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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Rapid and convenient synthesis of stable silver nanoparticles with kiwi juice and its novel application for detecting protease K

Bin Bin Chen,[a,b] Hai Liu,[a] Cheng Zhi Huang,[a] Jian Ling[c,*] and Jian Wang[a,*]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

In this work, kiwi juice functioned as the reducing and capping reagent to obtain stable silver nanoparticles (AgNPs), which was very effective and inexpensive. Importantly, it could be finished within 2 min at room temperature, making the synthesis much faster and easier. Compared with the most common AgNPs (such as sodium citrate coated AgNPs), the proposed AgNPs presented more excellent properties such as high stability. Moreover, a new analytical assay was established for protease K with colour turning from light yellow to brown when protease K was introduced into the AgNPs aqueous solution. It was found that the increase of AgNPs absorbance at 470 nm was in proportional to the concentrations of protease K in the range of 0.50 - 15 μg/mL with the limit of detection as low as 0.47 μg/mL. This selective and colorimetric assay has a great application prospect in the bioanalysis due to its simplicity and specificity for protease K detection.

1. Introduction

Metal nanomaterials have been greatly concerned since the 1980s due to their unique advantages,[1] such as excellent optical properties,[2, 3] high catalytic activity,[4, 5] good biocompatibility,[6, 7] *etc*. Therefore, they have presented a broad application prospect, especially in biological and chemical sensing,[8-10] cell imaging,[11, 12] drug delivery[13] and cancer treatment.[14-16]

Nowadays, considerable efforts have been made to develop enormous amount of methods of nanomaterials preparation to improve their properties. The most commonly used one is the sodium citrate reduction method.[17] However, its drawbacks limit further application in chemical and biological fields, especially in complex biological samples. For one thing, the traditional preparation method is complicated and time-consuming, which requires apparatus to heat reaction solution to boiling, increasing the cost and prolonging the reaction time. For another thing, the traditional metal nanomaterials display poor stability and are easy to aggregate in extreme acid/base conditions or in salty medium, which may induce false positive or negative results. Usually, surfactants or biomolecules are employed as the coating reagents to improve the stability. However, for the former, some surfactants (such as cetyltrimethylammonium bromide) present strong biological toxicity, which not only make the operation more complicated, but also are not conducive to biological samples such as cells or animals.[18] For the latter, the enhancement of stability as well as the specificity for biorecognition purpose could be achieved by chemically coupling better biocompatible biomolecules (nucleic acids, amino acids, proteins, *etc.*) onto the surface of nanomaterials.[19] However, the problem is, its cost is relatively high and requires the long-lasting and complex modification procedure, which also increases the risk of closing the active sites to reduce the reactivity.[20] Therefore, it is desirable to develop simple and green ways to obtain metal nanomaterials with excellent properties.

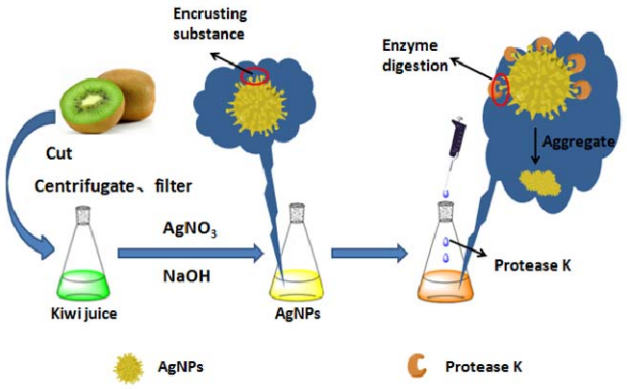
In recent years, it was found that fruit juice could act as the reducing reagent to prepare AgNPs, which possess the unique advantages compared with the traditional methods.[21] On one hand, numerous properties of the metal nanoparticles have been significantly improved, such as better stability, milder and shorter reaction, higher antibacterial activity,[22] *etc*. On the other hand, these stable metal nanoparticles have also achieved selective detection of biological molecules.[21]

In this work, kiwi juice acted as the reducing reagent to prepare AgNPs, which coul be completed within 2 min at room temperature. Compared with other ordinary methods, the propoded method was more simple and effective. Most importantly, the as-prepared AgNPs were stable in salty solution, which showed high selectivity for protease K analysis. Consequently, as a novel method to detect protease K, there will be a great potential application.

Hereby, protease K is a serine protease, which can digest carboxyl terminal peptide bonds of aromatic and aliphatic amino acids and is capable of degrading the native protein.[23] Therefore, protease K is particularly important in biology, including the preparation of PFGE chromosomal DNA[24] and western blotting,[25] as well as removal the nuclease in the process of preparing DNA and RNA.[26] Since protease K shows a strong ability to degrade protein in the biological samples, then, in the production practice, it is particularly necessary to detect the content of protease K existing in the enzyme and protein product.

2. Experimental section

2.1 Materials



Scheme 1 Schematic of the synthesis of AgNPs with kiwi juice and protease K detection.

Domestic kiwi was bought from Yonghui Supermarket (Beibei, Chongqing). Sodium hydroxide (NaOH) and silver nitrate ($AgNO_3$) were purchased from Chuandong Chemical Group Co., Ltd. (Chongqing, China) and Ruijinte Chemical Group Co., Ltd. (Tianjin, China), respectively. NaCl and Britton-Robinson (BR) buffer were employed to adjust the ionic strength and control the acidity, respectively.

Proteins including α-amylase and trypsin were commercially obtained from Xiya Chemical Group Co., Ltd. (Chengdu, China) and Thermo Fisher Scientific Co., Ltd. (Shanghai, China), respectively. Additionally, albumin and bovine serum albumin (BSA) were bought from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). The other proteins including papain, pepsin, protease K were all bought from GenView Co., Ltd. (Tianjin, China).

2.2 Instruments

An ESCALAB250 X-ray photoelectron spectroscopy (XPS) was used to determine the elemental composition of AgNPs. A FTIR-8400S Fourier Transform Infrared spectrometer (Hitachi, Japan) was for scanning infrared spectrum of AgNPs. JEOL JEM-2100 transmission electron microscopy (TEM, Tokyo, Japan) was for scanning the images of AgNPs. A Hitachi U-3010 spectrometer (Tokyo, Japan) was applied to record the plasmon resonance absorption (PRA) spectra. The dynamic light scattering (DLS) as well as Zeta potential of AgNPs were carried out with a Nano-zs zetasizer (Malvern, England). The color change of AgNPs was recorded by an E-510 Olympus camera (Tokyo, Japan). And the toxicity of AgNPs was assessed with a Biotek Microplate Reader (USA).

2.3 Synthesis of AgNPs with kiwi juice

Firstly, kiwi was peeled and cut into slices or chunks to get the light green juice. After centrifugation at 12000 rpm for 5 min, the collected juice was further purified with 0.45μm filter membrane to obtain the colourless solution.

Then, to effectively synthesize AgNPs using kiwi juice as the reducing and capping reagent, many factors, such as reaction time, pH and the concentrations of kiwi juice and $AgNO_3$, were taken into consideration. In this work, the volume ratios of 0.01 M $AgNO_3$ and as-obtained kiwi fruit juice were adjusted from 1:3 to 11:1 to obtain the high yield of AgNPs. Moreover, the concentrations of NaOH were adjusted within 0.2 M and the preparation process was monitored within 30 min.

Finally, AgNPs were obtained by following this protocol: 100 μL kiwi juice was mixed with 500 μL $AgNO_3$ (0.01M), and then the dark yellow AgNPs were observed within 2 min after the addition of 0.1 M NaOH at room temperature.

2.4 Preparation of AgNPs with sodium citrate

In order to compare some properties of the proposed AgNPs, the common AgNPs were synthesized using sodium citrate as the reducing reagent. Briefly, 50.0 mL solution containing 1.0 mM $AgNO_3$ was placed into a 100 mL conical flask, and was heated until boiling under continuous stirring. Then, 2.0 mL of 1% (*w/w*) trisodium citrate was introduced. The colloidal solution was kept boiling for about 30 min and was continuous stirred until it was cooled down to room temperature. The above aqueous mixture gradually changed from brown to yellow, indicating the formation of AgNPs.[27]

2.5 Operational processes of cytotoxicity test

100 μL 1×10^5 cells/mL Human epidermoid cancer cells (Hep-2) in Roswell Park Memorial Institute 1640 (RPMI 1640) medium, which was supplemented with 2% fetal bovine serum, were planted in each well of a 96-well plate. The cells were cultured first for 24 h in an incubator (37˚C, 5% CO_2), and followed by another 24 h incubation after the culture medium was replaced with 100 μL of RPMI 1640 containing 10 μL of AgNPs at different doses (1.0×10^{-13} M, 1.0×10^{-12} M, 1.0×10^{-11} M, 1.0×10^{-10} M, 1.0×10^{-9} M, 1.0×10^{-8} M). Then, followed by removing the culture medium, the mixture of 10 μL of Cell Counting Kit-8 (CCK-8) solution and 90 μL RPMI 1640 medium was added to each well and were further incubated for 1h. The optical density (OD) of the mixture was measured at 450 nm with a Microplate Reader Model. The cell viability was estimated according to the following equation:

Cell viability [%] = (OD treated / OD control) × 100 %

Where, OD control was obtained in the absence of AgNPs, and OD treated obtained in the presence of AgNPs.[28]

2.6 Standard operating procedure for detecting protease K

0.04 mL AgNPs, 0.10 mL BR buffer (pH 6.80) and appropriate amount of protease K solution were mixed fully in a 2.0 mL tube, which were then diluted to 1.00 mL with doubly distilled water and blended again. Finally, the absorption spectra were scanned on a U-3010 UV-vis spectrophotometer from 300 nm to 800 nm.

3. Results and discussion

3.1 Preparation and characteristics of AgNPs

Kiwi juice-capped AgNPs were effectively synthesized by optimizing the experimental conditions. According to the results (Fig. 1a), when the volume ratio of 0.01 M $AgNO_3$ and kiwi juice was 5:1, the absorbance of the AgNPs reached maximum, indicating the high yield of AgNPs.

$AgNO_3$ in alkaline condition shows stronger oxidizing capacity than in acidic medium. Thus, proper amount of NaOH in aqueous solution can significantly prompt the formation of AgNPs.[29] For one thing, it could negatively charge the reductant, which then further electrostatically attract silver ions to speed up the

reaction.[29] For another thing, it is also propitious to the alkaline-responsive phytochemicals mechanism, wherein, the amino acids, peptides and/or proteins in the kiwi juice, may play a role in the formation of nuclei during the reduction of silver ions and their secondary growth.[30] It was found that 0.1 M NaOH was appropriate (Fig. 1b) for the synthesis of AgNPs, and excessive NaOH may induce the aggregation of Ag_2O.[14]

Under the optimum conditions, $AgNO_3$ and kiwi juice could generate AgNPs within 2 min at room temperature (Fig. 1c), which is much faster than the previously reported methods, even much rapider than our previous one.[21] The proposed AgNPs were spherical with small size about 5-15 nm (Fig. 1d), which was based on the size statistics of more than 500 nanoparticles. Moreover, these AgNPs were well dispersed.

The surface elements of the as-prepared AgNPs can be analyzed by FTIR spectrum and XPS. As shown in Fig. 2a, the as-prepared AgNPs present characteristic absorption bands of O–H and N–H stretching vibrations at 3425 cm^{-1}, C=O stretching vibrations at 1597 cm^{-1} and C-O-C stretching vibrations at 1072 cm^{-1}, respectively.[31] These chemical groups may come from sugars, peptides or proteins of kiwi juice and coat on the surface of AgNPs after the reaction, which would be beneficial to improve the properties of AgNPs.

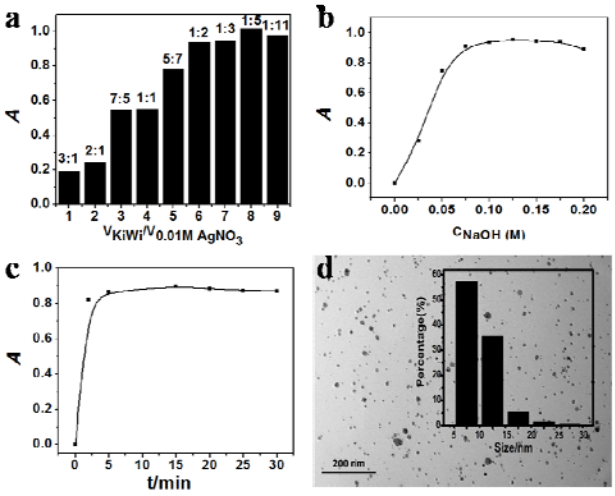


Fig. 1 The main factors during AgNPs synthesis. (a) The effect of kiwi juice and $AgNO_3$ concentrations on the synthesis of AgNPs. c_{NaOH}, 0.1 M; The volume fraction of kiwi juice were 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% and 0.01M $AgNO_3$ were 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, respectively. (b) The effect of NaOH concentration on the synthesis of AgNPs. Kiwi juice was used as obtained by keeping the 1:5 volume ratios to $AgNO_3$ (0.01 M). (c) The effect of reaction time on the synthesis of AgNPs. c_{NaOH}, 0.1 M; volume ratio of 0.01 M $AgNO_3$ and as-obtained kiwi juice was 5:1 (d) TEM imaging and size statistics of the as-prepared AgNPs.

The XPS spectrum (Fig. 2b) displays C_{1s}, O_{1s} and N_{1s} peaks at 285.0, 531.6 and 399.8 eV, respectively.[31] The other two peaks at 373.4 and 1071 eV are due to Ag_{3d} and Na_{1s},[31] suggesting that the surface of AgNPs is mainly consisted of carbon, oxygen and nitrogen as well as limited amount of Na and Ag elements. Carbon, oxygen and nitrogen mainly came from the sugars, peptides or proteins in the kiwi juice, while Na and Ag were due to the introduction of NaOH and $AgNO_3$. Moreover, the XPS spectrum (Fig. 4b) shows that the content of nitrogen is higher than silver, indicating that not all nitrogen from $AgNO_3$, but a portion of nitrogen atoms coming from peptides or proteins of kiwi juice bound to the surface of AgNPs. Importantly, the proportions of oxygen and nitrogen have risen to 34.95% and 2.87%, indicating the higher content of ingredients such as peptides and proteins in kiwi than in pear, so that kiwi juice possesses the stronger reducing ability than pear.[21] In our previous report, the pear juice was unstable that changed to brown within 2 h at room temperature owing the oxidation by oxygen. Fortunately, kiwi juice is much more stable than pear juice, which could stand for more than 24 hours at room temperature without significant change.

The C_{1s} spectrum (Fig. 2c) presents four peaks at 284.6, 286.0, 286.6 and 288.1 eV, owing to C–C, C–N, C–O, and C= N /C=O bonds, respectively.[31] The two peaks at 531.7 and 533.0 eV in O_{1s} spectrum (Fig. 2d) could be attributed to C=O and C–OH/C–O–C groups. Additionally, the peaks at 399.5 and 400.5 eV in N_{1s} spectrum (Fig. 2e) could be due to the C–N–C, N–$(C)_3$ groups.[31]

All the above results suggest that the as-prepared AgNPs are successfully synthesized and surrounded by some chemical groups on the surface, such as C=O, C-OH and C-O, *etc*.

3.2 The stability of the as-prepared AgNPs

Stability is one particularly important property of nanomaterials, which could avoid false positive/negative results in chemical and biological sensing.

Compared with the traditional AgNPs (sodium citrate coated AgNPs, *etc*), the proposed AgNPs showed the similar stability in different pH buffer solutions. However, the stabilities in salty medium and at different temperatures have been remarkably improved. Fig. 3a shows that kiwi juice-coated AgNPs are very stable within given temperature range, while the stability of citrate-coated AgNPs show an obvious decline in absorbance when the temperature rise to 37 ^oC, which is due to the aggregation or adsorption. Fig. 3b presents the stability of two kinds of AgNPs in salty medium, respectively. The absorbance of kiwi juice capped-AgNPs changed slightly when the concentration of NaCl was as high as 0.5 M. However, the

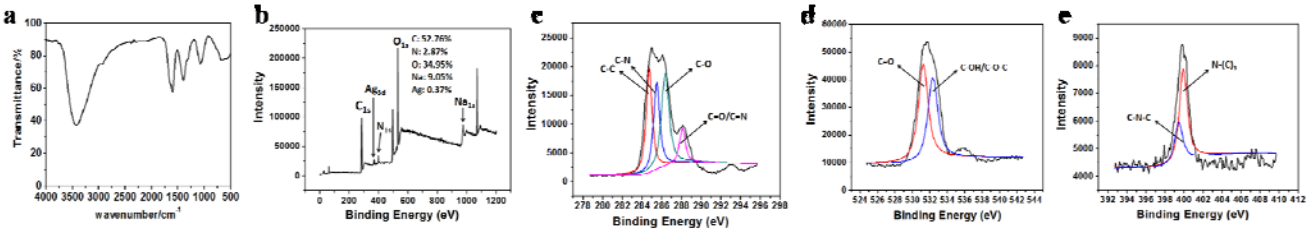


Fig. 2 The surface structure analysis of AgNPs. (a) FTIR analysis of AgNPs. (b)The XPS, (c) C_{1s}, (d) O_{1s} and (e) N_{1s} spectra of AgNPs.

absorbance of AgNPs that coated with sodium citrate decreased significantly as the concentration of NaCl reached 0.1 M, which is due to the aggregation. Therefore, the kiwi juice-prepared AgNPs were more stable, especially in high salty medium, which is in favour of the biosensing in complex samples.

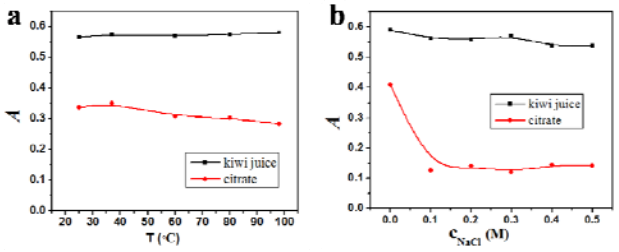


Fig. 3 The stability of AgNPs. (a) The thermal stability. $c_{\text{kiwi-AgNPs}}$, 1.0×10^{-4} M; $c_{\text{citrate-AgNPs}}$, 1.15×10^{-3} M. (b) The stability in salty medium. $c_{\text{kiwi-AgNPs}}$, 1.0×10^{-4} M; $c_{\text{citrate-AgNPs}}$, 1.15×10^{-3} M.

3.3 The cytotoxicity test

The toxicity of the as-prepared AgNPs was assessed through the cell viability of Hep-2 cells using a Cell Counting Kit-8 method (Fig. 4). The cytotoxicity of both kiwi-coated AgNPs and the control group (sodium citrate prepared AgNPs) are relatively low, and Hep-2 cells survival rate almost reaches 100%, indicating kiwi juice prepared AgNPs also present good biocompatibility, which is beneficial to biological applications.

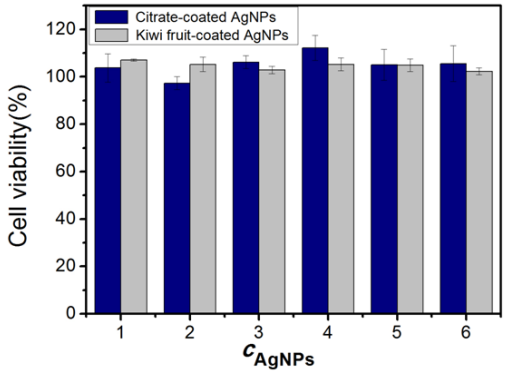


Fig. 4 Cytotoxicity test of the as-prepared AgNPs. The concentrations of AgNPs were 1, 1.0×10^{-14} M; 2, 1.0×10^{-13} M; 3, 1.0×10^{-12} M; 4, 1.0×10^{-11} M; 5, 1.0×10^{-10} M; 6, 1.0×10^{-9} M, respectively.

3.4 The detection mechanism

The mechanism of specifically sensing protease K with kiwi juice-capped AgNPs as optical probes, not only profits from the electrostatic attraction, but also derives from the unique property of protease K, which could digest and remove the functional groups surrounding on the surface of AgNPs, leading to the aggregation of AgNPs. As is shown in scheme 1, in the synthesis process of AgNPs, the sugars, peptides or proteins coming from the kiwi juice could be firstly adsorbed onto the surface of AgNPs through covalent or coordinate bonds. Following, the functional groups are removed by protease K through enzyme function to result in the aggregation of AgNPs.

3.5 The detection of protease K with as-prepared AgNPs

The as-prepared AgNPs show great importance in selective and colorimetric detecting protease K. Fig. 5a shows that the average hydrodynamic size of AgNPs was 38.44 nm and protease K was 628.8 nm in DLS, while the hydrodynamic size increased as huge as 4856 nm when mixed, suggesting the aggregation of AgNPs. Herein, the hydrodynamic size was much larger than that obtain from TEM because of the hydraulic size was measured by dynamic light scattering technique to assess the apparent particle size, including the surface adsorbed molecules, which is generally greater than the actual particle size obtained from TEM.

Moreover, the Zeta potential (Fig. 5b) shows that AgNPs was much negatively charged and the protease K displayed little positive potential. However, their mixture was less negatively charged, which could be concluded that the electrostatic interaction makes a great contribution to the aggregation.

TEM was carried out to further observe the protease K-induced aggregation. Fig. 5c shows that the as-prepared AgNPs was well dispersed. However, it can be found that the particle distance of AgNPs decreased when protease K was present (Fig. 5d), indicating that the aggregation of AgNPs was induced by protease K.

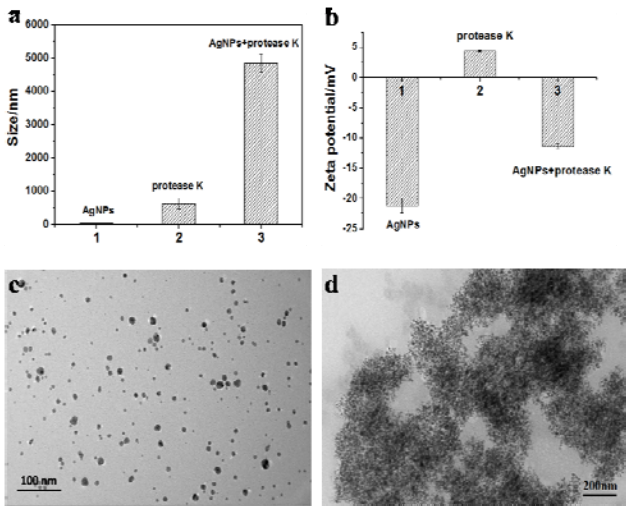


Fig. 5 The characteristics of AgNPs with protease K. (a) DLS results of AgNPs, protease K and their mixture; (b) The Zeta potential study of AgNPs mixed with protease K; (c) TEM of AgNPs; (d) TEM of the AgNPs-protease K mixture. $c_{\text{protease K}}$, 7.5 μg/mL; c_{AgNPs}, 5.0×10^{-4} M.

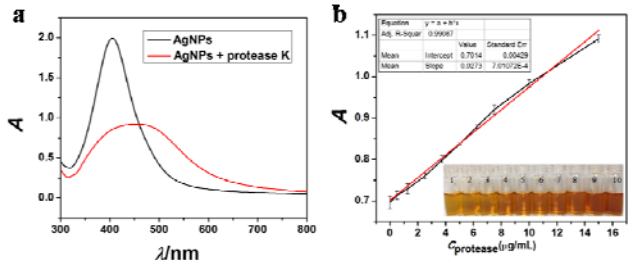


Fig. 6 The sensitivity of protease K detection. (a) The change of AgNPs absorption band. (b) The sensitivity of the protease K detection. pH 6.80; c_{AgNPs}, 5.0×10^{-4} M.

3.6 Sensitivity of protease K detection

The absorption band of AgNPs located at from 404 nm when protease K was absent. However, it shifted to 470 nm with lower absorbance when in the presence of protease K, owing to the aggregation of AgNPs induced by protease K (Fig. 6a). Fig. 6b shows that the absorbance of AgNPs at 470nm was in proportional to the concentrations of protease K in the range of 0.50-15 μg/mL with the limit of detection as 0.47 μg/mL. Accompanied with the concentration of protease K, the colour of AgNPs changed from light yellow to brown, which supplied a colorimetric assay for protease K detection.

3.6 Selectivity of protease K detection

The protease K-induced aggregation of AgNPs is highly specific. As Fig. 7a shows, the absorbance ratio (A_{470}/A_{404}) caused by protease K was extraordinarily larger than other proteins, indicating that the as-prepared AgNPs had high selectivity towards the protease K. Therefore, the selectivity property can be the basis for detecting protease K.

The selectivity was studied with the same proteins to compare sodium citrate and kiwi juice coated AgNPs. Fig. 7b shows that most proteins could induce the aggregation of sodium citrate capped-AgNPs. The pI of both α-amylase and albumin were lower than the working pH, therefore, these two kinds of proteins were negatively charged in solution. And sodium citrate capped-AgNPs surface presented negative charge as well, which avoided aggregation owing to the electrostatic repulsion between negative charges. But other proteins including papain, pepsin, BSA, protease K and trypsin were positive charged, so they were easy to induce aggregation of citrate coated-AgNPs through electrostatic attraction, which could not be able to sense protease K specifically.

Importantly, trypsin did not interfere with the sensing of protease K. The reason is that trypsin only can break down peptide bonds formed by lysine or arginine's carboxyl groups.[32] Compared with protease K, trypsin has a little limitation in degrading general proteins. Therefore, protease K possesses stronger ability to degrade general proteins on the surface of AgNPs.

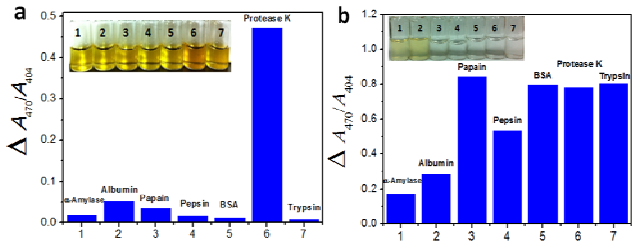


Fig. 7 The selectivity research of protease K detection. (a) AgNPs coated with kiwi juice; c_{AgNPs}: 5.0×10^{-4} M; $c_{proteins}$, 15 μg/mL; pH 6.80. (b) AgNPs coated with sodium citrate. c_{AgNPs}: 1.9×10^{-3} M; $c_{proteins}$, 15 μg/mL; pH 6.80.

4. Conclusions

In summary, a rapid and convenient synthesis method of AgNPs using kiwi juice as the reducing reagent and coating reagent was developed in this work, which much improved the properties, especially the stability. In addition, based on protease K-induced aggregation, the as-prepared AgNPs can be used for detecting protease K. Importantly, it possess so high selectivity for detecting protease K that it presents a broad application prospect in terms of detection of protease K in complex biological samples.

Acknowledgements

All authors herein are grateful to the financial support from the Natural Science Foundation Project of China (No. 21405123 and 21105087), Chongqing (cstc2014jcyjA50006) and SWU (SWU112092, 2013JY048), the Fundamental Research Funds for the Central Universities (XDJK2013C159, XDJK2013A025 and XDJK2013A015) as well as the Program for Innovative Research Team in University of Chongqing (2013).

Notes and references

[a] *Education Ministry Key Laboratory on Luminescence and Real-Time Analytical Chemistry, College of Pharmaceutical Sciences, Chongqing 400715, PR China. Fax: +86-23-68367257; Tel: +86-23-68254059; E-mail: wj123456@swu.edu.cn.*

[b] *College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, China.*

[c] *School of Chemical Science and Technology, Yunnan University, Kunming 650091, China*

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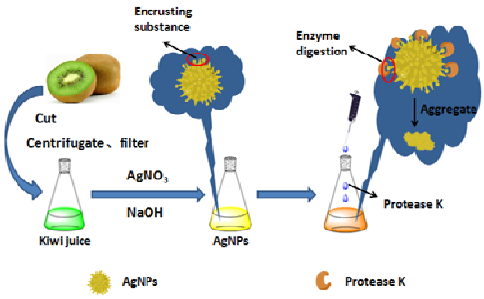
Graphic abstract
for

Rapid and convenient synthesis of stable silver nanoparticles with kiwi juice and its novel application for detecting protease K

Bin Bin Chen,[a,b] Hai Liu,[a] Cheng Zhi Huang,[a] Jian Ling[c,*] and Jian Wang[a,*]

Stable AgNPs were synthesized with a facile approach, which were further developed as colorimetric nanoplatform for selectively detecting protease K.



Schematic of the synthesis of AgNPs with kiwi juice and protease K detection.