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Protein stabilized fluorescent gold nanocubes as selective probe for alkaline phosphatase via inner filter effect

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ABSTRACT

We present a novel approach for the synthesis of unique template free protein stabilized gold nanocubes (PSGNCs) for sensing and quantification of alkaline phosphatase (ALP) in biological fluids. The unique wavelength independent excitation of PSGNCs and absorption of the final product of ALP catalysis, i.e., p-Nitrophenol (p-NP) at 405 nm was exploited for the inner filter effect (IFE) as a sensitive and efficient method to detect ALP. The limit of detection in aqueous solution and serum was found to be 1.616 U/L and 9.441 U/L, respectively.

1. Introduction

Despite several cardinal instigations for detection of serum alkaline phosphatase (ALP) [1–3], scientists have continually shown great attention to discover novel materials for excellent sensing capability. ALP is a metalloenzyme containing two Zn^{2+} and one Mg^{2+} in the active sites, which is found abundantly in the human blood [4] ranging from 25 to 100 U/L. However, increase and decrease in the serum concentration of ALP is associated with many diseases such as liver cirrhosis [5], hepatitis [6], bone disease [7,8] and cancer [9]. Therefore, it is essential to develop a sensitive and selective detection method for ALP, akin to many key enzyme biomarkers such as proteinase K, creatine kinases, glucose oxidase, etc. There are some fundamental challenges in the solution based detection of ALP such as instability of the enzymes, stringent parameters for enzyme activity as well as low detection limits. With the understanding of heroic roles of nanoparticles as sensitive probes for enzyme assays, many protocols have been reported using gold nanoparticles [10,11], silver nanoparticles [12,13], carbon dots [14,15], gold nanoclusters [16,17], modified electrodes [18,19], copper nanoparticles [20] and smart polymers [21], two dimensional transition metal oxides [22] etc. Among the various strategies, colorimetric detections of ALP based on aggregation of nanoparticles such as gold and silver under the influence of ATP phosphorylating/dephosphorylating reaction [12,23] have also been reported. However, the absorbance based colorimetric assays have a limitation of cross-reactivity with similar kind of analytes and hence the sensitivity and stability of such systems become unreliable. The inner filter effect (IFE), in contrast, is a powerful fluorescence-based strategy for detection ALP assay [3,23]. Gabor and Walt first reported IFE in 1991 [24] involving the participation of two chemical moieties where absorption of one species overlaps with the absorption of the other [25,26]. Other mechanisms based on the similar concept of fluorescence quenching are Forster (Fluorescence) Resonance Energy Transfer (FRET), and Photoinduced Electron Transfer (PET) [27–30] applied widely for sensing applications. Relatively, IFE is found to be superior to both FRET and PET as it is easily tunable with enhanced sensitivity of fluorescence signals. The associated simplicity and accuracy is a widely used method for detection of various types of analytes [31–34].

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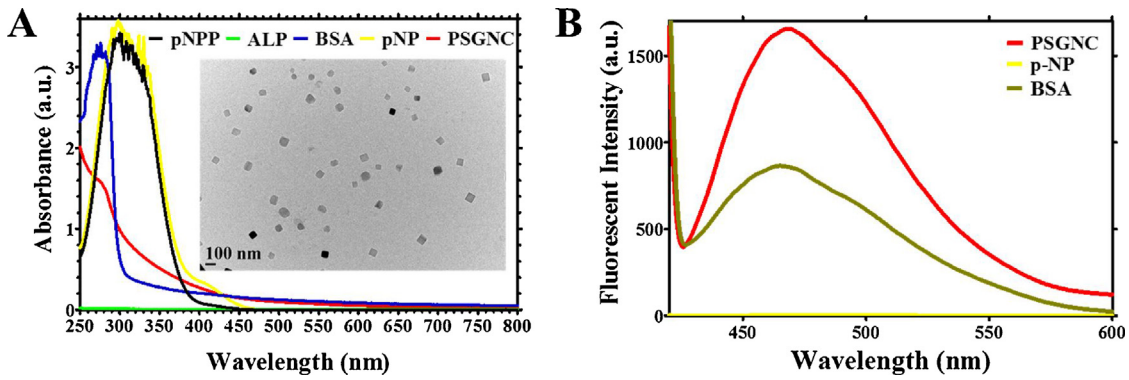

Fig. 1. Physical and Optical characteristics of PSGNC (A) UV–vis absorbance spectroscopy of individual components involved in the synthesis of PSGNC; Inset: TEM representative of PSGNC; (B) Fluorescence spectroscopy of PSGNC observed at an excitation wavelength of 370 nm.

ALP catalyzes the conversion of its substrate p-Nitrophenyl phosphate (p-NPP), a colorless solution, to yellow colored) with absorbance λ_{max} = 405 nm [35]. Existing strategies for sensing ALP have employed this property widely as the basis for detection. Exploring over these attributes, we have used a unique fluorescence property of Protein Stabilized Gold Nano Cubes (PSGNCs) and specific absorption of p-NP for sensitive detection of ALP in a biological fluid with high sensitivity and selectivity.

The PSGNCs have been synthesized via a novel method which is free of any amino acid template and incubating the mixture of gold salt ($HAuCl_4$) and bovine serum albumin (BSA) for 12 h. (*The experimental details are provided in Supplementary Material*). In an earlier report, Ding et al. have shown the successful synthesis of Protein-Gold Hybrid Nano Cubes in the presence of tryptophan as a template with λ_{max} = 480 nm [36]. The fluorescence characteristic of PSGNCs is applied here by the absorbance of p-NP to act as an inner filter for PSGNCs emission. The intensity of absorption of p-NP at λ_{max} = 405 nm, is proportional to the amount of ALP present in the reaction volume. In the presence of ALP, the absorption of emitted photons by the p-NP results in reduced fluorescence intensity of PSGNCs. Thus, this proves the IFE in the assay of ALP. In contrast to the earlier work [3], our method is highly sensitive to detect 1.616 U/L of ALP and 9.441 U/L in human serum with good reproducibility (S/N = 3). The current approach also opens new avenues for development of nanosensors for many other phosphatases.

2. Results and discussions

2.1. Synthesis and characterization of PSGNC

In the absence of any ironclad proof depicting the *modus operandi* of fluorescent gold nanocubes, we have following speculation as per earlier investigations [36] carried out to prove the fundamental mechanism of gold nanocube formation. During the process of synthesis of fluorescent gold nanocrystals, we did several modifications. Firstly, the pH was adjusted to 3.7 instead of alkaline condition as usually required for synthesis [37] of gold nanoclusters. Secondly, we kept the solution of BSA and gold salt without mechanical stirring at 37 °C for 72 h. The optical spectra (Fig. 1A) shows a spectral overlap between PSGNCs and p-NP with a hump at 300 nm in the case of PSGNCs. In accordance with the earlier findings for the formation of gold nanoclusters, the origin of the hump may be due to quantum sized homoleptic cluster formation (Au_{10-12}) [38] with a non-spherical structure (herein PSGNCs) [39]. The monolayer of the ligand on the metal core (here BSA) may be due to undisturbed and prolonged incubation time during the synthesis. This allowed generation of the necessary confined space and preventing the slow etching or chelation of the metal core nanocluster from the edges thereby resisting the formation of spherical clusters, but nanocubes. The inset of Fig. 1A is a TEM representation of the PSGNCs marking a size of up to 50–70 nm. As is evident from the literature, the excitation-emission behavior of fluorescent moieties is largely related to the highest occupied molecular orbitals and the lowest unoccupied molecular orbital (HOMO-LUMO) [40]. Also the emergence of fluorescence in metal nanoclusters is attributable prominently to three factors, viz. metal-metal electronic transitions, metal-ligand electronic transitions and ligand-ligand electronic transitions [38]. The change in pH of the solution from alkaline (as used conventionally) to acidic medium resulted in blue shift in the fluorescence response of metal nanocluster [41]. Thus, facilitating acidic medium results in the metal-ligand electronic transitions with an unusual absorbance property at 405 nm and emission at 468 nm. The relative quantum yield of the as-synthesized PSGNCs was 28.64% as calculated using quinine sulfate as reference (58%). To explain the phenomenon of IFE, the absorbance spectra of the as-synthesized PSGNCs and p-NP is shown in Fig. 1A. To deny the role of other components such as BSA, ALP, p-NPP, we recorded their individual optical spectra as shown in Fig. 1A and 1B. In contrast to the red fluorescence reported in the case of gold nanoclusters, the excitation and emission spectra of PSGNCs were 405 and 468 nm, respectively (Fig. 1B). The optical excitation was found to be independent of maximum absorption. The shift in the emission spectrum could be associated with the shape, density and the surface ligands of gold cores of PSGNCs [36] (Table 1).

2.2. Stability of the detection system

To assess the behavior of the detection system under different physical and physiological conditions, it was subjected to varying concentrations of saline, incubated under the influence of different metals, and exposed to a varied range of pH. It was observed that the reaction system was stable nearly at all concentrations of saline prominently at 0.05 M (Fig. S1) while the reaction system is found to be stable at pH 10. (Fig. S2) relating to the alkaline nature of ALP. The presence of Zn^{2+} resulted in reduced fluorescence intensity as compared to other metals (Fig. S3). This may be due to the inherent affinity of p-NPP towards Zn^{2+} ions which is also present in ALP.

2.3. Sensing alkaline phosphatase using PSGNCs

IFE is a label-free technique which is efficient in detecting smaller concentrations of analytes. p-NP has strong absorption at λ_{max} = 405 nm overlapping with PSGNCs. Owing to this, in the