Analytica Chimica Acta 829 (2014) 60–67

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Selective and sensitive homogenous assay of serum albumin with 1-anilinonaphthalene-8-sulphonate as a biosensor

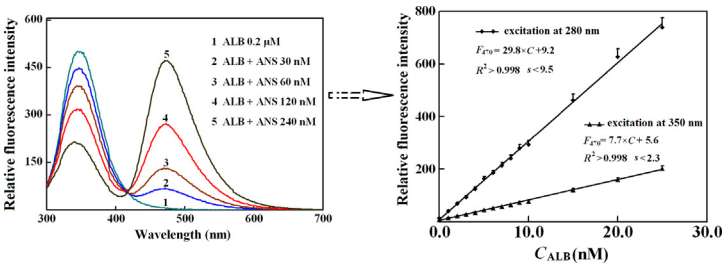
Jialin Qin [1], Yuanli Li [1], Chenxiong He [1], Xiaolan Yang, Yanling Xie, Xiaolei Hu, Chunyan Chen, Lu Wang, Jun Pu, Fei Liao *

Unit for Analytical Probes and Protein Biotechnology, Key Laboratory of Clinical Laboratory Diagnostics of the Education Ministry, College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China

HIGHLIGHTS

- 1-Anilinonaphthalene-8-sulphonate (ANS) emitted at 470 nm in organic solvents.
- The complex of ANS and albumin (ALB) was effectively excited at 280 and 350 nm.
- In 20.0 mM phosphate at pH 7.0, ANS < 0.65 μM bound to ALB at 1:1 with K_d of 0.13 μM.
- At 0.3 μM ANS in the buffer, fluorescence at 470 nm was proportional to ALB levels.
- New method had stronger resistance to interference and higher sensitivity than classical methods.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 21 April 2014
Accepted 23 April 2014
Available online 26 April 2014

Keywords:
Homogenous noncompetitive assay
Förster-resonance-energy-transfer
Serum albumin
1-Anilinonaphthalene-8-sulphonate

ABSTRACT

Homogenous selective assay of albumin (ALB) in clinical sera was tested with 1-anilinonaphthalene-8-sulphonate (ANS) as Förster-resonance-energy-transfer (FRET) acceptor of tryptophan residues and biosensor of ALB. Between the excitation at 280 and 350 nm, the ratio of the fluorescence at 470 nm of free ANS in ethanol was about 1.9 while that of the complexes of ALB and ANS was about 3.9, supporting FRET in complexes of ANS and ALB. ANS below 1.0 mM saturated one site of ALB with K_d of about 0.13 μM in 20 mM sodium phosphate buffer at pH 7.0. For selective assay of ALB, 0.30 μM ANS was used to quantify fluorescence of the complexes at 470 nm under the excitation at 280 nm. ALB from 1.8 to 25 nM was quantified, whose lower limit was below 1% than that by bromocresol green assay while one-third than that by immunoturbidimetric assay. Globular proteins at comparable levels gave negligible signals. This new method showed reasonable resistance to other interfering substances in clinical sera. Quantities of ALB in clinical sera by this method were consistent with those by bromocresol green assay and immunoturbidimetric assay. Hence, homogenous assay of ALB with ANS as FRET biosensor was effective.

Abbreviations: ALB, albumin; ANS, 1-anilinonaphthalene-8-sulphonate; CV, variation coefficient; BCG, bromocresol green; FRET, Förster-resonance-energy-transfer; LOQ, lower limit of quantification.

* Corresponding author at: Chongqing Medical University, College of Laboratory Medicine, No. 1, Yixueyuan Road, Daping, Chongqing 400016, China. Tel.: +86 23 68485240; fax: +86 23 68485111.
E-mail addresses: liaofeish@yahoo.com, liaofeish@yeah.net (F. Liao).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.aca.2014.04.047
0003-2670/

1. Introduction

Serum albumin (ALB) synthesized in liver functions as a nonspecific binder of hydrophobic substances in blood. ALB in sera is an important biomarker for laboratory diagnoses of diseases related to liver and kidney functions [1–3]. In clinical sera, ALB levels exhibit limited variations and homogenous noncompetitive methods of high precision are needed for ALB assay. In clinical laboratories, the spectrophotometric method via the binding of bromocresol green (BCG) to ALB is routinely practiced for ALB assay [1–3]. However, BCG assay of ALB tolerates the interference from globular non-ALB proteins in clinical sera, and is susceptible to variations of ionic strength and pH values of reaction mixtures [1–4]. Immunoturbidimetric assay of ALB has high precision and selectivity, but tolerate cost and nonlinear response. Hence, new homogenous noncompetitive assays of ALB are needed.

Förster-resonance-energy-transfer (FRET) is a fundamental principle for homogenous bioanalysis. For homogenous non-competitive assay of a protein of interest based on FRET, there are two methods. The first utilizes two fluorophores as a pair of donor and acceptor to label a pair of monoclonal antibodies against the protein of interest, and quantifies the fluorescence due to FRET in the complexes of the protein and those two labeled antibodies. This method has high sensitivity and satisfactory precision, but is hampered by heavy cost [5,6]. The second method employs a small fluorescent ligand as both an acceptor of tryptophan residues and FRET biosensor of the protein of interest, and quantifies the fluorescence of the bound biosensor by the excitation of tryptophan residues at 280 nm [7–10]. For favorable sensitivity, a suitable FRET biosensor of a protein should have high affinity to the protein, high quantum yield in complexes with the protein, an excitation peak close to 340 nm and an excitation valley close to 280 nm. This new method has much lower cost and linear response, but has not been applied to ALB due to inaccessibility to required FRET biosensors of ALB.

1-Anilinonaphthalene-8-sulphonate (ANS) is a classical nonspecific fluorescent ligand of ALB with moderate affinity; FRET between tryptophan residues in ALB and the bound ANS has long been observed [11–13]. These properties suggest that ANS may be FRET biosensor for homogenous noncompetitive assay of ALB. However, ANS has an excitation peak rather than a valley close to 270 nm; this spectral property devalues the use of ANS as FRET biosensor for homogenous noncompetitive assay of ALB due to potential unsatisfactory sensitivity. Moreover, the weak affinity of ANS to ALB reduces the selective binding of ANS to ALB against other proteins in sera. Nevertheless, the sensitivity and selectivity for homogenous assay of ALB with ANS as FRET biosensor may be enhanced via the following mechanisms. (1) Turning-on in complexes with proteins was a universal approach to fluorescent biosensors for protein detection [14]. ANS has moderate quantum yields in complexes with ALB while negligible signals in aqueous buffers [11–13]; the excitation of free ANS at 280 nm should thus cause no problems to quantify fluorescence of the complexes of ALB and ANS. (2) FRET in the complexes of ANS and ALB under the excitation at 280 nm should enhance both sensitivity and selectivity for ALB assay with ANS. (3) The binding of ANS to ALB involves hydrophobic and electrostatic interactions [15]; the use of buffers of lower ionic strength and slightly lower pH values may enhance the binding of ANS to ALB and thus sensitivity and selectivity to quantify ALB. Herein, we tested homogenous noncompetitive assay of ALB with ANS as FRET biosensor; the results supported that this new method was effective and advantageous for assay of ALB in clinical sera.

2. Materials and methods

2.1. Instrumentation

Cary Eclipse fluorospectrophotometer (Agilent, USA) was used for fluorescence assay. The band widths of the fluoro-spectrophotometer were 10 nm for both the excitation and the emission, and the sensitivity (PMT) was preset at high, unless otherwise stated. MAPADA UV 1600 PC was used for absorbance assay (http://www.mapada-sh.com/).

2.2. Materials and chemicals

The human serum albumin fraction V (ALB) and sodium bromocresol green (BCG) were from Sigma–Aldrich (St. Loius, MO, USA). Bilirubin, 1-anilinonaphthalene-8-sulphonate (ANS), hemoglobin and quinoline sulphate were from Aladdin Reagent Co., Ltd. (Shanghai, China). Mouse anti-6His tag monoclonal antibody was from Beijing Bioss Biotechnology Co., Ltd. (Bejing, China). Other chemicals were domestic reagents of analytical grade or better. The leftover sera after clinical laboratory analyses was randomly collected in the Department of Clinical Chemistry, the First Affiliated Hospital of Chongqing Medical University. Reagent kits for immunoturbidimetric assay of ALB and reference solutions of ALB were from Shanghai Kehua Bio-engineering Co., Ltd. (Shanghai, China; http://ft.skhb.com/contact.html).

2.3. BCG assay of ALB

The method followed that reported [16]. In brief, 0.15 mM sodium bromocresol green was used in sodium succinate buffer at pH 4.1, plus 1.6 mM sodium azide and 2.4% Tween-20. The standard solution of ALB was made in physiological saline and was calibrated with its absorptivity at 280 nm. To 1.0 mL of BCG reagent, a sample of 20 μL after dilution at 1:4 with 1.0 mM sodium phosphate buffer at pH 7.0 was added; absorbance at 628 nm was measured in 30 s after the addition of the sample. Interfering substances were mixed with sera during dilution and their equivalents in sera before dilution were indicated.

Lower sensitivity of BCG assay required higher concentrations of standard solutions of ALB. In order to examine consistency among methods, concentrations of standard solutions of ALB for BCG assay were calibrated by immunoturbidimetric assay to facilitate tracing. Each clinical sample was analyzed in duplicate, unless otherwise stated.

2.4. Fluorometric homogenous noncompetitive assay of ALB

All the assays of fluorescence with proteins utilized 20.0 mM sodium phosphate buffer at pH 7.0 and 25 °C. ANS was dissolved in dimethylsulfoxide at 0.30 M and diluted with the phosphate buffer or an organic solvent. Unless otherwise stated, the reaction mixture of 3.0 mL contained 50 μL solution of ANS and 50 μL solution of a diluted sample. Fluorescence was measured after 1.0 min lag since the addition of ANS. Signals of solvents were corrected.

To estimate quantum yields, quinine in 1.0 M hydrogen sulfate served as the reference with a quantum yield of 0.58. The fluorescence signals from 380 to 700 nm under the excitation at 350 nm were integrated. To quantify quantum yield of the complex of ALB and ANS, ALB was 6.5 μM while ANS was 0.30 μM for 98% binding of ANS. To estimate affinity, an aliquot of 10 μL solution of ALB or ANS was added each time to 3.0 mL solution of ANS at 0.30 μM or ALB at 0.20 μM in the phosphate buffer. The fluorescence at 470 nm under the excitation at 280 and 350 nm

was measured synchronously. The final levels of ALB and ANS were limited to 0.65 μM.

For tracing concentrations of ALB with the new homogenous assay, standard solutions of ALB were calibrated by immunoturbidimetric assay. To apply the new homogenous method, ALB in each clinical serum was determined in duplicate, unless otherwise stated.

2.5. Preparation of a recombinant uricase and mixture sample of non-ALB proteins

ALB has an isoelectric point of about 5.0. A uricase with an isoelectric point of about 5.0 was used as a model of globular non-ALB protein [17]. The uricase was produced via recombinant expression in *Escherichia coli* BL21 (DE3) and purified twice via DEAE-cellulose chromatography. The native states of the uricase and other proteins were checked by resonant-light-scattering signals of potential aggregates [18]. Meanwhile, cell lysate of *E. coli* BL21 (DE3) transformed with a blank *p*ET24a vector after induced expression was prepared via sonication treatment and centrifugation. Total proteins were determined by Bradford method [19], and used as a mixture sample of non-ALB proteins.

2.6. Immunoturbidimetric assay of ALB

The immunoturbidimetric assay of ALB with reagent kits was performed following the protocol stated by the provider with the primary wavelength of 340 nm and the auxiliary wavelength of 700 nm [20,21]. In brief, after mixing of 400 μL reagent A and 30 μL diluted serum sample or reference solution of ALB and incubation at 37 °C for 5 min, the difference in absorbance at 340 and 700 nm ($\Delta A1$) was determined for the first time. Then, 150 μL reagent B was added and the resulting mixture was further incubated 37 °C for 5 min. Finally, the difference in absorbance at 340 and 700 nm ($\Delta A2$) was determined. Response curve of net change of absorbance ($\Delta A = \Delta A2 - \Delta A1$) was constructed with five reference solutions of ALB and fit with a polygonal function; the concentration of ALB in sample was determined from the response curve. With each clinical serum, ALB content was measured in duplicate, unless otherwise stated.

2.7. Data processing and other analytical methods

The lower limit of quantitation (LOQ) was the smallest quantity of an analyte giving the change of signals over five-fold of the standard error of estimate with variation coefficient (CV) below 10%. The upper limit of quantitation was the highest quantity of an analyte giving signals limited by the measurable range of the instrument or signals with CV below 5% and deviation below twice the standard error of estimate. To estimate K_d, the reversible binding of ANS and ALB was analyzed by fitting Eq. (1) to fluorescence at 470 nm of reaction mixtures under the

$$f = s_1 \times \frac{\left((K_d + x + C_0) - \sqrt{(K_d + x + C_0)^2 - 4 \times C_0 \times x}\right)}{2} + s_2 \times (C_0 - x) \quad (1)$$

excitation at 280 or 350 nm. In Eq. (1), K_d was the dissociation constant, f was the fluorescence minus that of the buffer, s_1 and s_2 were slopes for the responses of fluorescence at 470 nm to concentrations of ALB and free ANS, respectively; C_0 was the concentration of ALB or ANS to be titrated with ANS or ALB at varying total concentrations as x. CFTool function in Matlab 6.5 was used for fitting. For the examination of consistency between any pair of methods with clinical sera, the results were compared by both regression analysis and Bland–Altman analysis [22]; regression analysis was run with MS Excel 6.0.

3. Results and discussion

3.1. Feasibility of ANS as a biosensor for homogenous noncompetitive assay of ALB

In ethanol, ANS under the excitation at 280 or 350 nm had only one emission peak close to 470 nm (Fig. 1a, Fig. S1 in Supporting Information). In ethanol, the fluorescence of ANS at 470 nm had an excitation peak close to 268 nm while a broad excitation peak from 330 to 390 nm (Fig. 1b); the emission under the excitation at 280 nm was about 1.9-fold of that at 350 nm. In neutral phosphate buffer, however, ANS had a very weak emission at 470 nm under the excitation at 280 or 350 nm. The quantum yields of ANS were about 0.52, 0.045 and 0.13 in the phosphate buffer at pH 7.0, ethanol and tetrahydrofuran, respectively. By the excitation at 280 nm, the emission of ALB overlapped with the excitation

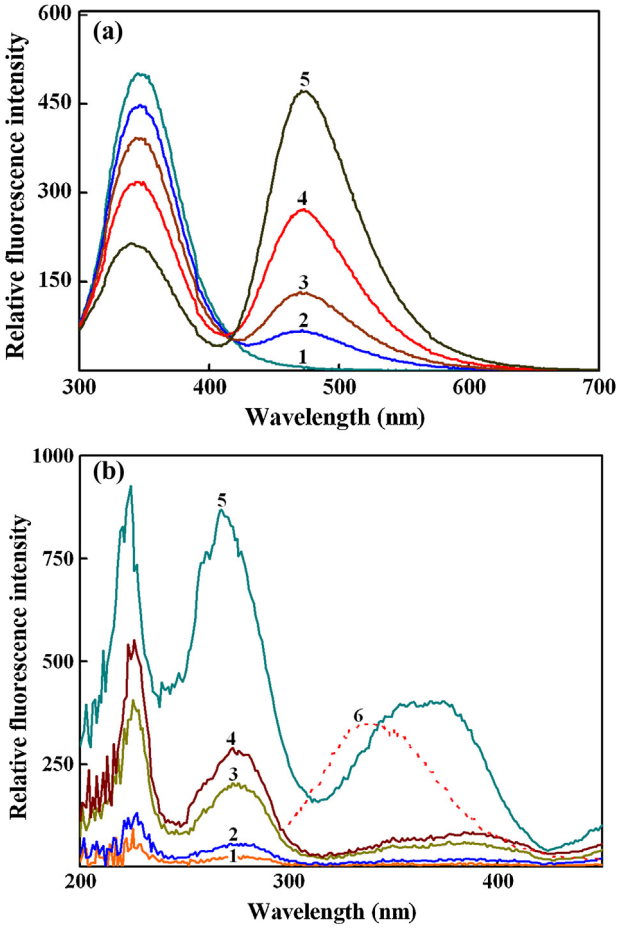


Fig. 1. The excitation and the emission spectra of ANS, ALB, and their reaction mixtures. (a) The emission spectra under the excitation at 280 nm (excitation band of 5 nm, emission band of 10 nm and PMT at medium). 1: 200 nM ALB alone; 2: 200 nM ALB + 30 nM ANS; 3: 200 nM ALB + 60 nM ANS; 4: 200 nM ALB + 120 nM ANS; 5: 200 nM ALB + 240 nM ANS. (b) The excitation spectra detected for fluorescence at 470 nm and the emission spectrum of ALB under the excitation at 280 nm (excitation band of 5 nm, emission band of 10 nm and PMT at high). 1: the excitation spectrum of 25 nM ALB alone; 2: the excitation spectrum of 0.30 μM ANS + 5 nM ALB; 3: the excitation spectrum of 0.30 μM ANS + 16 nM ALB; 4: the excitation spectrum of 0.30 μM ANS + 25 nM ALB; 5: the excitation spectrum of 0.30 μM ANS; 6: the emission spectrum of 25 nM ALB alone under the excitation at 280 nm.

spectrum of ANS in ethanol (Fig. 1b), supporting potential FRET in the complexes of ALB and ANS.

In the phosphate buffer, ALB at 200 nM or ANS at 300 nM alone had negligible fluorescence at 470 nm under the excitation at 280 or 350 nm. Under the excitation at 280 nm, there were continuous increase in the fluorescence at 470 nm but concomitant decrease in fluorescence at 340 nm *versus* increasing concentrations of ANS with ALB fixed at 200 nM (Fig. 1a). With 300 nM ANS in the phosphate buffer under the excitation at 280 nm, there were continuous increases in fluorescence at 470 nm *versus* increasing concentrations of ALB (Fig. S1 in Supporting Information). The excitation spectrum of the complexes of ALB and ANS were similar to that of ANS in ethanol, but the ratio of the emission under the excitation at 280 nm to that under the excitation at 350 nm was about 3:9, supporting FRET in the complexes. Moreover, the quantum yield of ANS in the complexes with ALB reached 0.027, nearly 52-fold increase *versus* that in the neutral phosphate buffer. Hence, spectral properties supported that ANS with an excitation peak close to 270 nm may still be a suitable FRET biosensor for homogenous noncompetitive assay of ALB in clinical sera when assay specificity is reached.

3.2. Rationality for homogenous selective assay of ALB with ANS as a biosensor

As a putative, a biosensor for noncompetitive assay of a protein can be applied at higher concentrations to form more complexes with the protein for higher sensitivity and wider quantitation ranges. However, there are multiple sites on ALB to bind ANS with different K_d, and such sites have different distances from tryptophan residues in ALB [11–14]. As a result, the binding of ANS to multiple sites of ALB may result in inconsistent responses of fluorescence at 470 nm of the bound ANS to ALB levels and thus narrows the quantitation ranges. More importantly, sera are rich in globular non-ALB proteins and there are more nonspecific bindings of ANS at higher concentrations to those globular non-ALB proteins [11–13]. Fortunately, two factors facilitate selective assay of ALB with ANS. One is the specific binding of ANS to ALB, which is expected for ANS at low levels but requires high affinity of ANS to just one site on ALB. The other is FRET in the complexes of ANS and ALB under the excitation at 280 nm, as observed above (Fig. 1a and b). FRET fluorescence of bound ANS is sensitive to distances between the donors and the acceptors, and abundance of tryptophan residues as donors around the binding sites. However, the binding sites of ANS on non-ALB proteins in sera and abundance of tryptophan residues nearby such sites are unknown. Hence, the affinity of ANS to ALB was estimated to optimize ANS levels for negligible nonspecific bindings; binding ratio of ANS to ALB under optimized conditions was estimated to examine whether ANS bind to just one site of ALB; the responses of fluorescence at 470 nm to concentrations of ALB and some globular non-ALB proteins were compared to test selectivity of homogenous noncompetitive assay of ALB with ANS.

The binding of ANS to ALB primarily depends on electrostatic interactions [15]. ALB is an acidic protein while ANS is usually negatively charged in neutral buffers. The lower ionic strength and slightly lower pH value (20 mM sodium phosphate buffer at pH 7.0) were utilized to promote the formation of complexes between ANS and ALB [11–13]. From the titration of 0.30 μM ANS by ALB below 0.65 μM under the excitation at 280 nm, saturated binding was observed; K_d of ANS to ALB was (0.14 ± 0.02) μM ($n = 2$, $R^2 > 0.9987$ with Eq. (1)). By analyzing the fluorescence of the same solutions under the excitation at 350 nm, K_d was (0.13 ± 0.02) μM ($n = 2$) (Fig. S2a in Supporting Information). When 0.20 μM ALB was titrated with ANS below 0.45 μM, there was also saturated binding; K_d from data under the excitation at 280 or 350 nm

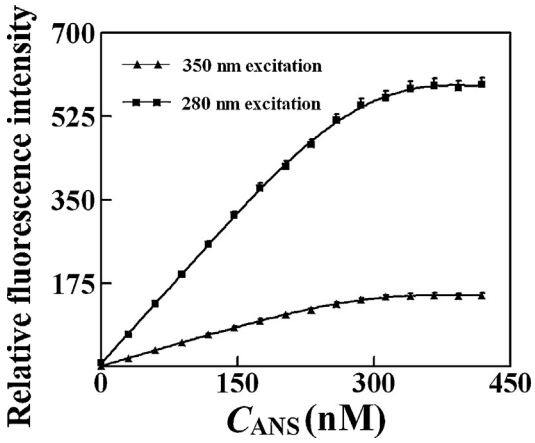


Fig. 2. The estimation of affinity via titration of 0.20 μM ALB with ANS below 0.45 μM. The excitation band of 5 nm, the emission band of 10 nm and the PMT at the medium were used.

was (0.10 ± 0.02) μM ($n = 2$) (Fig. 2). Under the stated conditions, the affinity of ANS to ALB was higher than that in 100 mM sodium phosphate buffer at pH 7.4 [11]. Moreover, from the titration curve of ALB with ANS below 0.45 μM, the intersection point of two plots for ALB and ANS in great excess, respectively, indicated the binding equivalency between ALB and ANS. The molar equivalency for the binding of ANS to ALB was thus (1.1 ± 0.2) ($n = 3$) (Fig. S2b in Supporting Information), supporting ANS within the tested range bound in a saturated manner to just one site on ALB. The saturated binding of ANS to one site on ALB with sufficient affinity supported consistent response of fluorescence at 470 nm to concentrations of ALB under the stated conditions.

For selective assay of ALB in sera, the slope for the responses of fluorescence at 470 nm of the bound ANS to ALB levels should be as larger as possible than those for the responses of fluorescence at 470 nm of the bound ANS to levels of non-ALB proteins. A (globular) uricase bearing the isoelectric point consistent with that of ALB [16], and a monoclonal antibody against 6His-tag, were used as models of globular non-ALB proteins to compare the responses of fluorescence at 470 nm at different ANS levels. Interestingly, with ANS at 0.30 μM, the ratio of the slope for the response of fluorescence at 470 nm under the excitation at 280 nm to uricase levels against that to ALB levels was below 0.7% (Table S1 in Supporting Information). At 0.90 and 3.0 μM ANS, such ratios under the excitation at 280 nm only increased slightly. Under the excitation at 350 nm, such ratios were larger but consistent at different ANS levels. When the antibody against 6His-tag was tested, the ratios displayed stronger increases at higher levels of ANS (Table S1 in Supporting Information). With both proteins, however, the largest one of such ratios under the excitation at 280 nm was below 2.0% than that with ALB at ANS below 3.0 μM, and smaller than those under the excitation at 350 nm. Hence, ANS at submicromolar levels selectively bound to one site on ALB and there should be a specific response of fluorescence at 470 nm to ALB levels under the excitation at 280 nm.

To explore origination of fluorescence at 470 nm of reaction mixtures of ANS at high levels and non-ALB proteins, resonant-light-scattering (RLS) signals were examined. The complexes at 3.0 μM ANS with either model of non-ALB proteins displayed RLS signals at 376 nm. Specially, with any model protein at higher levels, RLS signals at 376 nm were stronger at 3.0 μM ANS but negligible at 0.30 μM ANS (Fig. S3 in Supporting Information). These data suggested that ANS at higher concentrations bound nonspecifically to hydrophobic surfaces of those non-ALB proteins to potentially cause aggregation of their complexes and stronger

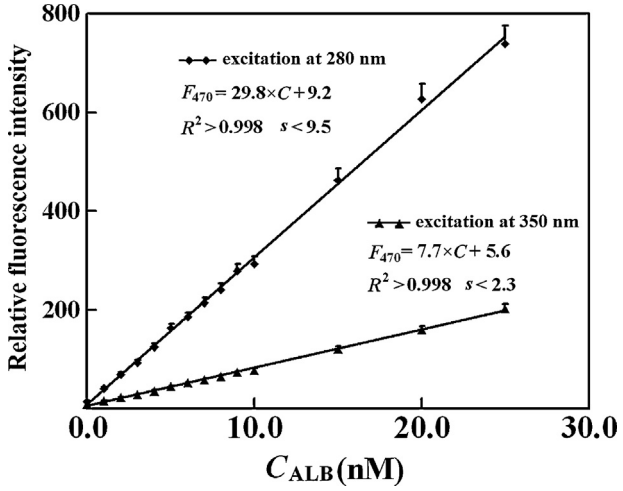


Fig. 3. The responses of relative fluorescence intensity at 470 nm to ALB concentrations in the presence of 0.30 μM ANS.

fluorescence at 470 nm [17]. Clearly, the use of ANS at low levels can alleviate nonspecific binding of ANS to globular non-ALB proteins and thus mitigate interference with the assay of fluorescence at 470 nm of their complexes. In theory, the use of ANS at 0.30 μM can provide a linear range of about 20-fold to quantify ALB considering its K_d of about 0.14 μM; this linear range is sufficient for the assay of ALB in clinical sera. Hence, ANS at submicromolar levels may be a suitable FRET biosensor for homogenous noncompetitive selective assay of ALB.

3.3. Response curve for homogenous selective assay of ALB with ANS

The reaction between ANS and proteins in sera reached equilibrium in 50 s and the fluorescence at 470 nm was stable in 20 min (Fig. S4 in Supporting Information); this property was a great advantage over BCG assay of ALB since absorbance of reaction mixture of BCG and serum proteins continued to increase after reaction for a long time. There was excellent linear response of fluorescence at 470 nm to ALB levels below 25 nM under the excitation at 280 or 350 nm (Fig. 3). The slope of linear response of fluorescence at 470 nm under the excitation at 280 nm was about 3.9-fold of that under the excitation at 350 nm; this difference was consistent with that estimated from the excitation spectrum and further supported FRET in the complexes of ANS and ALB (Fig. 1b). From the linear response under the excitation at 280 or 350 nm, the intercept was consistent with the fluorescence of 0.30 μM ANS alone in the buffer (Fig. 3). Linear responses ranged from about 1.8 to 25 nM under the excitation at 280 nm, and from 1.5 to 25 nM under the excitation at 350 nm. Meanwhile, BCG assay showed excellent linear response (Fig. S5 in Supporting Information), but immunoturbidimetric assay displayed nonlinear response (Fig. S6 in Supporting Information). For ALB assay with ANS under the excitation at 280 nm, LOQ was <0.7% of that under BCG assay, and below one-third assay, supporting much higher sensitivity of ALB assay with ANS. Hence, ANS was a suitable biosensor for homogenous assay of ALB in sera based on FRET between bound ANS and tryptophan residues in ALB.

3.4. Resistance to common interfering substances in clinical sera

For homogenous assay of ALB with final ANS at 0.30 μM, clinical sera were diluted by 50,000-fold with 20 mM sodium phosphate buffer at pH 7.0 into the final reaction mixtures. The fluorescence of the reaction mixtures at 470 nm before the final addition of ANS was measured for the correction of the effects of background signals from components in sera [7]. The immunoturbidimetric assay and BCG assay are established methods for the analysis of ALB in clinical laboratories, but it is a putative that the immunoturbidimetric assay of ALB has better selectivity than BCG assay [4,20,21]. Therefore, the potential interference for a candidate substance by the new homogenous method with ANS as FRET biosensor was compared against that without the interference by the same method and immunoturbidimetric assay.

Table 1
Comparison of interference from common substances in clinical sera.

Samples/interfering substances	ALB concentration ($g L^{-1}$)			
	New method under excitation at 280 nm	New method under excitation at 350 nm	BCG assay	Immunoturbidimetric assay
Serum A + phosphate buffer	50.5 ± 1.0 (3)	52.6 ± 0.6 (3)	51.1 ± 1.0 (3)	52.0 ± 0.7 (3)
Serum A + 0.3 mM bilirubin	49.9 ± 1.3 (5)	55.2 ± 0.3 (5)	55.8 ± 0.5* (3)	49.5 ± 1.2 (3)
Serum A + 1.6 mM uric acid	50.9 ± 2.0 (3)	53.2 ± 0.3 (3)	47.7 ± 0.4^{*,a} (3)	51.3 ± 2.1 (4)
Serum A + 7.8 μM hemoglobin	51.3 ± 0.9 (5)	50.3 ± 0.5 (5)	46.1 ± 0.8^{*,a} (3)	48.9 ± 0.9 (3)
Serum A + 0.7 mM penicillin G	52.1 ± 1.3 (3)	51.9 ± 0.7 (3)	52.5 ± 0.6 (3)	52.9 ± 0.3 (3)
Serum A + 12.5 U heparin	52.8 ± 0.4 (5)	52.2 ± 1.1 (5)	54.1 ± 1.7 (3)	58.3 ± 1.0^{a} (3)
Serum A + 0.4 mM uricase	49.4 ± 0.8 (5)	52.0 ± 0.6 (5)	65.9 ± 0.6^{**,b} (3)	49.6 ± 1.8 (5)
Serum A + 0.2 mM mouse mcAb	51.0 ± 0.7 (3)	53.1 ± 0.9 (3)	58.1 ± 0.8^{*,b} (3)	52.9 ± 0.3 (3)
Serum A + 3.0 $g L^{-1}$ bacterial protein$^{\#}$	51.0 ± 0.7 (3)	49.5 ± 0.4* (3)	51.4 ± 0.6 (3)	54.8 ± 0.8 (3)
Serum A + 6.0 $g L^{-1}$ bacterial protein$^{\#}$	52.0 ± 0.6 (5)	49.1 ± 0.3* (5)	58.5 ± 0.6^{*,a} (3)	50.4 ± 0.2 (3)
Serum A + 9.0 $g L^{-1}$ bacterial protein$^{\#}$	52.0 ± 0.8 (5)	53.3 ± 1.6 (5)	62.8 ± 0.7^{**,b} (3)	55.1 ± 0.4^{a} (3)
Serum B (TG 1.4 mM, HDL 1.9 mM, LDL 4.1 mM, TC 6.0 mM)	52.8 ± 1.0 (3)	48.4 ± 2.7 (3)	49.5 ± 0.8 (3)	47.2 ± 0.9 (3)
Solution of ALB 1 in phosphate buffer	10.6 ± 0.2 (3)	12.7 ± 1.4 (3)	8.3 ± 0.6^{a} (3)	11.0 ± 0.5 (3)
Serum B + ALB 1	62.4 ± 0.6 (5)	67.0 ± 1.6^{*,x} (5)	58.1 ± 0.2 (3)	53.2 ± 0.9^{*,x} (3)
Solution of ALB 2 in phosphate buffer	21.9 ± 0.4 (5)	24.5 ± 1.5 (5)	18.3 ± 0.7 (3)	21.0 ± 0.7 (3)
Serum B + ALB 2	79.0 ± 0.8^{*,x} (3)	79.5 ± 0.5^{*,x} (3)	66.5 ± 0.1 (3)	60.1 ± 0.6^{**,x} (3)

By the new method with ANS, two assays under excitation at 280 and 350 nm were measured for the same solution concomitantly. The number in parenthesis was that for independent assays. Serum A was a sample of clinical sera bearing no hemolysis, no heavy lipids or bilirubin. An indicated additional substance was mixed with Serum A during dilution to test potential interference and the concentration of the additional substance was presented as an equivalent in Serum A before dilution. Serum B is a sample of clinical sera rich in lipids as common interfering substances. TG: triglycerides; HDL: high-density-lipoprotein; LDL: low density lipoprotein. $^{\#}$ cell lysate of *E. coli* as mixture sample of non-ALB proteins. * and ** indicated $P < 0.05$ and $P < 0.01$ by *t*-test *versus* that without additional substance by the same method, ^{a} and ^{b} indicated $P < 0.05$ and $P < 0.01$ by *t*-test *versus* that by the immunoturbidimetric method, respectively. *x* indicated $P < 0.05$ for statistical comparison by *t*-test *versus* the sum of ALB in serum B and that in solution of ALB 1 or 2 prepared from a calibrated standard solution.

With clinical sera, CVs for assay of ALB from 11 to 62 g L^{-1} by the new homogenous method under the excitation at 280 or 350 nm were below 5%. After the addition of ALB for 10 and 20 g L^{-1} in clinical sera, the recovery ratios of ALB by the new homogenous fluorometric assay with ANS were (103 ± 4) and (105 ± 5) under the excitation at 280 nm, while (91 ± 4) and (96 ± 5) under the excitation at 350 nm, respectively (n = 5 for each). Under the same conditions, the recovery ratios of ALB in clinical sera were (106 ± 9) and (96 ± 5) by BCG assay, and (100 ± 8) and (92 ± 3) by immunoturbidimetric assay, respectively (n = 5 for each). Hence, the new homogenous fluorometric assay exhibited recovery ratios comparable to established methods.

The slow binding of BCG to globular non-ALB serum proteins causes interference with BCG assay of ALB [4,14]. As expected [4,20,21], BCG assay of ALB was sensitive to interference of non-ALB proteins while immunoturbidimetric assay was generally resistant to non-ALB proteins. In comparison to BCG assay, this new homogenous assay with ANS under the excitation at 280 nm had stronger resistance to non-ALB proteins while comparable resistance to other substances in sera (Table 1). When ratios of ALB to non-ALB proteins in sera were varied from 0.5 to 2.0, which covered common ranges of clinical significance, this new homogenous assay of ALB with ANS under the excitation at 280 nm to tested substances showed resistance comparable to immunoturbidimetric assay (Table 1). Specially, this new homogenous assay under the excitation at 280 nm displayed stronger resistance to globular proteins than that under the excitation at 350 nm; this difference further supported the enhancement of specificity of homogenous assay of ALB with ANS by FRET in the complexes of ALB and ANS. Hence, the new homogenous assay of

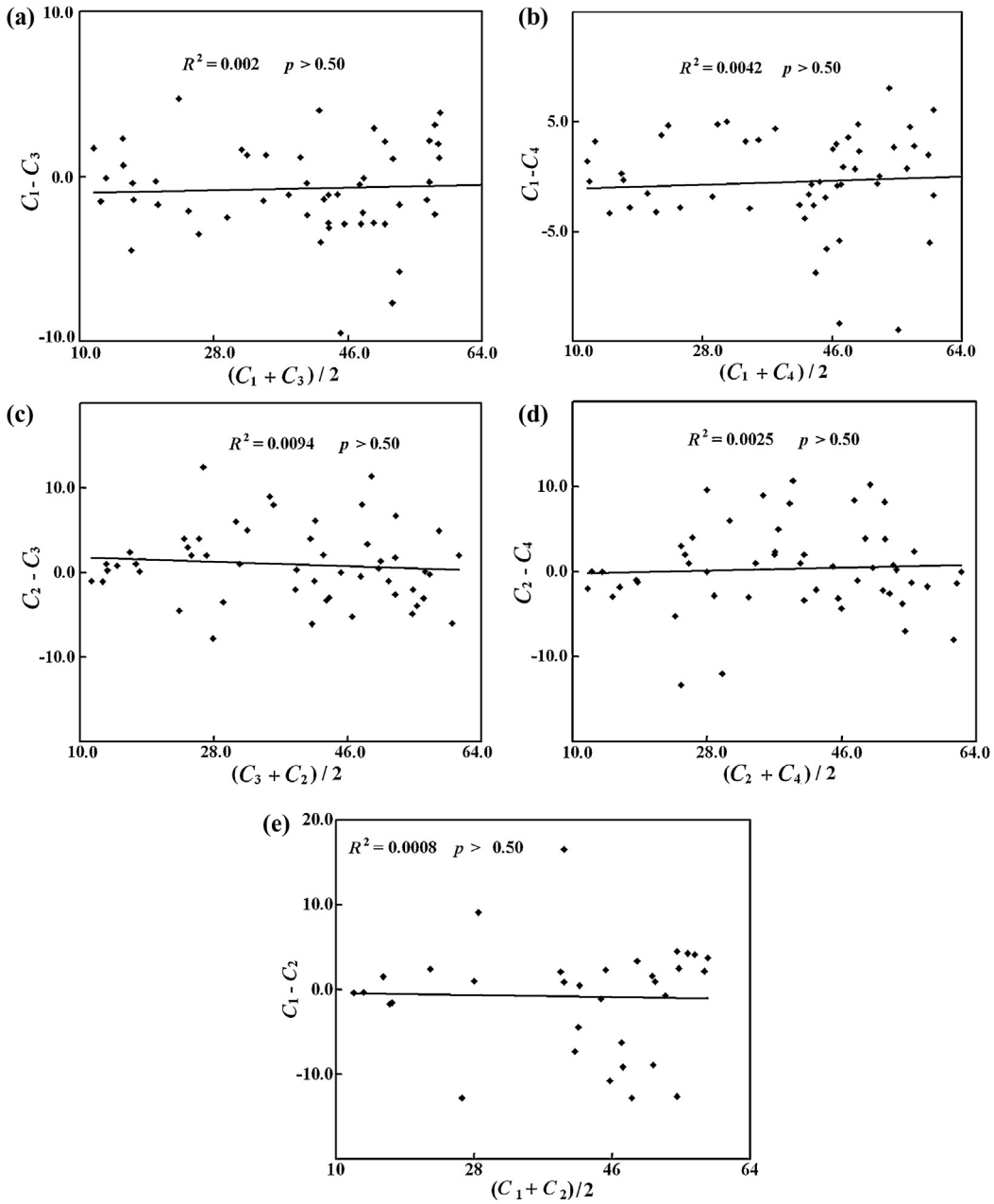


Fig. 4. The Bland–Altman analysis of consistency among methods for homogenous assay of ALB. C_1: immunoturbidimetric assay; C_2: BCG assay; C_3: this new homogenous assay with 0.30 μM ANS under the excitation at 280 nm; C_4: this new homogenous assay with 0.30 μM ANS under excitation at 350 nm.

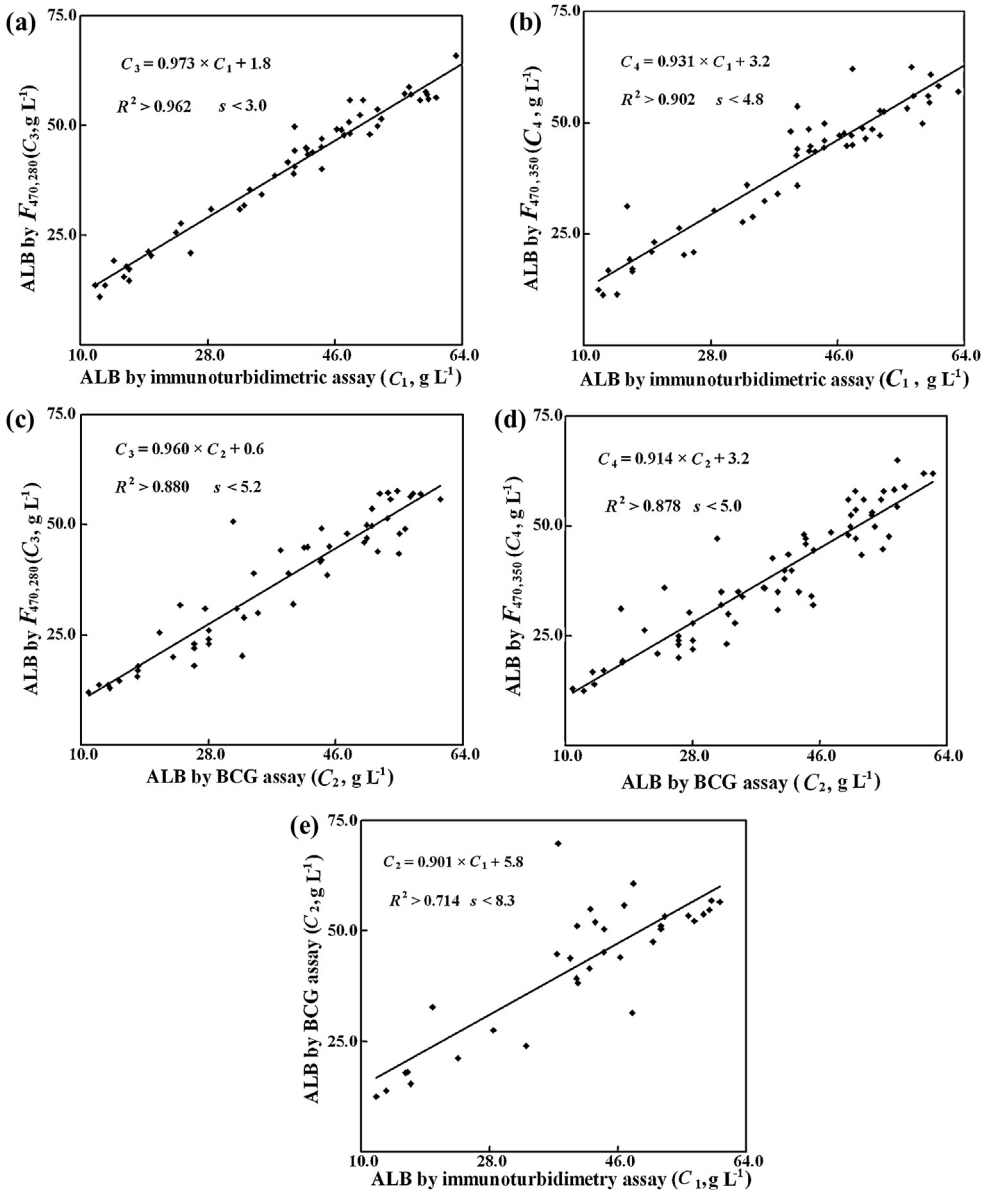


Fig. 5. The regression analysis of the results among tested methods for homogenous assay of ALB. C_1: immunoturbidimetric assay; C_2: BCG assay; C_3: this new homogenous assay with 0.30 μM ANS under the excitation at 280 nm; C_4: this new homogenous assay with 0.30 μM ANS under excitation at 350 nm.

ALB with ANS as FRET biosensor under the excitation at 280 nm was advantageous over BCG assay while comparable to immunoturbidimetric assay, and may be applicable.

3.5. Application of homogenous selective assay of ALB with ANS to clinical sera

With 50 clinical sera, ALB levels by this new homogenous assay with ANS as FRET biosensor under excitation at 280 nm (C_3) and 350 nm (C_4) were checked by regression and Bland–Altman analyses [22], against those by BCG assay (C_2) and immunoturbidimetric assay (C_1). By Bland–Altman analysis, the new homogenous noncompetitive assay under the excitation at 280 or 350 nm was consistent with either established method for the assay of ALB in clinical sera (Fig. 4). By regression analysis, this new homogenous assay with ANS as FRET biosensor showed good correlation with BCG assay and immunoturbidimetric assay of ALB, regardless of the excitation at 280 or 350 nm (Fig. 5). More importantly, results by this new homogenous assay with ANS under the excitation at 280 nm correlated with those by immunoturbidimetric assay by an equation of $C_3 = 0.973 \times C_1 + 1.8$ with $R^2 > 0.96$ while standard error of estimate (s) <3.0; the slope and R^2 were the largest whereas s was the smallest, among those for regression analyses of results by any pair of methods. These results should be associated with comparable resistance to common interfering substances in clinical sera between this new homogenous assay with ANS under the excitation at 280 nm and immunoturbidimetric assay, while susceptibility of BCG assay to non-ALB proteins.

4. Conclusion

A new homogenous fluorometric assay of ALB with ANS as FRET biosensor under the excitation at 280 nm was developed with advantages. In comparison to BCG assay of ALB, the new homogenous assay with ANS under the excitation at 280 nm showed stronger resistance to non-ALB proteins in clinical sera, much higher

sensitivity, simpler operation process while showed comparable analysis efficiency and cost. In comparison to immunoturbidimetric assay of ALB, it exhibited much lower cost, simpler operation and higher analysis efficiency while comparable resistance to non-ALB proteins and other common interfering substances in clinical sera. Hence, homogenous fluorometric assay of ALB with ANS under the excitation at 280 nm concomitantly possessed advantages of those two methods for the assay of ALB in clinical sera. Moreover, the effectiveness of this homogenous assay of ALB with ANS as FRET biosensor under the excitation at 280 nm relied on significant increase in quantum yield of ANS and FRET effect in its complex with ALB. The use of fluorophores bearing enhanced fluorescence in hydrophobic environment of binding sites of proteins has been proposed as a universal principle for design of fluorescent biosensors for selective detection of proteins [14]. The advantages of ANS as a biosensor of ALB under the excitation at 280 nm over the excitation at 350 nm thus supported the combination of fluorescence turning-on and FRET in complexes with proteins is a better approach to fluorescent biosensors for selective detection of proteins.

Acknowledgements

This work was supported by National Natural Science Foundation of China (grant no. 81071427), Natural Science Foundation Project of CQ (grant no. CSTC2012JJA0057) and the Education Ministry of China (grant no.20125503110007).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.04.047.

References

[1] B.T. Doumas, T. Peters Jr., Clinica Chimica Acta 258 (1997) 3–20.
[2] I. Infusino, M. Panteghini, Clinica Chimica Acta 419 (2013) 15–18.
[3] C.L. Snozek, A.K. Saenger, P.R. Greipp, S.C. Bryant, R.A. Kyle, S.V. Rajkumar, J.A. Katzmann, Clinical Chemistry 53 (2007) 1099–1103.
[4] Y. Xu, L. Wang, J. Wang, H. Liang, X. Jiang, British Journal of Biomedical Science 68 (2011) 120–125.
[5] K. Blomberg, P. Hurskainen, I. Hemmilä, Clinical Chemistry 45 (1999) 855–861.
[6] S.A. Grant, R.C. Stringer, S. Studer, D. Lichlyter, C.L. Lorenzen, Biosensors and Bioelectronics 21 (2005) 438–444.
[7] F. Liao, Y. Xie, X. Yang, P. Deng, Y. Chen, G. Xie, S. Zhu, B. Liu, H. Yuan, J. Liao, Y. Zhao, M. Yu, Biosensors and Bioelectronics 25 (2009) 112–117.
[8] Y. Xie, X. Yang, J. Pu, Y. Zhao, Y. Zhang, G. Xie, J. Zheng, H. Yuan, F. Liao, Spectrochimica Acta, Part A: Molecular and Biomolecular Spectroscopy 77 (2010) 869–876.
[9] Y. Xie, T. Maxson, Y. Tor, Journal of the American Chemical Society 132 (2010) 11896–11897.
[10] T. Li, J.Y. Byun, B.B. Kim, Y.B. Shin, M.G. Kim, Biosensors and Bioelectronics 42 (2013) 403–408.
[11] D. Essassi, R. Zini, J.P. Tillement, Journal of Pharmaceutical Sciences 79 (1990) 9–13.
[12] D.I. Cattoni, S.B. Kaufman, F.L. González Flecha, Biochimica et Biophysica Acta 1794 (2009) 1700–1708.
[13] I.M. Kuznetsova, A.I. Sulatskaya, O.I. Povarova, K.K. Turoverov, PLoS One 7 (2012) e40845.
[14] Y.D. Zhuang, P.Y. Chiang, C.W. Wang, K.T. Tan, Angewandte Chemie International Edition in English 52 (2013) 8124–8128.
[15] D. Matulis, R. Lovrien, Biophysical Journal 74 (1998) 422–429.
[16] J.E. Gustafsson, Clinical Chemistry 24 (1978) 369–373.
[17] C. Zhang, X. Yang, J. Feng, Y. Yuan, X. Li, Y. Bu, Y. Xie, H. Yuan, F. Liao, Bioscience, Biotechnology, and Biochemistry 74 (2010) 1298–1301.
[18] J. Feng, Y. Chen, J. Pu, X. Yang, C. Zhang, S. Zhu, Y. Zhao, Y. Yuan, H. Yuan, F. Liao, Analytical Biochemistry 409 (2011) 144–149.
[19] M.M. Bradford, Analytical Biochemistry 72 (1976) 248–254.
[20] G.F. Watts, J.E. Bennett, D.J. Rowe, R.W. Morris, W. Gatling, K.M. Shaw, A. Polak, Clinical Chemistry 32 (1986) 1544–1548.
[21] P.W. Mueller, M.L. MacNeil, S.J. Smith, D.T. Miller, Clinical Chemistry 37 (1991) 191–195.
[22] J.M. Bland, D.G. Altman, Lancet 346 (1995) 1085–1087.