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Fluorescence Signal Amplification by Cation Exchange in Ionic Nanocrystals**

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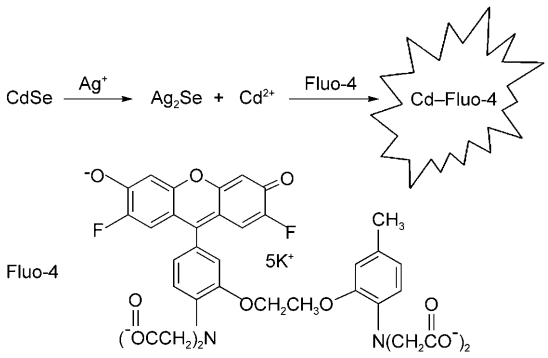
The sensitive detection of trace analytes is extremely useful in many areas, including systems biology, disease control and diagnostics, biodefense, and environment surveillance.[1–7] The most common detection scheme is to label the reporter molecules with fluorescent dyes, as fluorescence has many advantages over other technologies in biosensing, such as simplicity, sensitivity, availability of organic dyes with diverse spectral properties, and fast advancement in optical imaging.[8] However, further sensitivity improvements in fluorescence-based bioassays are hindered by the low luminescence intensity of organic dyes as well as the low dye-to-reporter molecule labeling ratio, which is limited by the availability of functional groups and the photoquenching problem accompanied with high fluorophore density. Thus, many schemes have been developed to amplify the fluorescence signals, including generation of highly luminescent nanocrystals,[9] synthesis of fluorescent polymers,[10] utilization of metal or metal oxide surfaces for fluorescence enhancement,[11,12] and encapsulation of multiple optical tags inside nanocarriers.[13,14] Such approaches either strictly rely on the optical properties of the synthesized materials, or require special instrumental design, or employ harsh detection conditions that are not compatible with the typical sensing platform. Herein, we present a much simpler and milder strategy to amplify fluorescence signals by using ionic nanocrystals with no special optical properties. We show that a cation-exchange reaction with ionic nanocrystals can release thousands of divalent cations, which can in turn trigger the fluorescence from thousands of nonfluorescent metal-sensitive dyes to obtain large fluorescence amplification. The nanocrystal–dye set of CdSe and Fluo-4 (Scheme 1) used in the present study led to a 60-fold enhancement of the fluorescence signal and a limit in protein detection 100 times lower than that of the organic fluorophore Alexa 488. This signal amplification scheme is fast and simple, with a large dye-to-reporter molecule labeling ratio, but does not affect the fluorescence quenching. Our study indicated that a large selection of nanocrystals and fluorophores could be chosen for further improvement in detection performance. In contrast to other detection schemes that utilize high-quality nanomaterials with special optical properties, our approach employs the nonfluorescent nanocrystals that could be available at a much reduced cost.



Scheme 1. Cation-exchange-based fluorescence amplification (CXFluoAmp) with Ag^+, CdSe nanocrystals, and Fluo-4.

The design of our cation-exchange-based fluorescence amplification (CXFluoAmp) that uses CdSe and Fluo-4 as the model nanocrystal and metal sensor is illustrated in Scheme 1. It has been discovered that cation-exchange (CX) reactions could occur completely and reversibly in ionic nanocrystals at room temperature with unusually fast reaction rates because of their large surface areas and small volumes.[15] Our design takes advantage of this special feature to release the cations from the nanocrystals, which in turn bind to the metal-responsive fluorophores and alter their structures to obtain much higher quantum yields.[16] Therefore, the non- or weakly fluorescent dye molecules become highly fluorescent. If the reporter molecules in bioassays are labeled by the nanocrystals in a 1:1 ratio, each of them can generate fluorescence from thousands of fluorophores. Such a high "dye to reporter molecule" labeling ratio should result in very high detection signals and assay sensitivity. Photoquenching is no longer an issue with our scheme as the fluorophores are present in the detection solution instead of being encapsulated with high density inside a narrow space and they are barely fluorescent before the ion-exchange reaction. The cation-release process happens instantaneously and involves no enzymatic or catalytic reagents except the cations added to initiate ion exchange. Hence, our process is fast, benign, and has no special requirements from the assay platform.

A demonstration of the CXFluoAmp technique is shown in Figure 1. Water-soluble CdSe nanocrystals were produced through a high-temperature ligand-exchange procedure that we reported previously.[17] The original fluorescence of the CdSe particles disappeared during ligand exchange with

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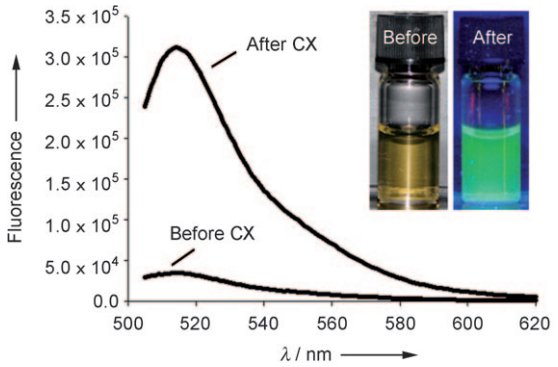

Figure 1. Fluorescence spectra measured before and after cation exchange (CX) reaction with Ag^+ ions in CdSe nanocrystals. Images of CdSe suspensions before and after CX illuminated by a handheld UV lamp are shown as insets.

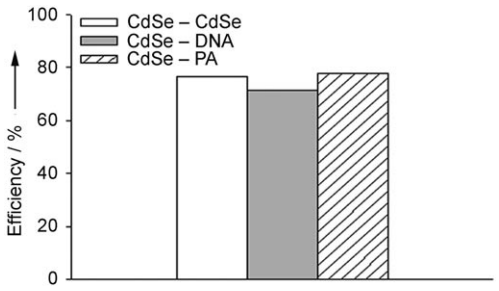

Figure 2. Comparison of cation-exchange efficiency in non-, DNA-, and protein A (PA) conjugated CdSe nanocrystals.

polyacrylic acid (PAA) because of the creation of surface defect sites. A solution of CdSe particles (5 nM) and Fluo-4 (10 μM) initially showed negligible fluorescence under UV illumination, but displayed bright fluorescence upon the addition of Ag^+ ions (500 μM; see images in Figure 1). The fluorescence emission spectra (Figure 1, excitation at 490 nm) also exhibited about ninefold fluorescence enhancement after CX. Moreover, the plot of fluorescence versus nanocrystal concentration was linear with a detection limit of 0.5 pM and a low average relative standard deviation (RSD) of 3% ($n = 3$), which indicated that CXFluoAmp could serve as a quantitative, sensitive, and reproducible detection method (see Figure S1 in the Supporting Information).

The propagation of the reaction front at the interface, that is, the surface of the nanocrystal, is no longer the rate-limiting process of the ion-exchange reaction in the nanometer-sized crystals as in the case of bulk material, because of the relatively small number of atomic layers within a few nanometers.[15] Instead, the accessibility of the reaction interface to the ion-exchange reagents could affect the reaction more, which may be impeded by the conjugation of nanocrystal to the reporter molecules.[15] Therefore, we compared the cation-exchange efficiency among the PAA-coated CdSe, the oligonucleotide-modified CdSe (27-nt, $M_w = 8250.4\ g\,mol^{-1}$), or the protein A ($M_w = 42$ kDa) modified CdSe under the same CX conditions. The amount of Cd^{2+} ions was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). The exchange efficiency was assessed as the percentage of Cd released from CdSe by CX. In an aqueous environment, more than 75% of the Cd^{2+} ions could be released within one minute from the three CdSe preparations, and biomolecule conjugation had negligible effect on the exchange efficiency (Figure 2; Table S1 and Figure S2 in the Supporting Information). This effect could be attributed to the high diffusivity of the metal ions, the loose structure of the PAA coating of the nanocrystal, and the large surface area of the nanocrystals.

To evaluate the performance of CXFluoAmp in the detection of biomolecules compared to traditional fluorescent dye labeling, protein A labeled with the CdSe nanocrystals or Alexa Fluor 488 was employed to detect human immunoglobulin G (IgG) immobilized on a microtiter plate. We compared the net fluorescence signals after background (IgG concentration = 0) subtraction from the captured protein A in Figure 3a. An approximately 60-fold fluorescence increase

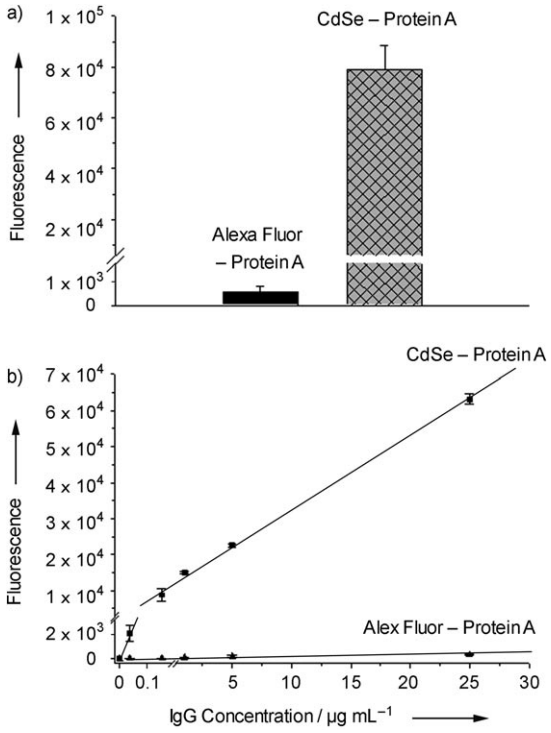

Figure 3. a) Bar plot of fluorescent intensities measured at 520 nm after the capture of Alexa Fluor 488 conjugated protein A or CdSe-conjugated protein A by immobilized IgG. b) Detection of IgG with protein A conjugated with Alexa Fluor 488 or CdSe.

was obtained with CXFluoAmp compared to the organic dye labeling. The high fluorescence intensity also resulted in a much steeper calibration curve, the fluorescence from the CdSe labeling rose much more rapidly with the IgG concentration than that of the Alexa 488 label (Figure 3b). Marginal fluorescence enhancement from the background signal was observed at an IgG concentration of 0.05 $\mu g\,mL^{-1}$ (which corresponds to 300 fM IgG) by using CXFluoAmp as the detection scheme, while the organic dye-based detection only led to the reliable detection of 5 $\mu g\,mL^{-1}$ IgG. The fluorescence amplification factor of 60 compared to the regular

organic dye, the higher detection sensitivity, and the 100-fold lower limit of detection obtained in our proof-of-principle study clearly demonstrate the power of the CXFluoAmp system as a superior detection method.

CdSe was chosen in the current study only because its complete conversion to Ag_2Se by cation exchange has been demonstrated in toluene,[15] and because numerous synthesis strategies have been developed together with various surface modification methods for improved water solubility and feasibility in chemical conjugations, which facilitates their applications in bioassays.[17–20] Toxicity could be a concern regarding the applications of CdSe in biosensing. However, CdSe is only employed in CXFluoAmp to detect bioanalytes that have been isolated from the biosystem on a detection platform such as a microarray. There is no direct potential toxic effect from CdSe to the biosystem. In addition, it has been demonstrated that the cation-exchange reaction can take place in nanocrystals with different shape, size, or composition. The metal-responsive fluorophore used in our study, Fluo-4, also coordinates to various divalent metal ions, such as Zn^{2+}, Pb^{2+}, Ni^{2+}, and Cu^{2+}, which can trigger intense fluorescence (Figure S3 in the Supporting Information).[21–24] Therefore, other types of nanocrystals could be chosen for our system. For example, we tested the cation exchange in PbS nanocrystals with Ag^+ ions, and observed a fluorescence enhancement of about fourfold in Fluo-4 (see Figure S4 in the Supporting Information). The results from this study suggested the use of other types of nanocrystals that allow higher biocompatibility of the system by selection of less toxic ions such as Zn^{2+}. Differences in the fluorescence gains obtained with different metal ions could be caused by variations in the binding affinity between M^{2+} ions and Fluo-4, and in the structural rigidity of Fluo-4 induced by binding that directly impacts its quantum yield.

As Fluo-4 or other metal indicators all have excitation and emission wavelengths compatible with the common optical detection platforms such as confocal microscopes, microtiter plate readers, and microarray scanners, CXFluoAmp could be a general reporting method for bioassays without special instrumental requirements. Multiplexed detection with CXFluoAmp is also possible since a variety of metal-responsive fluorescent metal sensors with distinguishable optical characteristics and affinity to metals are available.[16] Furthermore, by selecting different combinations of fluorophores and nanocrystals, we can improve the detection performance of CXFluoAmp.[15,16] For instance, the usage of Fluo-4 not only requires tedious water treatment with Chelex ion-exchange resin before preparing the detection solutions, but also needs a masking reagent, ethylene glycol tetraacetic acid (EGTA), to shield the residual Ca^{2+} ions by complexation. Otherwise, a high fluorescence background could be observed because of the high binding affinity of Fluo-4 for Ca^{2+} ions. Such treatments were avoided by replacing Fluo-4 with Rhod-5N, which has higher affinity for Cd^{2+} ions than Ca^{2+} ions.[16,25,26] A fluorescence intensity increase of about 10-fold was obtained when Rhod-5N was used as the indicator (Figure S5 in the Supporting Information).

In summary, CXFluoAmp takes advantage of the unique thermodynamic and thermokinetic properties of nanomaterials in chemical reactions to achieve a fast and simple but effective scheme of fluorescence signal amplification for sensitive detection in bioassays. The method is adaptable to the conventional bioassay formats. It is flexible in selection of the nanocrystal and metal-responsive fluorophore combination, which could further enhance its detection performance and make it more environmentally friendly.

Experimental Section

The procedure for the conjugation of the CdSe particles to biomolecules can be found in the Supporting Information. All fluorescence measurements were performed by using a Spex FluoroLog Tau-3 fluorescence spectrophotometer (HORIBA Jobin Yvon Inc., NJ) at excitation wavelengths of 490 nm (for Fluo-4) or 530 nm (for Rhod-5N). A suspension of CdSe in KOAc (0.1 M)/EGTA (10 μM) /Tween 20 (0.05 %) at pH 7.0 was first treated with Chelex 100 for 1 h, and then mixed with Fluo-4 and $AgNO_3$ to trigger the cation exchange reaction and fluorescence amplification. No Chelex treatment and EGTA were used with Rhod-5N. The immunoassay was performed in a 96-well plate, which was first coated with human IgG solutions for 1 h, blocked by 1 % BSA in 1 × PBS for 30 min, and then incubated with Alexa 488 (1 μM, 100 μL) labeled protein A or CdSe-labeled protein A in PBS/BSA (0.1 %) for 2 h (PBS = phosphate-buffered saline, BSA = bovine serum albumin). All incubations were conducted at room temperature. In the case of Alexa 488 labeled protein A, the captured protein A molecules were first released in glycine (0.1 M)/HCl/NaCl (0.1 M)/Tween 20 (0.05 %) buffer (120 μL) at pH 2.5, and the solution was then neutralized with NaOH and transferred to the FluoLog fluorometer for fluorescence measurements. It was confirmed that the fluorescence intensity of Alexa Fluor 488 was not reduced by the glycine treatment (data not shown). For detection with the CdSe-labeled protein A, a detection solution (120 μL) of KAc (0.1 M)/EGTA (10 μM/ Tween 20 (0.05 %) containing $AgNO_3$ (500 μM) and Fluo-4 (10 μM) was added to induce the fluorescence before measurement.

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