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Liquid Crystal Multiplexed Protease Assays Reporting Enzymatic Activities as Optical Bar Charts

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We describe a highly sensitive, substrate-specific, label-free, and multiplexed protease assay which reports proteases activities as an optical bar chart, allowing test results to be easily assessed by laymen with the naked eye. First, an oligopeptide microarray having six rows of immobilized oligopeptides, with well-controlled orientations and concentration gradients, is immersed in a buffer solution containing proteases. Then, a thin layer liquid crystal is supported on the microarray to transduce the oligopeptide cleavage event into an optical bar chart of different colors and lengths. This type of optical bar chart provides very rich information such as protease concentration, incubation time, surface densities of oligopeptides, etc. Both trypsin and chymotrypsin can be detected by using this assay within 3 h. The capability of the multiplexed protease assay opens up possibilities for detecting toxins such as botulinum neurotoxins which are known to cleave proteins and affect the docking and fusing synaptic vesicles.

Proteases are enzymes which can cleave peptide bonds in polypeptides or proteins. They are essential for many important biological processes, such as digestion, blood clotting, and apoptosis.[1] Dysfunction of proteases can lead to several well-known diseases including cancers,[2] viral infections (e.g., HIV[3,4]), and neurodegenerative disorders (e.g., Alzheimer's disease[5]), making them important therapeutic targets. Traditionally, proteases activities are determined by using radioactive or fluorogenic methods. For example, fluorescein isothiocyanate (FITC)-labeled casein was added to a solution containing proteases. Then, enzymatic activities of the proteases were quantified by the increase in the fluorescence. It was demonstrated that this assay can be used to detect approximately 5 ng of trypsin in a 10 μL solution. More examples of traditional proteases assays can be found in the literature.[6] Moreover, many new methods have been developed for monitoring enzymatic activities without using radioactive or fluorescence labels in recent years. For example, Sailor and Gooding's group demonstrated the use of optical properties of nanoporous silicon coated with a thin layer of protein substrates for the detection of proteases.[7] After the cleavage of the protein by proteases, the color of the nanoporous silicon changes and that can be easily observed with the naked eye.

Recently, more and more array-based protease assays have been developed because of their high-throughput nature.[8−11] Unlike traditional protease assays, immobilized oligopeptides on solid surfaces were used as protease substrates because oligopeptides with well-defined sequences and lengths can be custom-made by using solid-phase synthesis. When these immobilized oligopeptides are exposed to a solution containing proteases, they are recognized and cleaved by proteases, leading to a decrease in the oligopeptide length. Subsequently, surface-sensitive analytical techniques such as phosphorimaging,[8] surface plasmon resonance (SPR),[12,13] fluorescence,[1a] or matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry[14,15] are employed to transduce minute changes in surface properties into

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measurable signals. However, these methods either require labels or complicated instrumentation. Therefore, a convenient and label-free method that is able to report the proteases activities is highly desirable.

In the past, liquid crystals (LCs) have been used to amplify and transduce chemical and biological binding events on surfaces into optical signals.[16−22] The LC-based detection principle is viable because the anchoring energy of LCs is so small that an anchoring state of LCs can be easily disrupted by subtle changes on a solid surface. Another advantage of using LCs as imaging materials is that the signal output is in the form of optical signals which are clearly visible with the naked eye. Recently, on the basis of the unique properties of LCs mentioned above, Abbott and co-workers designed a LC-based sensor to report the protease activity of trypsin acting on an oligopeptide substrate (SNKTRIDEANNKAT-KML), which was covalently immobilized at an aqueous/LC interface.[17,23] It was found that when the oligopeptide was cleaved by trypsin, the optical textures of the LCs underneath changed from bright to dark. Despite the promise of this method, three major challenges remained. First, oligopeptides were immobilized at the aqueous/LC interface by reacting oligopeptides with carboxylic acid-terminated lipids self-assembled at aqueous/LC interfaces. Therefore, the reaction is not site-specific, and that can lead to multiple anchoring points at lysine residues and N-terminal amine of the oligopeptides. Second, the exact mechanism that leads to the disruption of LCs was not fully understood. As reported by the authors, cleavage of some well-known trypsin substrates, such as polylysine, did not cause any response in the LCs. Third, this sensor was built upon aqueous/LC interfaces rather than on traditional solid surfaces, which precluded the use of microarray techniques for preparing a high-density array with hundreds or thousands of oligopeptide probes.

In this paper, we report a LC-based protease assay which can potentially overcome the limitations mentioned above. First, we labeled oligopeptides with an N-terminal cysteine, which can react with aldehyde irreversibly and form thiazolidine. This reaction also proceeds much faster than the one between lysine and aldehydes. Thus, it is possible to immobilize an oligopeptide on an aldehyde-terminated surface with a single anchoring point at the N-terminal cysteine.[24] Second, the assay was built on a solid substrate in a microarray format. This configuration allows us to immobilize multiple oligopeptides (or the same oligopeptide with different surface densities) on the same surface for probing protease activities simultaneously. With dependence on the oligopeptide sequences, very rich information, such as surface densities of oligopeptides, protease concentrations, and incubation time, can therefore be obtained from these microarrays. Third, we combined the unique optical properties of LCs and the microarray techniques to create a multiplexed protease assay. In this assay, we used the orientational transitions of LCs to report the enzymatic activities. Our recent studies showed that orientations of LCs supported on glycine oligomers-modified surface were disrupted if the ellipsometric thickness exceeded 5 ± 1 Å.[25] This phenomenon suggests that the orientations of LCs are very sensitive to the lengths or surface densities of the immobilized oligopeptides and that forms the basis of using LCs for detecting proteases (if they can cleave immobilized oligopeptides and decrease their lengths or densities).

EXPERIMENTAL SECTION

Chemicals. Trypsin (from bovine pancreas, activity = 10 000−15 000 units/mg protein when *N*-α-benzoyl-*L*-arginine ethyl ester is used as the substrate), α-chymotrypsin (from bovine pancreas, type II, ≥40 units/mg protein), *N*,*N*-dimethyl-*n*-octadecyl-3-aminopropyltrimethoxysilyl chloride (DMOAP), and fluorescein isothiocyanate isomer I (FITC) were purchased from Sigma Aldrich (Singapore). Phosphate buffer saline (PBS) and sodium dodecyl sulfate (SDS) were purchased from 1st Base (Singapore). Triethoxysilane aldehyde (TEA) was purchased from United Chemical Technologies. Oligopeptides including CDRVYIHPF-HLK (**P1**), CDRVYIHPFHL (**P2**), CDHVYIHPFHLK (**P3**), CSNK-TRIDEANNKATKML (**P4**), CWHWQRPLMPVSI (**P5**), and CDYKDDDDK (**P6**) (with a purity of >90%) were synthesized by Research Biolabs and Sigma Aldrich (Singapore). Liquid crystal 4-cyano-4′-pentylbiphenyl (5CB) was purchased from Merck (Singapore). All solvents used in this study were HPLC grade. Water was purified by using a Milli-Q system (Millipore).

Surface Modifications. Glass slides (Sailboat, China) were cleaned three times by sonication in a 5% Decon-90 solution for 15 min. After this, they were immersed in 4 M of NaOH for 30 min and rinsed thoroughly with deionized water. Subsequently, the cleaned glass slides were immersed in an aqueous solution containing 0.1% (v/v) of DMOAP for 1 min and rinsed with copious amounts of deionized water. The DMOAP-coated glass slides were dried under a stream of nitrogen and then heated in a 100 °C vacuum oven for 15 min to allow the cross-linking of silanol groups. To introduce aldehyde functional groups to the surface, we immersed the glass slides into a methanolic solution containing 2% (v/v) of TEA for 2 h. After this, the glass slides were rinsed with copious amounts of methanol to remove residual TEA. Finally, they were dried under a stream of purified nitrogen and then heated in a 100 °C vacuum oven for 15 min. The characterization of aldehyde-terminated surface was described in the Supporting Information.

Fabrication of Oligopeptide Microarrays. First, different concentrations of oligopeptides solutions were prepared in PBS buffer (0.1 M, pH = 7.0) containing 0.001% (w/v) SDS. To prepare an oligopeptide microarray, oligopeptide solutions were dispensed onto an aldehyde-terminated surface by using a spotting robot (Biodot). There were two types of oligopeptide microarrays used in this study; both of them had 6 rows, each row had 30 spots, and each spot had a volume of 100 nL. The first type of microarray

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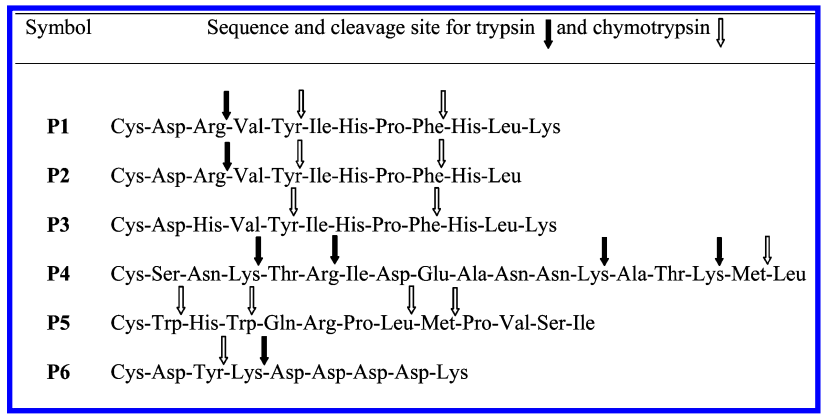
Table 1. Sequences of P1−P6 and Their Cleavage Sites for Trypsin and Chymotrypsin, Respectively[a]



[a] Trypsin hydrolyzes a peptide with arginine (Arg or R) or lysine (Lys or K) residue in its sequence unless they are followed by proline (Pro or P). Chymotrypsin hydrolyzes a peptide with tryptophan (Trp or W), tyrosine (Tyr or Y), phenylalanine (Phe or F), leucine (Leu or L), or methionine (Met or M) residue.[26,27]

had six different concentrations of **P1** in each row and the second type had immobilized oligopeptide **P1**−**P6** (40 μM) in each row. The sequences of oligopeptides and their enzymatic cleavage sites by trypsin and chymotrypsin, respectively, are shown in Table 1. After oligopeptide solutions were dispensed, these microarrays were incubated in a humid chamber for 12 h, allowing the covalent immobilization of oligopeptides on the surfaces. In the final step, all microarrays were washed thoroughly with PBS buffer to remove unreacted oligopeptides and dried under a stream of purified nitrogen.

Trypsin Microarrays. To prepare a trypsin microarray, different concentrations of trypsin solutions were dispensed onto an aldehyde-terminated surface by using the spotting robot. After this, the microarray was incubated in a humid chamber for 3 h and then washed thoroughly with deionized water and dried under a stream of purified nitrogen.

Characterization of Oligopeptide Density with Fluorescence. The oligopeptide microarray supported on a glass slide was incubated in 10 μg/mL of FITC in PBS buffer for 2 h. After this, the glass slide was washed with 1% SDS in PBS buffer. Fluorescence images of the microarray were then taken with a GenePix (4100A) microarray scanner manufactured by Molecular Devices. The same parameters (PMT gain = 600, pixel size = 10 μm) were used for all images and analysis. The fluorescence profiles were analyzed with Image J.

Cleavage of Immobilized Oligopeptides. To cleave immobilized oligopeptides with protease, we immersed the oligopeptide microarray in a protease solution by using two different experimental setups. In the mode of gradient immersion time, an oligopeptide microarray was mounted vertically inside a small vial (2.62 cm in diameter). Then, trypsin buffer (50 mM Tris-HCl, 20 mM $CaCl_2$, pH = 8.0) with 3 μg/mL (or 30 units/mL in terms of enzyme activity based on the data provided by the supplier) of trypsin was delivered to the vial at a flow rate of 70 μL/min for 10 h by using a peristaltic pump. The temperature of the solution was maintained at 37 °C by using a hot plate. Under this experimental condition, it took 7.7 min for the trypsin solution level to increase by 1 mm. In the mode of constant immersion time, an oligopeptide microarray was immersed in trypsin or chymotrypsin solutions at 37 °C for 3 h. After the immersion, the oligopeptide microarray was withdrawn from the solution, washed thoroughly with deionized water, and dried under a stream of purified nitrogen.

Fabrication of LC Cells. A hybrid LC cell was made by facing two glass slides, one is oligopeptide-modified and the other is DMOAP-coated glass, by using two strips of spacer (~6 μm) and two binder clips. After the LC cell was made, approximately 40 μL of 5CB was drawn into the cavity formed between the two glass slides by using capillary force. The optical appearance of the sample was observed by using a polarizing optical microscope (Nikon ECLIPSE LV100POL, Japan) in the transmission mode. Each image was captured by a digital camera mounted on the microscope with an exposure time of 25 ms.

RESULTS AND DISCUSSION

Preparation of Oligopeptide Microarrays. Our first goal was to build an oligopeptide microarray with well-orientated, covalently immobilized oligopeptides. The strategy we adopted was to label an oligopeptide **P1** with an N-terminal cysteine.[24] The N-terminal cysteine can react with an aldehyde-decorated surface and form a thiazolidine ring (in the absence of reducing agents) and prevent lysines from reacting with aldehydes. Figure 1a shows the green fluorescence image of the **P1** microarray after it was immersed in FITC solution (as a free amine marker). The green fluorescence in Figure 1a confirms the presence of free lysine groups on the surface and suggests that an oligopeptide with an N-terminal cysteine label can be immobilized on an aldehyde-terminated surface through a single anchoring point at the N-terminal cysteine label.[24] In contrast, when solutions of **P1** were applied to a surface without aldehyde groups, no fluorescence was observed (Figure 1b). This observation further confirms that the N-terminal cysteine-labeled oligopeptide reacted with the surface aldehyde

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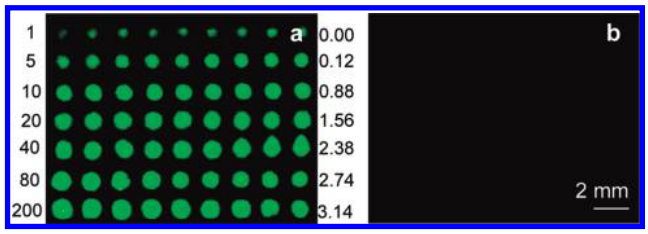

Figure 1. Fluorescence images of **P1** microarrays after they were immersed in 10 μg/mL of FITC (as a free lysine marker) for 2 h. These microarrays were built on (a) an aldehyde-terminated surface and (b) a DMOAP-coated surface. Numbers on the left of part a indicate concentrations (micromolar) of **P1** solution dispensed on the surface; numbers on the right were estimated surface densities of **P1** ($\times 10^{10}$/mm^2) based on a fluorescence intensity calibration curve (please see the Supporting Information).

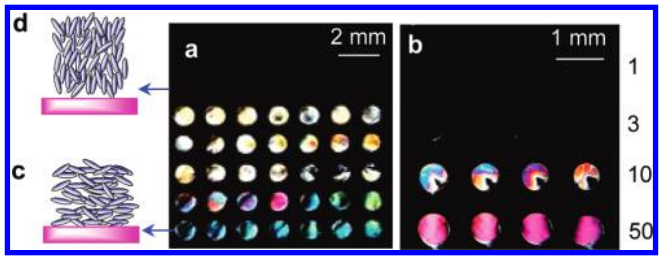

Figure 2. Optical textures (under crossed polars) of 5CB sandwiched between two DMOAP-coated glass slides. One of the DMOAP-coated glass slides was also functionalized with TEA and decorated with (a) a **P1** microarray and (b) trypsin microarray. Concentrations of **P1** are the same as those in Figure 1, and concentrations of trypsin (micrograms per milliliter) are indicated on the right. Schematics on the left are the proposed orientations of 5CB in the (c) bright region and (d) dark region.

groups. More experimental evidence for the cross-linking of the N-terminal cysteine label to the surface aldehyde groups can be found in the Supporting Information.

Optical Textures of LCs Supported on Oligopeptide Microarrays. Figure 2a shows an optical image of a LC made from a DMOAP-coated slide and a **P1** microarray, whose pattern is similar to the one shown in Figure 1. Unlike the continuous fluorescence profile in Figure 1a, the optical image of LCs shows a very clear-cutoff point when the oligopeptide concentration is below 10 μM (i.e., when the **P1** concentration is equal to or above 10 μM, 5CB appears bright, but when the **P1** concentration is below 10 μM, 5CB appears dark). The different optical textures of 5CB can be attributed to different orientations of 5CB supported on the surface. When the orientations of 5CB are disrupted by the surface immobilized **P1** as shown in Figure 2c, they give strong birefringence under crossed polarizers. In contrast, when orientations of 5CB are not disrupted by **P1** as shown in Figure 2d, 5CB gives no birefringence under crossed polarizers. The results in Figure 2 suggest that whether 5CB appears bright or dark is determined by the surface density of **P1.** More specifically, we can estimate that when the surface density of **P1** exceeds a critical value, 0.88×10^{10}/mm^2, the surface immobilized **P1** is able to disrupt 5CB. The sharp orientational transition of 5CB at the critical surface density of **P1** can be developed as a mechanism to detect protease, because proteases activities may cause the surface density of **P1** to fall below the critical value after the cleavage of **P1**.

Interactions between LCs and Proteases. Before developing a LC-based protease assay, we also need to understand the interactions between LCs and proteases. Herein, we selected trypsin as a model protease since it has been used in many studies. To determine whether trypsin can adsorb on the surface and influence the orientations of LCs, we prepared a trypsin microarray on an aldehyde-terminated surface and used it to make a LC cell. As shown in Figure 2b, if the concentration of trypsin is higher than 10 μg/mL, the optical texture of 5CB appears bright, suggesting that the orientation of 5CB can be disrupted by trypsin adsorbed on the surface if the initial trypsin concentration is too high. In contrast, 5CB remains dark if trypsin concentration is lower than 3 μg/mL. Therefore, we conclude that the maximum trypsin concentration can be used in the LCs protease assay is 3 μg/mL. If the trypsin concentration is higher than 10 μg/mL, it may adsorb on the surface and influence the orientations of LC.

Cleavage of Oligopeptides with Trypsin. Because the minimum concentration of **P1** that triggered the orientational transition of 5CB shown in Figure 2 was 10 μM (or a surface density of 0.88×10^{10}/mm^2), we created an oligopeptide microarray with **P1** concentrations ranging from 10 to 500 μM. Figure 3a shows that before cleavage of **P1**, the fluorescence images of all of **P1**-modified regions were green. Subsequently, we delivered 3 μg/mL of trypsin solution to the surface following the gradient immersion time procedure. Figure 3b shows that the fluorescence images on all of the regions appear dark after 10 h, suggesting that trypsin cleaves surface immobilized **P1** completely regardless of the surface density of **P1**. However, when the incubation time is only 2 h, the fluorescence intensities correlated with the original surface densities of **P1**. If the concentration of **P1** is lower than 40 μM (which corresponds to a surface density of 2.38×10^{10}/mm^2), the fluorescence images on **P1**-modified regions appears dark after cleavage. In contrast, if concentration of **P1** is higher than 80 μM (which corresponds to a surface density of 2.74×10^{10}/mm^2), the fluorescence images on **P1**-modified regions appear green after cleavage. These results indicate that trypsin does not cleave all of the surface immobilized **P1** within 2 h when the initial surface density of **P1** is higher than 2.74×10^{10}/mm^2. To further investigate the influence of incubation time on the cleavage of **P1**, we compared the fluorescence profiles of **P1** with two initial surface densities, 2.74×10^{10}/mm^2 and 3.47×10^{10}/mm^2, as a function of incubation time as shown in parts c and d of Figure 3. If we assume the fluorescence intensity is proportional to the surface density of **P1** (no fluorescence quenching), we can calculate the surface density of **P1** decrease to 0.91×10^{10}/mm^2 after a 2 h incubation time (Figure 3c). In contrast, Figure 3d shows that if the initial surface density of **P1** is 3.47×10^{10}/mm^2, the surface density of **P1** decrease to 2.70×10^{10}/mm^2 after the same incubation time.

LCs Protease Assays. To demonstrate that LCs can be used as an imaging material in a label-free trypsin assay, we prepared two oligopeptide microarrays with different densities of **P1** immobilized on the surface. One of the microarrays was incubated in trypsin buffer, while the other microarray was incubated in a 3 μg/mL trypsin solution (or 30 units/mL) following the gradient immersion time procedure. Parts a and b of Figure 4 show the images of LC cells made from these two microarrays. In Figure

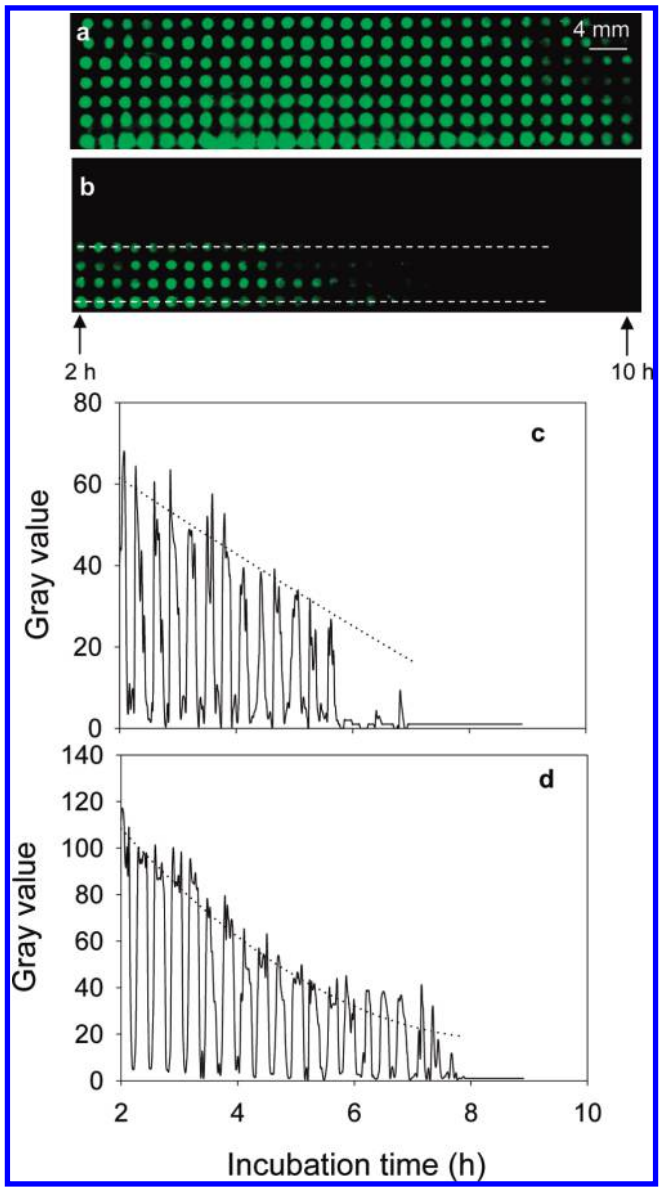

Figure 3. Fluorescence images of a complete **P1** microarray (a) before and (b) after 3 μg/mL of trypsin was delivered to the slide from right to left with a peristaltic pump with a flow rate 70 μL/min at 37 °C. Then, it was immersed in 10 μg/mL of FITC for 2 h. The concentrations of **P1** were 10, 20, 40, 80, 160, 320, and 500 μM from the top to the bottom. (c, d) Fluorescence intensity profiles obtained along the dashed line shown in part b. (c) 80 μM **P1** (surface density, 2.74×10^{10}/mm^2) and (d) 500 μM **P1** (3.47×10^{10}/mm^2).

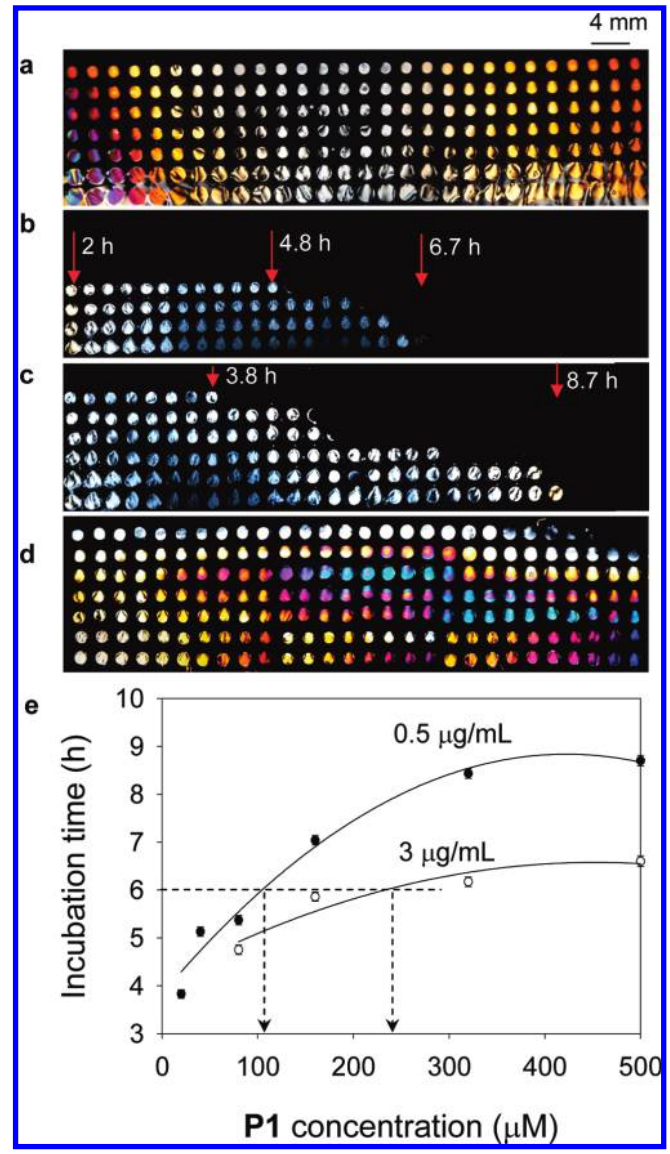

Figure 4. Optical textures (under crossed polars) of 5CB sandwiched between two DMOAP-coated glass slides. The bottom slide was also modified with TEA and droplets of **P1** solutions with various concentrations. Then (a) trypsin buffer, (b) 3 μg/mL (30 units/mL), (c) 0.5 μg/mL (5 units/mL), and (d) 0.05 μg/mL (0.5 units/mL) of trypsin were delivered to the slide, respectively, from right to left with a peristaltic pump with a flow rate 70 μL/min at 37 °C. Concentrations of **P1** are the same as those in Figure 3. (e) Minimum incubation time (in trypsin solution) required for changing a bright LC spot (caused by immobilized **P1**) to dark as a function of **P1** concentrations. Concentrations of trypsin solutions used in this experiment were 0.5 and 3 μg/mL, respectively.

4a, all **P1** spots remained bright, which suggests that the buffer solution did not affect the immobilized **P1**. In contrast, Figure 4b shows that in regions where the incubation time is longer, or where the surface density of **P1** is lower, some of the bright spots disappear. Unlike the continuous profile of fluorescence intensity in parts a or b of Figure 3, the LCs assay in Figure 4b exhibits a clear-cutoff point in each row. As a result, Figure 4b appears like an optical bar chart, which is very useful for determining the incubation time needed to cleave immobilized **P1** at different surface densities. Furthermore, when we decreased the trypsin concentration to 0.5 μg/mL (or 5 units/mL), more bright spots can be observed in Figure 4c compared to Figure 4b. These results indicate that the optical bar-chart feature of the LCs image is useful for the quantification of trypsin concentration, incubation time, and surface density of **P1**.

Detection Limit and Quantitative Analysis. We also tested the detection limit of the label-free protease assay by decreasing the concentration of trypsin further to 0.05 μg/mL (or 0.5 units/mL). As shown in Figure 4d, the images of 5CB supported on 10 μM **P1**-modified regions changed to dark after 8.7 h of incubation time. However, the images of 5CB supported on other **P1** spots remained bright. Therefore, the detection limit of this assay is at least 0.05 μg/mL, which is better than the sensitivity of a typical fluorescence array (~0.5 μg/mL).[6a] Next, we calculated the

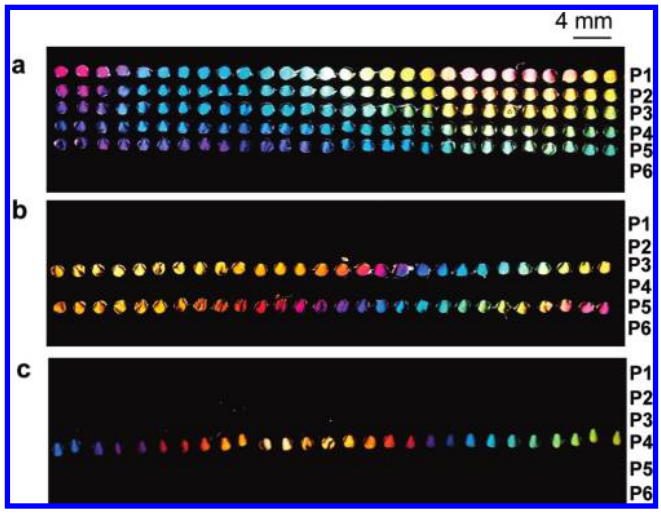

Figure 5. Optical textures (under crossed polars) of 5CB sandwiched between two DMOAP-coated glass slides. The bottom slide was also modified with TEA and circular domains of 40 μM **P1**, **P2**, **P3**, **P4**, **P5**, and **P6** in a microarray format and then incubated in (a) trypsin buffer, (b) 3 μg/mL (30 units/mL) of trypsin solution, and (c) 3 μg/mL (0.12 units/mL) of chymotrypsin solution at 37 °C for 3 h.

minimal incubation time needed to cause a spot to change from bright to dark for each **P1** concentration used to prepare the microarray. Figure 4e shows that when the trypsin concentration is 0.5 μg/mL, it takes 5.4 h to cleave immobilized **P1** having a density of 2.74×10^{10}/mm^2. In comparison, when the trypsin concentration is 3 μg/mL, only 4.7 h is needed to cleave immobilized **P1** of the same density. Figure 4e also can be used to estimate the concentration of trypsin in an unknown sample. For a **P1** microarray incubated in trypsin solution for 6 h, if 5CB supported on 160, 320, and 500 μM **P1** spots appear bright then we can estimate that the concentration of trypsin is about 0.5 μg/mL. On the other hand, if 5CB supported on 320 and 500 μM **P1** spots appear bright, while the images of 5CB supported on 160 μM **P1** spot appear dark, we can predict that the concentration of trypsin is about 3 μg/mL.

Assay Specificity. To examine the specificity of the trypsin assay, we prepared another type of oligopeptide microarray containing oligopeptides **P1**−**P6**. Among them, **P2** has the same sequence as **P1** except that **P2** does not have a C-terminal lysine residue. **P3** is also similar to **P1**, but the arginine residue, which is cleavable by trypsin, is replaced by a noncleavable histidine residue. **P5** also cannot be cleaved by trypsin because the arginine residue is followed by proline, which prevents the trypsin from cleaving the C-terminal arginine.[26] Both **P4** and **P6** can be cleaved by trypsin (their cleavage sites are detailed in Table 1).

After the microarray was prepared, we fabricated a LC cell from the microarray. Figure 5a shows that the only the last row modified with **P6** appears dark, whereas other rows modified with **P1**−**P5** appear bright. This is probably because **P6** only has nine amino acid residues (which is shorter than **P1**−**P5**) such that it cannot trigger the orientational transitions of LCs when it is immobilized on the surface. Next, we incubated another oligopeptide microarray in 3 μg/mL trypsin solution for 3 h (following the constant immersion time procedure) and then fabricated a LC cell to study whether immobilized oligopeptides **P1**−**P5** can be cleaved as expected. Figure 5b shows that the images of 5CB supported on **P1**, **P2**, and **P4** change to dark while the images on **P3** and **P5** remain bright. This result suggests that trypsin cleaves the immobilized **P1**, **P2**, and **P4** but does not cleave the immobilized **P3** and **P5** as expected. This result also implies that the cleavages of **P1**, **P2**, and **P4** by trypsin all lead to changes in the optical textures of 5CB supported on the oligopeptide microarray. These results, when combined, showcase the following two key features of the LC-based assay. First, the detection principle of this assay is general. Any oligopeptides containing specific residues and sequences may be cleaved by proteases and detected by LCs. Second, the cleavage of oligopeptide substrates by trypsin has high selectivity. It can discriminate two oligopeptide substrates (i.e., **P1** and **P3**), which only differ in one amino acid residue.

Detection of Chymotrypsin. To further test the generality of the LC-based protease assay, we selected chymotrypsin as our second model protease. Although chymotrypsin has a similar structure to trypsin, it prefers larger hydrophobic residues, including tryptophan, tyrosine, phenylalanine, leucine, and methionine. Cleavage sites on different oligopeptides available for chymotrypsin are shown in Table 1. Once again, we prepared an oligopeptide microarray with **P1**−**P6** and incubated the oligopeptide microarray in 3 μg/mL of chymotrypsin solution (or 0.12 units/mL) for 3 h before a LC cell was fabricated. Figure 5c shows that the images of 5CB supported on **P1**, **P2**, **P3**, and **P5** change to dark, while the images on **P4** remain bright. This result suggests that chymotrypsin cleaves the immobilized **P1**, **P2**, **P3**, and **P5** as expected. For **P4**, although chymotrypsin can also cleave it from the C-terminal methionine, the cleavage of one amino acid unit from **P4** does not greatly decrease the ellipsometric thickness (from 28 ± 4 to 25 ± 5 Å) and thus the orientations of 5CB remain planar. The different optical images in parts b and c of Figure 5 confirm that the LC-based protease assay is general, which clearly allows us to differentiate two proteases with similar structure, chymotrypsin and trypsin. Moreover, the combination of oligopeptide microarray and optical textures of LCs can be used to report the cleavage of oligopeptide substrates by proteases, and this procedure is simple and does not require any labels.

CONCLUSIONS

In this study, we have successfully demonstrated a simple and label-free LC-based multiplexed protease assay which can easily report enzymatic activities acting on immobilized oligopeptides. A key element of this assay is an oligopeptide microarray with well-orientated immobilized oligopeptides which were obtained by reacting N-terminal cysteine-labeled oligopeptides with an aldehyde-terminated surface. When the surface density of **P1** exceeded 0.88×10^{10}/ mm^2, the immobilized **P1** disrupted the orientations of LCs supported on the surface and changed the images of LCs from dark to bright. The dependence of orientations of LCs on the surface densities of **P1** was then exploited to detect protease activities. When a **P1** microarray was immersed in a trypsin solution by using the gradient immersion time mode, an interesting optical bar chart having clear-cutoff points was obtained. This optical bar chart is very different from the continuous intensity profile found in most fluorescence assays, and it allows us to determine the concentration of trypsin with high sensitivity and low detection limit (~0.05 μg/mL). This technique reported herein also can be

used for detecting other proteases such as chymotrypsin with high specificity or differentiating two oligopeptides with a difference in only one of the residues in their sequences.

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SUPPORTING INFORMATION AVAILABLE

Detailed description of surface characterization, HATR-FTIR, FITC array, and ellipsometry. This material is available free of charge via the Internet at http://pubs.acs.org.

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