Dual Emission Fluorescent Silver Nanoclusters for Sensitive

Detection of the Biological Coenzyme NAD^+/NADH

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ABSTRACT

Fluorescent silver nanoclusters (Ag NCs) displaying dual-excitation and dual-emission properties have been developed for the specific detection of NAD^+ . With the increase of NAD^+ concentrations, the longer wavelength emission (with the peak at 550 nm) was gradually quenched due to the strong interactions between the NAD^+ and Ag NCs, while the shorter wavelength emission (peaking at 395 nm) was linearly enhanced. More importantly, the dual-emission intensity ratio (I_{395}/I_{550}), fitting by a single-exponential decay function, can efficiently detect various NAD^+ levels from 100 μM to 4000 μM , as well as label NAD^+/NADH ratios in the range of 1 to 50.

Keywords: Dual-emission fluorescent silver nanoclusters, Dual-emission intensity ratio, Biological coenzyme NAD^+ , NAD^+/NADH ratios

Introduction

The biological coenzyme NAD^+ , and its reduced form NADH , are critical intermediates of cellular energy metabolism, mitochondrial function, gene expression, calcium homeostasis, cell death, aging, and carcinogenesis. [1-7] In light of the important roles that NAD^+ and NADH play in redox processes, it is of considerable significance to develop sensitive and specific sensors for monitoring NAD^+/NADH .

Thus far, various efficient and reproducible biochemical methods, such as high

performance liquid chromatography (HPLC), [8] capillary electrochromatography (CE), [9] and enzymatic cycling assay, [2] have been developed to detect NAD^+ levels. However, these methods have the drawbacks of high cost, time-consumption, and destructive sample processing. Unlike other polycyclic aromatic coenzymes with strong fluorescence (such as NADH, NADPH, and FAD), NAD^+ has no fluorescent chromophore.[10] Therefore, it is impossible to directly detect NAD^+ via fluorescence measurement. Many investigators have created genetically encoded fluorescent protein biosensors (Rex, cpFPs, Peredox, Frex/Frexh) to report NAD^+ levels.[2-3, 6, 11-13] It is interesting to note that the protein sensors demonstrate stronger affinity to NADH than NAD^+ . In fact, genetically encoded fluorescent proteins are not specific sensors for NAD^+ . Moreover, in living cells, the level of NAD^+ dominates in the total levels of NAD^+/NADH . Therefore, a specific and sensitive NAD^+ sensor would be more preferred and valuable. Although these protein biosensors open up new avenues in developments of high performance sensors for the sensitive detection of NAD^+/NADH , there is still a great need for the development of fluorescent nano-sensors with not only high sensitivity and selectivity but also low operation cost. Until now, there are few reports about sensitive sensors that directly detect the biological molecule NAD^+ .

Recently, silver nanoclusters (Ag NCs) have attracted considerable attention because of their excellent photo-stability, non-toxicity, low steric hindrance properties, and tunable fluorescence emissions. In contrast to the large size of Ag nanoparticles (Ag NPs), the Ag NCs can be synthesized in many stabilizing scaffolds, such as dendrimers,[14] polymers,[15-16] proteins,[17] and DNA.[18] Among those templates, DNA, oligonucleotide, and nucleobases-templated Ag NCs have received special attention, because some nucleobases are excellent ligands for the Ag NCs.[18-20] Since NAD^+ contains active nitrogen atoms and π electron systems (adenine, nicotinamide moieties), it has been proved that they can strongly interact with the metal nanoparticles. Here we reported a sensitive probe of NAD^+ , named dual-emission fluorescent Ag NCs, which can be used to monitor NAD^+ levels and various NAD^+/NADH ratios, and to facilitate practical biological coenzyme detection.

Materials and methods

Materials

All the reagents were used without any further purification. Nicotinamide adenine dinucleotide disodium salts (NAD^+ (98%), and its reduced form, NADH (98%)) were purchased from Roche Company. Silver nitrate (AgNO_3), and ascorbic acid were bought from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cysteamine (96%) was bought from J&K Chemical Ltd (Shanghai, China). Ultrapure water (18.2 M Ω) was used throughout all the experiments.

Synthesis of Ag NCs

The Ag NCs were prepared in two steps: the modification of Ag NPs with cysteamine, and the second etching of NAD^+ . The Ag NPs were fabricated as follows: at room temperature, 5 mL distilled water and freshly prepared ascorbic acid (75 μL , 10 mM) were injected in a clean beaker under vigorous stirring. Silver nitrate solution (3 mL, 0.5 mM) was added into the reaction mixture solution drop by drop. 5 minutes later, the Ag NPs were completed. The prepared Ag NPs (900 μL) were modified by cysteamine solution (100 μL , 50 μM) with a molar ratio of (1:8). 3 hours later, the cysteamine capped Ag NCs formed. In order to allow for efficient interactions, NAD^+ with different concentrations and cysteamine capped Ag NCs were mixed and equilibrated 3 hours before spectral measurements.

Instrumental measurements

At room temperature, the steady fluorescence spectra were measured by a Hitachi F-4500 spectrofluorometer with 5 nm slits, PMT voltage at 700 V, and 240 nm/min scanning speed.

Results and discussion

The procedure for NAD^+ detection via dual-emission fluorescent Ag NCs

The procedure for the detection of NAD^+ via fluorescent Ag NCs is shown in Scheme 1. The Ag NCs were prepared and synthesized by two etching processes. After the first modification of the small thiol molecule cysteamine, the initial fluorescence emission peak of cysteamine capped Ag NCs locates around 550 nm

(Fig.1). Due to the positive charge on the surface of Ag NCs, it has a high affinity to negative NAD^+ ligands, which are good electron donors. The interactions between the Ag NCs and the NAD^+ ligands caused an obvious change in the fluorescence emission spectrum. The levels of NAD^+ can be calculated by measuring the dual-emission of Ag NCs.

Scheme 1 Schematic representation of the procedure for the detection of NAD^+ via dual-emission Ag NCs

Fig.1 The normalized steady state excitation (dash red curve) and emission (solid blue curve) spectra of cysteamine capped Ag NCs

Sensitive detection of NAD^+ via dual-emission fluorescent Ag NCs

Figure 2 shows the relation of dual-emission (550 nm, 395 nm) fluorescence intensity with NAD^+ concentrations. There are strong interactions between Ag NCs and NAD^+ molecules, which caused the quenching of the longer wavelength emission (550 nm, Fig.2(A)), and produced a new and enhanced short wavelength emission (395 nm, Fig.2(C)). The fluorescence intensity at longer wavelength was quenched

quickly when the NAD^+ concentration was in the range of 0.1-500 μM (Fig.2(B)). There is no significant appearance of new fluorescence emissions with NAD^+ concentrations lower than 100 μM . However, a new fluorescence peak at 395 nm increases linearly with NAD^+ concentrations from 100 μM to 4 mM (Fig.2(C) & Fig.2(D)). Since NAD^+ acts as an etching ligand to cysteamine capped Ag NCs, another type of Ag NCs was formed, which has a fluorescence emission at 395 nm.

Fig.2 The relation of dual-emission steady state fluorescence intensity with NAD^+ concentrations. (EM 550 nm, Fig.2(A)&Fig.2(B), EX 510 nm; EM 395 nm, Fig.2(C)&Fig.2(D), EX 290 nm)

The fluorescence emissions at 395 and 550 nm showed different dependences on the NAD^+ concentration. The fluorescence intensity ratio (I_{395}/I_{550}) curve can be described by an exponential function of NAD^+ concentrations, as shown in Fig.3. The curve of the fluorescence intensity ratio (I_{395}/I_{550}) against various NAD^+ concentrations shows a good mono-exponential dependence from 100 μM to 4000 μM . Actually, exponential decay function models have already been used to explain the fluorescence intensity quenching of some metal nanoparticles, due to the interactions

between analytes and nanoparticles with binding constant.[21-22] Reported actual concentrations of NAD^+ are ~ 300 mM in animal cells [8, 23] and ~ 1 mM in yeast.[24] Therefore, the efficient detection limit of dual-emission Ag NCs can satisfy the requirements of NAD^+ detection in living cells and cell extracts. In addition, compared with single fluorescence emission detection, the dual-emission Ag NCs can avoid some experimental errors from current measurement systems and enhance the precision of detection methods. The equation of fit (Fig.3) shows that the detection precision can be elevated by a factor of almost 18. Therefore, the dual-emission intensity ratio can efficiently label the NAD^+ concentrations.

Fig.3 The responses of the fluorescence intensity ratio (I_{395}/I_{550}) against various NAD^+ concentrations from 100 μM to 4000 μM .

Specificity detection of NAD^+ via dual-emission fluorescent Ag NCs

Besides the sensitivity of the fluorescent Ag NC sensor, high specificity is also important in real sample detections. To evaluate the specificity of fluorescent Ag NCs, we selected NADH as the coordinating ligand to measure the fluorescence changes of Ag NCs under the same conditions. However, there is no significant fluorescence emission located at 395 nm (Fig.4). The difference between NAD^+ and NADH is that NADH has reduced nicotinamide moiety, causing the poor electron-transfer capacity

between Ag NCs and the coordinating ligands, which cannot support the luminescence of Ag NCs. Thus, the fluorescent Ag NC sensor shows a good selectivity for NAD^+ . Although the fluorescent Ag NCs are not sensitive to the presence of NADH, it provides the possibility to label NAD^+/NADH ratios.

Fig.4 The steady state fluorescence spectra of Ag NCs vs various NADH concentrations. The excitation wavelength is 295 nm.

The feasibility of NAD^+/NADH ratio detection via dual-emission fluorescent Ag NCs

The NAD^+/NADH ratio plays a crucial role in regulating the redox state, which is an important indicator of metabolic state. For many years, investigators have estimated the ratios of NAD^+/NADH as follows: 3 in yeast, 4-10 in *Escherichia coli*, 3-10 in mammals, 7-8 in mitochondria, and ~ 700 -1 in cytosol.[3, 25-28] In this paper, we further studied whether Ag NCs can be used to label NAD^+/NADH ratios when the total concentration of NAD^+ and NADH is approximately 500 μM . Fig.5 shows the response of the dual-emission intensity ratio (I_{395}/I_{550}) versus NAD^+/NADH ratios. The dual-emission intensity ratio curve is modeled by a single-exponential decay function with NAD^+/NADH ratios ranging from 1 to 300. However, the efficient detection range is 1-50. There is a competitive interaction mechanism between NAD^+

and NADH. NAD^+ competes with NADH in interacting with Ag NCs, and increasing the NAD^+/NADH ratio efficiently strengthens the interactions between NAD^+ and Ag NCs. When NAD^+/NADH ratios range from 1 to 50, the corresponding dual-emission intensity ratios increase significantly. However, when NAD^+/NADH ratios exceed 50, the dual-emission intensity ratios tend to become stable, because NAD^+ completely dominated in the competition with NADH. Therefore, the Ag NCs can also probe NAD^+/NADH ratios from 1 to 50.

Fig. 5 The responses of the fluorescence intensity ratio (I_{395}/I_{550}) *versus* various NAD^+/NADH ratios from 1 to 300. The total concentration of NAD^+ and NADH is about 500 μM

Conclusions

In the paper, we have designed and created novel dual-emission fluorescence Ag NCs, which are ideal for the sensitive detection of NAD^+ and NAD^+/NADH ratios. The dual-emission intensity ratio can eliminate the experimental error from detection methods. More importantly, the fitted single-exponential decay curves of the fluorescence emission intensity ratio (I_{395}/I_{550}) can efficiently label the NAD^+ concentrations and NAD^+/NADH ratios from 1 to 50.

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