Calcium Sensors

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# Bioinspired Colorimetric Detection of Calcium(II) Ions in Serum Using Calsequestrin-Functionalized Gold Nanoparticles**

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The calcium(II) ion is the most abundant cation in the body, and participates in various biological activities such as skeletal mineralization, blood coagulation, neurotransmission, excitation of skeletal and cardiac muscle, and stimulus-mediated hormone secretion.[1] Blood calcium levels range from 2.1 mM to 2.6 mM in healthy humans; this level is strictly maintained and varies by 3% at most. Severe fluctuations in calcium levels are associated with many diseases. For instance, the common causes of hypercalcemia are primary hyperparathyroidism, malignant tumors, and hyperthyroidism.[2] Therefore, the accurate and fast estimation of blood calcium levels is of great importance.

Several techniques are available to assess blood calcium levels; these include atomic absorption spectroscopy, the use of ion-selective electrodes, and chromophore-based spectrophotometric methods.[3–5] Both the atomic absorption and ion-selective electrode detection methods are, however, complicated and require both expensive instruments and careful maintenance. Chromophore-based spectrophotometric methods often lack selectivity; artifactually high readings that arise from the presence of other divalent metals such as $Mg^{2+}$ in blood samples may be problematic.[6] Nevertheless, chromophore-based spectrophotometry is the most widely used method to detect calcium ions because of its simplicity. Recently, gold nanoparticle (GNP) based colorimetric detection has been devised for a variety of targets including metal ions,[7,8] DNA,[9] bacterial toxins,[10] proteins,[11] and enzyme activity.[12] The aggregation of ligand-functionalized GNPs upon binding to a target results in a colorimetric response that is indicated by broadening and shifting of a surface plasmon resonance peak. Colorimetric methods are convenient and attractive because the color changes can be easily discerned with the naked eye, hence there is no need for any instrumentation. To date, few attempts have been made to detect $Ca^{2+}$ ions using GNPs. Lactose-functionalized GNPs that undergo self-aggregation by $Ca^{2+}$ ion mediated interactions between the carbohydrate moieties were first used for $Ca^{2+}$ ion detection; a colorimetric change resulted from aggregation.[13] However, the linear dynamic ranges of detectable $Ca^{2+}$ ion concentration were 10–35 mM or 0.8–2.0 mM, which lie outside the blood calcium level of 2.2–2.6 mM.[14] To overcome this limitation, we developed a bioinspired GNP-based colorimetric calcium sensor that shows high specificity, can distinguish between normal and abnormal calcium levels with the naked eye, and works under physiological conditions.

The key element of the sensor system is calsequestrin (CSQ) functionalized GNPs, which have an approximate size of 13 nm, and can form aggregates in the presence of appropriate amounts of calcium ions. The aggregation results in a colorimetric change (Figure 1a). CSQ is the most abundant calcium-binding protein and is an endogenous $Ca^{2+}$ ion sensor in the sarcoplasmic reticulum. CSQ binds and releases large amounts of $Ca^{2+}$ ions because of a high capacity (40–50 binding sites per one molecule) and relatively low affinity for $Ca^{2+}$ ions ($K_d \approx 1$ mM).[15] Because of the calcium buffering capacity afforded by CSQ in the luminal space, the concentration of free $Ca^{2+}$ ions in the sarcoplasmic reticulum

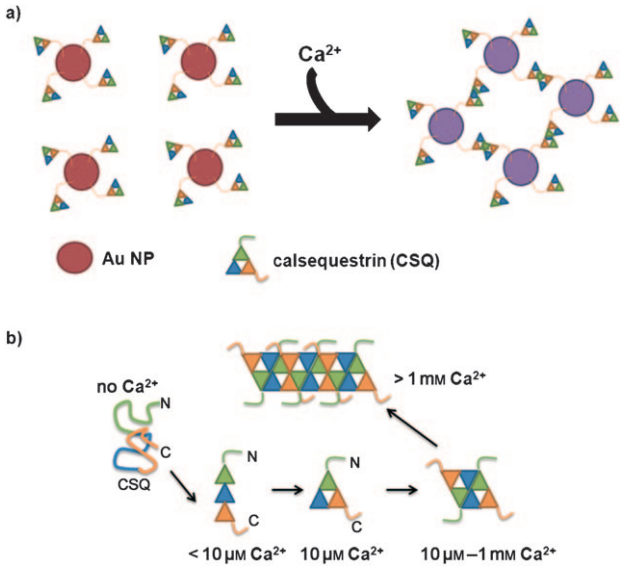


***Figure 1.*** a) Schematic representation of the calcium ion sensor: the aggregation of calsequestrin (CSQ) functionalized gold nanoparticles caused by binding of $Ca^{2+}$ ions results in the color change. b) Calcium-dependent conformational changes and interactions of CSQ molecules, which underlie the aggregation effect of the nanoparticles.

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can be maintained below 1 mM. CSQ undergoes a conformational change as a function of $Ca^{2+}$ ion concentration (Figure 1b). In the absence of $Ca^{2+}$ ions, CSQ adopts an unfolded form. When the $Ca^{2+}$ ion concentration gradually increases from 10 μM to 0.01–1 mM, the randomly coiled CSQ condenses into a compact monomer, which subsequently undergoes dimerization and then polymerization (Figure 1b).[16] We expected that CSQ-functionalized GNPs might form aggregates through CSQ-induced dimerization or polymerization above a threshold $Ca^{2+}$ ion concentration, thus resulting in a red-to-blue color change that arises from changes in the surface plasmon resonance upon GNP binding.

Cysteine-mediated protein immobilization on a gold surface has been widely explored.[17] Therefore, we genetically engineered human cardiac muscle CSQ to contain two cysteine residues at the C terminus (see the Supporting Information). This modified CSQ was immobilized onto GNPs with an average diameter of 13 nm by incubation in an aqueous solution of excess (200-fold) modified CSQ for 12 h. Unbound CSQ was removed by repeated washing and centrifugation. The overall negative charge of CSQ (isoelectric point pI = 4.2) at physiological pH values allowed the resulting GNPs to be well-dispersed in aqueous solution; no aggregation was observed.

The color change of the GNP-based sensor in the presence of $Ca^{2+}$ ions was monitored by UV/Vis spectroscopy (Figure 2a). When $Ca^{2+}$ ions (5 mM) were added to CSQ–GNP conjugates dispersed in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; 20 mM, pH 7.0) at room temperature, the color of the solution began to change to purple, and a precipitate eventually appeared. UV/Vis spectroscopy showed that the plasmon band at 530 nm moved to 575 nm because of the calcium-mediated aggregation (the TEM image of aggregated particles after incubation with 5 mM $Ca^{2+}$ is shown in Figure S1 in the Supporting Information). This result suggests that the CSQ on the surface of GNPs changed conformation upon addition of $Ca^{2+}$ ions, and the CSQ molecules became able to oligomerize or otherwise interact, which led to aggregation. To examine whether the calcium-mediated CSQ–CSQ interaction is reversible, an excess of ethylenediaminetetraacetate (EDTA; 50 mM) was added to the aggregate. The aggregated material immediately returned to the original red-colored, dispersed state, as also confirmed by the UV/Vis spectrum, which showed the return of the initial plasmon peak at 530 nm. This aggregation/dispersion cycle could be repeated up to three times (Figure 2b).

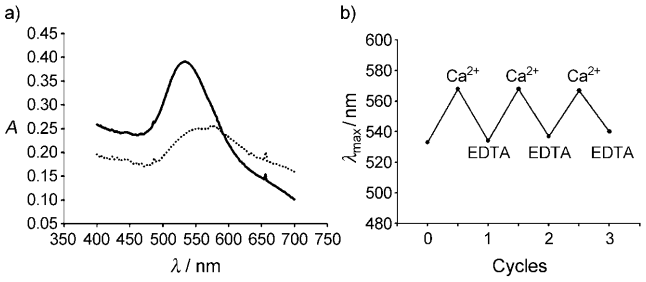


***Figure 2.*** a) UV/Vis spectra of CSQ–GNPs in the absence (solid line) and the presence (dotted line) of $Ca^{2+}$ ions (5 mM). b) Maximum absorption wavelength ($\lambda_{max}$) of CSQ–GNPs with $Ca^{2+}$ ions (5 mM) and EDTA (50 mM). $\lambda_{max}$ was restored to 530 nm upon complexation of $Ca^{2+}$ ions with an excess of EDTA, and repetitive addition of $Ca^{2+}$ ions caused re-aggregation.

The ion selectivity of the CSQ–GNPs was evaluated using a variety of metal ions that might interfere with $Ca^{2+}$ ion detection in biological samples, including divalent ions ($Mg^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Hg^{2+}$, $Cd^{2+}$, $Ba^{2+}$, and $Sr^{2+}$) and monovalent ions ($K^+$ and $Na^+$). Human blood contains $Ca^{2+}$ (2.14–2.56 mM), $K^+$ (3.50–5.10 mM), $Mg^{2+}$ (385–544 μM), and $Na^+$ (136–145 mM). The levels of other metal ions in blood are very low, ranging from nanomolar to micromolar concentrations ($Zn^{2+}$, ca. 30 μM; $Sr^{2+}$, ca. 400 nM; and $Ba^{2+}$, ca. 500 nM). We used ion concentrations much higher than those in blood in our selectivity test: $Mg^{2+}$ (5 mM), $K^+$ (10 mM), $Na^+$ (200 mM), and all other ions ($Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Hg^{2+}$, $Cd^{2+}$, $Ba^{2+}$, and $Sr^{2+}$) at 100 μM. Out of all the ions tested (Figure 3), only incubation with $Ca^{2+}$ ions led to a color change, which indicates the high specificity of the CSQ–GNP sensor system. Although $Mg^{2+}$ is the second most abundant divalent cation in blood and is known to severely interfere with $Ca^{2+}$ ion sensing using chromophore-based sensors, the CSQ–GNP system did not respond to $Mg^{2+}$ ions even at a much higher concentrations (10-fold) than the normal blood level. This result clearly indicates that the CSQ–GNP system is more specific than any existing chromophore-based calcium-sensing methods. No aggregation was observed in the presence of other divalent ions ($Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Hg^{2+}$, $Cd^{2+}$, $Ba^{2+}$, or $Sr^{2+}$) or in the presence of monovalent ions such as $Na^+$ and $K^+$, at concentrations much higher than normal blood levels, which indicates that these ions do not interfere with $Ca^{2+}$ ion detection by the CSQ–GNP sensor system.

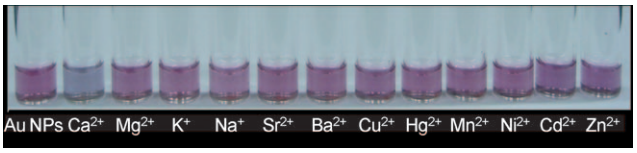


***Figure 3.*** Colorimetric responses of CSQ–GNPs to different metal ions whose concentration levels are greater than seen in blood: $Ca^{2+}$ (5 mM), $Mg^{2+}$ (5 mM), $K^+$ (10 mM), $Na^+$ (200 mM); $Sr^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Cd^{2+}$, and $Zn^{2+}$ (all 100 μM).

To examine the utility of the CSQ–GNP sensor, the sensitivity and linear detection concentration range was evaluated by UV/Vis spectroscopy (Figure 4). The ratio of absorbance intensities at 630 and 530 nm ($A_{630}/A_{530}$) was used to assess the degree of aggregation; a larger value indicates a higher degree of aggregation. Various $Ca^{2+}$ ion concentrations spiked in phosphate buffered saline (PBS; pH 7.4) were tested. The sensor showed linear detection from 1–4 mM $Ca^{2+}$, which is well matched to the physiological concentration range of the ion (Figure 4b). Furthermore, as seen in Figure 4a (the optical image), a clear color change from red to purple along with precipitation was observed by the naked

  

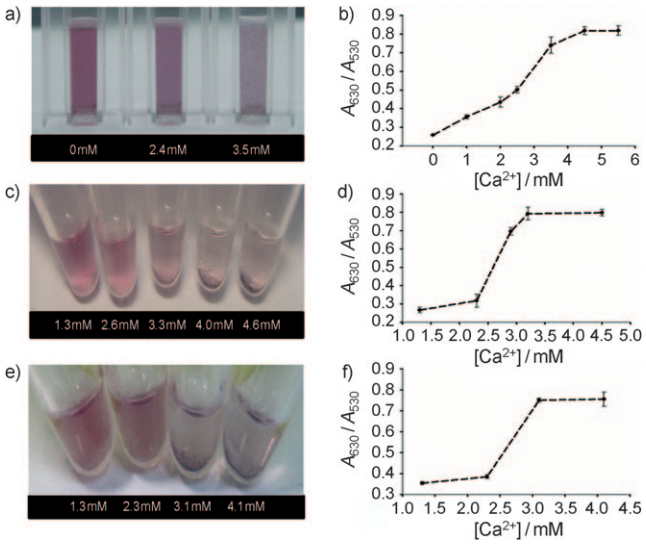


***Figure 4.*** a) Colorimetric responses of CSQ–GNPs to different $Ca^{2+}$ ion concentrations (0, 2.4, and 3.5 mM) in PBS (pH 7.4). b) $A_{630}/A_{530}$ of CSQ–GNPs systems at various $Ca^{2+}$ ion concentrations (1, 2, 2.5, 3.5, 4.5, and 5.5 mM) in PBS. c) Colorimetric responses of CSQ–GNPs to different total calcium ion concentrations (1.3, 2.6, 3.3, 4.0, and 4.6 mM) prepared from FBS. d) $A_{630}/A_{530}$ of CSQ–GNPs systems at various $Ca^{2+}$ ion concentrations of FBS (1.3, 2.3, 2.9, 3.2, and 4.5 mM). e) Colorimetric responses of CSQ–GNPs to different calcium ion concentrations (1.3, 2.3, 3.1 and 4.1 mM) prepared from rat serum. f) $A_{630}/A_{530}$ of CSQ–GNPs systems at various $Ca^{2+}$ ion concentrations of rat serum (1.3, 2.3, 3.1, and 4.1 mM).

eyes when $Ca^{2+}$ concentration changed from 2.4 to 3.5 mM. This result suggests that the sensor may distinguish between normal (2.4 mM) and hypercalcemic (3.5 mM) conditions frequently found in patients with malignancies. Encouraged by these findings, we applied the sensor to detect $Ca^{2+}$ ions in physiological samples. $Ca^{2+}$ ions in human serum exit in the forms of unbound (free $Ca^{2+}$ ions) and complexed with proteins (mostly albumin) in an approximately equal ratio. It is also known that the bound, complexed $Ca^{2+}$ ions can be released by lowering the pH value to 3. In our experiment, fetal bovine serum (FBS) and rat serum were tested as models of human serum. For the preparation of hypercalcemia samples, additional $Ca^{2+}$ ions at determined concentrations were spiked into each FBS and rat serum containing a normal level of $Ca^{2+}$ ions. To measure the total $Ca^{2+}$ ion concentration in the serum, FBS and rat serum containing various concentrations of $Ca^{2+}$ were treated with HCl to adjust the pH value to 2 and release the bound $Ca^{2+}$ ions, followed by filtration using a spin filter of 10 kDa pore size to remove proteins. The pH value of the resulting filtrate was restored the physiological value using Tris buffer (1M, pH 8.5) before $Ca^{2+}$ ion detection using the CSQ–GNPs sensor. The total $Ca^{2+}$ ion concentration in each filtrate was measured by using an inductively coupled plasma optical emission spectrometer (ICP-OES), which revealed that hypocalcemic (1.3 mM for both FBS and rat serum), normal (2.6 mM for FBS; 2.3 mM for rat serum), and hypercalcemic (3.3 mM, 4.0 mM, and 4.6 mM for FBS; 3.1 mM and 4.1 mM for rat serum) samples were prepared. With the CSQ–GNPs sensor system, no color change was seen from the hypocalcemic and normal samples, whereas the color changed from red to purple and finally clear precipitation was clearly seen from hypercalcemia levels (Figure 4c,e for FBS and rat serum, respectively).

Similar to $Ca^{2+}$ ion detection in PBS (Figure 4b), UV/Vis spectroscopy measurements led to clear differences in the ratio of absorption intensities at 630 and 530 nm only with hypercalcemic samples (Figure 4d,f for FBS and rat serum, respectively). Encouraged by these results, we tested whether the CSQ–GNPs work with real human serum in a preliminary trial. The CSQ–GNPs showed a clear color change from pink to purple with a hypercalcemic patient sample (3.0 mM; see Figure S2 in the Supporting Information), which indicated that our system has potential for use in practical $Ca^{2+}$ ion sensors. This result indicates that the CSQ–GNPs system can distinguish between normal and abnormal (hypercalcemia) calcium levels under the physiological conditions by the naked eye without the aid of any instruments.

In conclusion, we have developed a simple and rapid colorimetric method for the detection of $Ca^{2+}$ ions with high specificity using calsequestrin-functionalized GNPs. The technique does not require specialized equipment because test results can be easily seen by the naked eye. Unlike most chemical chromophore-based $Ca^{2+}$ ion sensors that show poor selectivity for $Ca^{2+}$ ions over $Mg^{2+}$ ions and other divalent cations, the CSQ–GNP sensor shows high specificity for $Ca^{2+}$ ions. More importantly, the CSQ–GNP sensor can also distinguish between normal and abnormal (hypercalcemia) $Ca^{2+}$ ion levels in serum, by showing a clear color change from red to purple along with precipitation for abnormal $Ca^{2+}$ ion levels. This bioinspired sensing system, which allows visualization of changes in blood calcium levels, may be useful in the detection or monitoring of several diseases associated with hypercalcemia, such as malignant tumors.

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