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ORIGINAL PAPER

Functionalized gold nanoparticles for the detection of nitrates in water

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Abstract A rapid and sensitive colorimetric assay was developed using cysteamine modified gold nanoparticles for the direct detection of nitrates in water samples. Gold nanoparticles stabilized with citrate were modified with cysteamine that has excellent affinity for nitrates, and its capacity to capture nitrates was evaluated, in comparison with other anions. The presence of nitrates in water samples could be tracked by naked eye with a color variation of the colloidal suspension from red to gray, and these results were confirmed through ultraviolet–visible measurements within a nitrate concentration of 35 ppm. In field analysis was performed in underground water extracted from wells during the year 2012 in Arborea area (Italy), a nitrate vulnerable zone, and information of nitrate concentration in the range of the recommended nitrate level in water was studied. This simple assay can be used for onsite detection of nitrates in water without the need for skilled personnel, sample pretreatment or expensive instrumentation.

Keywords Nanoparticles · Nitrates · Ultraviolet–visible · Colorimetric test kit · Water

Introduction

Nitrate (NO_3^-) and nitrite (NO_2^-) anions are found in the environment and can be produced in soil, water and plants through the oxidation of nitrogen by microorganisms (Addiscott and Benjamin 2004; Environment Canada 2003; Kirmeyer et al. 1995; NRC 1995; US EPA 2002). Their presence in the environment is due to their use as inorganic fertilizers, food preservatives, component of explosives and products of chemical industries; they are very stable and different nitrogenous compounds are converted into nitrates. In particular, nitrate salts are very soluble and mobile in soil and can migrate to the water table when present in excess to be used as plant nutrients (Adam 1980; Agriculture Canada 1991; Fanning 2000). The presence of nitrates in water supplies can therefore be used as an index of water pollution due to the excessive use of fertilizers, livestock waste and pollution from sewage on agricultural effluents. The problem of groundwater contamination by

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nitrate ion is experienced by several countries worldwide, and different studies suggest that the nitrate concentration in water is above the limits allowed by the European Community for drinking water (50 ppm, maximum concentration admissible CMA) and exceeds the maximum contaminant level MCL (45 ppm NO_3) determined by the Environmental Protection Agency (EPA) (US EPA 1995; US EPA 1999; Zuane 1996). Nitrate contamination of groundwater is a problem because of its effect on birth defects, abortions, methaemoglobinaemia known as blue baby syndrome (Beatson 1978) and problems of the central nervous system (Brender and Olive 2004) in humans (Bruningfann and Kaneene 1993) and animals. Different methods exist to test the nitrate concentration based on an indirect determination after reduction to nitrites or using different techniques comprising of spectrophotometry, spectrofluorimetry, colorimetry (Griess reaction), gas chromatography–mass spectrometry, Raman spectrometry, high-performance liquid chromatography, ion chromatography, capillary electrophoresis, chemiluminescence and electrochemistry (Shaviv et al. 2003; Wang et al. 2009; Xiao et al. 2011). All of these methods are selective and sensitive but require sample pretreatment, and use of toxic reagents, furthermore they suffer from interferences, is time consuming and require sophisticated expensive instruments and highly trained technicians for the measurements. The classical methods of analysis of nitrates can not be applied in an on-site setting, in rural and remote areas where these tests are necessary. To overcome these limitations, a procedure to monitor nitrates in situ, without sample preparation, in real time, not affected by interference from competing molecules and being able to detect ppm concentrations, will be useful and practical. The earlier findings about nitrate detection provided the analysis in well-equipped laboratories, and only two recent works applied a colorimetric method for the detection of nitrites infield (Daniel et al. 2009; Xiao and Yu 2010). In these works, a direct method was used for the detection of nitrites while an indirect and complicated method was applied for the detection of nitrates. In fact in most colorimetric assays, nitrate is reduced to nitrite with enzymes or catalysts and detected indirectly with a long reaction time. The aim of the present work therefore is to develop a simple, sensitive, direct, low-cost colorimetric test kit for routine analysis to evaluate water quality and to determine ultra trace amount of nitrates using nanotechnology. Recently, gold nanoparticles have attracted great attention because of their biological compatibility and non-toxic nature (Poole and Owens 2003; Sharma et al. 2009). This interest has led to the development of biological and chemical sensors that utilize nanoparticles in different fields (catalysis, biotechnology, electronic, photonic and medicine) (Mosier-Boss and Lieberman 2002; Stewart and Anderton 2008; Tan et al. 2010; Vongsvivut et al. 2010). Particle size and shape in fact influence physical and chemical properties of nanomaterial and provide a unique optical behavior that, according to the state of aggregation of nanoparticles, produces changes in color (Michota et al. 2002; Sharma et al. 2010; Yu et al. 2007). In fact, the greater λ_{max} the larger NPs dimension; larger is the peak and wider is the NPs distribution (Doering and Nie 2002; Handley 1989; Keating et al. 1999). In this effort, in this study, gold nanoparticles (AuNPs) were functionalized with a thiol (cysteamine) and their properties were studied in the presence of nitrates. The mercapto group of cysteamine could be easily attached to the surface of the AuNPs by the formation of Au–S bonds while the NH_2 groups exposed to the outer surface of NPs can interact with nitrates present in water samples. In this way, functionalized gold nanoparticles in the presence of nitrates form crosslinks that induce their aggregation and precipitation, causing a distinct change in color, visible with the naked eye. This property was exploited to develop a new disposable fast kit, composed of functionalized nanoparticles for the analysis of water samples. Taking advantage to the optical properties of functionalized nanoparticles, nitrates were also detected selectively and in a fast and simple way with ultraviolet–visible (UV–vis) measurements of water solutions. Information of nitrate concentration in the range of the recommended nitrates level in water was studied in underground water extracted from wells during the year 2012 in Arborea area (Italy), a nitrate vulnerable zone.

Materials and methods

Chemicals

Gold (III) chloride hydrate (puriss. p.a., ACS reagent, >49 % Au basis), sodium citrate dihydrate (>99 %), calcium phosphate (purum, p.a. >96 %), sodium carbonate (bioXtra, >99 %), sodium bicarbonate (reagentplus >99.5 %), sodium chloride (bioXtra, >99.5 %), potassium nitrate (cell culture tested), calcium nitrate tetrahydrate (>99 %), cysteamine hydrochloride (>98 %) and acetone (reagent grade >99.8 %) were purchased from Sigma-Aldrich. Deionized water (18 MΩ) was used for the preparation of buffer solutions. All solvents and reagents were used without further purification.

Synthesis of citrate-stabilized gold nanoparticles (AuNPs)

To obtain monodispersed nanoparticles, citrate-stabilized AuNPs were synthesized with a simple equipment and with a modified method based on a Lee and Meisel's (1982) standard procedure. A solution of $HAuCl_4$ (1 mM) in water

was prepared, stirred and heated to boiling, under reflux. Then, 5 mL of a sodium citrate solution (38.8 mM) was rapidly added to the solution of $HAuCl_4$. The color of the solution changed from yellow to black, lavender and finally red. Part of this solution was continually boiled for 30 min and another for 3 h. Finally, these solutions were filtered through a 0.22 mm Millipore syringe filter to remove the precipitate and then characterized with UV–vis and transmission electron microscopy (TEM) measurements. The filtrate was stored in refrigerator at 4 °C. The synthesis of AuNPs was based on the reduction of $HAuCl_4$ by sodium citrate. This method was used for the first time by Turkevitch in 1951 because sodium citrate acts as a reducing agent and stabilizes the anion. In fact after their formation, AuNPs are surrounded by a layer of citrate, sodium and chloride ions. Modifying citrate concentrations, nanoparticles (NPs) with a diameter between 15 and 150 nm can be produced, but to obtain reproducible NPs with the same size, it was important to control all the experimental conditions and the cleanliness of glassware. In the present work, the reaction was conducted at high temperature (100 °C), with intense stirring, under reflux, and during the different steps, the change in the color of the solution was noted until the appearance of a red wine color. These color changes are due to the size and shape of NPs but also due to the refractive index of the surrounding medium and can be explained by considering the adsorption and scattering of light in the visible, both related to the resonance phenomenon of surface plasmons. Among other metals, the choice of gold is due to the fact that gold is a noble metal, inert, has a low tendency to oxidize with fewer defects compared with other metals, excellent for the formation of self-assembled monolayers (SAMs), binds the thiols with strong interactions and is biocompatible (Swaminathan et al. 2005). A functionalization step was introduced to prevent the aggregation of nanoparticles in solution. Alkyl thiols on gold have a high affinity to the surface of the metal and allow the formation of well-defined organic surfaces with variable chemical functionalities that determine the properties of the interface exposed to the external environment.

Functionalization of AuNPs with cysteamine hydrochloride (Cy HCl)

For the final detection of nitrates, the citrate-stabilized AuNPs were functionalized with cysteamine, a chemical linker revealed selective for nitrate capture. This functionalization was obtained by mixing 0.001 M cysteamine HCl solution in water with the AuNPs solution at a volume ratio of 1:100 and stirring for 12 h. The final solution (AuCyNPs) appeared as a deep red color, and it was stable for several months at 4 °C. UV–vis measurements were carried out in the range 400–700 nm to determine the maximum wavelength (λ_{max}) of adsorption of AuNPs and AuCyNPs in order to correlate the results with the dimensions and distribution of the NPs obtained.

Detection of nitrates

AuNPs functionalized with cysteamine (AuCyNPs) were used to link and precipitate nitrates mixing these NPs to different nitrate solutions at various concentrations and to underground water samples.

In particular, to assess the relationship between agricultural practices and quality of groundwater affected by farm nitrate contamination, different groundwater samples were taken in the specific context of the nitrate vulnerable zone (NVZ) of Arborea (Italy). In this area, characterized by sandy soils and shallow water table, zootechnical (milk production with more than 50,000 cows) and agricultural (corn and ryegrass) activities affect a fragile territory (Cau and Paniconi 2007). Since 2 years (Idrisk Project), within NVZ, a specific groundwater monitoring activity is carried out, including the wells A, B and C (Fig. 7). They have been selected for their particular pollution source properties. In fact, hydrogeological surveys, chemical and isotopical analysis suggests a direct relation between farm waste and groundwater pollution. Conventional techniques and new colorimetric techniques based on the use of functionalized gold nanoparticles were carried out. The sampling was carried out in three different wells indicated as A, B and C, from which groundwater samples were collected as illustrated in Fig. 7. The depth of the wells was A 14 m; B 27 m; and C 20 m, and the distance between sites was A–B 1,400 m; B–C 730 m; A–C 1,900 m. Two samples from each site were collected at the well-pump outflow and filtered (0.45 μm filter) into 1 L polythene bottles thoroughly prewashed with distilled water. The samples were transported in low-temperature thermal bags and stored under refrigeration. Standard methods (APHA 1992) were applied to quantify the presence of major ions. In particular, cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) were determined by atomic absorption spectrometry (Perkin Elmer model AAnalist 200) while the anions (F^-, Cl^-, Br^-, NO^{3-}, $PO_4{}^{3-}$, $SO_4{}^{2-}$) were determined by ion chromatography (anion column 20 Alltech model allsep anion 7 μm, 100 mm). Carbonate and bicarbonate contents were obtained by titration methods, whereas $NH_4{}^+$, $NO_2{}^-$ and SiO_2 content by colorimetric methods. The ion balance errors for the analyses were within ±5 %. While for the innovative analysis with nanopartices, 0.5 mL of AuCyNPs (3 h) were mixed with 0.5 mL of water samples containing nitrates at different concentrations (8–8,000 ppm), obtained dissolving KNO_3 in deionized water. Different ratios of AuNPs, AuCyNPs and nitrates were tried, and also the use of aggregating agents as NaCl 0.05 M was tested to decrease the time of reaction. In fact, in some cases, it is possible to initiate aggregation of the colloids only with the analyte,

while, in other cases, the analyte alone is not sufficient to initiate aggregation and anionic aggregating agents are necessary (Lee and Irudayaraj 2009). To study the possible presence of interfering solutes, AuCyNPs were added to salt solutions of other anions that are usually present in water samples (phosphates, chlorides, carbonates and bicarbonates) and UV–vis spectra were analyzed. Colorimetric, UV–vis and TEM images of samples after the capture of nitrates at different concentrations were collected, and the limit of detection of the present method was evaluated on solution of nitrates and on samples of underground water collected in Arborea area. For the detection of nitrates, 0.5 mL of AuCyNPs (3 h) were mixed with 0.5 mL of underground water and the colorimetric response was analyzed.

Material characterization

UV–visible adsorption spectra were obtained using a Nicolet Evolution 300 spectrophotometer. The spectra of nitrates and other anions were acquired from 200 to 800 nm; a water sample was used for the background. Size and shape of AuNPs were characterized with TEM FEI Tecnai 12, and the voltage used was 120 kV. For each sample (AuNPs, AuCyNPs, AuCyNPs with nitrates), one drop of the dispersion was deposited on a copper grid. Before proceeding to the observation, the liquid was completely evaporated in 16 h.

Results and discussion

Preparation and functionalization of AuNPs

The red gold NPs were studied with UV–visible spectroscopy and their morphology by direct visualization with TEM, so that their size and shape can be assessed. In Fig. 1, the characteristic gold peak at 520 nm that indicates the formation of stable AuNPs which corresponds to radii in the range 10–30 nm, with a narrow peak that confirms the homogeneity of size distribution can be observed. The absorbance values after 30 min or 3 h of boiling (100 °C) indicate the different concentration of colloids prepared with different time of reaction but with the same dimension. The observed full width at half height of these gold colloidal suspensions was within 50–60 nm. The size values and morphology of NPs were confirmed through TEM images as illustrated in Fig. 2a. The image shows monodispersed and spherical particles with an average diameter of around 10–15 nm in size. Gold NPs, once stabilized with sodium citrate, were modified with cysteamine (thiolated molecule) at a critical concentration, to decrease the repulsion between AuNPs to form aggregates upon sensing the presence of nitrates. As illustrated in Fig. 2b, after functionalization with cysteamine, the NPs are closer but exhibit good dispersion, while after the adsorption of high concentrations of nitrates, a strong aggregation can be noted (Fig. 2c) that can lead to NPs precipitation. In fact, after a few minutes of interaction between AuCyNPs and nitrates, the color changes can be observed either by the naked eye or from the UV spectra.

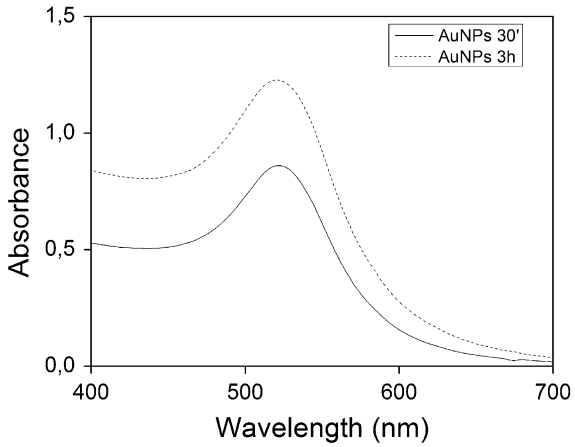


Fig. 1 UV–vis spectra of AuNPs after 30 min (*solid line*) and 3 h (*dot line*) of boiling

First trials for the detection of nitrates

Different UV–vis assays were performed to compare the sensitivity of the cysteamine-modified AuNPs with the direct spectra of nitrates. As reported in SI Fig. 1a (inset), UV spectra of calcium nitrate and potassium nitrate were measured at very high concentration and a peak at 300 nm can be observed. Decreasing the concentration of nitrates, this characteristic peak disappears, and with this method, the direct detection of nitrates at low concentration is not possible. The same test was repeated with AuCyNPs 30′ (nanoparticles of gold and cysteamine boiled 30 min), and also in this case, the peak at 300 nm was visible only at a concentration of nitrate of 8,000 ppm (SI Fig. 1b), but observing the peak due to AuNPs at 520 nm at this concentration disappears as a result of formation of cluster aggregates (SI Fig. 1c), leading to a change in the color of the solution from red to gray. This property of the solution exhibiting a change in color was studied in the next sections, evaluating also the possible use of aggregating agents to facilitate the detection of nitrates at low concentration.

Selectivity of the assay toward other anions

To test the selectivity of the assay, other relevant anions (phosphates, chlorides, carbonates, bicarbonates) possibly present in water samples at high concentration were evaluated. In SI Fig. 2a is reported the UV spectrum of NaCl

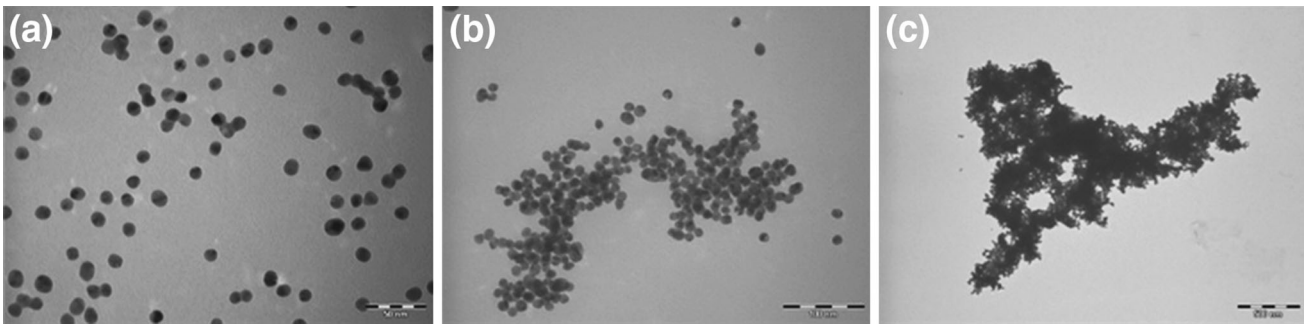


Fig. 2 TEM images of **a** AuNPs, **b** AuCyNPs, **c** AuCyNPs after the capture of nitrates at different magnification (the *bar* indicates 50 nm in fig. 2a; 100 nm in fig. 2b and 500 nm in fig. 2c)

alone without significant peaks. Adding this anion also to AuCyNPs, no changes were observed in the spectra (SI Fig. 2b) for low wavelength, while at high concentrations of chlorides (8,000 ppm) (SI Fig. 2c), the disappearance of the peak at 520 nm could be noted but without a color change of the solution. In SI Fig. 3a, the phosphate spectra were reported before and after the addition to AuCyNPs solution (SI Fig. 3b), and in this case at high concentration, a peak at 270 nm was observed that does not have the same wavelength of the peak of nitrates, therefore not interfering. Also in this case, when this anion is added at high concentrations (8,000 ppm) to AuCyNPs solution, the peak at 520 nm disappeared (SI Fig. 3c) but without any notable change in the color of the solution or other modifications. Other experiments were carried out with carbonates and bicarbonates, but the spectra were not reported because they were not significant. The developed system demonstrated a high capacity to detect nitrate ions with definite UV–vis peaks and color changes that cannot interfere with other anions present in water.

Aggregation properties of AuNPs

As illustrated in the previous sections, a significant peak and a change in color of solutions were obtained only with AuCyNPs in the presence of high concentrations of nitrates. However, the purpose of our work was to detect low concentrations of nitrates with this method. To initiate the aggregation process of nanoparticles at low concentrations of nitrates, different concentrations of aggregating agents were added to the NPs solutions. In fact, AuNPs in the colloidal suspension are negatively charged, repel each other and stay in solution. Salts such as NaCl shield the negative charges allowing the particles to crowd. For this reason to start the aggregation of NPs and to obtain a quick response also toward small amount of nitrates in the next experiment, AuCyNPs were added with a small amount of NaCl that creates a local modification of the ionic strength and enhances the interaction of AuCyNPs with KNO_3 even at low concentration.

Detection of nitrates with AuCyNPs

Different trials with functionalized nanoparticles were carried out using different cysteamine molar ratios. In fact, the modification with high concentrations of cysteamine decreases the specific surface plasmon resonance of the AuNPs at 520 nm, which leads to a minor stability of the AuNPs. Based on these results, modified AuNPs at a volume ratio cysteamine (0.001 M)/AuNP 1:100 were used in the following experiments. In the first experiment, 0.5 mL of AuCyNPs (30 min) were mixed with 250 µL of KNO_3 solutions at different concentration in water (8–8,000 ppm) and 125 µL of NaCl (0.05 M); in this case, addition of the aggregating agent was necessary because without NaCl only high nitrate concentrations could be sensed with a long reaction time. As illustrated in Fig. 3, AuCyNPs

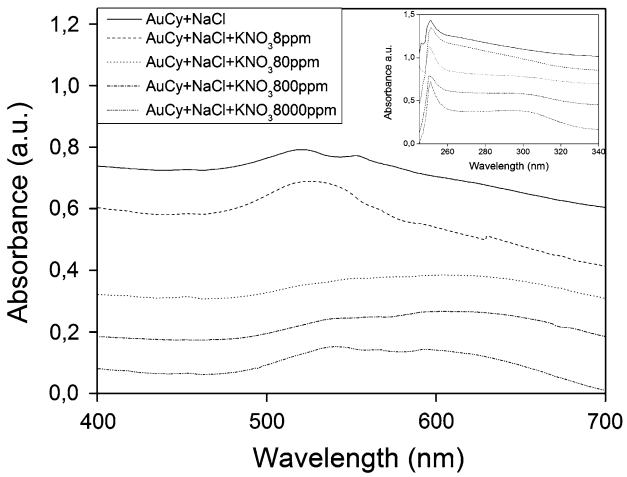


Fig. 3 UV–vis spectra of AuCyNPs boiled 30 min after the addition of chlorides and nitrates at different concentrations (0–8,000 ppm from the top to the bottom) in the range 400–700 nm. Inset depicts the same measurements in the range 250–340 nm

solutions present a peak at 520 nm due to AuNPs and a shoulder at 555 nm due to coupled plasmon resonance, indicating the attachment of cysteamine onto the AuNP. Increasing the concentration of nitrates, a shift of the peak at highest wavelength could be observed. From a concentration of nitrates of 80 ppm, the disappearance of the peak at 520 nm can be noted. At the same time, as illustrated in the inset in Fig. 3, the appearance of a peak at 300 nm at a concentration of 80 ppm characteristic of nitrates can be noted. Considering the final volume of the solution with this experiment, it was possible to reach a detection limit of 35 ppm for nitrates that is near the limits for drinking water stated by the American and European Community. This was possible using AuCyNPs with a small addition of NaCl as aggregating agent. This result was also confirmed colorimetrically (Fig. 5a), considering the change in the color of solutions from the reference (red AuCyNPs) with the addition of nitrates at different concentrations. A slight variation in color was also observed with the addition of 8 ppm nitrates. This result is interesting because the proposed detection kit can be used for common situations, as in urban areas, where most of nitrate concentrations, exceeding the regulated values, are in the range 50–300 ppm. Thus, water samples at very low concentration of nitrates can be analyzed, and contaminated water can be identified in few minutes with a clear color variation of the solution, using the developed approach. The objective of the second experiment was to demonstrate low detection limits of nitrates without the need for any aggregating agent. After different trials, it was determined that using AuNPs (boiled for 3 h) it was possible to detect nitrates directly using the highest concentration of nanoparticles that are sensitive to small amounts of nitrates. In fact, as reported in Fig. 4, with the growing presence of

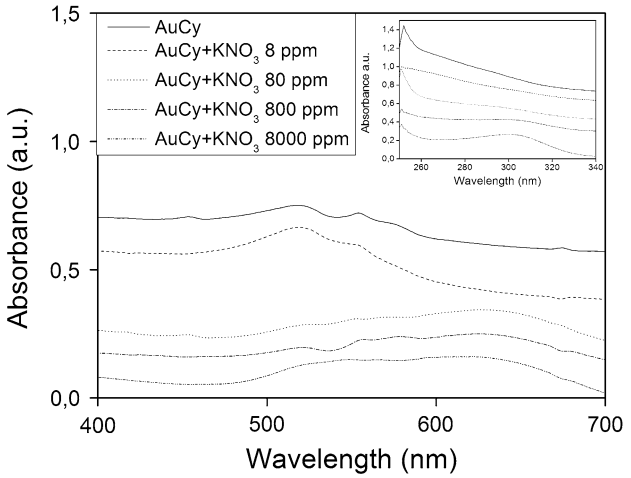


Fig. 4 UV–vis spectra of AuCyNPs boiled 3 h after the addition of nitrates at different concentrations (0–8,000 ppm from the top to the bottom) in the range 400–700 nm. In the inset, the same measurements are depicted in the range 250–340 nm

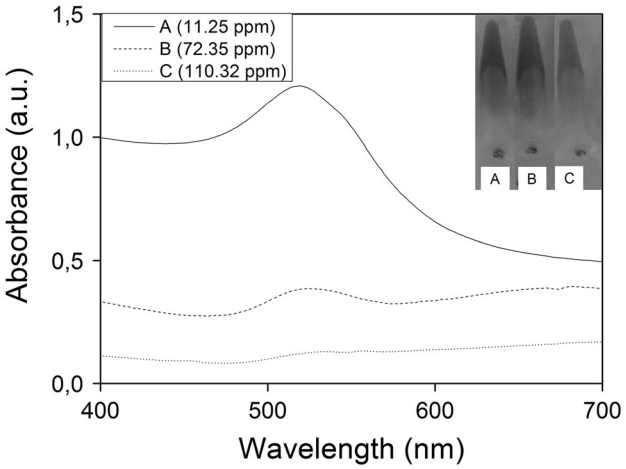


Fig. 6 UV–vis spectra of AuCyNPs boiled 3 h after the addition of three water samples (*A*, *B* and *C*) with different concentration of nitrates in the range 400–700 nm. In the *inset,* the colorimetric image of the samples

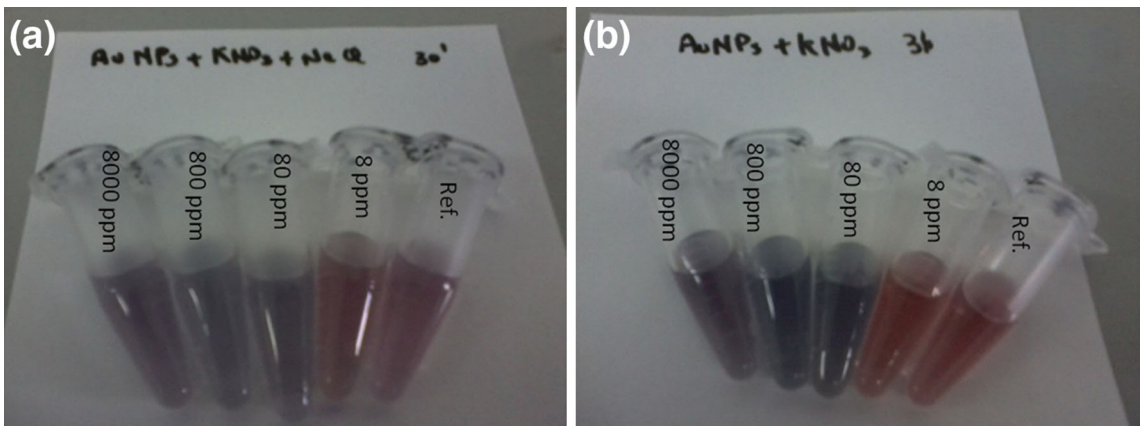
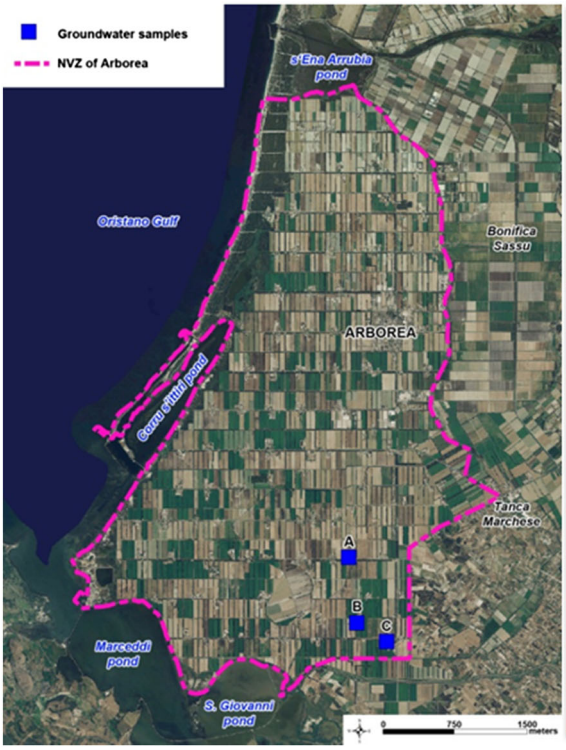


Fig. 5 Pictures of AuCyNPs alone (Ref.) and **a** with NaCl and different additions of nitrates solutions (NPs boiled 30 min) **b** with nitrates at different concentrations (NPs boiled 3 h)



	A	B	C
Ca^{2+}	27	82	218
Mg^{2+}	17	60	125
Na^{+}	185	360	875
K^{+}	7.6	18	23.75
F^{-}	0	0	0
Cl^{-}	195.48	611.02	1809.92
Br^{-}	0	0	0
NO_3^{-}	11.25	72.35	110.32
PO_4^{3-}	0	0	0
SO_4^{2-}	60.57	123.25	343.5
HCO_3^{-}	296.85	275.13	222.59
CO_3^{2-}	0	0	0
NH_4^{+}	0	0	0.07
NO_2^{-}	0	0	0.06
SiO_2	39.23	77.99	55.29

Fig. 7 Map of Arborea NVZ with the wells analyzed on the *left* and table of cations and anions concentrations of three water samples (*A*, *B* and *C*) expressed in mg/L analyzed with standard methods, on the *right*

nitrates in solution (up to 80 ppm), the disappearance of the peak at 520 nm with the simultaneous appearance of the nitrate peak at 300 nm was observed (inset, Fig. 4). This particle aggregation can be observed at the same time with TEM (Fig. 2c) and colorimetric analysis (Fig. 5b), with more intense color variations compared with the previous experiment. To validate our results, the same experiment with AuCyNPs (3 h) was carried out on three underground water samples collected from wells in a nitrate vulnerable zone. As illustrated by standard analysis (Fig. 7), these samples contain different anions and cations and therefore are useful to study the behavior of functionalized nanoparticles in presence of nitrates and other competing ions simultaneously present in solutions. The same conditions used previously with single-ion solutions were applied. As reported in Fig. 6, also in this experiment with underground water samples containing different ions, increasing the concentration of nitrates from sample A to sample C, a shift at highest wavelength in the UV spectra can be observed from a concentration of nitrates of 72–110 ppm, with a progressive disappearance of the peak at 520 nm. Considering the final volume of suspensions also in this case, the limit of detection is 36 ppm, in accordance with the previous results. Furthermore, a color change can be observed from water samples with a nitrate concentration of 11 ppm (inset, Fig. 6, sample A, red) to 72 and 110 ppm (sample B and C, gray), able to discriminate water samples with a nitrate concentration over the limit for drinking water.

Conclusion

The present work consists in synthesizing gold nanoparticles functionalized with cysteamine for a direct detection of nitrates with a simple and fast method (2 min for the analysis) not requiring other collateral or enzymatic reactions. AuCyNPs were synthesized and used for UV–vis and colorimetric detection of nitrates. The appeal in this colorimetric-based nanoparticle assay is its simplicity for onsite measurements. The approach does not require any instrumentation because the presence of nitrates up to 35 ppm can be observed with a simple color change of the solution and detected by naked eye. In fact, colorimetric assay is desirable for infield monitoring due to its simplicity and portability, and with our colorimetric test kit, these results were obtained. The assay can be used to detect nitrates directly in a field setting without the need for specialized training and sample pretreatment. This proposed approach is simple, robust, sensitive, low cost, specific, selective and can be utilized for routine testing of water in the field conditions.

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