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A sensitive, universal and homogeneous method for determination of biomarkers in biofluids by resonance light scattering correlation spectroscopy (RLSCS)

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ABSTRACT

In this paper, we reported a sensitive, universal and homogeneous method for assay of biomarkers by combining resonance light scattering correlation spectroscopy (RLSCS) with silver nanoparticles (SNPs) as labeling probes. In the homogeneous assay, the competitive immunoreaction mode was used, and antibody and antigen (or hapten) were labeled with SNPs with strong plasmonic scattering property, respectively. The antibody-labeled SNPs were firstly mixed with a sample containing antigens, and the part of antibody-labeled SNPs was bound to antigens of interest in the sample. And then, the antigen-labeled SNPs were added into the mixed solution above, and they were bound to free antibody-labeled SNPs (excess) to form dimers (or oligomers), which led to the significant increase in the characteristic diffusion time of SNPs in the tiny detection volume (about 0.5 fL). In the competitive mode, the characteristic diffusion time of SNPs decreased with an increase of antigen concentration. The RLSCS is a novel single particle method and can sensitively detect the changes in the characteristic diffusion time of SNPs before and after the immunoreactions. In order to demonstrate the universality of this new method, small biomolecules, 17-β estradiol (E2), and biomacromolecules, liver cancer antigen alpha-fetoprotein (AFP), were used as assay models. In the optimal conditions, the linear ranges of this method were from 10 pM to 10 nM for E2 and 100 pM to 10 nM for AFP, respectively, and the detection limits were 10 pM for E2 and 100 pM for AFP, respectively. The presented method was successfully used to the determination of E2 levels in human urine and AFP levels in human sera, and the results obtained were in good agreement with conventional ELISA assays.

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1. Introduction

Immunoassays have been becoming a very important bioanalysis method since it was firstly introduced by Yalow and Berson about 50 years ago [1]. Currently, immunoassays are extensively applied in clinical diagnostics, food safety testing and environmental analysis due to its high specificity and sensitivity [2–6]. In principle, immunoassay modes can be divided into direct immunoassays and competitive immunoassays [7]. In direct immunoassays, the sandwich mode is generally used. This mode needs two antibodies and is only suitable for analysis of biomacromolecules (such as proteins) with two binding sites. For small molecules such as hormones, drugs, environmental pollutants and chemical warfare agents, competitive immunoassays are used because these small molecules contain only one binding site. Compared to the sandwich mode, competitive immunoassays show its universality, and theoretically, it can be applied to assays of any compounds from biomacromolecules to small molecules. Generally, the sensitivity of competitive immunoassays are lower than that of direct immunoassays. Although immunoassay is a mature analysis technique to date, there are still certain unsolved issues that limit some applications. Firstly, the current immunoassays mostly employ heterogeneous procedure, and this procedure (such as ELISA) requires antibody immobilization, immune reaction, washing steps and color development, and thus this assay mode is labor-intensive and time-consuming [8,9]. In contrast to heterogeneous mode, homogeneous immunoassay is an attractive detection format because it is amenable to automation, reduced the risk of contamination and shorten analysis time [10,11]. In homogeneous immunoassays, the key issue is how to quantitatively and sensitively distinguish antibodies (or antigen) and antigen–antibody complexes in the reaction solution. So far, several detection strategies have been used in homogeneous immunoassays, such as colorimetric, chemiluminescent, fluorescence polarization [12], fluorescence and chemiluminescence resonance energy transfer [13,14], time-resolved fluorescence [15], etc. However, these detection

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methods still show unsatisfactory sensitivity. In order to improve the sensitivity, enzyme signal amplification technique is absolutely necessary in current immunoassays [16]. Therefore, to develop sensitive and direct detection techniques (without signal amplification), and design universal and homogeneous strategies are very important topics in current immunoassays.

Recently, nanotechnologies provide tremendous opportunities for the development of highly sensitive and selective detection methods for chemical analysis and bioassays such as immunoassays and DNA assays [17–20]. Especially in homogeneous immunoassays, there is no need to separate and wash like ELISA, meanwhile, nanoparticle labeling technologies provide a simple, low-cost and sensitive detection format. Some noble metal nanoparticles, such as gold nanoparticles (GNPs) and silver nanoparticles (SNPs), as labeling probes and building blocks of nanosensors have opened the door to design new approaches for sensing and imaging due to their excellent chemical and physical properties such as, ease of synthesis and functionalization, excellent chemical and photo stability, and good biocompatibility [21–23]. The most exciting optical property of noble metal nanoparticles is their strong resonance light scattering that is orders of magnitude higher than light emission from strongly fluorescent dye molecules [24,25], making them to be ideal optical labels for chemical, biological and clinical applications [26–28]. In bio-labeling or bioassays, GNPs have more widely been employed than SNPs due to their good chemical stability, but SNPs exhibit certain advantages such as higher extinction coefficients, sharper extinction bands and higher ratio of scattering to extinction. More recently, SNPs are rapidly gaining popularity as a consequence, and some research groups have been developing several strategies for optical sensors and imaging techniques using SNPs as building blocks and labeling probes [29–31].

In this study, we described a sensitive, universal and homogeneous assay of biomarkers by using resonance light scattering correlation spectroscopy (RLSCS) and SNPs as labeling probes. The RLSCS is a novel single particle method and can sensitively characterize the hydrodynamic diameters of nanoparticles similar to fluorescence correlation spectroscopy (FCS) [32] and confocal correlation spectroscopy (CCS) technology [33]. Competitive immunoreaction and silver nanoparticle labeling technique are used in homogeneous assay. The principle of this method is based on changes in diameters of SNPs before and after the competitive reactions using RLSCS technique. The change in diameters of SNPs can reflect the characteristic diffusion time of SNPs in the detection volume. In order to demonstrate the universality of this new assay, small biomolecules, 17-β estradiol (E2) and biomacromolecules, liver cancer biomarker alpha fetoprotein (AFP), were used as assay models. The experimental conditions were optimized, and the quantitative relations were investigated. The presented method was successfully applied for direct determination of E2 levels in human urine and AFP levels in human sera. The results obtained were in good agreement with ELISA assays.

2. Materials and methods

2.1. Chemicals and materials

E2 was purchased from Westingarea Co. Ltd (Shanghai, China). ELISA kits for human E2 were the product of Yanhui of Biological Technology Co. Ltd (Shanghai, China). Rabbit anti-E2 antibody, β-estradiol 6-(O-carboxymethyl) oxime: BSA (BSA–E2, hapten), bovine serum albumin (BSA), O-[2-(3-mercaptopropionylamino) ethyl]-O′-methylpolyethylene glycol 5000 (PEG-SH, 5 kD) and 4, 7, 10, 13, 16, 19, 22, 25, 32, 35, 38, 41, 44, 47, 50, 53-hexadecaoxa-28, 29-dithiahexapentacontanedioic acid di-*N*-succinimidyl ester (NHS–PEG disulfide) were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, USA). Mouse anti-human monoclonal AFP antibody, AFP protein and ELISA kits for human AFP were purchased from Beijing North Institute of Biological Technology (Beijing, China). Sodium citrate, disodium hydrogen phosphate and sodium dihydrogen phosphate were products of Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). The 20 nm silver colloid solution was purchased from Ted Pella, Inc (Redding, USA). Rhodamine Green was purchased from Invitrogen Co. (California, USA). Ultra-pure water (18.2 MΩ) was obtained from the Millipore Simplicity System (Millipore, Bedford, MA, USA). All other reagents were products of Sigma-Aldrich Chemical Co. (Milwaukee, USA).

Five human urine samples were provided by Shanghai Jiaotong University Affiliated Shanghai Xinhua Hospital. Four urine samples were from pregnant women and one sample from a healthy man. All subjects were healthy people, not taking any exogenous hormones. These urine samples were collected in the morning, and centrifuged (Beckman Coulter, USA) for 20 min at 3000 rpm, and the supernatant were carefully collected and stored in a refrigerator (−20 °C) for further use. The hepatic carcinoma patient serum samples (cancer patient samples 1–3) and two healthy human serum samples (normal samples 1, 2) were provided by Shanghai Jiaotong University Affiliated Shanghai First People's Hospital. Serum samples were clotted for 30 min at room temperature and centrifuged (Beckman Coulter, USA) at 2500 rpm for 10 min, and the serum samples were stored at 4 °C for further use. All experiments were performed in compliance with the relevant laws and institutional guidelines.

2.2. Instrumentations

The setup of RLSCS is shown in Fig. S1, which is based on an inverted Olympus IX 71 microscope (Japan). The exciting radiation provided by a 488 nm laser beam from an argon ion laser (ILT Technology, Shanghai, China) was attenuated to 20 μW by a neutral density filter. The laser line was focused on the sample solution by a water immersion objective (UplanApo, 60 × NA1.2, Olympus). The sample was placed on a coverslip (thickness: 0.13–0.17 mm). The scattering light was collected by the same objective, and passed through the dichroic mirror (505DRLP, Omega Optical, USA) and the 35 μm pinhole into an avalanche photodiode (SPCM-AQR14, Perkin-Elmer EG&G, Canada). The signals obtained were recorded by a real time digital correlator (Flex02–12D/C, Correlator.com, USA). All data were analyzed with the standard Eq. (2) for particles diffusing in a three-dimensional Gaussian volume element and nonlinearly fitted with the Origin 6.0 software package based on the Levenberg–Marquardt algorithm.

2.3. Conjugation of proteins (antibody and antigen) to SNPs

In the conjugation of SNPs with proteins, the antibodies (anti-E2 antibody, anti-human AFP antibody) and the antigen (AFP protein) were first modified with NHS–PEG disulfide through the reaction between the succinimidyl ester and amino group of proteins, and then linked to the surface of SNPs through the covalent interactions between the disulfide and SNPs, respectively. And the BSA–E2 was linked to the surface of SNPs by electrostatic adsorption mode. The conjugation procedure of AFP and E2 antibodies to SNPs were described in Fig. S2. In this study, 20 nm SNPs were used as labeling probes.

Disulfide-functionalized proteins were prepared by adding 10 μL protein solution (1 mg/mL for antibody solution or 0.3 mg/mL for antigen solution) and 15 μL NHS–PEG disulfide (0.005 M) into 75 μL 0.01 M PB buffer (pH 7.4). The resultant mixture was allowed to react for 8 h at room temperature. To remove excess NHS-PEG disulfide, the disulfide-functionalized proteins were purified and washed by ultrafiltration (Millipore ultrafiltration tube, cutoff of 50 kDa) 3 times with the 0.01 M PB buffer. The

products were redispersed in the 0.01 M PB buffer and were stored at 4 °C for further use.

Protein-functionalized SNPs were prepared by adding 4 μL 0.1 M NaOH and disulfide-functionalized proteins (4 μL 50 nM for disulfide-functionalized antibody, 3 μL 75 nM for disulfide-functionalized antigen and 4 μL 50 nM for BSA–E2, respectively) into 400 μL 112 pM SNPs solution. After incubated at 4 °C for 8 h, 4 μL 0.02% PEG5000 was added into the resultant mixture for 1 h at room temperature. In order to remove excess antibody, the SNP@protein conjugates were purified and washed by centrifugation at 14,000 rpm for 8 min. The centrifuged conjugates were redispersed in 80 μL 0.01 M PB buffer (containing 0.002% PEG5000, 10 mM, pH 7.4) and were stored at 4 °C for further use.

2.4. Homogeneous competitive immunoassay procedures

The procedure of homogeneous competitive immunoassay is shown in Fig. 1. In the immunoassay of AFP, 10 μL of SNP@Ab (AFP antibody) solution and 20 μL AFP solutions of different concentrations, or human sera, were mixed, and incubated at 37 °C for 60 min. The cancer patient sera were first diluted 100 times and normal human sera were without dilution. Then, 10 μL of SNP@Ag solution was added into the mixture above, mixed intensively, and incubated at 37 °C for 60 min. The measurements of sample solutions were carried out in a home-built RLSCS system. The recording time per sample was 300 s, and the assay of each sample repeated three times.

In the immunoassay of E2, 10 μL of SNP@Ab (E2 antibody) solution and 20 μL of E2 solution of different concentrations, or human urine samples, which were not diluted, were mixed together, and incubated at 37 °C for 60 min. Then, 10 μL SNP@BSA–E2 solution was added into the mixture above, mixed intensively, and incubated at 37 °C for 60 min. The measurements of sample solutions were the same as the immunoassay of AFP.

2.5. ELISA method (control experiments).

The ELISA procedures for assays of E2 and AFP were described in Supporting Information.

3. Results and discussion

3.1. Principle of homogeneous competitive immunoassay by RLSCS

The principle of homogeneous assay by RLSCS is illustrated in Fig. 1. In the assay, the competitive immunoreaction is used since this mode only needs one antibody and shows a good universality.

In the procedure of immunoassay, SNPs as optical probes are used to label antibody and antigen (or hapten), the antibodies-labeled SNPs are firstly added into a sample containing antigens, and the part of antibodies-labeled SNPs will bind to antigens. And then, the antigen labeled SNPs are added into the above solution, and they will bind to free antibodies-labeled SNPs (excess), which will cause SNPs to form dimers (or oligomers). The formation of immunocomplexes will induce the significant change in the characteristic diffusion time of SNPs in the detection volume. RLSCS can be used to sensitively detect the changes in the characteristic diffusion time of SNPs before and after the competitive reactions.

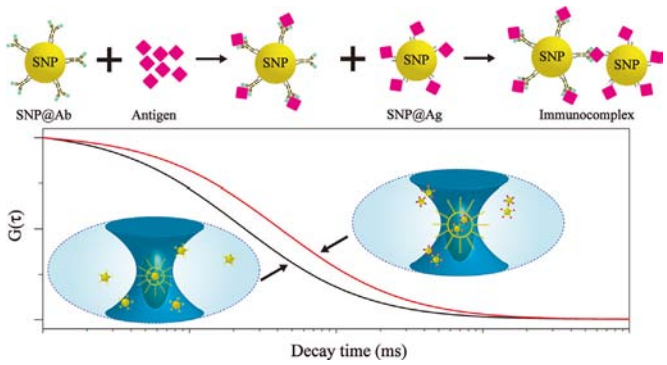

Fig. 1. The procedure of homogeneous competitive immunoassay by RLSCS. SNP@Ab and SNP@Ag represent the SNPs conjugated with antibodies and antigens, respectively.

The RLSCS is a novel single particle method, and the principle and setup of RLSCS is similar to FCS system except no emission filter. As shown in Fig. S1, when single SNPs diffuses into or out of the highly focused illumination volume due to Brownian motion, the scattering light fluctuations in this detection system can be real-time monitored by an avalanche photodiode. Like FCS, the scattering light intensity of SNPs in a tiny detection volume is correlated to obtain information about the processes that generate fluctuations in the scattering light intensity of nanoparticles. The autocorrelation function $G(\tau)$ in RLSCS is defined as:

$$G(\tau) = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2} \tag{1}$$

The diffusion of SNPs through the elliptical Gaussian-shaped confocal excitation/detection volume also can be described by a 3D-diffusion model as follows [34,35].

$$G(\tau) = \frac{1}{N} \cdot \frac{1}{(1+(\tau/\tau_D))} \cdot \frac{1}{\sqrt{1((\omega_{xy}/\omega_z))^2(\tau/\tau_D)}} \tag{2}$$

where N is the average number of nanoparticles in the confocal detection volume, and τ_D is the characteristic diffusion time of nanoparticles. Here ω_{xy} and ω_z are the lateral and axial radii of the detection volume at the e^{-2} point of the Gaussian laser beam intensity. The hydrodynamic radius R of GNPs can be calculated by Stokes–Einstein Eq. (3).

$$\tau_D = \frac{\omega_{xy}^2}{4D} \tag{3}$$

$$D = \frac{k_B T}{6\pi\eta R} \tag{4}$$

$$\tau_D = \frac{3\pi\eta\omega_{xy}^2 R}{2k_B T} \tag{5}$$

where D is the diffusion coefficient of nanoparticles, k_B is Boltzmann constant, T expresses solution temperature, and η is the viscosity of the solution. Under the given conditions, T, η and ω_{xy} are constants, and the characteristic diffusion time τ_D should be proportional to the hydrodynamic radius R.

In this study, SNPs are used as labeling probes due to their strong plasma scattering intensity over the same size GNPs. More importantly, the plasmonic scattering intensity of metal nanoparticles dramatically depends on the illumination wavelength, which is different from nonmetal nanoparticles such as polymers and silica. When the illumination wavelength is close to the maximum absorption values of metal nanoparticles, these nanoparticles show strong plasmonic scattering effect (or known as resonance light scattering). Ar ion laser with 488 nm well matches with the illumination wavelength of SNPs (Fig. S3 in Supporting Information) and was used as the illumination source in RLSCS system. When compared to long wavelength lasers such as 633 nm laser, which is usually used in detection of GNPs, the use of 488 nm laser can markedly reduce the detection volume, which can significantly decrease the effects of background and improve the sensitivity of RLSCS system. The detection volume of RLSCS system was less than 1 fL. This demonstrates that RLSCS is a highly sensitive single particle detection method for characterizing the diffusion behaviors of nanoparticles in solution.

3.2. The conjugation of SNPs with antibody (or hapten)

In this study, we chose the narrow size distribution of SNPs as probes, and the characterization of transmission electron microscopy (TEM) is shown in Fig. S4. This result documented that SNPs showed a good monodispersity. The procedure for conjugation of SNPs to proteins (antibody or antigen) is described in Fig. S2. RLSCS was used for the characterization of SNPs-protein conjugates. Fig. 2 shows the autocorrelation curves, the fitting curves and the fitting residuals of SNPs, SNPs labeled with antibody and SNPs labeled with hapten (BSA–E2). The autocorrelation curves of SNPs were well fitted by Eq. (2), and the correlation coefficients (R^2) were 0.9988, 0.9992 and 0.9992 for SNPs, SNP@Ab (E2) and SNP@BSA–E2, respectively. The fitting residuals were very low, which were all lower than 0.06. The characteristic diffusion times were 0.988 ms, 1.09 ms and 1.08 ms for SNPs, SNP@Ab (E2) and SNP@BSA–E2, respectively. The characteristic diffusion times of SNP@Ab (E2) and SNP@BSA–E2 were longer than that of free SNPs, and this data demonstrated that SNPs were successfully linked with proteins.

The AFP antibody and AFP antigen were linked with SNPs according to the same procedure above. The autocorrelation curves, the fitting curves and the fitting residuals of SNPs, SNPs labeled with antibody and SNPs labeled with AFP antigen were shown in Fig. S5 in Supporting Information. This result also showed that the proteins were conjugated with SNPs successfully.

As is known to us, nanoparticles have a trend of aggregation in biofluids, which interferes with the assay of bio-samples such as serum samples. In this study, O-[2-(3-mercaptopropionylamino) ethyl]-O′-methylpolyethylene glycol 5000 (PEG-SH) was used to suppress the aggregation of SNPs because the PEG-SH was able to bind to the surface of SNPs. PEG-SH modified gold nanoparticles (GNPs) and SNPs were observed to be very stable in solution of high salt [36]. Herein, we also found that PEG-SH was able to efficiently suppress the aggregation of SNPs.

3.3. Optimizing the competitive immunoassay of E2

It is very difficult to directly link E2 to SNPs because E2 is a small molecule. Therefore, we used E2 hapten (BSA–E2) to substitute immunogen E2 to construct a homogeneous competitive immunoassay since SNPs were easily labeled with BSA–E2.

In order to obtain the optimal reaction conditions, we studied the effects of certain factors such as the concentration of SNPs and the molar ratio of SNPs to proteins. Fig. 3A displays the effect of the molar ratio of SNPs to proteins (antibody or antigen). When the molar ratio of SNPs to protein is 1:5, the relation between the characteristic diffusion time (τ_D) and the logarithm of the E2 concentration (log C_{E2}) shows a good linearity and reproducibility. In principle, the high molar ratio of SNPs to protein should be benefited for the formation of the immunocomplexes and improvement in sensitivity. However, as shown in Fig. 3A, in the presence of high ratio of SNPs to protein (1:10), the detection system shows a poor reproducibility due to the formation of larger aggregates of SNPs in solution. Therefore, we choose the molar ratio of SNPs to protein 1:5 in subsequent experiments.

The effects of the concentration of SNPs are shown in Fig. 3B. When compared to low concentration of SNPs, the calibration curve exhibits a good linearity and reproducibility in the presence of 125 pM SNPs. In principle, when the antibody–antigen binding constant is large enough, the lower concentration of SNPs should be benefited for enhancement of sensitivity, which is in line with our experimental data. However, as shown in Fig. 3B, the poor reproducibility is obtained in the presence of the low concentration of SNPs. This phenomenon is mainly attributed to two reasons. Firstly, RLSCS is a single molecule statistical method, and the increase of data can significantly reduce the deviation of results. When the detection

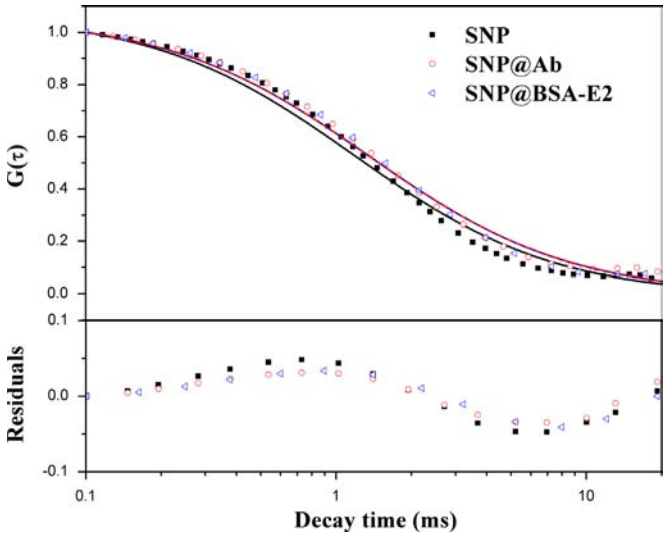

Fig. 2. Characterization of SNPs, SNP@Ab, SNP@BSA–E2 conjugates by RLSCS. The concentrations of SNP@Ab and SNP@BSA–E2 were 500 pM, the ratio of SNP to protein or hapten was 1:5 and the size of SNPs used in this study was 20 nm. The measurement time was 300 s and each sample was detected three times.

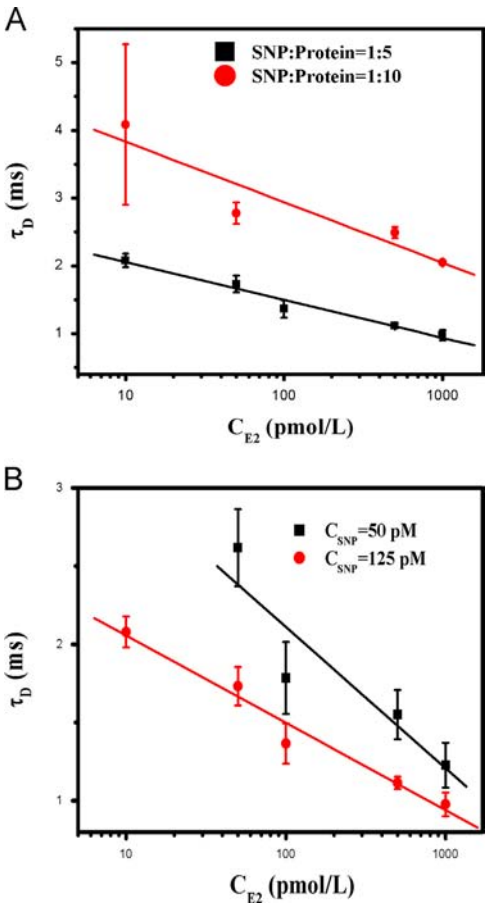

Fig. 3. Effects of the molar ratio of SNPs to proteins (A) and the concentration of SNPs (B). The concentrations of SNP@Ab and SNP@BSA–E2 in Fig. 3A were 125 pM, the ratio of SNP to protein or hapten in Fig. 3B was 1:5, and the incubation temperature was 37 °C. The size of SNPs used in this study was 20 nm. The measurement time was 300 s. The error bars represent the standard deviation of 3 time measurements.

volume is kept at the single particle level, the high concentration of particles should significantly improve the reproducibility of results, which is similar to the increase of data. Secondly, the high concentration of particles can considerably attenuate the effects from the adsorption of SNPs to the reaction tube and the sample wells for RLSCS measurements. In experiments it is very difficult to completely suppress the adsorption of SNPs. Therefore, 125 pM SNPs is used in subsequent experiments.

3.4. Homogeneous competitive immunoassay of E2 in urine samples

E2 is a sex hormone that is responsible for the development and maintenance of the female reproductive organs, female secondary gender characteristics, pregnancy and long-bone maturation [37]. Estradiol is also present in males, being produced as an active metabolic product of testosterone. Dysregulation in E2 metabolism has been implicated in infertility, obesity, and several human diseases including prostate cancer, Alzheimer's disease and osteoporosis [38]. Additionally, there is a considerable evidence that this endogenous estrogen plays an important role in the development of postmenopausal breast cancer [39]. Free estrogens in urine are usually found at extremely low levels [40]. Therefore, the accurate quantification of the E2 level from the urine sample is very important in the clinical diagnosis.

In this study, we want to explore the possibility for applying the RLSCS method to determine the E2 level in human urine samples. Fig. 4A shows the autocorrelation curves of SNPs, the fitting curves and the fitting residuals under different concentrations of E2. The results illustrated that autocorrelation curves of SNPs were well fitted by Eq. (2), the correlation coefficients (R^2) were from 0.9961 to 0.9993, and the fitting residuals were all lower than 0.15. Fig. 4B reflects the good linear relation between the characteristic diffusion time (τ_D) of SNPs and log E2 concentration (log C_{E2}), and the linear regression equation is described as $\tau_D = 2.61 - 0.56 \times \log C_{E2}$, and the correlation coefficient (R^2) is 0.985. The calibration curve of E2 has a linear range over two orders of magnitude from 10 pM to 1 nM, and the detection limit is 10 pM for E2.

This method was successfully applied for direct determination of E2 level in urine from normal man and pregnant women. In measurements, healthy man urine samples were 2 times diluted with buffer, pregnant women urine samples were 20 times diluted with buffer, and the results are shown in Table 1. The results obtained by both methods showed that the E2 level in urine from normal man was much lower than that from pregnant women, and these results were consistent with the actual situation [41]. The results obtained with present method were in good agreement with ELISA assays. As can be seen in Table 1, the RSDs are less than 20%, which are acceptable in homogeneous assays. Table S1 displays the recovery results of E2 competitive immunoassays by RLSCS. The recoveries by linear fitting method are 104–113%.

3.5. Homogeneous competitive immunoassay of AFP in serum samples

AFP is an oncofetal glycoprotein with a single-chain alpha globulin of 591 amino acids [42] and 3.4% carbohydrate [43], and is considered to be a major plasma protein produced by the yolk sac and the liver [44]. AFP has been widely used as a diagnostic biomarker for liver tumors [45,46]. The detection of this biomarker will play a significant role in the early diagnosis of liver cancer and therapy monitoring [47].

We used AFP as the macromolecular antigen and constructed a homogeneous competitive immunoassay to document the universality of the present method. The procedure for conjugation of SNPs to proteins (antibody or antigen) was described in the experimental section. Fig. 5A shows the autocorrelation curves of SNPs, their fitting curves and fitting residuals under different concentrations of AFP antigen. The autocorrelation curves of SNPs are well fitted by Eq. (2), the correlation coefficients (R^2) are from 0.9973 to 0.9995, and the fitting residuals are lower than 0.12. The linear relation for competitive assay is shown in Fig. 5B, which reflects the good linear relations between the characteristic diffusion time (τ_D) of SNPs and log AFP concentration (log C_{AFP}), and the linear regression equation is described as $\tau_D = 5.0 - 0.86 \times \log C_{AFP}$, and the correlation coefficient (R^2) is 0.991. The calibration curve of AFP has a wide linear range over two orders of magnitude from 100 pM to 10 nM and the detection limit is 100 pM for AFP.

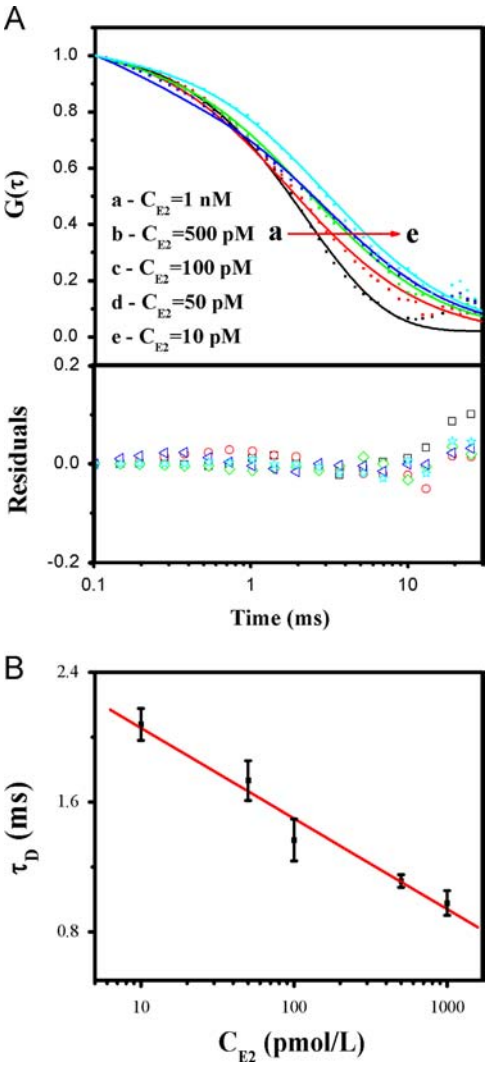

Fig. 4. The autocorrelation curves, their fitting curves and fitting residuals of SNPs (A) at different concentrations of E2, and the linear relation between the characteristic diffusion time of SNPs and log E2 concentration (B). The concentrations of SNPs@Ab and SNPs@BSA–E2 were 125 pM, the ratio of SNPs to hapten was 1:5 and the size of SNPs used in this study was 20 nm. The incubation temperature was 37 °C. The measurement time was 300 s. The error bars represent the standard deviation of 3 time measurements.

Table 1
The E2 levels in urine samples measured by ELISA and RLSCS ($n=3$).

Samples	ELISA (mol/L)	RLSCS (mol/L)	RSD (%)
Normal male	3.2×10^{-12}	6.5×10^{-12}	16.3
Female 1	3.3×10^{-11}	2.9×10^{-11}	17.7
Female 2	4.4×10^{-11}	5.9×10^{-11}	15.4
Female 3	3.0×10^{-11}	2.8×10^{-11}	9.7
Female 4	3.6×10^{-11}	3.2×10^{-11}	4.3

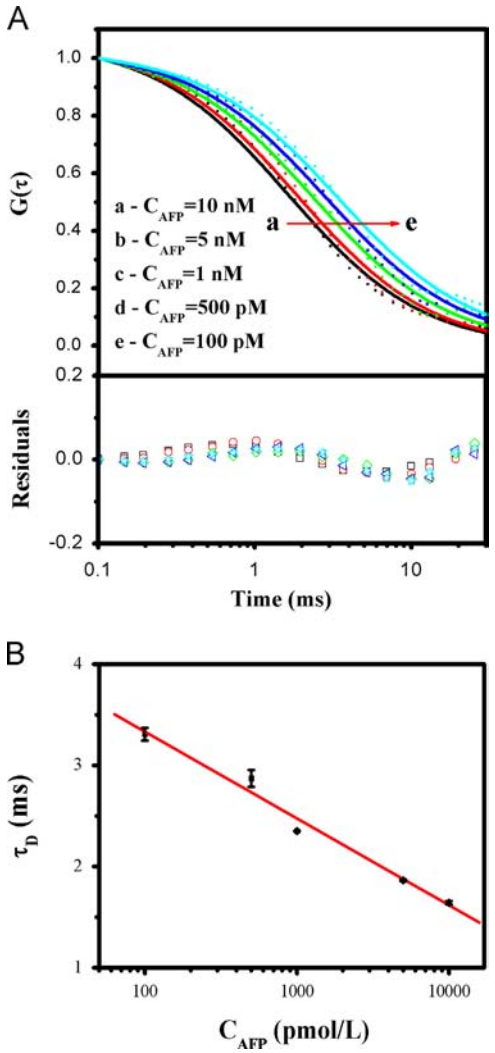

Fig. 5. (A) The autocorrelation curves, their fitting curves and fitting residuals of SNPs at different concentrations of AFP antigen, and (B) the linear relations between the characteristic diffusion time of SNPs and log AFP concentration. The concentrations of SNPs-antibody and SNPs-antigen were 125 pM, the ratio of SNP to protein was 1:5 and the size of SNPs used in this study was 20 nm. The incubation temperature was 37 °C. The measurement time was 300 s. The error bars represent the standard deviation of 3 time measurements.

This method was successfully applied for the direct determination of the AFP level in sera from healthy subjects and cancer patients. In measurements, patient serum samples were diluted 200 times with buffer, while healthy serum samples were diluted 2 times with buffer, and the results are shown in Table 2. We observed that the AFP serum levels in cancer patients were much higher than those in healthy subjects. The control experiments were preformed with ELISA, and the results obtained with the present method were in good agreement with ELISA assays. As shown in Table 2, the RSDs are less than 30% for healthy serum samples, and are less than 11% for patient serum samples. This result is probably attributed to the effects from certain interfering substances of normal serum samples with low dilution times.

4. Conclusion

In this work, we described a novel, universal and homogeneous immunoassay of biomarkers in biofluids by the RLSCS method. The principle of the method was described. The experimental conditions were optimized, and the quantitative relations were investigated. The universality of this method was successfully demonstrated by two assay models including analysis of small molecule E2 and biomacromolecule AFP. This new method was used to directly determine the E2 and AFP levels from clinical samples, and the results obtained were in good agreement with ELISA assays. When compared to the conventional methods such as ELISA, our method shows high sensitivity, simplicity and a short analysis time, and it exhibits promising potential applications in clinical diagnosis, food safety testing, and analysis of environments. It should be pointed out that the reproducibility of this assay is not good as ELISA when AFP in healthy samples were determined. The next work is to develop a simple treatment method to reduce the matrix interference of serum samples.

Table 2
The AFP levels in serum samples measured by ELISA and RLSCS ($n=3$).

Samples	ELISA (mol/L)	RLSCS (mol/L)	RSD (%)
Cancer 1	1.0×10^{-8}	1.1×10^{-8}	1.4
Cancer 2	5.1×10^{-8}	3.4×10^{-8}	11.0
Cancer 3	5.4×10^{-8}	6.6×10^{-8}	8.4
Normal 1	8.9×10^{-10}	2.2×10^{-10}	28.7
Normal 2	4.4×10^{-10}	3.0×10^{-10}	24.7

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Appendix A. Supplementry material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.07.024.

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