

Accepted Manuscript

Analytical methods

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PII: S0308-8146(13)01586-0

DOI: <http://dx.doi.org/10.1016/j.foodchem.2013.10.137>

Reference: FOCH 14923

To appear in: *Food Chemistry*

Received Date: 29 December 2012

Revised Date: 27 September 2013

Accepted Date: 26 October 2013



Please cite this article as: Yang, A., Zheng, Y., Long, C., Chen, H., Liu, B., Li, X., Yuan, J., Cheng, F., Fluorescent immunosorbent assay for the detection of alpha lactalbumin in dairy products with monoclonal antibody bioconjugated with CdSe/ZnS quantum dots, *Food Chemistry* (2013), doi: <http://dx.doi.org/10.1016/j.foodchem.2013.10.137>

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Fluorescent immunosorbent assay for the detection of alpha lactalbumin in dairy products with monoclonal antibody bioconjugated with CdSe/ZnS quantum dots

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Abstract

In this work, a new method termed competitive fluorescence-linked immunosorbent assay (FLISA) was developed for specifically quantification of bovine α -lactalbumin (α -La) in dairy products. The monoclonal antibodies (mAbs) against α -La were produced through hybridoma technology, and the mAbs were covalently conjugated with the CdSe/ZnS quantum dots (QDs) using the crossing-linking reagents. Moreover, a competitive FLISA based on QD-mAb conjugates was established to detect α -La in dairy products. It was shown that there was a good linear relationship between inhibition efficiency, and logarithm of α -La concentration after the detection parameters were optimized in which the

concentration of α -La varied from 0.1 to 1000 ng/mL. The value of IC_{50} was 0.03 μ g/mL, and the FLISA method exhibited high sensitivity with the LOD at 0.1 ng/mL. The developed FLISA has been successfully applied to determine α -La in commercial dairy products, providing more sensitive analysis compared with the ELISA method.

Keywords: α -lactalbumin; milk allergens; monoclonal antibody; quantum dots; fluorescence-linked immunosorbent assay

1. Introduction

Currently, food allergy is recognized as a worldwide problem, and the number of people suffered from food allergic disease has gradually increased. Dairy products are widely utilized in food industries due to their high protein concentration and balanced nutrition. However, survey showed that the incidence of disease induced by cow milk allergy (CMA) was 0.3-7.5% in the population all over the world (Pessler & Nejat, 2004), and cow milk is one of the top eight kinds of major allergic foods in our daily diet including milk, eggs, peanuts, tree nuts, fish, shellfish, soy and wheat. Among cow milk proteins, casein, beta-lactoglobulin (BLG) and α -La are recognized as the most common cow milk allergens, and the high cross-reactivity exists between milk allergens from different mammalian species (Carroccio, Cavataio, & Iacono, 1999).

Regarding to bovine α -La, it belongs to the lysozyme superfamily, consisting of 123 amino acid residues and forming a compact globular structure with four disulfide bonds, and its complete amino acid sequence is extensive homology with human α -La because 74% of the residues are identical and another 6% chemically similar (Farrell

et al, 2004). Nevertheless, bovine α -La has been highlighted as a major cow's milk allergen, and Adamsi's studies have indicated that 75% of the CMA serum containing α -La specific antibodies (Adams, Barnett, Walsh, Pearce, Hill, & Howden, 1991).

It is generally acknowledged that even some allergenic foods present in trace amount, such as milk, can cause severe and fatal reactions (El-Agamy, 2007). However, strict avoidance of food allergy is difficult because food labeling is still not standardized (Careri, Elviri, Lagos, Mangia, Speroni, & Terenghi, 2008), and contamination of allergenic ingredients in food can easily occur during processing and transport. For these reasons, there is a growing interest in the detection of hidden allergens in food. Appropriate analytical methods are necessary for the reliable and specific allergen detection to verify food labeling requirements and protect the allergic consumer. As far as the identification of bovine allergens is concerned, some approaches developed for the analysis of allergens were mostly based on protein separation and characterization techniques. The popular methods for protein separation included two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), reversed-phase liquid chromatography (RP-HPLC) and multidimensional liquid chromatography combined to mass spectrometric detection (LC-MS) (Wu, Black, North, & Rodrigo, 2012; Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008; Ren, Han, Chu, Zhang, Cai, & Wu, 2010). Among them, 2D-PAGE has been successfully applied for milk proteins analysis, including low-abundance protein identification and high resolution (Hsieh & Pan, 2012). However, the protein identification ability of such methods is still insufficient, related to the co-elution

phenomenon (Croley, White, Callahan, & Musser, 2012). Some analysis requires pre-treated samples and long analysis time, which lead to high cost. Moreover, most of the detection methods are inaccurate in quantification and identification of proteins because of a low sensitivity in complex matrices (Chassaigne, Ngaard, & van Hengel, 2007). In addition, the existing chromatographic technologies are unable to identify protein denaturation or modifications which may occur during processing and storage.

In recent years, the majority of methods used to detect α -La in dairy products are based on immunochemical protocols such as radio-allergosorbent tests (RAST), enzyme allergosorbent tests (EAST), Western blotting, rocket immuno-electrophoresis (RIE) and enzyme-linked immunosorbent assays (ELISA) (Monaci, Tregoat, van Hengel, & Anklaam, 2006; Monaci, Brohee, Tregoat, & van Hengel, 2011; van Esch et al., 2011). Among these techniques, ELISA is the technique mostly utilized for the routine analysis of allergens in foods for its low detection limit, high sensitivity, and suitability for analysis of several samples simultaneously (Paulie, Perlmann, & Perlmann, 2006). However, it is still tedious, time-consuming, and unexpected cross-reaction for the use of polyclonal antibodies. Beyond that, polymerase chain reaction (PCR) techniques based on the DNA level have frequently been reported as a promising alternative for ELISA (Takashi, Satoshi, Yusuke, Masayuki, & Hisanori, 2009), but there is no fixed correlation between the amount of DNA and protein allergens, which may lead to false positive or false negative results. Therefore, it is needed to find a more sensitive, time-saving and accurate approach to detect the milk

89 allergens.

90 The antibody plays a key important role in immunological detection methods.
91 Monoclonal antibodies (mAbs) were developed almost 40 years ago. Since mAbs
92 with strong affinity can specially recognize the target antigens, it is widely accepted
93 that mAbs have provided a valuable tool in modern immunological determination. Ma
94 et al reported that the competitive ELISA based on the mAbs against glycinin (a main
95 allergen) exhibited high-sensitivity with a low detection limit at 0.3 ng/mL of glycinin
96 in soybean products (Ma et al., 2010).

97 In recent years, organic fluorescent dyes have been used in biomedical imaging
98 and detection. However, organic fluorescent dyes are generally vulnerable to the
99 physiological environment and quickly photobleached under normal imaging
100 conditions (Yu, Chang, Drezek, & Colvin, 2006). In view of the defects of
101 conventional organic fluorescent dyes, quantum dots (QDs), semiconductor
102 fluorescent nanoparticles, have recently been used as fluorescent labels in
103 immunoassays (Yang, Gu, Xu, & Xu, 2011). QDs, owing to the quantum-size
104 confinement effects, have unique optical and electronic properties such as wide and
105 continuous absorption spectra, narrow emission spectra, water solubility and high light
106 stability, resulting in being widely used for biological labeling. Since QDs have been
107 applied in fluorescence labeling for cellular imaging, DNA mutations, and tumor cell
108 identification (Smith, Duan, Mohs, & Nie, 2008; Medintz, Mattoussi, & Clapp,
109 2008), it can be extended for detection of specific allergens if appropriate methods are
110 established.

111 In this work, high-affinity mAbs against α -La were produced using purified α -La as
112 the immunogen, and the generated mAbs were conjugated with QDs by using
113 N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) as an
114 activator and N-hydroxysuccinimide (NHS) as a coupling reagent (Watanabe et al.,
115 2011). A new approach called fluorescence-linked immunosorbent assay (FLISA) for
116 the detection of α -La was established. It was suggested that the developed FLISA is a
117 promising alternative way to detect α -La in food, and it only relies on simple labeling
118 procedures and allows rapid detection of characteristic antigens. Moreover, the new
119 technology also may offer a simpler platform for the detection of other allergens in
120 food.

121 **2. Materials and methods**

122 *2.1. Materials*

123 Bovine α -La (purity>95%) was produced in our laboratory, and it was identified by
124 SDS-PAGE and mass spectrometry (MS). The reference material of α -La
125 (purity>85%), EDC, NHS, gelatin, o-phenylenediamine (OPD), hypoxanthine-
126 aminopterin-thymidine (HAT) medium and hypoxanthine-thymidine (HT) medium
127 were purchased from Sigma. CdSe/ZnS QDs was kindly provided by Dr. Xu in
128 Nanchang University. Ten brands of dairy products were purchased from the local
129 supermarket in Nanchang, China. Other reagents were of analytical grade and used
130 without further purification.

131 Female Balb/c mice (20 ± 2 g) were obtained from Jiangxi Laboratory Animal
132 Center (Nanchang, China, the permission number is “SYXK (Gan) 2008-003”). All

133 mice were allowed access to water and food ad libitum under the controlled
134 conditions of temperature (22 ± 2 °C), humidity ($55 \pm 5\%$) and light (12 h of
135 light and dark, respectively). They were housed in a plastic cage containing sterile
136 paddy husk (procured locally) as bedding throughout the experiment. The animals
137 were maintained and treated according to the European Directive 2010/63/EU
138 concerning the protection of animals used for experimental and other scientific
139 purposes.

140 2.2. Preparation of mAbs against α -La

141 The mAbs against α -La were generated according to previous work (Richter &
142 Evers, 1984). Hybridoma cells were prepared by fusion of the spleen cells isolated
143 from an immunized mouse against the purified α -La and mouse myeloma cell. Then
144 the strong positive hybridoma cells were further diluted, subcloned and detected until
145 all the wells showed anti- α -La activity. At last, one mAb with highest activity was
146 selected and cultured to reach a certain amount of cells. The preparation of the ascites
147 containing a large number of mAbs was carried out by injecting the collected cells
148 into the abdominal cavity of mice. The crude ascites were defatted by centrifugation,
149 followed by precipitation using caprylic acid and ammonium sulfate. The extracted
150 antibodies were further purified with dialysis membrane (MWCO, 5,000Da). All
151 samples in the processes were subjected to SDS-PAGE.

152 2.3. Characterization of mAbs

153 The concentration of antibody was preliminary determined by a SmartSpecTM Plus
154 Spectrophotometer (Bio-Rad, Hercules, CA, USA).

155 *2.3.1. SDS-PAGE and western blotting*

156 Western blotting was performed to assess the specificity of the mAbs against α -La.
157 Bovine whey protein and α -La were subjected to sodium dodecyl
158 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a minigel apparatus
159 (BioRad Laboratories, Hertfordshire, UK) with 15% acrylamide separating gel and
160 4% acrylamide stacking gel. The separated proteins were transferred onto a
161 polyvinylidene difluoride (PVDF) membrane. Then the membrane was blocked with
162 1% gelatin in Tris-Buffered Saline Tween-20 (TBST) buffer for 1 h at 37°C, followed
163 by incubating with monoclonal antibody (1:2,000 dilutions in TBS) overnight at 4°C.
164 Subsequently, the membrane was washed with TBST buffer and incubated with
165 horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000 dilutions) for
166 1h at 37°C. Finally, the bands of antibody-binding were detected with an Enhanced
167 chemiluminescence kits (Amersham, ECL kits).

168 *2.3.2. Indirect competitive ELISA*

169 Polystyrene microtiter plate (Canda Jet Bio-Chemicals Int., Guangzhou, China)
170 were used as a solid support, and each well were coated with 100 μ L of antigens at 0.5
171 μ g/mL in 0.05 M bicarbonate buffer (pH 9.6), followed by incubation overnight at
172 4°C. The plates were washed with phosphate-buffered saline solution (PBS)
173 containing 0.05% Tween-20, and residual free binding sites were blocked with 1%
174 gelatin in PBS for 1 h at 37°C to prevent non-specific binding. Serial dilutions of the
175 α -La standard were incubated with mAbs previously diluted in PBS, and 100 μ L of
176 incubated solution was added to each well. After 1 h of incubation, 100 μ L of

177 HRP-conjugated goat anti-mouse IgG (1:5,000 dilution) was added into each well and
178 incubated for 1 h. Finally, OPD was used as substrate. After 15 min of incubation, the
179 reaction was stopped with 2 M sulphuric acid, and optical density (OD) was measured
180 at 490 nm. A negative control (neither competitive antigen nor mAbs) and positive
181 control (without competitive antigen but with mAbs) were included in each plate.

182 2.3.3. Cross-reactivity of mAbs

183 The cross-reactivity was assessed by determining the 50% inhibition concentration
184 (IC_{50}) value in the competitive ELISA described above. α -La and bovine
185 β -lactoglobulin (β -lg) were respectively dissolved in the assay buffer and examined as
186 a competitive antigen. The serial concentrations of competitive antigen were used for
187 determining the IC_{50} values. Cross reactivity (CR) value is calculated according to the
188 following formula:

$$189 \quad CR = \frac{IC_{50}(\alpha-La)}{IC_{50}(\beta-lg)}$$

190 2.4. Conjugation of mAbs with QDs

191 CdSe/ZnS QDs were activated with EDC for 5 min at room temperature, then NHS
192 was added to the solution, followed by incubation for 5 min again. The conjugation
193 step was performed by mixing the mAbs and activated QDs for 2 h at 37°C. At the
194 end of the conjugated reaction, the conjugates were collected by ultrafiltrating with
195 Millipore filter (MWCO 30 kDa, Millipore, USA) for several times to remove the
196 unreacted excess EDC and NHS, followed by further purification by gel filtration
197 chromatography (Sephadex-G200) to remove any unreacted excess QDs and mAbs.

198 2.5. Evaluation of anti- α -La mAbs conjugated with QDs

199 Dot-immunoblotting was performed to determine antibody activity of the
200 conjugates (Mahmoud et al., 2011). The α -La samples in a quantity range from 1 to 10
201 μ g were loaded on the nitrocellulose (NC) membrane, and the membrane was
202 incubated for 1 h at 37°C, followed by blocking with 1% gelatin in TBS for 1 h at
203 37°C. The membrane was washed three times with 5% Tween-20 in TBS and loaded
204 with 3 μ L of QD-mAbs conjugates for 2 h at 37°C. The fluorescence intensity was
205 analyzed with the ultraviolet imaging system (Universal Hood II, Bio-Rad, Hercules,
206 CA, USA).

207 The emission spectra of QDs and conjugates were measured by fluorescence
208 spectroscopy. Fluorescence spectra between 350 and 700 nm (excitation, 365nm)
209 were recorded at room temperature on a 960 CRT fluorescence spectrophotometer (Jin
210 He instruments, Shanghai, China).

211 *2.6. Establishment and assessment of the determination method*

212 *2.6.1. Establishment of the determination method*

213 A competitive FLISA based on the prepared QD-mAbs were performed to establish
214 an inhibition calibration curve for α -La. Briefly, polystyrene microtiter plates were
215 coated with a solution (100 μ L/well) containing purified α -La (1 μ g/mL) in 0.05 M
216 bicarbonate buffer (pH 9.6), followed by incubation overnight at 4°C. Afterwards, the
217 plates were washed and then blocked with 1% gelatin for 1 h at 37°C. Serial dilutions
218 of the commercial α -La were incubated for 1 h with QD-mAbs previously diluted in
219 PBS, and 100 μ L was added to each well. After 1 h of incubation, fluorescence
220 intensity in the plate containing QD-mAbs was measured by Tecan Genios (Thermo

221 Fisher), and compared with the indirect competitive ELISA for sample analysis
 222 described in Section 2.3.2. The inhibition rate (%) for α -La in the competitive ELISA
 223 /FLISA was calculated (You et al., 2008).

224 2.6.2. Assessment of the developed determination method

225 The major parameters evaluated in the validation and reliability study (Ma et al.,
 226 2010; Le, Yu, Guo, Ngom, Shen, & Bi, 2009) included (a) recovery of α -La spiked
 227 in PBS, (b) intra-assay coefficient of variation (CV) for precision within an assay, (c)
 228 inter-assay CV for precision between assays, (d) Inhibitory concentration (IC_{50})
 229 values and (e) LOD (limit of detection) of the assay.

230 Competitive curves were generated by plotting inhibition values versus the
 231 logarithm of α -La concentration. In the curves, IC_{50} was defined as the concentration
 232 of analyte that provided a 50% of the maximum inhibition values, and the LOD of the
 233 assay is determined by calculating the mean value of blank samples plus three times
 234 standard deviations on the standard curve.

235 2.6.3. Quantitative determination of α -La in dairy products

236 The samples (liquid or powdered) were dissolved at 0.1 g/mL in PBS, and
 237 precipitates were removed by centrifugation. The supernatants, containing α -La, were
 238 diluted (1:100) in PBS and used as test samples. α -La in test samples were detected by
 239 the established competitive FLISA and ELISA , respectively.

240 2.7. Statistical analysis

241 All data were obtained from three independent experiments. Our experimental data
 242 were presented as the mean and standard deviation (SD), which were analyzed with

the "Descriptive Statistics" procedure in SPSS 11.0. Comparisons among all groups were performed with the two-tailed Student's t-test. In all cases, $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Preparation and characterization of mAbs against α -La

The titer of mAb from ascites was about 3×10^5 defined by ELISA. Fig. 1A shows the SDS-PAGE patterns of the purified ascites. High contents of antibodies were found in original ascites, heavy and light chains of antibodies were clearly visible in the gels (lane 1). It should be noted that precipitation by caprylic acid and ammonium sulfate significantly reduced the content of impurities (lane 2) and the chains were also clearly visible after purification with ultrafiltration (lane 3). Pure mAbs were obtained after purification in spite of the weight loss of antibody. The concentration of the prepared antibody was sufficiently high for further experiments.

The specific binding of the mAbs to α -La was assessed by Western blotting. Fig. 1B(a) shows the electrophoretic pattern of purified α -La and bovine whey protein, and bovine whey protein in lane 2 is mainly composed of α -La and β -lg band. Fig. 1B(b) shows the corresponding immunoblotting complexes of α -La and bovine whey protein with mAbs against α -La. It indicated that mAbs against α -La could only specifically bind α -La in whey rather than β -lg or the other whey protein. It confirmed that the mAbs had a good specificity against α -La.

The specificity of the mAb against α -La was also evaluated by a cross-reaction assay. The inhibitory concentration (IC_{50}) values for α -La and β -Lg were 75 ng/mL

265 and 27,200 ng/mL, respectively. The CR value was only 0.275%, indicating that there
266 was no cross-reactivity of mAbs with β -Lg.

267 3.2. Conjugation of mAbs with QDs

268 CdSe/ZnS QDs used to couple with mAbs was modified with carboxyl groups, and
269 it will yield the desired functionality and water solubility to the QDs. It could
270 covalently bind with amino groups on the surface of antibodies when EDC and NHS
271 were existed (Fig.2A). In order to optimize the condition of conjugation of mAbs with
272 QDs, a range of QD-carboxyl/EDC/NHS/mAbs ratios were investigated. It was
273 observed that precipitation or fluorescence quenching of quantum dots appeared upon
274 EDC/NHS excess. Finally, we confirmed that the optimized ratio of
275 QD/EDC/NHS/mAbs was 1:10:6:4. Under the condition, no deposit but the mixture
276 of QD-mAbs and free mAbs were found in reaction solution. As shown in Fig. 2A, the
277 fluorescence intensity of conjugates was high and uniform. It suggested that the
278 conjugation reaction could occur successfully under the optimized condition.

279 The gel filtration chromatography (GFC) was used to rapidly remove unconjugated
280 antibodies from conjugates after coupling of mAb with QDs. Two elution peaks “a”
281 and “b” (monitored by UV detector at 280 nm) were clearly obtained after purified by
282 GFC (Fig. 2B). Since the molecular weight of QD-mAbs was greater than
283 unconjugated antibody, this was excluded faster out of the gel granule than that of free
284 antibodies. Two eluting peaks were collected and concentrated through ultrafiltration.
285 It was found that only peak “a” containing QD-mAbs could emit strong fluorescence
286 under UV lamp (exciting at 365 nm) because the QDs can usually emit fluorescence

287 under ultraviolet excitation. It indicated that QD-mAbs was successfully purified from
288 the mixture of conjugates.

289 3.3. Characterization of QD-mAbs

290 The absorption spectra of QDs are continuous, while the emission spectra are
291 narrow. Excitation-emission matrix (EEM) reveals that free QDs always emit the
292 same fluorescence within all the excitation wavelength. Fluorescence
293 spectrophotometer was used to analyze the difference in emission spectra between
294 QDs and QD-mAbs. As shown in Fig.3A, the spectra of conjugates (curve b) were
295 characterized by strengthening and a slight blue shift of emission peak (4 nm)
296 compared with that of QDs (curve a). This change could be due to the covalent
297 attachment between the carboxyl and amino groups, which would reduce the surface
298 charge and the interaction between dipoles of QDs, leading to blue shift of emission
299 peak (Chen et al., 2005). It further confirmed that QDs were successfully coupled
300 with mAbs.

301 The dot-immunoblotting assay was used to estimate the activity of the QD-mAbs.
302 For this purpose, the α -La and PBS (blank control) were spotted on the PVDF
303 membrane. As shown in Fig.3B, the more protein spotted on PVDF membrane, the
304 stronger fluorescence intensity was. This implied that the purified QD-mAbs could
305 specifically recognize α -La, and QD-mAbs would bind more allergen protein with the
306 increase of α -La. Moreover, it was found that the side immobilized QD-mAbs was
307 bright, while the side without immobilized QD-mAbs was dark. Obviously, the
308 antibodies in the conjugates still remained strongly active, ensuring the

immunological detection requirements.

3.4. Establishment of the detection method

QD-mAbs can directly, rapidly and specifically recognize and qualitatively detect α -La in dairy products, and the content of α -La in food was analysed by fluorescence intensity of the QDs. In order to validate the detection, the purified QD-mAbs was used to establish a calibration curve for α -La in the competitive FLISA. The optimal concentration of the coated α -La, the optimal volume ratio of antibody and competitive allergen, and the optimal dilution ratio of the QD-mAbs were 1.0 μ g/mL, 1:1 and 1:3,200, respectively. The competitive inhibition curves were shown in Fig.4, and there was a good linear relationship between inhibition efficiency and logarithm of α -La concentration when the concentration of α -La varied from 0.1 to 1000 ng/mL. In contrast, a competitive ELISA was developed on the basis of the mAbs, and goat anti-mouse IgG-HRP was used as the second antibody diluted to 1:3,000. To determine the recoveries, 0.50, 1.00 and 1.50 mg of α -La spiked in PBS were measured in triplicate. As shown in Table 1, the percentages of recovery were within 97.83-102.11% and 90.75-92.34% for competitive FLISA and competitive ELISA, respectively. The mean intra- and inter-assay CV of α -La FLISA were 4.47% and 11.53%, respectively, while the mean intra- and inter-assay CV of α -La ELISA were 4.85% and 12.83%, respectively. Moreover, the intra- and inter-assay CV of the competitive FLISA were lower than those of competitive ELISA, indicating that the competitive FLISA based on the QD-mAbs was more accurate than the competitive ELISA based on the mAbs.

331 In the inhibition curves, IC_{50} was defined as the concentration of analyte that
332 provided a 50% of the maximum inhibition values. The IC_{50} value and limit of
333 detection (LOD) of the competitive FLISA were 30 ng/mL and 0.1 ng/mL,
334 respectively (Table 1). Compared with the competitive ELISA, the competitive FLISA
335 had lower IC_{50} and LOD. It indicated that the FLISA method based on the QD-mAbs
336 exhibited higher sensitivity than the competitive ELISA based on the mAbs.

337 3.5. Quantitative detection of α -La in dairy products

338 The α -La in dairy products was quantified by the established competitive FLISA
339 and ELISA respectively. According to the analysis of different samples (Table 2), the
340 content of α -La in the sample of grain dojo flavor soymilk was the lowest, while α -La
341 in the sample of mengniu yogurt was the highest. Comparing the two detection
342 methods, the concentration of α -La in all samples detected by the competitive FLISA
343 were higher than those measured by the competitive ELISA, while they were not
344 significantly different except for the samples of grain dojo flavor soymilk and
345 mengniu yogurt ($P < 0.05$), which may be due to a too high or too low concentration
346 of α -La in the two samples, respectively. Furthermore, the results were corroborated
347 by the spike experiment in Table 1, which showed that the percentages of recovery for
348 competitive FLISA were higher than those of competitive ELISA.

349 Since QDs are superior to any conventional organic fluorescent dyes, and the
350 fluorescence of QDs can be scanned even at very low concentration, QDs are helpful
351 for measuring the related composition by conjugation with the suitable tracer
352 molecule (Wang, Ma, Li, Li, & Su, 2007). Actually, QDs was one of the foremost

353 relevant tools in food safety detection due to their rapidity, convenience and
354 sensitivity, and QDs can also exhibit their specificity from biological binding
355 reactions including antigen/antibody, enzyme/substrate, and receptor/ligand (Thakur
356 & Karanth, 2003). In the other hand, mAb has become a powerful bioanalytical tool
357 for specific detection in food safety. Therefore, the combination of mAbs and
358 quantum dots makes FLISA highly attractive tool for biological analysis, especially
359 for food allergen in food safety.

360 **4. Conclusions**

361 In this study, a high-affinity mAbs against α -La was generated based on the
362 principle of hybridoma antibody. A competitive FLISA based on the QD-mAbs was
363 established to detect α -La in commercial dairy products. Compared with most of the
364 methods for food allergens analysis, the newly developed method possesses many
365 advantages, including simplicity, high flexibility and good sensitivity. FLISA assay
366 exhibited high-sensitivity with the minimum LOD at 0.1 ng/mL and IC₅₀ value at
367 0.03 μ g/mL of α -La, and it only required simple pre-handling to detect the
368 corresponding allergen in complex food matrices without using enzymatic label
369 second antibody. Consequently, the FLISA method can be used for reliable detection
370 of α -La in commercial dairy products, providing more sensitive detection compared
371 with the ELISA method.

372

373 **Acknowledgements**

374 The authors are grateful for financial support of National High Technology

375 Research and Development Program of China (863 Program, No. 2013AA102205),
 376 the International Science & Technology Cooperation Program of China (No.
 377 2013DFG31380), National Science and Technology Support Program
 378 (NO.2012BAK17B02, 2011BAK10B03), National Natural Science Foundation of
 379 China (No. 31171716), Foundation for Natural Science Foundation of Jiangxi
 380 Province (No. 20122BAB204001), Young Scientist Training Program of Jiangxi
 381 Province (20122BCB23006), Research Program of State Key Laboratory of Food
 382 Science and Technology (SKLF-KF-201007, SKLF-ZZA-201302 and
 383 SKLF-ZZB-201302), and Program for Graduate innovation(YC10A015) of Jiangxi
 384 Province.

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481 **Figure Captions**

482 **Fig.1.** Purification and specific analysis of mAbs against α -La. A: SDS-PAGE
483 patterns of the purified ascites (Lane M: standard molecular marker; Lane 1: ascites
484 from mouse; Lane 2: ascites precipitated by caprylic acid and ammonium sulfate;
485 Lane 3: samples further purified with ultrafiltration). B: SDS-PAGE patterns (a) and
486 Western blotting (b) of mAbs with α -La and whey protein (Lanes M: standard
487 molecular marker; Lanes 1: α -La; Lanes 2: whey protein).

488
489 **Fig.2.** Conjugation of mAbs with QDs. A: Schematic illustration of QDs coupling
490 with mAbs and fluorescence images of conjugates under UV 365 nm lamp. B: The
491 conjugates of QD-mAbs purified by GFC and its fluorescent analysis.

492
493 **Fig.3.** Characterization of QD-mAbs. A: Fluorescence emission spectra of CdSe/ZnS
494 QDs (a) and QD-mAbs bioconjugates (b). B: Fluorescence images for
495 dot-immunoblotting of the QD-mAbs conjugates with α -La with different content.

496
497 **Fig.4.** Inhibition curves for detection of α -La by indirect competitive FLISA and
498 ELISA.

Fig. 1

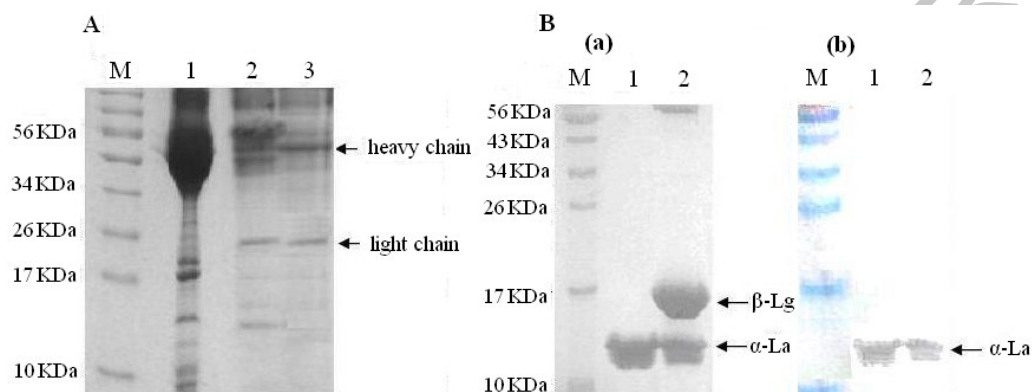
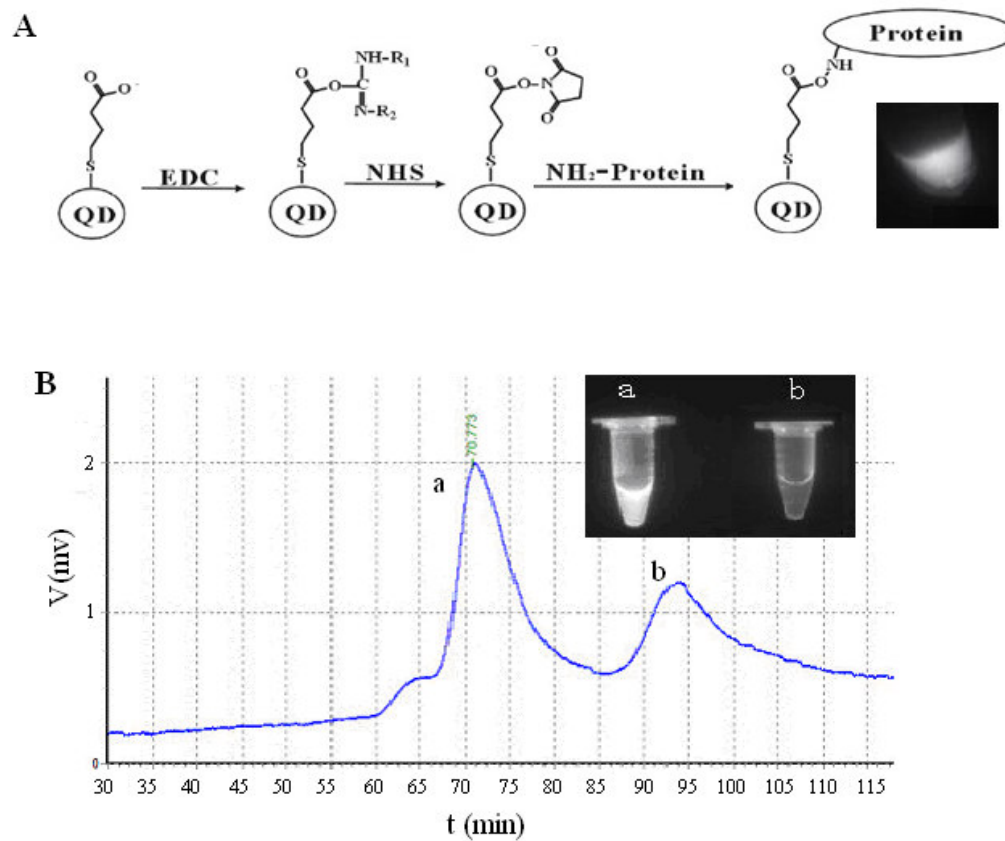


Fig.2



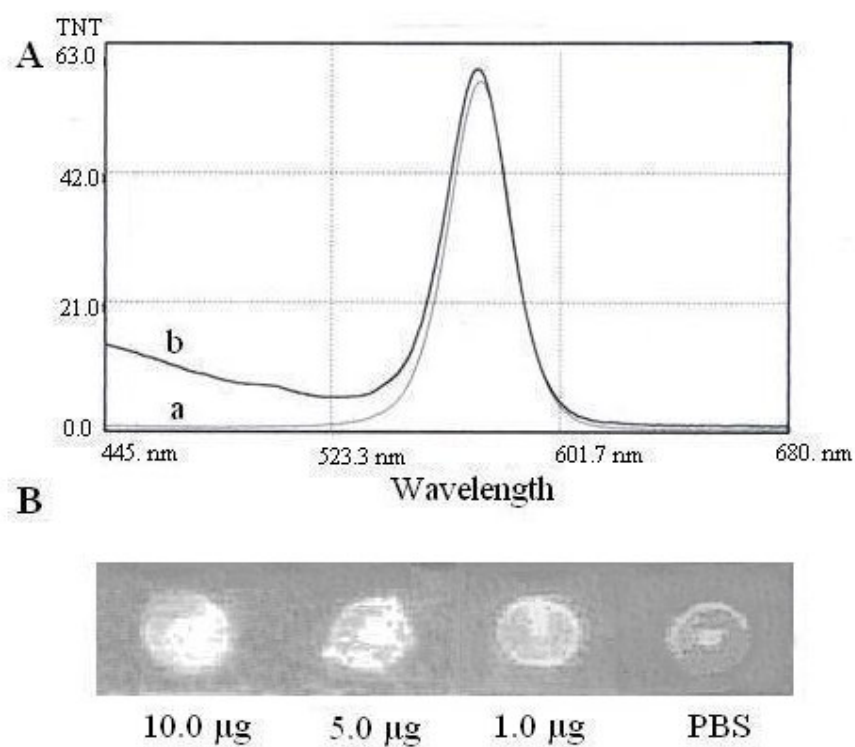
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541 **Fig.3**

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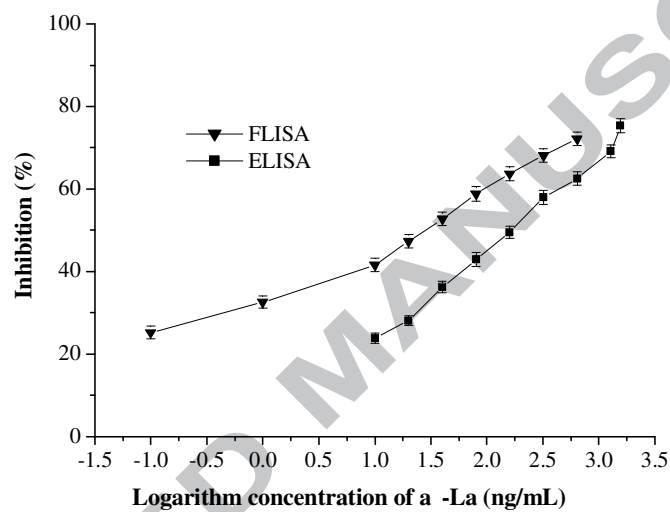
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558 **Fig.4**

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Table 1 Assessment of the competitive FLISA and ELISA for α -La.

Method	Spike (mg)	Mean recovery (%)	Intra-assay CV (%)	Inter-assay CV (%)	IC ₅₀ (ng/mL)	LOD (ng/mL)
FLISA	0.50	102.11				
	1.00	99.26	4.47	11.53	30	0.1
	1.50	97.83				
ELISA	0.50	92.34				
	1.00	90.52	4.85	12.83	150	1.0
	1.50	90.75				

Table 2 Quantification of α -La in dairy products with the competitive FLISA and ELISA

Samples	FLISA (g/L)	ELISA (g/L)
Xiang-yue milk tea	0.144±0.024	0.117±0.050
Xiang-yue milk coffee	0.135±0.047	0.116±0.012
Grain dojo flavor soymilk	0.031±0.010 ^a	0.020±0.010
U-loveit milk tea	0.229±0.041	0.203±0.081
Mengniu yogurt	2.481±0.466 ^a	1.755±0.455
Mengniu pure milk	1.580±0.335	1.214±0.128
Assam milk tea	0.383±0.039	0.314±0.087
Sunshine peanut milk	1.124±0.160	0.998±0.121
Bright peanut milk	0.564±0.047	0.334±0.175
Yinlu peanut milk	0.059±0.018	0.056±0.023

^a $P < 0.05$ compared to ELISA for α -La in dairy products.

590

591 **Highlights**

592 > The monoclonal antibodies against α -La were produced by using hybridoma technology. >

593 CdSe/ZnS quantum dots were conjugated with mAbs using cross-linking reagents. > A

594 competitive FLISA based on QD-mAb conjugates was established. > The developed FLISA can

595 sensitively detect α -La in dairy products.

596

597