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A microarray-based resonance light scattering assay for detecting thrombin generation in human plasma by gold nanoparticle probes†

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A peptide microarray-based resonance light scattering (RLS) assay with gold nanoparticle probes has been developed for monitoring thrombin generation in human plasma. Thrombophilia, hypocoagulability and normal plasma have been successfully differentiated by the assay.

Thrombin, a serine protease, plays an important role in haemostasis and thrombosis since it produces insoluble fibrin through the proteolytic cleavage of soluble fibrinogen in the last step of the blood coagulation cascade.[1] More than 95% of thrombin generation occurs after the initial formation of fibrin, which means that traditional coagulation tests including the prothrombin time (PT) and the activated partial thromboplastin time (APTT) only detect as little as 5% of the overall thrombin potential.[2] The situation with shortened APTT values can be associated with an increased risk of thrombosis.[3] Therefore, thrombin generation tests as useful diagnostic tools for hemostatic disorders are currently under intense investigation since they may represent the future of coagulation laboratory testing.[4] There are a variety of assays including aptasensor and fibrin degradation product (D-dimer)-based assays available to measure thrombin generation in the laboratory.[5] For instance, aptasensors have been used to detect thrombin activity with high sensitivity in human serum, spiked serum, diluted human plasma and spiked human plasma.[5c–e] Although these assays have high sensitivities, the use of the assays as prognostic markers of hypercoagulability in general remains to be fully defined. According to the properties of coagulation cascades, human plasma and human blood are appropriate samples.[6] Adding tissue factor, phospholipids and $CaCl_2$ to activate human plasma samples will be the advisable method to mimic *in vivo* clotting of the system.[7] There are few reports on applications of biosensors for detecting thrombin generation in activated human plasma samples with high dilution ($\leq$10% (v/v)).[5d,5e,8]

As a vital tool for high-throughput screening, peptide microarrays have been employed to study the functionalities and inhibitions of enzymes including kinases, proteases and phosphatases.[9] These approaches allow for measurable comparisons to be made across samples, facilitating the differentiation of closely related proteins. In addition, convenient solid phase peptide synthesis methods and automated synthesizers have led to greater library availability, both from research and commercial avenues, devoting the technology to take on greater roles in detection and diagnostics. Because of their unique optical property (*i.e.*, surface plasma resonance (SPR) absorption and resonance light scattering (RLS)), gold nanoparticles (GNPs) have been extensively explored as probes for sensing or imaging wide ranges of analytes or targets, such as heavy metallic cations, nucleic acids, proteins and cells, *etc.*[10] For example, microarray-based RLS assays with GNP probes have been employed for detecting DNAs and proteins.[11] Compared to the conventional fluorescence readout format, the RLS assays with GNP probes normally have lower detection limits for analytes combined with a larger dynamic range.

The goal of this study is to fabricate a peptide microarray-based RLS assay with GNP probes for detecting thrombin generation in human plasma. Because proteases in the blood coagulation cascade have overlapping specificities, the optimal peptide substrate of thrombin was firstly screened by the RLS assay. With the optimal peptide substrate, thrombophilia, hypocoagulability, and normal plasma samples have been successfully differentiated by the approach.

In this assay, biotinylated peptides were spotted and immobilized on commercial aldehyde 3-D slides using a standard robotic procedure.[11a] The aldehyde group on the slide surface reacts readily with the N-terminal of the peptide to form a Schiff base linkage. The binding scheme and detection principle of the assay is schematically shown in Fig. 1. Generally, the

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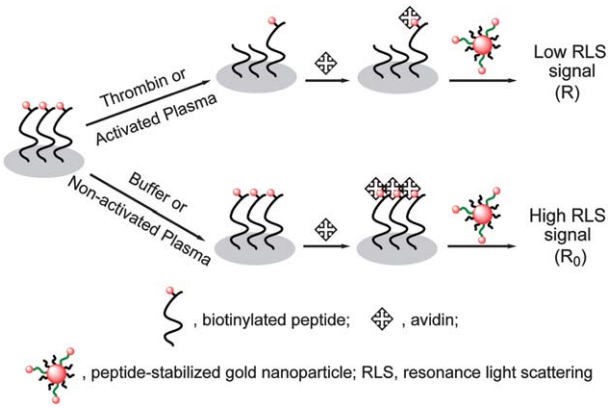

Fig. 1 Schematic representation of a peptide microarray-based RLS assay for detecting pure thrombin or thrombin generation in human plasma by gold nanoparticle probes.

spots on the microarray can generate strong RLS signals when the biotinylated peptides are labeled by the GNP probes through a biotin–avidin reaction. In the presence of pure thrombin or human plasma generated thrombin, the peptide fragments carrying biotin sites depart from the slide through the hydrolysis reaction. As a result, the RLS intensity is decreased by increasing the concentration of protease. Therefore, the protease activity can be determined by the change of RLS intensity. The RLS signal changes (ΔR) were calculated using the following equation: $\Delta R = (R_0 - R)/R_0 \times 100\%$, where R and R_0 are the mean RLS intensities of spots on the microarray with and without treatment of pure proteases or thrombin generated in human plasma, respectively. In this case, 30 nm peptide-stabilized GNPs (named as GNP probes) were employed since they have relatively good monodispersibility, excellent colloidal stability and strong light scattering properties.[10b] The characterizations of GNP probes are shown in Fig. S1 in the ESI.†

Thrombin and Factor Xa (FXa) normally have the same substrate, and to obtain a specific peptide substrate of thrombin, we investigated the hydrolysis efficiencies of nine peptide substrates (S01–S09, see Table S1 in the ESI†) including eight thrombin substrates and one FXa substrate which were derived from literature reports.[8a,12] As shown in Fig. 2, the peptide substrates were screened by thrombin and FXa. Under the same experimental conditions, S07 shows the strongest hydrolysis efficiency with thrombin and relatively low hydrolysis efficiency with FXa, while S09 shows the strongest hydrolysis efficiency with FXa and relatively low hydrolysis efficiency with thrombin. The hydrolysis efficiencies of S07 and S09 with different amounts of thrombin or FXa are shown in Fig. S2 in the ESI.† For achieving good assay performance (*i.e.*, sensitivity and selectivity) of thrombin detection, S07 was used as a specific thrombin substrate and S09 was chosen as the FXa substrate for monitoring the amount of FXa in the reaction mixture.

A set of experiments were designed to determine the detection limits of the described method. A series of standard thrombin solutions with various concentrations were prepared and applied to hydrolyze S07 on microarrays. As shown in Fig. 3, an optimal change of RLS intensity (three times the standard deviation of the control sample) could be detected using thrombin at a concentration as low as 19.7 pM. And the change of RLS intensity is linearly increased with the logarithm of the thrombin concentration from 2.7×10^{-2} to 810 nM, indicating a dynamic range of more than 4 orders of magnitude. The sensitivity and dynamic range of the assay meet the testing requirement of thrombin in practical samples because the peak concentration of thrombin is about 458 nM in 67% diluted human plasma, which is the maximum plasma concentration that has been used in thrombin generation tests.[13]

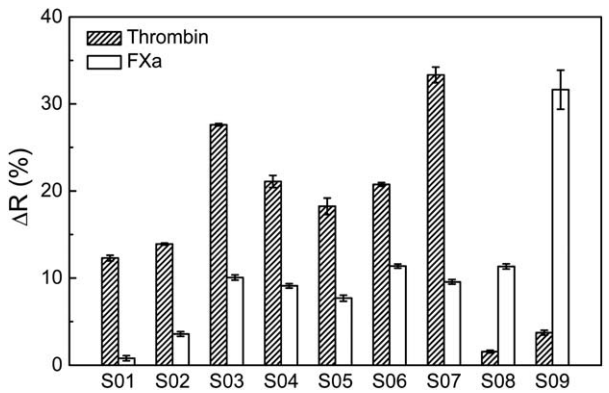

Fig. 2 Quantitative analysis of RLS signals of 9 peptide substrates hydrolyzed by thrombin and FXa. The concentration of thrombin and FXa is 27 nM. The concentration of peptide substrates in spotting solution is 0.5 mg mL^{-1}.

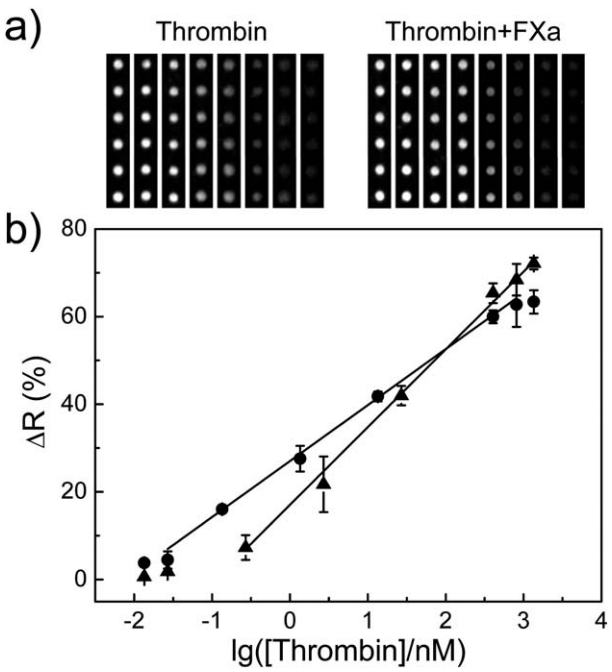

Fig. 3 (a) RLS images and (b) the corresponding logarithmic plots of integrated RLS intensity change as a function of the concentration of pure thrombin (●), or the corresponding thrombin concentration in a protease mixture (▲). The concentrations of pure thrombin are 0.0135, 0.027, 0.135, 1.35, 13.5, 405, 810 and 1350 nM. The corresponding concentrations of thrombin in the protease mixture are 0.0135, 0.027, 0.27, 2.7, 27, 405, 810 and 1350 nM. The molar ratio of thrombin and FXa in the protease mixture is 10:1. The concentration of S07 in spotting solution is 0.5 mg mL^{-1}.

S07 and S09 have also been hydrolyzed by a mixture of thrombin and FXa. For mimicking the real concentration ratio of thrombin and FXa in human plasma, the molar ratio of thrombin and FXa in the protease mixture was fixed at 10 : 1.[14] As anticipated, the ΔR of S07 is higher than that of S09 (as shown in Fig. 3 and S3 in the ESI†). Because there are mutual interferences between the proteases, the hydrolysis efficiencies of thrombin and FXa in the protease mixture are poorer than those of pure thrombin and FXa when the total protease concentration of the protease mixture is less than 110 nM (the intersection point of two logarithmic plots in Fig. 3). Corresponding concentration of FXa (*ca.* 10 nM) in the protease mixture is higher than the peak concentration of FXa (*ca.* 5 nM) in clotting human plasma.[15] The experimental results of the pure protease assay and protease mixture assay indicate that the peptide microarray-based RLS assay can be used to detect thrombin in practical samples (*e.g.*, human plasma) if S07 is employed as a substrate.

To further prove the veracity of the peptide microarray-based RLS assay, the thrombin generation in human plasma was examined. For mimicking the blood coagulation process, the plasma clotting cascade was activated from the extrinsic pathway by adding trigger solution (see the Experimental section in the ESI† for details) and $CaCl_2$.[7] Different concentrations of activated or non-activated human plasma were applied to microarrays. The largest change of RLS intensity was obtained for the reaction mixture containing 33% (v/v) human plasma (see Fig. S4 in the ESI†). Under the optimized human plasma concentration (33%), 24 human plasma samples were assayed (see Table S2 in the ESI†). The samples contain 13 cases of normal plasma, 4 cases of patient plasma with a history of idiopathic venous thrombosis (thrombophilia samples) and 7 cases of normal plasma with 7.5 μM thrombin inhibitor Human Antithrombin-III (hypocoagulability samples). The corresponding non-activated human plasmas were used as control samples (see Fig. S5 in the ESI†). As expected, the changes of RLS signal intensities of the samples follow the order of thrombophilia samples > normal samples > hypocoagulability samples (as shown in Fig. 4), which are consistent with literature reports.[4c,16] The experimental result confirms that the peptide microarray-based assay could be used to evaluate thrombin generation in human plasma. In addition, high concentration (33%) human plasma samples were used for testing in this assay, reducing the effects of buffer elements on endogenous thrombin generation.[17]

To evaluate the reproducibility of the assay, six trials were run, using arrays from several batches, fabricated at different days. The RLS intensities of 3 different human plasmas are shown in Fig. S6.† As a result, the assay-to-assay precision coefficient of variation (CV) of the RLS signal intensity was below 10% in all experimental datasets. Such a precision was considered to be within an acceptable range, indicating that the assay has reasonable reproducibility.

In summary, we have developed a peptide microarray-based RLS assay for detecting thrombin generation in human plasma with relatively high selectivity and reasonable reproducibility. Under the optimized plasma concentration (33%), thrombophilia, hypocoagulability, and normal plasma have been successfully differentiated by the assay. These experimental results demonstrate that the peptide microarray-based RLS assay has great potential to detect proteolytic activity of protease in complex samples, such as human plasma. In particular, the assay is capable of multiplexing since, in principle, the detection of different human plasma sample or protease activities can be carried out simultaneously by simply immobilizing different kinds of substrates on the same microarray.

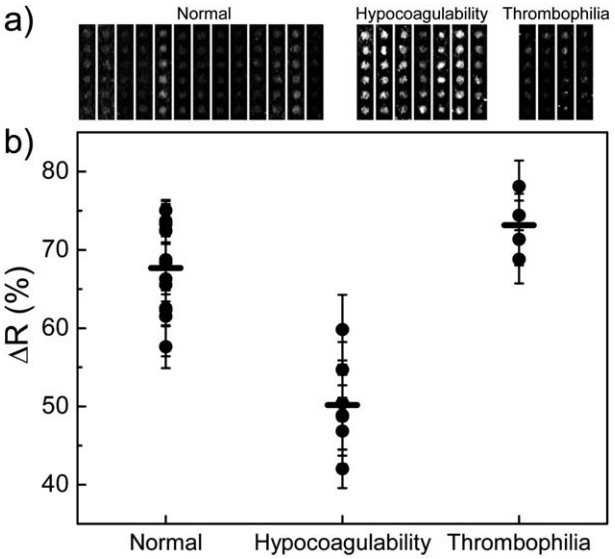

Fig. 4 (a) RLS images and (b) the corresponding RLS intensity changes of normal, hypocoagulability, and thrombophilia plasma samples. The mean signal changes of normal, hypocoagulability and thrombophilia samples are 67.7%, 50.2% and 73.2%, respectively. Horizontal bars indicate the mean values. The concentration of S07 in spotting solution is 0.5 mg mL^{-1}.

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