A bead-based fluorescence immunosensing technique enabled by the integration of Förster resonance energy transfer and optoelectrokinetic concentration

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Bead-based immunosensing has been growing as a promising technology in the point-of-care diagnostics due to great flexibility. For dilute samples, functionalized particles can be used to collect dispersed analytes and act as carriers for particle manipulation. To realize rapid and visual immunosensing, Förster resonance energy transfer (FRET) was used herein to ensure only the diabetic biomarker, lipocalin 1, to be detected. The measurement was made in an aqueous droplet sandwiched between two parallel plate electrodes. With an electric field and a focused laser beam applying on the microchip simultaneously, the immunocomplexes in the droplet were further concentrated to enhance the FRET fluorescent signal. The optoelectrokinetic technique, termed rapid electrokinetic patterning (REP), has been proven to be excellent in dynamic and programmable particle manipulation. Therefore, the detection can be complete within several tens of seconds. The lower detection limit of the REP-enabled bead-based diagnosis reached nearly 5 nM. The combinative use of FRET and the optoelectrokinetic technique for the bead-based immunosensing enables a rapid measure to diagnose early stage diseases and dilute analytes. © 2016 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4940938>]

I. INTRODUCTION

Early diagnosis of diseases is a goal of preventive medicine. However, poor signal due to low-abundance of pathogenic factors in clinical samples forms a barrier in the detections. Concentration of analytes or cells is a simple measure to tackle the problem. Accompanying with bead-based immunosensing, target molecules can be detected.^{1,2} Bead-based immunosensing, as opposed to conventional bioassays, features not only simplicity but also flexibility and high efficiency.^{1,3–6} To visualize unlabeled targets in such a bead-based system, Förster resonance energy transfer (FRET) is commonly used for this purpose.⁷ Similar bio-analysis approaches have been reported in hybridization assays for oligonucleotides,^{8,9} quantum dot (QD)-based molecular beacons for aptamer binding assays,^{10,11} and immunoassays.^{12,13} Among them, Liu *et al.*¹⁴ reported a novel detection of gold nanoparticles (AuNPs) functionalized with fluorescently labelled dsDNA by forming plasmonic hotspots through glass nanocones. A minimum concentration of 1 fM was achieved, and the intensity reached a maximum after minutes of packing time. Later, the same group advanced the signal detection by another technique,¹⁵ termed singular scattering, using silica fiber tips. Instead of functionalized AuNPs, amin-modified oligo probes were coated on the fiber tips. Subsequently, as low as 100 molecules of ssDNA targets (Dengue genome) were concentrated and measured through the fibers under a

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microscope. In addition to a minimum measurable concentration of 1 fM, the technique also featured a simultaneous detection of up to 70 000 targets. In spite of the lower detection limit (LDL), both methods require sophisticated fabrications and lack flexibility (i.e., addressable manipulation). In this study, particles coated with the dye-labeled antibodies and the QD-conjugated antibodies were separately prepared in liquid media. The use of QDs has been recognized a novel way to enhance the efficacy of FRET.¹⁶ When target antigens are present, both antibodies will bind to the antigens, bringing the QDs and the dye molecules in close proximity for the FRET response to take place. On the contrary, however, no FRET signal will be observed when target antigens are absent.

By labeling particles with special fluorescent molecules, the signal intensity can be processed by manipulating the particles. Membrane or microstructure filtration^{17–19} has been frequently used to condense target particles in microfluidic chips over the past years due to simplicity. However, both designs suffer from serious clogging after a long operation and require a minimum amount of liquid to drive the flow. The latter requirement also limits the possible use of some sample fluids, such as tears or saliva. Alternatively, some research efforts turn to focus on developing techniques for maneuvering micro-/nano-sized particles.^{20–27} However, only few of them are capable of harnessing particles programmably, i.e., addressable manipulation. Among the technologies, optoelectronic tweezers were first incorporated into a bead-based immunoassay for a surface-enhanced Raman scattering (SERS) detection.²⁸ A LDL concentration of 98 pg/ml and a total sample volume of 500 nl were achieved. The attempt successfully demonstrated such an integrated system for a sensitive detection. Later, an optoelectrokinetic technique, termed rapid electrokinetic patterning (REP),^{29–32} has also been developed to implement intricate particle manipulation. Programmability enables REP to effectively concentrate dilute analytes.³⁰ REP is a typical rendition utilizing parallel plate electrodes and a highly focused laser beam to create localized “hot-spots” on the electrodes. The localized hot-spots then induce electrothermal flows, which allow rapid collection of analytes at any prescribed location on the electrode surface.

An assessment of signal enhancement by concentrating immunocomplexed particles using REP was conducted in this study. Only a small drop of sample ($\sim 1 \mu\text{l}$) was required for the operation. The signal increased rapidly within several tens of seconds after concentration. In an attempt to demonstrate a practical diagnosis, FRET was used to detect the presence of lipocalin 1 (LCN1). LCN1 is considered one of the biomarkers existing in the tears of patients suffering from diabetic retinopathy.³³ The normal concentration level of LCN1 for a healthy human is approximately $70 \mu\text{M}$.³⁴ In contrast, the concentration level of LCN1 for a patient with diabetic retinopathy usually deviates from the normal concentration by 3–5 folds.^{33,35} Therefore, a LDL of at least $1 \mu\text{M}$ is required to diagnose the disease. Our result proved that the FRET signal was relevant to the presence of LCN1. When further concentrating the immunocomplexed particles by REP, the signal was enhanced. A LDL concentration of 5 nM was eventually achieved. Compared with conventional substrate-based immunosensors,^{36–39} this approach is more flexible and can be extended to different measurements when particles are conjugated with different antibodies. As a result, the bead-based fluorescence immunosensing enabled by the optoelectrokinetic technique provides insight to the possibility of developing early diagnoses for more other diseases.

II. METHODS AND MATERIALS

A. The optoelectric system

As shown in Figs. 1(a) and 1(b), the optoelectric microchip is composed of two parallel plate electrodes separated by a $50 \mu\text{m}$ spacer. By simply placing two strips of 3 M Scotch tapes on one of the plate electrodes, the spacer is formed. The bottom electrode is a glass slide coated with a gold film, and the top electrode is an indium tin oxide (ITO) glass. The gold electrode was made by depositing a thin layer of 20-nm chromium and a 150-nm gold film on a glass slide using an E-beam evaporator (VT1–10CE, ULVAC). The target analytes and QD-conjugated antibodies were incubated with the dye-labeled antibodies on particles to form sandwiched immunocomplexes. Subsequently, a drop of the colloidal suspension was loaded onto the gold electrode with a pipette. The final microchip was complete by clamping the two plate electrodes with paper clips. During operations, the microchip

was fixed on a holder with the electrodes connected to an electrical input. The input signal was generated from a function generator (GFG-3015, GWINSTEK) with its voltage amplified by a power amplifier (2340, TEGAM). A tunable solid-state IR laser beam (300 mW, 1064 nm, LD-WL206, Optoelectronics Tech.) was irradiated and focused on the bottom electrode of the microchip. The $1/e$ diameter of the laser beam projected on the electrode was measured to be $200\ \mu\text{m}$. A motorized mirror (T-MM2, Zaber Technologies) was arranged in the light path to steer the laser beam for programmable scanning (see the supplementary material, Fig. S1). The concept of the signal enhancement was realized by binding the target analytes on the functionalized particles based on the immune reaction, followed by the REP-enabled concentration as shown in Fig. 1(c). In addition, the programmability enabled the optoelectric system to concentrate more analytes in a dilute sample. Above the microchip, an upright fluorescent microscope (BX51, Olympus) was used to visualize the particle movement and aggregation. A $10\times$ objective lens, proper filter cubes (i.e., QD fluorescence (Ex:488 nm/Em:515 nm) and FRET fluorescence (Ex:488 nm/Em:605 nm)), and a fast CMOS camera (FL3-03-13S2C-CS, PointGrey) were used to capture the particle images. The FRET fluorescence was observed only in the presence of the LCN1 antigens. When there was no LCN1, however, the QDs and dye molecules were not in close proximity to induce the FRET fluorescence.

B. Rapid optoelectrokinetic concentration

An empirical model interpreting the mechanism of REP is shown in Fig. 2(a). From a global viewpoint, the electrothermal effect drives the fluid toward the center of irradiation,

FIG. 1. (a) Schematic of the experimental setup of the optoelectric system. (b) Top-view and side-view images of the experimental microchip. The bottom substrate is a gold electrode and the top plate is an ITO electrode. Noted that the depth in the side-view sub-image is increased to $200\ \mu\text{m}$ to facilitate visualization. (c) Schematic of the bead-based FRET fluorescence immunosensing enabled by REP. Dispersed immunocomplexed particles are concentrated by REP within several tens of seconds. The concentrated particles with LCN1 show significant fluorescent signal.

resulting in a toroidal flow. Consequently, particles are brought to the center by the fluid drag. The location of trapped particles can be changed with the irradiated spot. From a local viewpoint, the optoelectrokinetic behavior of particles during concentration is a dynamically balanced state of multiple forces, including a lift force, particle-particle interactions, and particle-electrode adhesion. The lift force is a combination of several sophisticated phenomena, which is capable of moving particles away from the bottom electrode. Nevertheless, the bulk electrothermal flow is well known the major factor to the lift force.⁴⁰ The particle-particle interactions include attraction due to local electrohydrodynamic (EHD) flow and repulsion due to the induced dipole effect. When particles are aligned perpendicular to the electric field, the induced dipole will cause repulsion between particles. When particles are aligned in parallel with the electric field, the induced dipole will cause adhesion, resulting in particle-electrode adhesion.

Taking all the mentioned electrohydrodynamic and electrokinetic effects into consideration, the particle behavior turns to be a function of electrical frequency, electric potential, light intensity, medium conductivity, and particle size. The detailed information of the particle behavior in response to different applied parameters can be referred to the prior literature.³⁰ In this study, only the translation and concentration were used to collect dispersed particles and enhance the final signal by optoelectrokinetic concentration. 3- μm immunocomplexed particles and a medium of conductivity maintained under 1 mS/m were used. When an electric field was applied on the microchip and a focused laser beam was irradiated on the bottom electrode, particles were rapidly concentrated within the irradiated region (Fig. 2(b)). Notably, the operational parameters (electrical frequency and electric potential) are subjected to the medium conductivity and particle size. The optimal parameters vary accordingly when either one of them changes. To raise the signal-to-noise ratio, the irradiated spot was scanned over the microchip by a motorized mirror to collect dispersed particles until the fluorescent signal saturated (Fig. 2(c)). The collection step is particularly useful for very dilute analytes. The final particle aggregate can therefore grow bigger and brighter than that in the initial stage (Fig. 2(d)).

C. Sample preparation for the dye-labeled antibodies on particles

The mouse anti-LCN1 monoclonal IgG (ab76611, Abcam), termed capture antibody, was labeled with the Alexa 546 dye (A20183, Ex:556 nm/Em:573 nm, Life Technologies), treated as an acceptor in the FRET reaction. To this end, 100 μl of 1 M sodium bicarbonate was mixed with the dye in a vial. Then, the vial was capped and gently agitated until the dye was fully

FIG. 2. Schematic of the system configuration and particle concentration by the optoelectrokinetic technique. (a) 3- μm particle concentration at 5 kHz, 16.5 V_{pp}; (b) 3- μm particle translation at 5 kHz, 16.5 V_{pp} with an irradiated spot steered by a motorized mirror. (c) A bigger particle aggregate is formed after scanning over the microchip.

dissolved in the buffer. Subsequently, 2.5 μl of the dissolved dye molecules was mixed with 5 μl of 0.5 mg/ml antibodies. The whole solution was finally incubated at room temperature (25 °C) in a thermal shaker (Clubio CB1702, Medclub Scientific) for an hour.

Amine-modified polystyrene particles (3 μm , Polyscience Asia Pacific Inc.) and the dye-labeled monoclonal antibodies were covalently coupled through the amide bond formation. First, the total 7.5 μl of the 0.5 mg/ml dye-labeled antibodies was thoroughly mixed with 1 μl of 10 mg/ml Ethyl(dimethylaminopropyl) Carbodiimide (EDC), 10 μl of 2-(*N*-morpholino)ethanesulfonic acid (MES), and 1.5 μl of 10 mg/ml *N*-Hydroxysuccinimide (NHS). The additions of EDC and NHS activated the carboxyl group on the antibodies. Subsequently, the activated dye-labeled antibodies were added into the amine-modified particles (5 μl , 1 680 000 #/ μl) suspension, and then the whole solution was incubated at 4 °C in a thermal shaker for 4 h. The mixed solution was washed four times with Phosphate Buffered Saline Tween-20 (PBST) and then four times with deionized (DI) water by centrifugation (8000 rpm for 6 min per run), followed by sonication for 15 min. The final conductivity of the washed solution was approximately under 1 mS/m. The solution was eventually aliquoted into four vials before use. The formation of particle-antibody binding was later verified with the FITC-labeled secondary antibodies.

D. Sample preparation for the QD-conjugated antibody

In this step, the CdSe QDs (QD525 ITK, Em:525 nm, Life Technologies) were conjugated with the rabbit anti-LCN1 polyclonal IgG (ab87786, Abcam), termed probe antibody, through the amide bond formation. First, 2.5 μl of 4.8×10^{12} #/ μl QD suspension was thoroughly mixed with 80 μl 1X Phosphate Buffered Saline (PBS), 1 μl of 1 mg/ml EDC, and 4 μl of 10 mg/ml NHS for 15 min. Similarly, the mixing was to activate the carboxyl groups that were coated on the QDs. Subsequently, 2.5 μl of 1 mg/ml polyclonal antibodies were added into the activated QD suspension and then incubated at the room temperature (25 °C) in a thermal shaker for an hour. Limited to the size of the QDs, however, the antibody-conjugated and unconjugated QDs were not further separated in this phase. They were separated when later the antibody-conjugated QDs bound to the functionalized particles. Additionally, ethanolamine (*a.k.a.*, ETA) (E9508, Sigma-Aldrich) was used to block the free carboxyl groups on the QDs and the probe antibodies to avoid unexpected binding on the amine-modified particles. The colloidal suspension was then stored at 4 °C for 48 h. The last step was to ensure all carboxyl groups were inactivated. The solution was eventually aliquoted into four vials before use.

E. Bead-based FRET fluorescence immunosensing

FRET is a technology capable of transferring energy between two closely spaced (<10 nm) light sensitive molecules. Two-step fluorescent transfer is involved in the process. Initially, the donor molecules absorb the incident light at a shorter wavelength and then emit fluorescence. Next, the adjacent acceptor molecules will absorb the emission from the donor and then emit new fluorescence at a longer wavelength. The technology was used herein to enable a visual detection. The target antigen was human lipocalin 1 (ab116833, Abcam), which is one of the biomarkers used in the diagnosis of diabetic retinopathy.³³ The immunocomplexed configuration on particles was designed as a sandwiched structure—capture antibody-antigen-probe antibody to enable the FRET effect (Fig. 3). The amine-modified polystyrene particles were coated with the dye-labeled (Alexa 546) mouse anti-LCN1 monoclonal antibodies. As mentioned in Sections IID and IIE, the roles of the dye and QDs in the FRET effect were treated as “acceptor” and “donor,” respectively. When LCN1 was present in the probe solution the dye-labeled capture antibodies and the QD-conjugated probe antibodies would spontaneously bind to the target antigen, bringing the donor in close proximity to the acceptor. To form the final sandwiched structure, the aliquoted dye-labeled capture antibodies were incubated with the LCN1 antigens at the room temperature (25 °C) for 1 h, followed by another hour at the same room temperature with the aliquoted QD-conjugated probe antibodies in a thermal shaker. For a concentration calibration, LCN1 was diluted in half from 611 nM to 5 nM. The mixed solution was then washed four times with DI water by centrifugation (8000 rpm for 6 min per run),

FIG. 3. Illustration of the bead-based immunocomplex. The sandwiched configuration forms a capture antibody-antigen-probe antibody structure, hence enabling FRET signal by transferring energy from the green CdSe QDs to the red Alexa 546 dye molecules.

followed by sonication for 15 min. Both the dye-labeled and QD-conjugated antibodies were mixed at a volumetric ratio of 1:1. By exciting the QDs with 488 nm light, the emitted energy will be transferred to the Alexa 546 dye, producing the FRET fluorescence at 572 nm. A fluorescent filter set (Ex:488 nm/Em:605 nm) was used to visualize the FRET images. As a result, particles with red fluorescence were indicative of the presence of the target antigens and vice versa.

III. RESULTS AND DISCUSSION

A. Signal enhancement for a bead-based immunoassay

To assess the signal enhancement of the technique, the designed particles carrying the sandwiched structure, capture antibody-antigen-probe antibody, were used. To visualize the particles, the green QDs were conjugated on the probe antibodies. The colloidal suspension of 1.2×10^7 #/ml was mixed with a high concentration ($2.44 \mu\text{M}$) of the antigen LCN1 in a volume of $100 \mu\text{l}$ vial to ensure the particle surfaces were fully covered with the immunocomplexes. When the particles were pipetted onto the microchip, the dispersed particles were concentrated within several tens of seconds due to the rapid electrothermal flow generated by REP at $16.5 \text{ V}_{\text{pp}}$ and 5 kHz (Figs. 4(a) and 4(b)). When the process was complete, a strong glow of green fluorescence from the particle aggregate could be observed through a microscope (Fig. 4(c)). Although the surfaces of the immunocomplexed particles were not as smooth as those of plain polystyrene particles, the modification seemed not to affect their response to REP. The fluorescent intensity was expressed in terms of gray level per pixel (Fig. 4(d)). Evaluations of the fluorescent intensity for single particles and aggregation were elucidated in the supporting information (see the supplementary material, Figs. S2 and S3).⁴¹ In addition, the signal was found constantly elevated as the functionalized particles were further diluted (data not shown). The signal increase was because the dilution allows each particle to capture more target analytes. Direct evidences from transmission electron microscopy (TEM) images show that the surfaces of the functionalized particles are covered by an exogenous matrix. Before a TEM measurement, $4 \mu\text{l}$ samples were dropped on a TEM grid and then stained with 2% uranyl acetate (UA) for 1 min. Proteins stained without the UA show dark gray color under TEM (Fig. 4(e)); whereas those proteins stained with UA show white color under TEM (Fig. 4(f)). The surfaces

FIG. 4. (a) and (b) Images showing the responses of the immunocomplexed particles ($d_p = 3 \mu\text{m}$) before and after REP concentration. (c) Fluorescent image of the same particle aggregate shown in (b). The green fluorescence indicates the successful sandwiched structure formed on the particles. (d) Plot of the particle count and signal intensity of the concentrated particle aggregate with respect to time. The error bars represent standard deviations. (e) TEM image of a particle without immunocomplexes on surface. The dark gray color indicates no UA coated on the particle. (f) TEM image of particles with immunocomplexes on surfaces. The white color indicates UA coated on the particles.

of the particles covered with the immunocomplexes are brighter than those without valid conjugations.

B. Specific binding effect of LCN1 on the bead-based system

The presence of the capture antibodies on particles was verified by incubating the colloidal suspension with the FITC-labeled donkey anti-mouse secondary antibodies (Fig. 5(a)). Compared with the control group without the capture antibodies on particles, the former shows brighter green fluorescence, implying successful binding between the capture antibodies and the particles (Fig. 5(b)). In addition, the expected sandwiched immunocomplex—capture antibody-antigen-probe antibody was also verified. The target antigens (LCN1) were initially incubated with the capture antibodies at room temperature (25°C) in a thermal shaker for an hour. Subsequently, the particles coated with the antigen-capture antibody complexes were incubated with the probe antibodies at the same room temperature for 1 h to form the final capture antibody (mouse anti-LCN1 IgG)-antigen (human LCN1)-probe antibody (rabbit anti-LCN1 IgG) structure (Fig. 5(c)). At last, the solution was incubated with the donkey anti-rabbit secondary antibodies to show the binding specificity. In the control group, only the antibody-conjugated particles and the secondary antibodies exist in the solution. In the experimental group, LCN1 was added to the solution in addition to the antibody-conjugated particles and the secondary antibodies. The fluorescent images are depicted in Fig. 5(d). The result shows that the fluorescent intensity of the experimental group is significantly higher than the control group, indicating that the expected immunocomplex forms specific binding rather than non-specific binding.

C. Detection of LCN1 based on the FRET immunosensing

To perform an immunosensing application, the dye-labeled capture antibodies were first mixed with the LCN1 antigens ($10 \mu\text{l}$, 611 nM) for 1 h at the room temperature (25°C).

FIG. 5. (a) Configuration of the functionalized particle to verify the presence of the capture antibodies. (b) Quantitative comparison of particles with and without the capture antibodies. The corresponding fluorescent images are shown in the insets. The symbol “***” denotes $p < 0.001$ under the student t test ($n = 17$). The error bars represent standard deviations. (c) Configuration of the functionalized particle to verify the presence of the probe antibodies. (d) Quantitative comparison of particles with and without the probe antibodies. The corresponding fluorescent images are shown in the insets. The symbol “***” denotes $p < 0.001$ under the student t test ($n = 17$). The error bars represent standard deviations.

FIG. 6. Assessment of the signals with and without LCN1 at a concentration of 611 nM. (a) The QD (Ex:488 nm/Em:515 nm) and (b) the FRET (Ex:488 nm/Em:605 nm) fluorescent images of the functionalized particles in the presence of LCN1. (c) The QD (Ex:488 nm/Em:515 nm) and (d) the FRET (Ex:488 nm/Em:605 nm) fluorescent images of the functionalized particles in the absence of LCN1. (e) Quantitative comparison of the QD and FRET signals. Comparisons at both emission wavelengths prove the FRET effect and the presence of LCN1. The symbol “***” denotes $p < 0.001$ under the student t test ($n = 21$). The error bars represent standard deviations.

FIG. 7. (a) The fluorescent signals are improved over time as particles with and without LCN1 are concentrated by REP. The representative plot was obtained at 611 nM LCN1. Both curves reach their maxima in nearly 10 s ($n = 6$). The error bars represent standard deviations. (b) The fluorescent images are a series of exposures showing the particle concentration in 40 s at 38 nM LCN1. Both measurements with and without LCN1 show intensity increases over time. The scale bar is 40 μm . (c) The intensity of the FRET fluorescence was measured from 611 nM to 5 nM. ($n = 6$) The error bars represent standard deviations.

Subsequently, the antigen-antibody complexes were mixed with the QD-conjugated probe antibodies (22.8 μl , 0.5 mg/ml) for another hour at the same room temperature. By the innate immune reaction, the donor QDs and the acceptor Alexa 546 dye were brought in close proximity to each other, resulting in a strong FRET effect. The particle fluorescence was analyzed individually to examine the FRET signal of the sandwiched immunocomplexes before concentration. In Fig. 6(a), QDs are observed on particles due to the presence of LCN1. Therefore, exciting QDs induce the FRET signal as shown in Fig. 6(b). Compared with a control measurement in the absence of LCN1, dimmer QDs and FRET particle images were observed (Figs. 6(c) and 6(d)). The existence of the QD and FRET fluorescence without LCN1 was likely attributed to non-specific binding from the adsorption of the QD-conjugated probe antibodies.⁴² A bar chart quantifying the observation is shown in Fig. 6(e). Despite the non-zero intensity in the control, the result indicates that the FRET intensity in the presence of LCN1 remains higher than that in the absence of LCN1. A preliminary study (see the supplementary material, Fig. S4)⁴¹ showed that more wash steps accompanying with sonication can effectively remove most non-specific binding from the QD-conjugated probe antibodies. However, more wash steps also cause loss of particles, which increases the difficulty to collect particles for subsequent signal enhancement.

To enhance the fluorescent signal especially for detecting dilute analytes, the immunocomplexed particles were further concentrated by REP as shown in Fig. 7(a). The result shows that the FRET signals with and without LCN1 are improved noticeably in roughly 10 s. The corresponding images below Fig. 7(a) reveal the particle aggregation over time (Fig. 7(b) and also see the supplementary material Movie S1).⁴¹ Obviously, the REP-enabled concentration can effectively boost the visibility and fluorescent signal for dilute analytes. To estimate the LDL concentration of LCN1, LCN1 was sequentially diluted from 611 nM to 5 nM (Fig. 7(c)). In order to avoid possible variations due to slightly different condition in every measurement,

relative intensity was used here. The relative intensity is defined as a ratio of the fluorescent intensity difference between the experimental and control groups divided by the control. The LDL concentration of LCN1 in this study approximately reaches 5 nM as the cut-off relative intensity sets to be 0.7. Below 0.7, the linearity between the intensity and concentration will decrease. Despite the background noise, the signal intensity in the presence of LCN1 remains higher than that in the absence of LCN1. The overall result implies that the REP-enabled bead-based FRET immunosensing is feasible for the rapid diagnosis of dilute analytes.

IV. CONCLUSIONS

In this paper, a biosignal enhancement system was established using REP and bead-based FRET fluorescence immunosensing. REP is an optoelectrokinetic technique, excellent in micro/nano particle manipulation.^{29–32,40} The microchip for REP requires no complicated fabrication but only two parallel plate electrodes separated by a spacer. Successful particle concentration occurs when laser irradiation and an electric field are simultaneously applied on the microchip.

A proof-of-concept demonstration utilizing immunocomplexed particles was used to assess the bead-based signal enhancement enabled by REP. For the first time, we showed that REP was capable of manipulating not only plain particles but also particles coupled with functional structures. In addition, this technique allowed collecting dispersed particles within the microchip to enhance signal for dilute samples. Before the signal saturated, the fluorescent intensity was rapidly elevated as the particles were concentrated in several tens of seconds.

A practical immunoassay was conducted for detecting LCN1. LCN1 is one of the biomarkers in the tears of patients with diabetic retinopathy. To enable a visual detection, FRET was used. Non-specific binding resulting from the adsorption of the QD-conjugated probe antibodies was reduced by additionally washing and sonicating the immunocomplexed particles. In our design, FRET signal occurred when the dye-labeled antibodies, the QD-conjugated antibodies, and the LCN1 antigens were brought in close proximity by the immune reaction. Although the non-specific binding on particles was inevitable, the background intensity remained lower than the signal of interest. The final LDL concentration achieved herein was 5 nM, which is much lower than the threshold concentration level for the diagnosis of diabetic retinopathy. The successful signal enhancement of the dilute biomarkers proved the concept of a rapid and non-invasive diagnosis of diabetic retinopathy. Moreover, the technique provides insight to detecting other diseases in the early stage.

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- ¹B. Teste, A. Ali-Cherif, J. L. Viovy, and L. Malaquin, *Lab Chip* **13**, 2344–2349 (2013).
- ²H.-W. Yu, A. Jang, L. H. Kim, S.-J. Kim, and I. S. Kim, *Environ. Sci. Technol.* **45**, 7804–7811 (2011).
- ³D. Horejsh, F. Martini, F. Poccia, G. Lppolito, A. D. Caro, and M. R. Capobianchi, *Nucl. Acids Res.* **33**, e13 (2005).
- ⁴S. Miraglia, E. E. Swartzman, J. Mellentin-Michelotti, L. Evangelista, C. Smith, I. I. Gunawan, K. Lohman, E. M. Goldberg, B. Manian, and P. M. Yuan, *J. Biomol. Screening* **4**, 193–204 (1999).
- ⁵J. A. Thompson and H. H. Bau, *J. Chromatogr. B* **878**, 228–236 (2010).
- ⁶X. Yu, M. Hartmann, Q. Wang, O. Poetz, N. Schneiderhan-Marra, D. Stoll, C. Kazmaier, and T. O. Joos, *PLoS One* **5**, e13125 (2010).
- ⁷S. S. Varghese, Y. Zhu, T. J. Davisab, and S. C. Trowell, *Lab Chip* **10**, 1355–1364 (2010).
- ⁸C. H. Zhang, H. C. Yeh, M. T. Kuroki, and T. H. Wang, *Nat. Mater.* **4**, 826–831 (2005).
- ⁹W. R. Algar and U. J. Krull, *Anal. Chim. Acta* **581**, 193–201 (2007).
- ¹⁰J. H. Kim, D. Morikis, and M. Ozkan, *Sens. Actuators, B* **102**, 315–319 (2004).
- ¹¹M. Levy, S. F. Carter, and A. D. Ellington, *Chem. Bio. Chem.* **6**, 2163–2166 (2005).
- ¹²E. R. Goldman, I. L. Medintz, J. L. Whitley, A. Hayhurst, A. R. Clapp, H. Y. Uyeda, J. R. Deschamps, M. E. Lassman, and H. Mattoussi, *J. Am. Chem. Soc.* **127**, 6744–6751 (2005).
- ¹³H. Park, M. Lee, G. H. Seong, J. Choo, E. K. Lee, J. Y. Park, S. Lee, K. H. Lee, and Y. W. Choi, *Bull. Korean Chem. Soc.* **29**, 1297–1298 (2008).
- ¹⁴S. Liu, Y. Yan, Y. Wang, S. Senapati, and H. C. Chang, *Biomicrofluidics* **7**, 061102 (2013).
- ¹⁵Y. Wang, T. C. Chang, P. R. Stoddart, and H. C. Chang, *Biomicrofluidics* **8**, 021101 (2014).
- ¹⁶W. R. Algar and U. J. Krull, *Anal. Bioanal. Chem.* **391**, 1609–1618 (2008).
- ¹⁷Y. Xu, K. Sirkar, X. P. Dai, and R. G. Luo, *Biotechnol. Prog.* **21**, 590–597 (2005).

- ¹⁸H. M. Hegab, M. Soliman, S. Ebrahim, and M. Op de Beeck, *J. Biosens. Bioelectron.* **4**, 1000140 (2013).
- ¹⁹X. Chen, D. Cui, and J. Chen, *Electrophoresis* **30**, 3168–3173 (2009).
- ²⁰S. Agastin, M. R. King, and T. B. Jones, *Lab Chip* **9**, 2319–2325 (2009).
- ²¹M. Javanmard, S. Emaminejad, R. W. Dutton, and R. W. Davis, *Anal. Chem.* **84**, 1432–1438 (2012).
- ²²Z. T. Kuo and W. H. Hsieh, *Sens. Actuators, B* **141**, 293–300 (2009).
- ²³K. M. Mayer, F. Hao, S. Lee, P. Nordlander, and J. H. Hafner, *Nanotechnology* **21**, 255503 (2010).
- ²⁴H. S. Chuang, T. Y. Chung, and Y. Li, *J. Micromech. Microeng.* **24**, 125016 (2014).
- ²⁵H. S. Chuang, S. C. Jacobson, and S. T. Wereley, *Microfluid. Nanofluid.* **9**, 743–753 (2010).
- ²⁶I. F. Cheng, S. C. Chiang, C. C. Chung, T. M. Yeh, and H. C. Chang, *Biomicrofluidics* **8**, 061102 (2014).
- ²⁷R. Vaidyanathan, S. Dey, L. G. Carrascosa, M. J. A. Shiddiky, and M. Trau, *Biomicrofluidics* **9**, 061501 (2015).
- ²⁸H. Hwang, H. Chon, J. Choo, and J. K. Park, *Anal. Chem.* **82**, 7603–7610 (2010).
- ²⁹S. J. Williams, A. Kumar, and S. T. Wereley, *Lab Chip* **8**, 1879–1882 (2008).
- ³⁰K. C. Wang, A. Kumar, S. J. Williams, N. G. Green, K. C. Kim, and H. S. Chuang, *Lab Chip* **14**, 3958–3967 (2014).
- ³¹A. Kumar, H. S. Chuang, and S. T. Wereley, *Langmuir* **26**, 7656–7660 (2010).
- ³²A. Kumar, S. J. Williams, H. S. Chuang, N. G. Green, and S. T. Wereley, *Lab Chip* **11**, 2135–2148 (2011).
- ³³É. Csösza, P. Borossa, A. Csutakb, A. Bertab, F. Tótha, S. Pólskac, Z. Töröke, and J. Tózséra, *J. Proteomics* **75**, 2196–2204 (2012).
- ³⁴R. J. Fullard and D. M. Kissner, *Curr. Eye Res.* **10**, 613–628 (1991).
- ³⁵H. J. Kim, P. K. Kim, H. S. Yoo, and C. W. Kim, *Clin. Biochem.* **45**, 60–67 (2012).
- ³⁶H. Wang, S. Meng, K. Guo, Y. Liu, P. Y. Yang, W. Zhong, and B. Liu, *Electrochem. Commun.* **10**, 447–450 (2008).
- ³⁷F. Darain, K. L. Gan, and C. S. Tjin, *Biomed. Microdevices* **11**, 653–661 (2009).
- ³⁸F. Darain, P. Yager, K. L. Gana, and S. C. Tjin, *Biosens. Bioelectron.* **24**, 1744–1750 (2009).
- ³⁹L. Yu, C. M. Li, Y. S. Liu, J. Gao, W. Wang, and Y. Gan, *Lab Chip* **9**, 1243–1247 (2009).
- ⁴⁰A. Kumar, S. J. Williams, and S. T. Wereley, *Microfluid. Nanofluid.* **6**, 637–646 (2009).
- ⁴¹See supplementary material at <http://dx.doi.org/10.1063/1.4940938> for the movie of signal enhancement by REP concentration, the evaluations of fluorescent intensity, and the removal of non-specific binding.
- ⁴²S. J. Rosenthal, J. C. Chang, O. Kovtun, J. R. McBride, and I. D. Tomlinson, *Chem. Biol.* **18**, 10–24 (2011).