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Analytical Methods

# Gold nanoparticle aggregation-based colorimetric assay for β-casein detection in bovine milk samples

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ABSTRACT

Traditional Kjeldahl method, used for quality evaluation of bovine milk, has intrinsic defects of time-consuming sample preparation and two analyses to determine the difference between non-protein nitrogen content and total protein nitrogen content. Herein, based upon antibody functionalized gold nanoparticles (AuNPs), we described a colorimetric method for β-casein (β-CN) detection in bovine milk samples. The linear dynamic range and the LOD were 0.08–250 μg $mL^{-1}$, and 0.03 μg $mL^{-1}$ respectively. In addition, the real content of β-CN in bovine milk was measured by using the developed assay. The results are closely correlated with those from Kjeldahl method. The advantages of β-CN triggered AuNP aggregation-based colorimetric assay are simple signal generation, the high sensitivity and specificity as well as no need of complicated sample preparation, which make it for on-site detection of β-CN in bovine milk samples.



## 1. Introduction

There are four major caseins (CNs) in bovine milk, namely as1-, as2-, β-, and κ-CN. β-CN makes up 37% of the casein content and is steady (Johansson et al., 2009). Therefore, the quantity of β-CN could be used as an index to evaluate the quality of bovine milk. Kjeldahl method is the officially recognized standard method for quality evaluation of bovine milk (Kamizake, Gonçalves, Zaia, & Dimas, 2003). However, it has two main problems: the relatively long testing time and the necessity to carry out two analyses to determine the difference between non-protein nitrogen content and total protein nitrogen content. Other reported methods, such as optical immunosensor based assay (Muller-Renaud, Dupont, & Dulieu, 2004), electrospray-ionisation mass spectrometric detection (Gaucheron, Mollé, Léonil, & Maubois, 1995), and chromatography method (Bramanti, Sortino, Onor, Beni, & Raspi, 2003; Bramanti, Sortino, & Raspi, 2002) require complicated handling procedures or technical expertise.

Mono-dispersed colloidal gold nanoparticles (AuNPs) solution appears red, which has high extinction coefficients and different colour (red to blue) in the visible region of the spectrum when the AuNPs are well-spaced in comparison with when they are aggregated (Vilela, González, & Escarpa, 2012). Therefore, the chemical reactions between the analyte and AuNPs surroundings can lead to a change of colour, which allows the colorimetric assay of the target. Recently, various AuNPs aggregation-based strategies have been widely employed in colorimetric analysis, such as thiol-functionalized cyanuric acid derivative stabilized AuNPs for melamine (Ai, Liu, & Lu, 2009), peptide-capped AuNPs for lysozyme and cancer diagnosis (Huang, Zhang, Luo, & Zhao, 2012; Kang et al., 2010), thioctic acid functionalized AuNPs for fumarate (Youk, Kim, Chatterjee, & Ahn, 2008), N-benzyl-4-(pyridin-4-ylmethyl) aniline ligand functionalized AuNPs for Cr(III) (Zhao, Jin, Yan, Liu, & Zhu, 2012), 4′-(4-mercaptophenyl)-2,2′:6′,2″-ter-pyridine zinc(II) complex functionalized AuNPs for $PO_4^{3+}$ (He, Zhao, Chen, Liu, & Zhu, 2013), sialic acid stabilized AuNPs for viral detection (Lee, Gaston, Weiss, & Zhang, 2013), aptamer modified AuNPs for bisphenol A (Mei et al., 2013), and ethylenediamine-capped AuNPs for trinitrotoluene (Lin et al., 2012).

Herein, we utilized the mono- (McAb) and polyclonal (PcAb) antibodies to functionalize AuNPs as colorimetric probe and developed an AuNPs aggregation-based colorimetric assay for the detection of β-CN in bovine milk samples. Fig. 1 illustrates the design strategy. To synthesized probes, AuNPs (20 nm) are functionalized with McAb and PcAb, respectively. In the presence of β-CN, the probe 1 (AuNP-McAb) would firstly bind with β-CN. After addition of probe 2 (PcAb-AuNP), the structure of AuNP-McAb–β-CN–PcAb-AuNP would be formed. Consequently the wine red colour of the suspension would be accordingly changed to a blue colour. The degree of colour change is directly related to the amount of β-CN which can be easily seen by naked eye and semiquantitative

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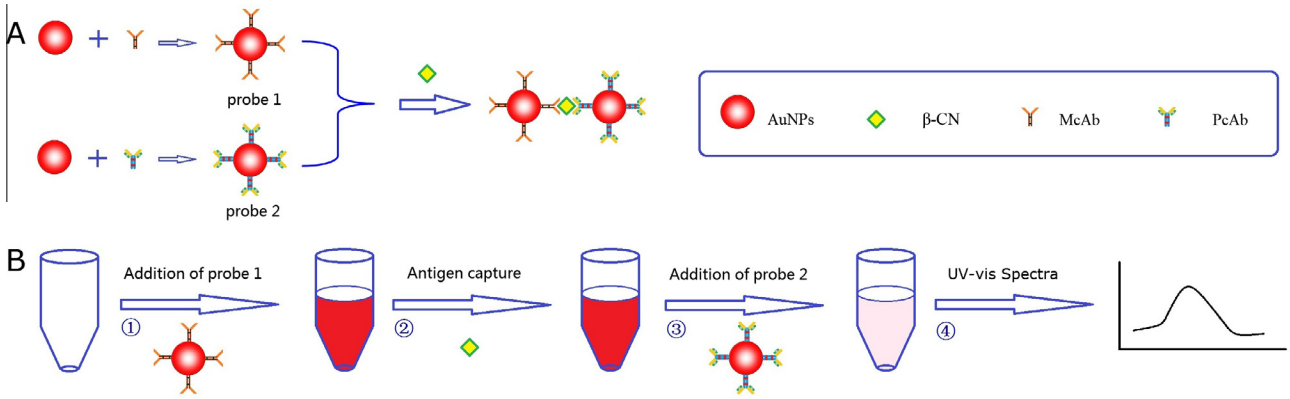


**Fig. 1.** Schematic diagram of preparation of the probes and AuNP probe-based colorimetric assay for β-CN. (A) Preparation of the probes and detection mechanism for β-CN. (B) Protocol of the assay. ① 300 μL of probe 1 was added in a tube. ② 100 μL sample solution was added and incubated for 5 min. ③ 700 μL of probe 2 was added and incubated for another 5 min. ④ The degree of colour change was monitored by UV–vis spectrometer (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

monitored by UV–vis spectrometer. This system may be used as a colorimetric sensor for on-site and real-time detection of β-CN in aqueous solution without the need of any advanced instrument.

## 2. Experiments

### 2.1. Materials and reagents

Bovine serum albumin (BSA), foetal bovine serum (FBS), as1-, as2-, β-, and κ-CN were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Chloroauric acid ($HAuCl_4$) and trisodium citrate were obtained from Shanghai Chemical Reagents (Shanghai, China). The anti-β-CN mono- (McAb) and polyclonal (PcAb) antibodies were prepared previously by the researchers themselves (Song et al., 2011; Zhou et al., 2013). Other reagents were of analytical purity. All the glassware used in the following procedure was thoroughly washed with aqua regia ($HCl/HNO_3$ (v/v) = 3:1), rinsed with Milli-Q water, and oven-dried prior to use. Doubly distilled water was used throughout all the experiments.

### 2.2. Synthesis of AuNPs

AuNPs were synthesized by reducing tetrachloroauric acid with trisodium citrate according to the method by the researchers themselves (Zhou et al., 2009). The obtained AuNPs were characterized using a combination of an ultraviolet spectrophotometer (UV-4802) at 400–660 nm and a transmission electron microscope (TEM, H-7650) operating at 80 kV.

### 2.3. Preparation of McAb-AuNPs and PcAb-AuNPs conjugate

The preparation reaction of McAb-AuNPs and PcAb-AuNPs was performed at room temperature by mixing the as-prepared AuNPs with McAb and PcAb, respectively (Zhou et al., 2009). The pH of AuNPs solution for McAb and PcAb conjugation was firstly adjusted to 8.5 with 0.1 M $K_2CO_3$. Then, with gently stirring, 1.0 mL of McAb and PcAb (0.5 mg $mL^{-1}$, respectively) was added drop by drop to 100 mL pH adjusted AuNPs solution, respectively. The mixture was gently mixed for 10 min, blocked by 10 mL of 1% BSA solution for 30 min. After being centrifuged at 21,885×*g* for 30 min, the pellets were suspended in 10 mL dilution buffer [20 mM Tris/HCl buffer (pH 8.2) containing 1% (w/v) BSA], and then stored at 4 °C for use.

### 2.4. Analysis of standard solution

The conjugates of McAb-AuNPs (300 μL) and PcAb-AuNPs (700 μL) were firstly added into a tube. Then the sample (100 μL) was added into the tube and incubated for 10 min. The standard β-CN solutions with different concentrations of 0.08, 0.4, 2.0, 10, 50, and 250 μg $mL^{-1}$ were detected with the system. All steps were carried out at room temperature. β-CN induced aggregation kinetics of the mixed probe was finally measured by UV–vis spectroscopy at 400–660 nm.

### 2.5. Selectivity of the colorimetric assay

Other proteins, including α-CN, κ-CN, α-lactalbumin, BSA and OVA were tested for selectivity study. Each protein solution (100 μL) at concentration of 2.5 mg $mL^{-1}$, were investigated respectively.

### 2.6. Analysis of real samples

To evaluate if matrixes influence the developed assay, five brand bovine milk samples brought from local supermarket were analysed by the developed assay after $1 \times 10^3$ times dilution of each sample, The results were also compared with those obtained from Kjeldahl method (Lynch, Barbano, & Fleming, 1998).

## 3. Results and discussion

### 3.1. Sensitivity of the probe for naked-eye detection

To demonstrate the performance of the assay for naked-eye detection of β-CN, 100 μL β-CN standard stock solution at different concentrations were added to 1 mL aqueous solution of the probes (300 μL McAb-AuNPs and 700 μL PcAb-AuNPs). After culturing for approximately 5 min, the results were directly detected with naked-eye observation. As shown in Fig. 2(I), there was a gradual colour change due to the addition of β-CN. With an increase in the concentration of added β-CN, the colour changed to purple at 10 μg $mL^{-1}$ (tube 4) and to blue at 50 μg $mL^{-1}$ (tube 6). The β-CN-stimulated aggregation of the probes was also evidenced by TEM images that revealed monodisperse AuNPs in the absence of β-CN and significant aggregation of AuNPs probe in the presence of 250 μg $mL^{-1}$ β-CN (Fig. 2(II)).

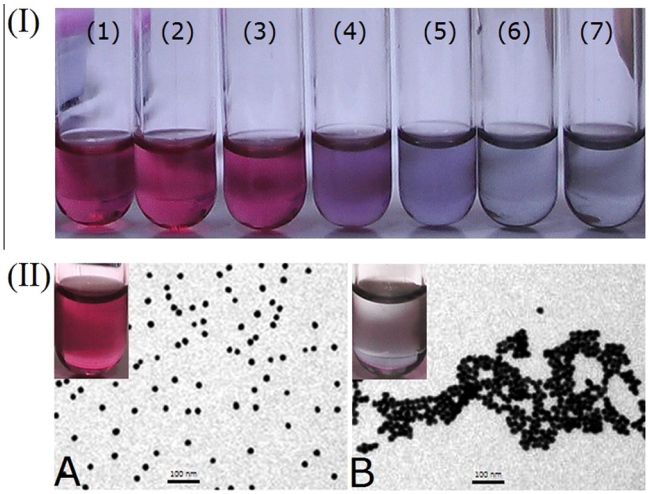


**Fig. 2.** (I) Visual colour change of the antibody functionalized AuNPs upon addition of β-CN with different concentrations (from left to right: (0, 0.08, 0.4, 2.0, 10, 50, 250 μg mL$^{-1}$). (II) TEM image of the probe suspension with the addition of β-CN at concentration of 0 μg mL$^{-1}$ (A) and 250 μg mL$^{-1}$ (B) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

### 3.2. Sensitivity of the assay for UV–vis spectroscopic measurements

The colour change of the probe solution triggered by β-CN was monitored by UV–vis spectrometer. As shown in Fig. 3A, upon addition of increasing concentrations of β-CN (0.08, 0.4, 2.0, 10, 50, 250 μg mL$^{-1}$), an increase in the absorbance at 612 nm region along with a concomitant decrease of the SPR peak at 523 nm was observed. These two wavelengths were employed to represent the relative amounts of mono-dispersed and aggregated AuNPs, respectively. For quantitative determination of β-CN, the ratio of absorption at 612 nm and 523 nm was used. There was a good linear relationship between the absorption ratio values ($A_{612}/A_{523}$) and the concentrations of β-CN in the range of 0.08–250 μg mL$^{-1}$ (Fig. 3B). The linear ranges were wider than those of optical immunosensor based detection (Muller-Renaud et al., 2004) and double-antibody based immunoassay (Zhou et al., 2013). The limit of detection (LOD) of the assay was 0.03 μg mL$^{-1}$, according to the rule of 3 times standard deviations of the blank responses (Mei et al., 2013). It was close to the sensitivity of surface plasmon resonance assay (Hiep et al., 2007) and lower than mass spectrometry (Mollé & Leonil, 2005). Although the developed method was not accurate and showed higher LOD than optical immunosensor based assay (Muller-Renaud et al., 2004) and chromatography method (Bramanti et al., 2002, 2003), it did not require complicated sample preparation or technical expertise.

The real concentration of β-CN in bovine milk is about 8.5 mg mL$^{-1}$ (Johansson et al., 2009). Before measurement with the developed assay, the milk sample was diluted by $1 \times 10^3$ times with bidistilled water. The concentration of β-CN was consequently about 8.5 μg mL$^{-1}$ which is in the linear dynamic range of the assay and higher than the LOD of the assay. Therefore, the proposed assay could be used for detection of β-CN in bovine milk.

### 3.3. Reaction dynamics of the probes

Considering that the probes having a fast response at room temperature is highly preferred for on-site and real-time detection. The aggregation reaction dynamics of the probes with different β-CN concentrations by monitoring the absorption ratio value ($A_{612}/A_{523}$) was studied. As shown in Fig. 4, the absorption ratio value

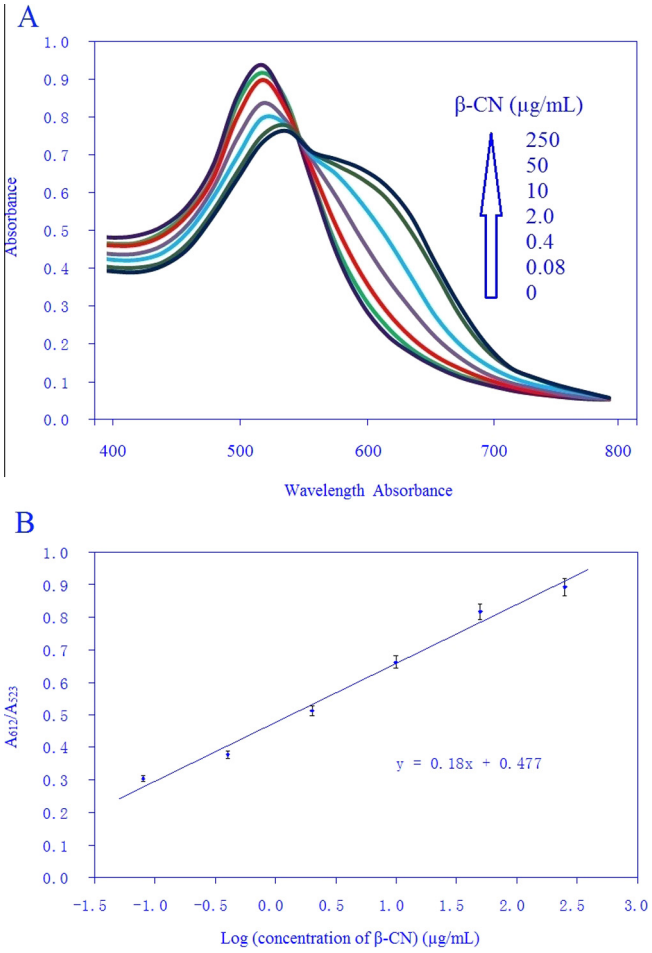


**Fig. 3.** (A) UV–vis absorption spectra of the AuNPs probes upon addition of different concentrations of β-CN (0–250 μg mL$^{-1}$). (B) The corresponding calibration curve. The absorption ratio of $A_{612}/A_{523}$ is linear with logarithm of β-CN concentration over the range from 0.08 to 250 μg mL$^{-1}$. Values are means ± SD ($n = 3$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

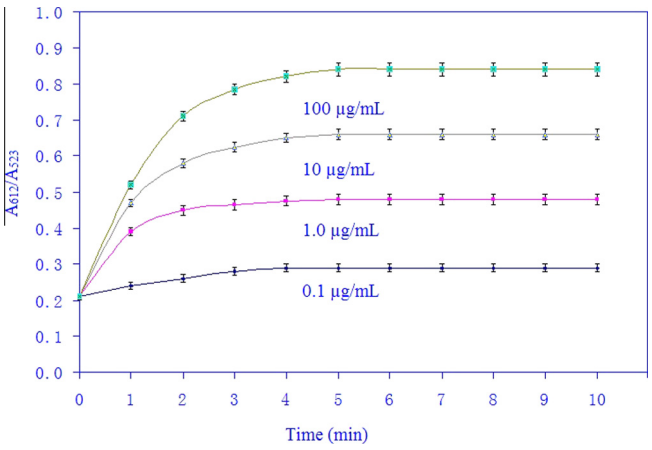


**Fig. 4.** The dynamics of the time-dependent absorption ratio of $A_{612}/A_{523}$ over 12 min in the presence of varying concentrations of β-CN.

($A_{612}/A_{523}$) increases gradually as soon as β-CN at different concentrations was added and then reaches a maximum. All absorption ratio values with different β-CN concentrations display saturation within 5 min, which indicates that the detection after 5 min incubation can produce a stable signal.

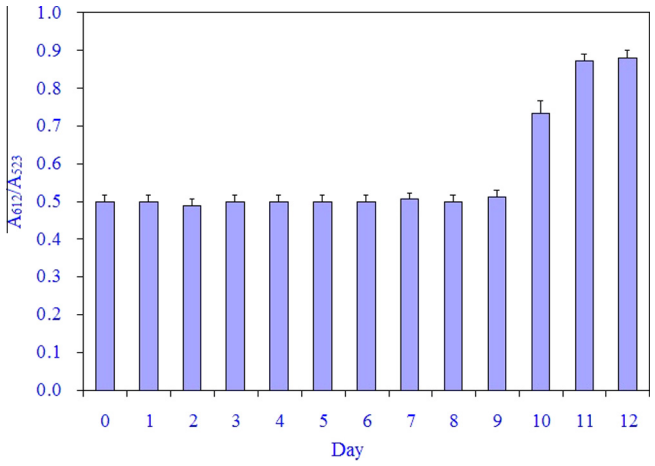


**Fig. 5.** The stability of the probes. No loss activity of the probes was observed within 9 days storage at 4 °C.

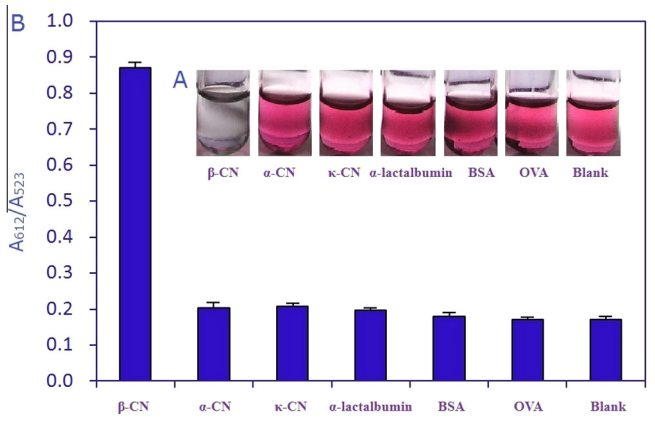


**Fig. 6.** Selectivity of the assay. (A) Colour change of the functionalized AuNPs upon addition of 2.5 mg $mL^{-1}$ of β-CN and other proteins, respectively. (B) The absorption ratio ($A_{612}/A_{523}$) change for β-CN and other proteins (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

### 3.4. Stability of the probes

For the practical application of the probes, the stability is very essential. It was examined using 1.0 μg $mL^{-1}$ of β-CN by running the procedure described in Section 2.4 in the storage of 1–12 days at 4 °C. As shown in Fig. 5, the value of $A_{612}/A_{523}$ did not changed within 9 days, which indicated that the probes can be used for repeated measurement.

**Table 1**
Application study of the developed assay.

| Samples | Concentration of β-casein (mg $mL^{-1}$)[a] | |
|---|---|---|
| | Kjeldahl method[b] | Developed assay[c] |
| Mengniu | 8.69 ± 0.14 | 8.71 ± 0.16 |
| Huishan | 8.61 ± 0.12 | 8.63 ± 0.13 |
| Guangze | 8.67 ± 0.11 | 8.68 ± 0.15 |
| Yili | 8.68 ± 0.13 | 8.69 ± 0.11 |
| Wandashan | 8.53 ± 0.09 | 8.51 ± 0.08 |

[a] Data are the means of triplicates. Values are means ± SD.
[b] The pH of the sample was firstly adjusted to 4.6 with trichloroacetic acid (150 mg/mL). Then the precipitate was collected and determined by Kjeldahl (Tang, Lv, Fu, Ye, & Liu 2009).
[c] Each sample was firstly diluted diluted by $1 \times 10^3$ times with bidistilled water, then measured by using the developed assay.

### 3.5. Selectivity of the assay

In order to examine the selectivity of the proposed method toward β-CN and other proteins (α-CN, κ-CN, α-lactalbumin, BSA and OVA), 2.5 mg $mL^{-1}$ of various proteins were respectively added into 1 mL probe dispersion. As shown in Fig. 6A, the colour of the probe dispersion with β-CN is quite different with that of probe dispersion with other proteins. And no significant change of the $A_{612}/A_{523}$ value was observed upon the addition of other proteins (Fig. 6B). The results indicated that the developed assay could be used for selective detection of β-CN in bovine milk samples.

### 3.6. Applicability of the assay

To demonstrate the potential practical application of the assay for β-CN in real-world samples, bovine milk samples of five different brands purchased from local supermarket were analysed. As shown in Table 1, the correlation coefficient ($R^2$) of the results obtained from the developed assay and Kjeldahl method (Lynch et al., 1998) was 0.992, indicating a good correlation between the two methods. Comparatively, the developed assay did not require complicated equipment and trivial pretreatment of the sample except for $1 \times 10^3$ times dilution with bidistilled water. Therefore, the developed assay could be used as an optional platform for β-CN detection in milk.

## 4. Conclusion

In this study, an AuNPs aggregation-based colorimetric assay for β-CN detection has been developed by using McAb and PcAb functionalized AuNPs. The experimental results showed that β-CN can trigger the aggregation of the AuNPs, which in turn, resulted in an obvious colour changes from red to blue. Furthermore, the degree of the colour changes triggered by β-CN also can be monitored by UV–vis spectrometer, which offers a linear detection range from 0.08 to 250 μg $mL^{-1}$. The application results reinforced that the colorimetric assay is applicable for β-CN detection in real bovine milk samples. Due to its advantages of the simplicity of the performance, high sensitivity and specificity as well as without the need of time-consuming sample preparation, the developed colorimetric assay provides a potential tool for rapid and convenient detection of β-CN in bovine milk samples on-site.

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