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Stable and Oxidant Responsive Zwitterionic Nanoclusters

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Abstract

Zwitterions are promising ligands for the fabrication of non-toxic and non-interacting biomaterials. Sulfonamide-based monothiol zwitterionic ligands on gold nanocluster (AuNC) surfaces provide nanomaterials with stable colloidal properties and intense red emission in biological environments. The fluorescence intensity of the nanocluster can be modulated by reactive oxygen species (e.g. •OH), allowing for quantitative and selective sensing of antioxidants (e.g. ascorbic acid) in real time.

Gold nanoclusters are composed of several to dozens of gold atoms, with dimensions between those of atoms and nanoparticles. Different from metallic gold nanoparticles, these nanostructures do not exhibit surface plasmon resonance (SPR) absorption in the visible region, but have fluorescence in the visible to near-infrared (NIR) region and exhibit excellent photo-physical properties. They have been used as fluorescent motifs and show wide applications in biochemical sensing and imaging fields.^{1–4} Two parameters of AuNCs, their fluorescent properties and stability, are key factors for determining their usefulness in bio-applications. Chemical reduction,⁵ photo-reduction,⁶ and chemical etching⁷ are the most common approaches in generating clusters with fluorescence. However, this fluorescence can be compromised by reduced colloidal stability of the nanoclusters in the presence of excessive salts, varied solution pH, and thiol-containing reducing agents that commonly exist in biological environments.^{8,9} An optimized design of the surface chemistry of clusters, such as introducing densely packed ligands and non-interactive functionalities, should greatly improve their stability, biocompatibility, of AuNCs, enhancing their utility for biomedical applications.

Neutrally charged zwitterions are promising ligands for fabricating multifunctional materials for a variety of biological applications.^{10–13} Nanoparticles with zwitterionic surfaces feature

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Conflicts of interest

There are no conflicts to declare.

excellent water solubility, biocompatibility, and stability in complex physiological environments that include high ionic strength, complex protein mixtures (e.g. serum) and varied pH.^{14,15} The resulting metallic nanoparticles feature highly organized ligand coverage on its surface compared as compared to smaller particles,¹⁶ offering excellent stability and antimicrobial properties.

We hypothesized that zwitterionic ligands could be used to fabricate fluorescent NCs featuring greater stability in biological milieus. We report here the use of sulfonamide-based monothiol zwitterionic ligands to create fluorescent AuNCs that were stable over a broad pH range (1–14), in high salinity, and in biologically relevant solutions. These sulfonamide-coated zwitterionic AuNC are emissive and oxidant-responsive, making them useful as probe for bioanalysis, with the ability to screen antioxidants in complex biological environments.

Experimental

Synthesis of zwitterionic nanoclusters

Chloroauric acid (5.0 mg in 500 μ L) and sulfonamide-based monothiol zwitterionic ligand (Fig. 1A, 30.0 mg) were dissolved in 7 mL of methanol/acetic acid 6:1 (v/v). 100 μ L of ice-cold water contained sodium borohydride (10.0 mg) was added dropwise with rapid stirring. After continuous stirring for 3 h, the solvent was then removed under vacuum at 40 $^{\circ}$ C. The residue was dissolved in de-ionized water, and the pH was adjusted to about 1 by addition of HCl (1 M). The solution was then dialyzed (dialysis membrane, MWCO=1000 kD) for 72 h against deionized water that was changed every 8 h.

Characterization of as-synthesized AuNC

The morphology and core size of AuNC were characterised using a JEOL-2010 microscope with an accelerating voltage of 200 kV. The hydrodynamic diameter of the AuNCs was measured on a Malvern Zetasizer (Malvern Instruments Inc, USA) with a He-Ne laser (633 nm) and a backscattering angle of 173 $^{\circ}$. Weight peak of ligand on the surface of AuNC was tested by laser-desorption/ionization mass spectrometry (Bruker Daltonics, Bremen, Germany). X-Ray Photoelectron Spectroscopy (XPS) spectrum of AuNC was measured by a Physical Electronics Model 5100 spectrophotometer.

All the excitation and fluorescence measurements were performed on a SpectraMax M5 spectrometer/plate reader (25 $^{\circ}$ C, Molecular Devices, USA). All the emission spectra were recorded upon excitation at 375 nm.

Responsiveness of reactive oxygen species

For $\bullet$ OH quenching study, 100 μ L AuNC (0.5 μ M) were mixed with 400 μ L of Fe²⁺/H₂O₂ with different concentrations. After 5 min incubation, the changes in fluorescence behavior of the solutions were observed using a fluorescence spectrometer.

Responsiveness of antioxidant

For antioxidant detection, 100 μL AuNC (0.5 μM) were mixed with 300 μL of different concentrations of ascorbic acid (AA) in 10 mM PBS (pH 5.0), followed by the addition of 50 μL of 800 μM Fe^{2+} and 50 μL of 800 μM H_2O_2 , respectively. After incubation at room temperature for 5 min, fluorescence spectra of the solutions were measured upon being excited at 375 nm.

Results and discussion

Synthesis and characterization of zwitterionic gold nanoclusters

Monothiol-based zwitterionic ligands¹¹ (Fig. 1A, and supporting information) were chosen as the capping agent to provide greater ligand coverage, enhancing the stability and biocompatibility of the AuNCs for use in biological environments.^{16,17} Sulfonamide-based monothiol zwitterionic AuNCs were fabricated using a modified Brust-Schiffrin protocol through direct reduction of gold precursors in the presence of the zwitterionic ligands using excess NaBH_4 .¹⁸ As revealed by transmission electron microscopy (TEM, Fig. 1B), the resulting average core size of obtained AuNC was < 2 nm, with a narrow size distribution when a 3:1 molar ratio of surface ligand to gold salt was used. The size of as-prepared AuNC was statistically analyzed to be 1.7 ± 0.4 nm (Fig. S1). Dynamic light scattering (DLS, Fig. 1B inset) analysis showed that the hydrodynamic size of the zwitterionic ligand-covered AuNC was 4.4 nm, consistent with the predicted size of the AuNC. The ligand on the AuNCs was analyzed using laser desorption/ionization mass spectrometry (LDI-MS, Fig. 1C). The molecular peak observed at m/z had a value of 601.2, consistent with the exact molecular weight of the zwitterionic ligand.

We next explored the optical properties of as-synthesized AuNCs. (Fig. 2A) Upon excitation at 375 nm, the AuNCs produced a strong emission band centred at 656 nm. This fluorescence is usually attributed to the mixed valence states of gold (I and 0) presented on the surface of the AuNC due to the incomplete reduction of Au(III) to Au(0).¹⁹ We carried out an XPS measurement to analysis the gold valence states with the obtained AuNC. The XPS spectra showed that the binding energies (BE) of Au $4f_{7/2}$ can be deconvoluted into two distinct components, as shown by the red fitting curve in Fig. 2B. This indicates the co-existence of Au(0) and Au(I) in the AuNCs. Moreover, the asymmetric band can be fitted into two peaks at around 84.0 eV and 85.4 eV, which were assigned to Au(0) and Au(I), respectively (pink curve and green curve in Fig. 2B).^{20,21} The red emission of our NC can thus be attributed to their small sizes and the presence of fractions of Au(0) and Au(I)-ligand complexes on the surface of the clusters.²²

We next determined the colloidal and fluorescent stability of the AuNC at different concentrations, and dispersed in several biological media at room temperature over a period of one month. As shown in Fig. 2C, AuNC emitted concentration-dependent bright red fluorescence on Day 1 and maintained their intensity over the course of the month at all concentrations tested. Moreover, the clusters also can retain both their small sizes (Fig. S2) and bright fluorescence emission in the presence of large excesses of salt (PBS) and against competition from the natural reducing agent glutathione (0.5 M GSH), which always poses a

hurdle to the positively charged nanoclusters.²³ The excellent stability probably resulted from the dense packing of zwitterionic ligands on the surface of AuNC.¹⁶ Furthermore, we challenged the colloidal stability of the AuNC in the presence of 10% FBS (Fetal Bovine Serum)-containing cell culture medium.²⁴ Corona formation in serum typically masks the nanoparticle surface, changes its physico-chemical properties, and eventually induces aggregation and precipitation. However, our data (Fig. 2 and Fig. S2) indicated that the NC remained quite stable in this medium for one month, and there was no obvious fluorescence change being observed. Here, it should be noted that the DMEM media itself exhibits a pronounced blue fluorescence, which is due to the presence of amino acids bearing aromatic residues,²⁵ which altered the overall emission of the dispersed cluster from red to bright pink, as shown in Fig. 2C. The long-term stability of synthesized AuNC exposed to the cell culture media can be attributed to the corona-minimizing properties of the neutrally charged zwitterionic surfaces,¹⁵ which can prevent proteins attachment on the clusters. Additionally, there is no detectable change of the fluorescence from the as-prepared nanoclusters when exposed to either extreme acidic (pH 1.0) or alkaline (pH 14.0) conditions, which meets the essentially all real-world sensing pH values.

Detection of reactive oxygen species (ROS)

Reactive oxygen species play important roles in cell homeostasis and signalling, and high ROS levels may result in significant damage to cellular structures and functions.^{26–28} Previous studies have shown that the oxidative properties of ROS can change gold valence states and induce fluorescence quenching, providing an indicator for these species. We next explored whether the zwitterionic AuNCs can be used as a ROS indicator. To this end, the Fenton reaction was used to generate the most reactive oxygen species, •OH. And the fluorescence change of the AuNCs in the presence of •OH was monitored by a fluorescence spectrometer.²² As shown by the fluorescence image shown in Fig. 3A, the red emission of AuNC solution faded gradually when the concentration of Fe²⁺/H₂O₂ (1:1 molar ratio) was increased from 5 to 80 μM. Quantitatively, the emission intensity of NC was quenched to 85% by 5 μM Fe²⁺/H₂O₂ (curve b) compared to the control (curve a). When increasing the concentration of generated •OH, the fluorescence was almost fully quenched (7%) when the Fe²⁺/H₂O₂ concentration increased to 80 μM (curve f). Moreover, we observed a linear relationship between the fluorescence intensity and the Fe²⁺/H₂O₂ concentration over a concentration range of 5–80 μM, highlighting the potential use of this AuNC as only a cellular ROS sensor. This fluorescence quenching phenomenon most likely arose from the oxidation of Au(0) to Au(I) in the clusters, as documented by previous reports.^{22,28}

Antioxidant testing using AuNCs

Having demonstrated the responsiveness of AuNC cluster emission to reactive oxygen species, we next tested this system for screening of antioxidants. Ascorbic acid (vitamin C), is an important antioxidant, inactivating ROS, and preventing cell damage.²⁹ In this work, ascorbic acid was chosen as a model antioxidant to investigate the use of the AuNCs for screening antioxidant activity. Zwitterionic AuNC (100 nM) were pre-mixed with different concentrations of AA, followed by addition of Fe²⁺/H₂O₂ (80 μM). After incubation at room temperature for 5 min, fluorescence spectra of the solutions were measured as described above, the fluorescence of NC was almost completely quenched (curve f in Fig. 3A) by the

presence of $\bullet\text{OH}$ ($\sim 80\ \mu\text{M}$). However, with ascorbic acid pre-treatment, the quenching effect was greatly reduced and it was observed that fluorescence was dependent upon ascorbate concentration. As shown in Fig. 3B, the fluorescence intensity of NC was nearly fully recovered when increasing the concentration of ascorbic acid to $200\ \mu\text{M}$, while $50\ \mu\text{M}$ of AA could also restore 28% (curve e) of the fluorescence quenched by $\bullet\text{OH}$. Moreover, we observed a linear increase of the fluorescence intensity with increasing concentration of AA over $0\text{--}200\ \mu\text{M}$ range ($R^2=0.998$). The fast, simple, and reliable AuNC/ $\bullet\text{OH}$ /AA platform offers a promising fluorescent probe for analysis or screening of antioxidants in biological samples.²⁸

To evaluate the selectivity of AuNC/ $\bullet\text{OH}$ system towards AA, various biological relevant bio-molecules (bovine serum albumin, glutathione, and glucose) and metal ions (K^+ , Na^+ , Ca^{2+} , Zn^{2+} , and Fe^{3+}) were tested and compared to AA. As shown in Figure S4, only the addition of AA caused pronounced fluorescence recovery of the AuNC, indicating the selectivity of AuNC/ $\bullet\text{OH}$ in detecting antioxidants. Followed, the potential application of the AuNC was demonstrated by testing the antioxidant (mainly in vitamin C or AA) in a commercial orange juice sample. The tested results of dilutions of orange juice were expressed in AA concentration (Table S1), which was close to the indicated value. The results confirm that the AuNC/ $\bullet\text{OH}$ system has great potential application in AA determination in complex environments.

Mechanism for AuNC based sensing

To determine whether oxidation or aggregation induced fluorescence quenching of the AuNCs, we used TEM to closely examine the morphological changes of AuNC after $\bullet\text{OH}$ and $\bullet\text{OH}$ /AA treatment. Compared with the well-dispersed AuNC (Fig. 1B), there were no significant changes observed after the addition of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ and AA, as shown in Fig. 4. Additionally, the XPS measurement of NCs being treated with $\bullet\text{OH}$, showed a decreased percentage of Au(0) and an increased percentage of Au(I) (Fig. S4). Taken together, the decreased fluorescence intensity of AuNC can be ascribed to the change in oxidative state of the gold by the generated $\bullet\text{OH}$ rather than the NC aggregation induced quenching effect, which is consistent with previous reports.²⁸

Conclusions

In summary, we reported the fabrication of sulfonamide-based monothiol zwitterionic gold nanoclusters. The zwitterionic ligands provide NC with excellent stability and strong fluorescence in a wide range of biological media. The fluorescence intensity of these AuNCs is responsive to reactive oxygen species, allowing for quantitative detection of ROS, and conversely antioxidants (e.g. ascorbic acid). This study highlights the potential of using zwitterionic ligand as a molecular tool to synthesize stable and multifunctional nanostructures for biological sensing and screening in complex environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Characterization of as-synthesized fluorescent zwitterionic AuNC. (A) General structure of the AuNC modified by sulfonamide-based zwitterionic ligand, which with “stealth” zwitterionic headgroup appended to a non-interacting OEG-functionalized interior. (B) Representative TEM images of AuNC (bar=5 nm), DLS analysis graph inset. (C) LDI-MS spectrum of AuNC with ligand modification (m/z: 601.2)

Fig. 2.

Fluorescence and stability characterization of as-synthesized fluorescent zwitterionic AuNC. (A) Excitation (purple curve) and emission (red curve) fluorescence spectra of AuNC. (B) X-Ray photoelectron spectroscopy (XPS) measurements of AuNC as prepared, base line (blue curve), measured line of AuNC (black curve), fitting line of AuNC (red curve), fitting line of Au(I) (green curve), and fitting line of Au(0) (pink curve). (C) Bright and fluorescence images of AuNC at varied concentrations (from 25 to 500 nM), in different buffers (Milli-Q water, PBS, GSH, and 10% FBS-containing DMEM), and pH (1.0 and 14.0), under bright light and UV light exposure for 1 day and 30 days (kept in dark at room temperature).

Fig. 3.

Responsiveness of AuNC to highly reactive oxygen species and antioxidant. (A) Fluorescence intensity of 100 nM AuNC in the presence of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ from 5, 10, 20, 40, to 80 μM in water. Inset: the linear relationship between the fluorescence intensity and the concentration of $\text{Fe}^{2+}/\text{H}_2\text{O}_2$. (B) Fluorescence intensity of AuNC (100 nM) / $\cdot\text{OH}$ (80 μM) system in the presence of AA from 0, 50, 100, 150, to 200 μM in 10 mM PBS. Inset: the linear relationship between the fluorescence intensity and the concentration of AA. Incubation was conducted at room temperature for 5 min. Fluorescent images inset correspond to the curve from a to f, respectively.

Fig. 4. Representative TEM images of AuNC after being treated with $\bullet\text{OH}$ and with $\bullet\text{OH}/\text{AA}$. (A) 100 nM AuNC in the presence of 80 μM $\text{Fe}^{2+}/\text{H}_2\text{O}_2$. (B) Mixture of 100 nM AuNC and 200 μM AA in the presence of 80 μM $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ (bar=20 nm).