binding Energy −13.34 kJ) (B) Response of α-MoO$_{3-x}$ in presence of different concentration of ALP. Inset shows linearity of the response. (C) Day-light photograph of solutions in presence of different concentrations of ALP.
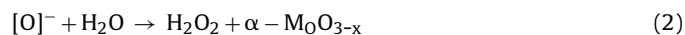
superior that is only via naked eye-selective detection of ALP in biological fluids. The use of molybdenum oxide nanomaterials, in this case, offers many advantages in comparison to other materials due to the absence of toxicity and tunable surface plasmon resonance [26,27].

TEM image (Fig. S1 A and B in Supporting Information) shows the formation of nano-flakes. The lattice fringe width of 0.37 nm supports the formation of nanoflakes [28] of α-MoO$_{3-x}$ nano-flakes. The X-ray photoelectron spectroscopy of nanoflakes (see Supporting Information Fig. S1C and D for details) was performed to understand their sub-stoichiometry revealing the transformation of α-MoO$_3$ to α-MoO$_{3-x}$ nano-flakes.

The optical properties of nanoflakes were investigated using an UV–vis spectroscopy (see Fig. 1). The α-MoO$_{3-x}$ nanoflakes exhibit characteristic absorption at ∼740 nm which extend till 1050 nm. Due to such broad absorption spectra, these two-dimensional nanomaterials can be used for a variety of sensing applications. After addition of zinc oxide (ZnO), the peak was found to be slightly attenuated in terms of intensity with no obvious shift in wavelength. Moreover, after addition of the ALP, the absorption peak became flat as it was evident from the figure. Another reason for attenuation of the peak intensity might be the agglomeration of nano-flakes after interaction with ALP and ZnO conjugate. This is evident from the DLS studies (Table 1). Another very interesting aspect of the experiment was a visible alteration in color due to the presence of ALP. As shown in the left inset of Fig. 1A, the change in the color of solution from blue to colorless only in the presence of ALP combined with ZnO. To show the generation of hydrogen peroxide, we added potassium iodide (KI) into the solution, which reacted with hydrogen peroxide and forms yellow-colored compound. To comprehend the mechanism of ALP detection, we performed molecular docking studies using *Autodock 4.2.6* (right inset in Fig. 1A). Based on the results, the affinity of ALP and Zn^{+2} towards α-MoO$_{3-x}$ (Binding energy = −13.34 kJ) was found in the highly thermodynamic way. Fig. 1B shows the detection of various concentrations of ALP in solution. The inset shows linearity of the detection (R^2 = 0.9469) and Limit of detection was found to be

0.1 μM (S/N = 3). Fig. 1C shows daylight photograph of solutions in presence of different concentrations of ALP.

The mechanisms of detecting ALP is outlined in Fig. 2. In accordance with the previous work, ZnO in aqueous solution dissociates to Zn^{+2} and OH$^-$ ions [29]. In our case, this stimulation might be catalyzed by ALP itself [30]. Due to the inherent affinity of ALP (zinc ions accommodate themselves in a groove associated with ALP) towards zinc, the ZnO probably splits into Zn^{+2} and nascent oxygen 2[O]$^-$ (as shown in Eq. (1)). Nascent oxygen attacks on water molecules and forms H$_2$O$_2$. The interaction with α-MoO$_{3-x}$ nanoflakes and H$_2$O$_2$ oxidized the nano-flakes to α-MoO$_3$ (Eq. (2)), thus resulting in a faint blue colored solution. The presence of H$_2$O$_2$ in the solution was further checked by adding KI in a solution containing ALP and ZnO. As shown in the inset of Fig. 1A, the color of the solution was changed to yellow due of the release of iodine (Eq. (3)).

$$ALP + ZnO \rightarrow Zn^{2+} + [O]^- \tag{1}$$

$$[O]^- + H_2O \rightarrow H_2O_2 + \alpha - M_OO_{3-x} \tag{2}$$

$$H_2O_2 + 2KI \rightarrow 2KOH + I_2 \tag{3}$$

To assess the ability of our sensor for detection of ALP in real samples, various concentrations of ALP was spiked into serum and the optical response of the sample was studied using UV–vis spectroscopy (Fig. 3A). As shown in the figure, there was proportional depletion of the intensity of the peak in presence of ALP. Following the earlier trend, the relation between relative absorbance intensity (I/I$_0$) vs concentration of ALP (Fig. 3B, R^2 = 0.8644). In serum, the detection needs further improvement due to the high affinity of these nanoflakes toward negatively charged components of serum. Fig. 3C shows the daylight photograph of color transformation in response to various concentrations of ALP in serum.

The results obtained using the proposed method have been compared to earlier reports on colorimetric detection of ALP (as shown in Table 1). The materials used in the literature involved tedious synthesis process. However, our approach on synthesis of α-MoO$_{3-x}$ is relatively very easy and the detection system is simplified. This justifies the relevance of the proposed method as a rapid detec-