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Time-resolved luminescent lateral flow assay technology

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ABSTRACT

We here report a detection technology that integrates highly sensitive time-resolved luminescence technique into lateral flow assay platform to achieve excellent detection performance with low cost. We have developed very bright, surface-functionalized and mono-dispersed phosphorescent nanoparticles of long lifetime under ambient conditions. The phosphorescent nanoparticles have been used to conjugate with monoclonal antibody for C-reactive protein (CRP), an inflammatory biomarker. Lateral flow immunoassay devices have been developed using the conjugate for highly sensitive detection of CRP. The CRP assay can achieve a detection sensitivity of <0.2 ng mL^{-1} in serum with a linear response from 0.2 to 200 ng mL^{-1} CRP. We have also developed a low cost time-resolved luminescence reader for the lateral flow immunoassay (LFIA) devices. The reader does not use expensive band pass filter and still provide very low detection background and high detection sensitivity on solid substrates such as nitrocellulose membranes. The reader can detect less than 2.5 ng phosphorescent particles captured on a nitrocellulose membrane strip with more than three orders of magnitude linear detection dynamic range. The technology should find a number of applications, ranging from clinical diagnostics, detection of chemical and biological warfare agents, to food and environmental monitoring.

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1. Introduction

Membrane-based lateral flow immunoassay (LFIA) technology has seen amazing commercial success in recent years because of its simplicity, low cost and user friendliness [1–7]. The technology is particularly suitable for point-of-care (POC) and over-the-counter (OTC) test markets. It has been utilized for detection of a large number of analytes from small molecules, macromolecules to microorganisms. Diagnostic products for pregnancy, infectious diseases, cardiovascular disorders, cancers to drugs-of-abuse have been commercialized. However, those products are limited to qualitative and semi-quantitative tests. In most cases, color particles such as gold nanoparticles and dyed latex particles are used to provide signals.

Much effort has recently been shifted to develop the technology for analyte quantification [8,9]. A number of platforms are being pursued through using different particles and various detection techniques. Colored particles [10], quantum dots [11,12], fluorescent latex particles [1–3], lipsome-based probes [13,14], magnetic particles [15], and Raman-active tags [16,17] are being explored by various companies to integrate with LFIA to achieve analyte quantification. Each of those detection techniques has its advantages and shortcomings. Absorbance based detection techniques often lack desirable detection sensitivity, while fluorescence techniques often require complex and expensive instrumentation. Lateral flow devices (LFDs) for magnetic-field measurements cannot be sealed inside a plastic housing, making it not acceptable for OTC markets. Like conventional fluorescence,

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Raman-based detection technology also needs expensive instrument.

So far, the only successfully commercialized system that can accomplish analyte quantification has been fluorescence-based LFIA technology (e.g., RAMP system by Response Biomedical Co., British Columbia, Canada). However, conventional fluorescence can have limitations due to possible high background caused by Tyndall, Rayleigh, or Raman scattering. The high background can also be caused by the instrument's optics, the sample matrix (e.g., blood or serum), and the test device itself. In particular, membrane-based immunochromatographic, or lateral flow, formats often have very high background fluorescence measured by a conventional fluorescence system due to the scattering caused by the membrane itself. These interferences due to light scattering and sample matrices are worsened by the small Stokes shifts of conventional fluorophores, which are typically 10–30 nm. Nevertheless, the technology platform has been proven to provide very sensitive detection of anthrax and west Nile virus in a portable and field-friendly format. The technology has also been successfully commercialized for detection of cardiac markers such as Troponin I and NT-proBNP. Despite its remarkable performance, the technology still has some drawbacks such as limited detection sensitivity and relatively high cost of the reader.

Time-resolved fluorescence detection techniques have been known to have potentially higher detection sensitivity than conventional fluorescence because of lower background noise [18–28]. In contrast to standard methods that only use optical filters to separate the fluorescence from background light through wavelength differences, the time-resolved fluorescence technique separates the fluorescence of interest from the background through lifetime differences. Basically, the technique involves exciting a fluorescent label of a long lifetime with a short pulse of light, then waiting a period of time for the background and other unwanted fluorescence to decay to a low level before collecting the remaining long-lived fluorescent signal. The excitation, delay and collecting processes can be cycled and summed to further improve the signal/noise ratio. This allows the short-lived signals, such as that from interfering components (e.g., blood) and scattered light to be eliminated from the signal so that only the long-lived fluorescence from the label can be read. The ability to eliminate the background is especially important for fluorescence measurements from lateral flow test strips, since its membrane-based structure tends to highly scatter the excitation light. Overall, time-resolved methods have been reported to increase the detection sensitivity by two or more orders of magnitudes over conventional fluorescence in liquid samples [18].

The time-resolved fluorescence detection technique has been successfully utilized for chemical and biological assays, particularly using europium chelates as probes [29,30]. Most of those techniques typically involve solution-based assays and often require multiple steps and involve very complex and expensive instruments, making them not suitable for POC and OTC applications.

In this article, we report a detection technology that integrates highly sensitive time-resolved luminescence detection technique into low cost and user-friendly LFIA technology to achieve remarkable detection performance with low cost. We used both commercial Europium-containing particles (EPs) and in house developed phosphorescent nanoparticles (KCPs) [31] as probes to demonstrate the power of the technology. We have also developed a low cost and portable time-resolved luminescence reader that does not use expensive optical filters which is a must for conventional fluorescence detection technique. The reader uses a time-resolved approach to measure either fluorescent or phosphorescent particles captured on a lateral flow test strip. The highly sensitive time-resolved luminescent lateral flow assay should be much cheaper than other related technologies using conventional fluorescent probes, surface enhanced Raman tags and paramagnetic particles. The cost of constructing a time-resolved luminescence reader is comparable to the absorbance-based reader, yet with much higher potential detection sensitivity. The drawback of the technology is a limited availability of the probes. The technology should find a number of applications, ranging from clinical diagnostics, detection of chemical and biological warfare agents, to food and environmental monitoring.

2. Methods

2.1. Materials

The materials used to assemble the lateral flow devices are purchased from Millipore Inc. Those materials include nitrocellulose membranes, plastic supporting cards, cellulose wicking pads, cellulose sampling pads and glass fiber pads. HF12002 Millipore nitrocellulose membranes are used. Europium-containing particles (EPs) were purchased from Molecular Probes Inc. In-house prepared phosphorescent nanoparticles (KCPs) were used as phosphorescence probes [31]. C-reactive protein (CRP) antigen was obtained from Biogenesis Inc. All the chemicals are obtained from Sigma Aldrich, unless specified.

2.2. Time-resolved luminescence measurements

Time-resolved luminescence was measured by Fluorolog III fluorimeter (JoBin YVon, Horiba Group) with time-resolved phosphorescence measurement capability (Fluorolog SPEX 1934D Phosphorimeter). Time-resolved phosphorescence on lateral flow devices was also measured by an in-house developed time-resolved luminescence reader. Luminescence intensity (either fluorescence or phosphorescence) is referred as time-resolved luminescence intensity unless specified. Front face mode was used for time-resolved luminescence measurements on lateral flow devices with Fluorolog III. For samples and devices using EPs, the fluorescence at 615 nm was recorded when excited at 370 nm. For samples and devices using KCPs, the phosphorescence at 650 nm (excited at 395 nm for Pt-containing particles: Pt-KCPs) or 670 nm (excited at 410 nm for Pd-containing particles, Pd-KCPs) was recorded. For measurements in solutions, right angle mode was used. For luminescence measurement of the detection line and control line on a lateral flow device, a black thin paper-board card with a rectangular hole matching the size and shape of the

detection line at the center was used to block the rest portion of the device while exposing the detection line to the excitation beam for luminescence collection. For background measurement, the hole of the card was moved to the background position close to the detection line and was aligned to be parallel to the detection line so that the excitation beam can be shined on the background position only. The partially blocked sample device was placed on a sample holder. The angle of the sample holder relative to the excitation beam can be adjusted. The sample device was aligned so that the detection line was positioned at the center of the excitation beam. For most of the luminescence measurements, the angle between the sample surface and excitation beam is ~45° and the emission-collection angle is ~60° relative to the sample surface. The typical parameters used for the time-resolved luminescence measurements involved in EPs are 10–50 μs of initial time delay, 50 ms per flash, 3 μs of sample window, and 10 flashes per measurements. The typical parameters used for the time-resolved luminescence measurements involved in KCPs are 40 μs initial delay, 50 ms time-per-flash, 20 flashes per measurement, 5 nm slit width for both excitation and emission. Usually, only one scan was recorded. Phosphorescence decay is typically obtained using the same parameters with exception of initial delay time of 10 μs.

2.3. Preparation of antibody conjugates of EPs and KCPs

To 500 μL of carboxylated europium-containing particles (0.2 μm, 0.5% solid) was added 100 μL Mes buffer (0.1 M, pH 6.1, 2-(*N*-morpholino)ethanesulfonic acid). 3 mg carbodiimide (purchased from Polysicences, Inc.) dissolved in 40 μL Mes was added to the particle suspension. The reaction mixture was shaken gently for 30 min. The activated particles were then washed twice by borate buffer through centrifugation. The washed particles were re-suspended in 200 μL borate buffer by 2 min bath sonication. Then 30 μL anti CRP monoclonal antibody (Mab1, cat# A5811, 4.9 mg mL^{-1} from BioSpacific Inc.) was added and the mixture was shaken overnight. 100 μL ethanoamine solution (0.1 M) was added and mixed for 30 min. The particles were then washed twice by HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer). The particles were then probe-sonicated in HEPES buffer for 30 s under ice bath and stored at 4 °C. The conjugate is designated as Mab1-EP.

The procedures of preparing Mab1 conjugate of KCPs are the same as above except using KCPs to replace EPs. The conjugate is designated as Mab1-KCP.

2.4. Preparation of lateral flow devices using Mab1-EP and Mab1-KCP

General procedures to assemble lateral flow devices were well known [1–7]. They normally consist of four components, a membrane strip with detection and control zones, a sample pad, a conjugate pad and a wicking pad. Typical conjugate pads are made by soaking a 0.4 cm × 10 cm glass fiber pad with a solution of 200 μL of Mab1-EP or Mab1-KCP (3 mg mL^{-1}), 200 μL of 2% Tween 20, and 200 μL of 20% sucrose, all in HEPES, and then being dried in an oven for 1.5 h at 37 °C. Nitrocellulose membranes with both detection and control lines were obtained by striping with 2.3 mg mL^{-1} anti CRP monoclonal antibody (Mab2, Cat# A5804, BioSpacific, Inc.) to form a detection line and polylysine solution (10 mg mL^{-1} in water) to form a control line with 1 h drying at 37 °C. A glass fiber pad soaked with 1% Tween 20 and dried under 37 °C for 2 h was used as a sample pad. The conjugate pad, sample pad and wicking pad (cellulose pad) were laminated with membrane cards. The assembled full card was then cut into 4 mm wide full devices. Unlike the typical opaque supporting cards for nitrocellulose membrane, a polyester backing card (<5% transmittance for <450 nm light and >95% transmittance for >450 nm light, from GML Inc.) was used to act as both a supporting card and a UV filter.

3. Results and discussions

3.1. EP probes

Time-resolved luminescent assay technology consists of two major components, a disposable lateral flow assay device and a re-usable reader. The physical, chemical and spectroscopic properties of the probe dictate many aspects of the reader development, including cost, size, complexity and performance. For time-resolved luminescent LFAs, the particle probes are preferred to have strong luminescence of long lifetime with a large Stoke shift under ambient conditions. They should be effectively excited by low cost light sources such as light emitting diodes (LED), preferably at visible range. The probes are also desired to be monodispersed with right sizes and have surface functional groups for surface covalent tagging of recognition molecules such as antibodies. They also should be chemically, physically and photochemically stable. Unfortunately, the probe particles with all those properties are not available.

EPs are obvious choice for initial investigation. EPs have very strong absorption at ~365 nm and strong fluorescence at ~615 nm with a long lifetime of ~500 μs and a huge Stokes shift of ~245 nm. They are monodispersed with surface functionalizable groups such as carboxylic acid. However, two major properties have made it less than ideal for time-resolved luminescent LFAs. On one hand, the particles are subject to severe photobleaching under 365 nm irradiation; on the other hand, the particles can be effectively excited only by ~365 nm UV light source which is not cheaply available. The UV light is also not suitable for consumer-based diagnostic products for safety concern. Nevertheless, they are still useful for time-resolved luminescent LFAs.

Nitrocellulose membranes commonly used for LFAs exhibit strong fluorescence and scattered light themselves when excited by a broad range of light, particularly UV. Time-resolved technique can easily eliminate those backgrounds. Fig. 1 shows the time-resolved luminescence spectra of Mab1-EP on nitrocellulose membrane at different delay times. The broad fluorescence peak at ~415 nm (attributable to the combination of scattered light, fluorescence from nitrocellulose membrane and ligands of Europium chelates in the particles) is actually much more intense than the peak at 615 nm. The broad peak can be completely eliminated at 40 μs delay, at

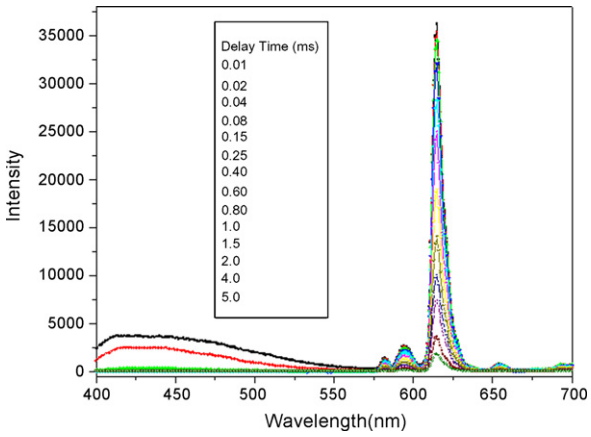

Fig. 1 – Time-resolved fluorescence spectra of Mab1-EP on nitrocellulose membranes.

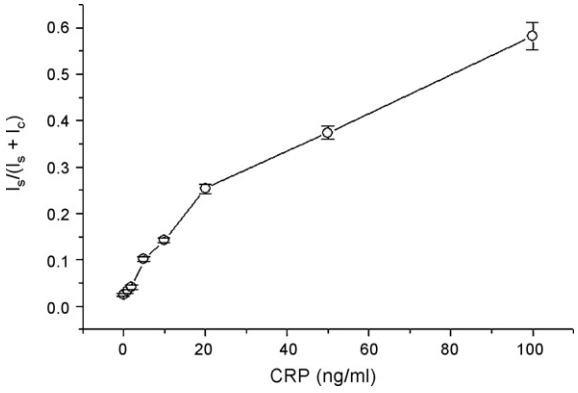

Fig. 2 – Time-resolved luminescence intensity at 100 μs delay as a function of CRP concentration in serum.

which the 615 nm peak is still strong. Our experiments have also demonstrated that the auto-fluorescence of blood and serum can also be completely rejected by the time-resolved luminescence measurements. Such a complete background elimination can not be achieved through conventional fluorescence measurements where wavelength differences are used to separate the luminescence of interest from the background because of the wavelength overlap.

3.2. *Detection of CRP using EPs*

In order to demonstrate the application of time-resolved luminescence LFAs, anti CRP monoclonal antibody was conjugated to EPs and the conjugate was then used to make eight lateral flow devices with both a detection line and a control line. The control line can capture all the conjugates that pass through the detection line. Each device was directly applied with a 40 μL CRP solution of different concentrations in serum, ranging from 0, 1, 2, 5, 10, 20, 50 and 100 ng mL^{-1}. The devices were allowed to develop for 30 min and time-resolved fluorescence on both detection line and calibration line were measured by Fluorolog SPEX 1934D Phosphorimeter. The development was visually observed to be complete within about 15 min. However, enough time was used to guarantee full development before the fluorescence measurements to eliminate the inconsistency of the development time for the different devices. Fig. 2 shows the time-resolved luminescence intensity at 40 μs delay as a function of CRP concentration in serum. A linear relation between the intensity and the concentration was observed from 0.2 to100 ng mL^{-1}. The luminescence signal was observed to level off beyond 200 ng mL^{-1}.

3.3. *Phosphorescent probes*

During development of a portable and low-cost time-resolved luminescence reader for the lateral flow device using EPs, 365 nm UV LEDs from Nichia were found to be not powerful enough, resulting in low detection sensitivity. In order to make the reader more compact and cheaper, a transmission measurement is preferred than reflectance mode. However, 365 nm UV light has little transmission through nitrocellulose membranes, making the transmission mode measurement relatively insensitive. Furthermore, EPs have severe photo-bleaching when exposed to ~365 nm light, which may compromise analyte quantification.

Because of the limitation of EPs and other fluorescent particles for time-resolved luminescent LFAs, phosphorescent particles were explored as an alternative because phosphorescence is well known for its long lifetime [32–42]. Much effort has been reported to apply phosphorescence for chemical and biological detection and analysis. Yet, little commercial success in the field has been achieved mainly due to oxygen quenching. In almost all the cases, oxygen free environment is needed for generating strong phosphorescence. We have recently developed a new class of phosphorescent nanoparticles with excellent physical and spectral properties for time-resolved biological detection techniques [31]. The particles exhibit strong phosphorescence at 650 nm of ~100 μs lifetime for Pt-based particles (ex at 390–400 nm) and 670 nm of ~500 μs lifetime for Pd-based particles (ex at 410–420 nm) and a large Stoke shift (~280 nm for both particles) under ambient conditions. The advantages of those particles over EPs include minimal photo-bleaching and availability of cheap and powerful light sources such as 390, 400 and 410 nm LEDs for effective excitation. Another advantage is that the background fluorescence is lower for most of biological matrices and nitrocellulose membranes when excited at 390–420 nm in comparison with the background excited at 365 nm. Although the transmission of 390–420 nm light through nitrocellulose membranes is also higher than 365 nm light, making it more suitable for transmission mode measurements.

3.4. *Reader development*

After vigorous testing and evaluation, transmission measurement mode was selected for use in a proof-of-concept reader intended to demonstrate the feasibility of low cost time-resolved luminescent LFAs. Although this mode results in some loss of luminescence by scattering in the nitrocellulose membrane, it nevertheless allows improved collection efficiency in a practical device. Furthermore, the simple layout of the optical components afforded by the transmission mode promises to facilitate low-cost implementation in mass

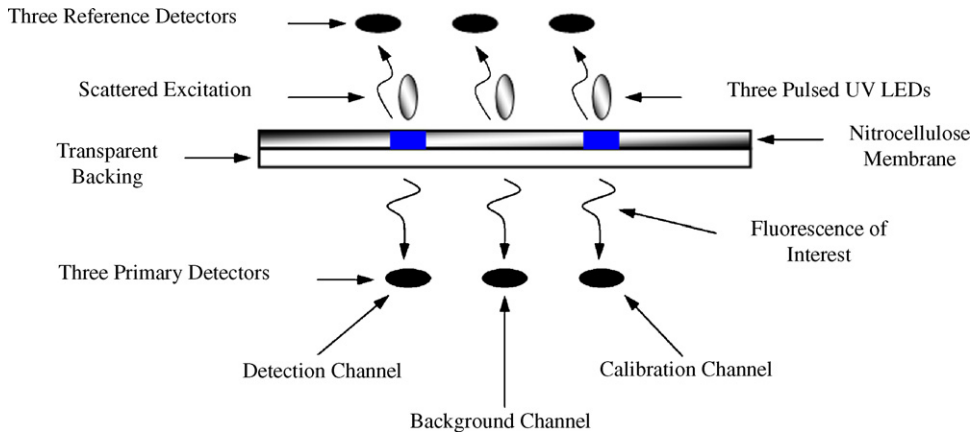

Fig. 3 – Layout for the optical heads.

production. To improve the transmission efficiency of the test strip in our implementation, the nitrocellulose membrane is attached to a supporting backing that is largely opaque to the UV excitation but transparent to the luminescent emission. Thus, the backing acts as a cheap cut-off filter for the scattered UV excitation and short wavelength non-signal fluorescence. By selecting one of several inexpensive backing materials that are commercially available as a blocking filter that is integral to the disposable test strip the need for external filters is eliminated.

Because the in-house developed phosphorescent particles (KCPs) have strong absorption spectra from 390 to 420 nm, low cost 395 nm LEDs (ETG, Inc, Los Angeles) were used as excitation light sources. Standard silicon photodiodes which are largely insensitive below 400 nm were used for detection. The optical head design involved three channels as shown in Fig. 3: the detection line, membrane background, and calibration line on a lateral flow test strip. Each channel was equipped with an LED on the membrane side for excitation and two photodiode detectors. The LED is driven by a programmable current source that can be pulsed with a rise or fall time of ~10 ns. The excitation waveform typically has rectangular shape with a duty cycle of ~10% and a repetition frequency of ~2 kHz. A photodiode on the backing side is used to measure the signal luminescence. This detector is connected to a high gain trans-impedance amplifier ($\sim 10^5$ V A^{-1}) designed to have a bandwidth exceeding 10 MHz. A second photodiode on the excitation side measures non-signal emission from the nitrocellulose membrane from the reflection side for the purpose of compensating for variations in the LEDs output power. It is connected to a low gain trans-impedance amplifier ($\sim 10^3$ V A^{-1}).

The prototype consists of an analog circuit board that is connected to an optical head that contains the LEDs, detectors, and a receiver slot for the test strip. A computer interfaced signal generator and data acquisition board is connected to the analog circuit board via breakout connectors.

The reader prototype consists of three key parts: optical heads, signal conditioning electronics, and data acquisition equipment. (1) *Optical heads*: The optical head includes a test strip mount, photodiode holder, LED holder, and custom optical components designed to maximize production and collection of fluorescence. (2) *Signal conditioning electronics*: The signal conditioning electronics includes the LED drivers, photodiode output conditioning circuitry, and interface to the data acquisition equipment (computer). It also contains the power supply and/or battery, that will power the unit. The electronics controls up to six photodiodes and three LEDs that are organized into three groups to monitor three specific areas of a fluorescent test strip: control line area, test line area, and membrane background. Each group consists of two photodiodes and an LED—one photodiode collects fluorescence from the targeted area, and one monitors the brightness of the LED in the group. (3) *Data acquisition equipment*: The data acquisition equipment includes an oscilloscope and a signal generator. The signal generator outputs waveforms to the LED drivers in the signal conditioning electronics to control the LEDs. The oscilloscope captures outputs from the signal conditioning electronics and transfers the captured data to PC for further processing. The unit has been assembled with a working analog-to-digital converter for measurements. A prototype reader (a computer for data collection and processing is not shown) is shown in Fig. 4. The bill of material for constructing the device is less than $20.

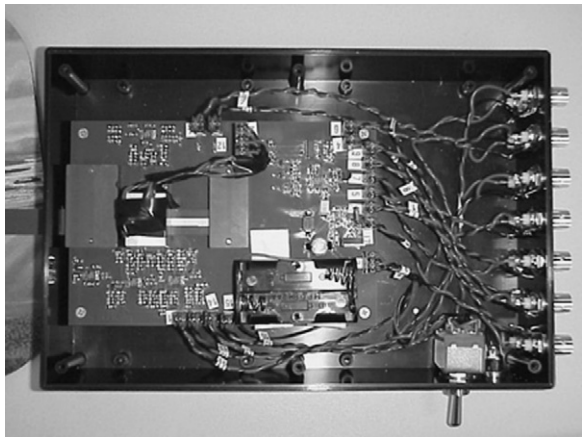

Fig. 4 – Prototype reader.

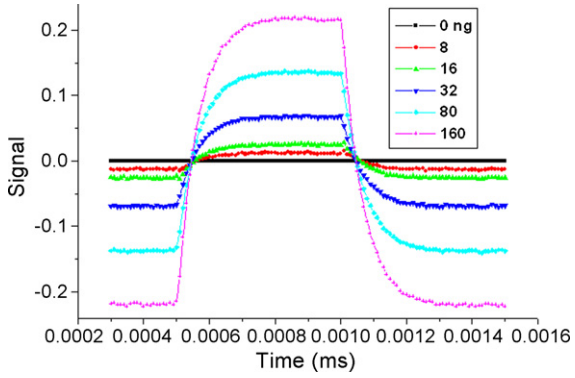

Fig. 5 – Time-dependent signal profile for Pt-KCP on lateral flow devices.

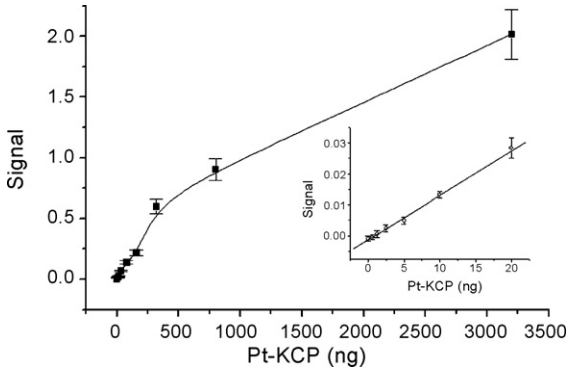

Fig. 6 – Time-resolved luminescence intensity at 40 μs delay as a function of the amount of Pt-KCP.

3.5. *Performance of prototype reader*

The quality of excitation square pulses from the prototype reader with 395 nm LEDs was found to be very good. The square pulses decay rapidly to background within less than 10 μs for the reader upon switch-off, which is much faster than the lifetime of in-house developed phosphorescent particles. Only the channel for detection line was used to collect data for evaluation. A series of lateral flow devices with a different amount of KCPs captured on the detection line by polylysine (from 0, 8, 16, 32, 80, 160, 320, 800 and 3200 ng per device) were prepared. The devices were placed into a sample holder and the polylysine line was aligned with the middle channel of the holder with nitrocellulose membrane facing the top and the transparent backing facing the bottom. The sample holder was then slided into the reader for time-gated phosphorescence measurements. Fig. 5 shows the time-dependent signal profile of one pulse for those devices using Pt-KCP (data for the three devices captured with 320, 800 and 3200 ng per device were not shown). The signals in the decaying region show excellent dose response for more than three orders of magnitudes.

In order to evaluate the reader's detection sensitivity for Pt-KCPs, ten duplicates of lateral flow devices with each of the following amount of Pt-KCP captured on the detection line per device were measured for the time-resolved phosphorescence signal at 40 and 120 μs delay: 0, 0.625, 1.25, 2.5, 5, 10 and 20 ng. Fig. 6 shows the signal at 40 μs delay subtracted by the signal at120 μs delay versus the amount of Pt-KCP. The detection sensitivity of the reader was estimated to be ~2.5 ng Pt-KCP per device.

In order to demonstrate the application of the time-resolved luminescent LFA for analyte quantitation, forty lateral flow devices were prepared using Mab1-KCP as conjugate. The forty full lateral devices were divided into eight groups with five duplicates for each group. A 60 μL serum sample of different CRP concentrations, ranging from 0, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng mL^{-1}, was applied to each device of each group, respectively, to allow 30 min of signal development. CRP in the serum was removed by magnetic particles tagged with anti CRP monoclonal antibody before the known amount of CRP was added. The time-resolved fluorescence on the detection lines and background for each device was then measured by the reader. The time-resolved luminescence signals at 40 μs delay time show a linear relation with CRP concentrations. The detection sensitivity for CRP is estimated to be 0.2 ng mL^{-1}.

4. Conclusions

Time-resolved luminescent LFA technology has been demonstrated to be capable of providing very sensitive analyte quantification. Lateral flow devices using EPs as time-resolved fluorescence probes were developed that are capable of detecting 0.2 ng mL^{-1} CRP in serum. However, an expensive UV excitation light source and sensitive detector are needed to achieve high detection sensitivity. We have also developed time-resolved luminescent lateral flow assays that use KCPs as probes. The assay technology consists of a disposable strip and a reusable low cost reader. The low cost and portable assay can also achieve a detection sensitivity of 0.2 ng mL^{-1} CRP in serum.

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