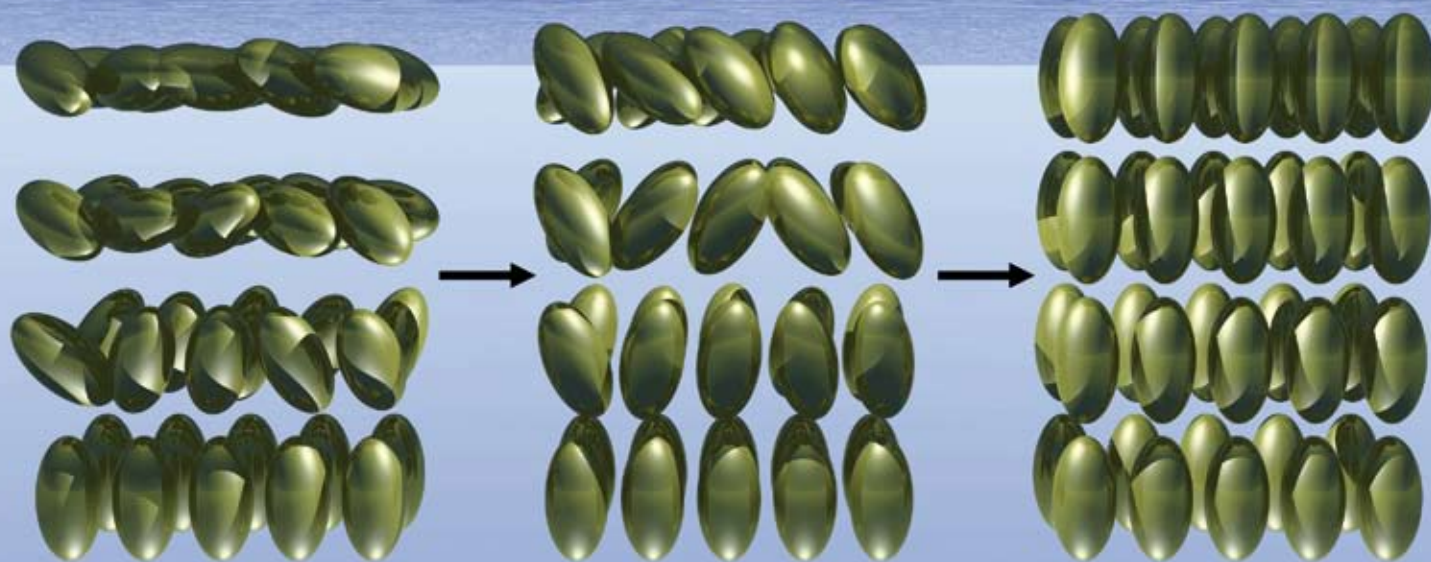


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A hydrogel-based sensor for screening protease specificity has been developed that combines the versatility of solid-phase synthesis (SPS) with the simplicity of liquid crystal display (LCD) technology.

Proteases constitute an estimated 2–3% of the mammalian proteome¹ and catalyse a myriad of fundamental biological processes. Proteases hydrolyse peptide bonds with substrate specificities defined by their preference for particular amino acid residues flanking the scissile peptide bond.² Due to their central biological role they are important in many disease states, such as cancer, HIV and Hepatitis C, which makes them important targets for the pharmaceutical industry.³

Given the medical and commercial importance of proteases, versatile screening and detection methods are required. Recent advances in this field have relied on fluorimetric detection,⁴ such as microarrays⁵ and FRET-based quantum-dot–peptide conjugates,⁶ or colorimetric detection, for example the dispersion of peptide-functionalised nanoparticle aggregates.⁷ SERRS-based detection has also been used to rapidly screen the activities of several hydrolases.⁸ However there is a need for low-cost portable sensors that will allow detection using the naked eye without requiring specialist equipment. A significant advance in methods for measuring enzyme activity was made in 2003, when Brake and Abbott demonstrated that enzyme activity could be detected using surfactant-modified liquid crystal displays (LCDs) based on 4'-n-pentyl-4-cyano-biphenyl (5CB).⁹ When in contact with water, 5CB liquid crystals align parallel at the interface and appear bright when viewed through crossed polarisers, since the 5CB layer twists incoming plane-polarised light (Fig. 1a). However adsorption of a surfactant at the aqueous–LC interface changes LC alignment from planar to perpendicular;¹⁰ incoming polarised light cannot be reorientated and the 5CB layer appears dark (Fig. 1b). Changing the surface activity of the species adsorbed at the aqueous–LC interface changes their ability to re-orientate the 5CB,¹¹ and this effect was used by Abbott *et al.* to monitor the hydrolysis of adsorbed phospholipids by phospholipase A₂.⁹ Similarly, Hoogboom *et al.* used LC realignment to measure lipase catalysed hydrolysis of esters immobilised on a glass slide.¹² Non-interfacial enzymes, like

proteases, have been sensed indirectly *via* the hydrolysis of an oligopeptide barrier membrane that had prevented surfactants from adsorbing on the LC surface.¹³

Inspired by this work, we wished to combine LCD technology with solid-phase synthesis (SPS) to develop a simple and cheap sensor system for protease activity (Fig. 1c). Using SPS removes the need to isolate and purify labelled peptides, gives access to large numbers of sequences using cheap reagents, and potentially allows automation. Only protease-catalysed release of a peptide from the SPS surface will cause LC realignment and the LC response will not depend on subtle changes in the surfactant properties of the analyte. Spatial separation of detector and analyte means that the enzyme does not have to act at an LC interface, which may be important for preventing the denaturation of non-interfacial enzymes. Herein we report proof-of-concept studies showing that a combined SPS–LCD system can discriminate between three different proteases.

Trypsin (EC 3.4.21.4), elastase (EC 3.4.21.36) and thermolysin (EC 3.4.24.27) were the proteases chosen for this trial as they have different but complementary specificities for the amino acids flanking the scissile peptide bond (Fig. 2). A polyethylene glycol acrylamide (PEGA) hydrogel was used as the SPS support. PEGA is compatible with both organic and aqueous solvents, which allowed SPS and bioanalysis to be carried out on the same platform. The three proteases chosen are small enough to penetrate the PEGA matrix¹⁴ and are known to cleave peptide sequences immobilised on PEGA beads.¹⁵ Two sequences were devised for these studies: Fmoc-Phe-Phe-Lys and Fmoc-Phe-Ala-Ala. Trypsin should

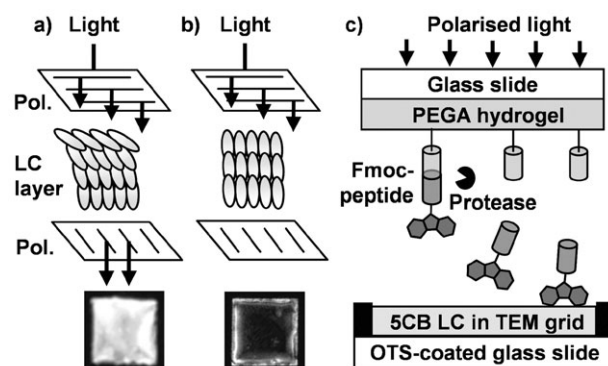


Fig. 1 (a) Plane-polarised light is transmitted through an LC layer that is aligned parallel to the surface by contact with an aqueous phase. (b) Homeotropic alignment caused by adsorption of a surfactant (e.g. sodium dodecyl sulfate) at the aqueous–LC interface causes the LC well to appear dark (shown underneath) when viewed through crossed polarisers (Pol.). (c) Schematic representation of the sensor chamber in our combined SPS–LCD protease sensor.

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† Electronic supplementary information (ESI) available: Experimental details, including the preparation of LC chambers, pixel-counting histograms and HPLC–LCMS analyses. See DOI: 10.1039/b805321a

Table 2 Percentage of Fmoc groups lost from Fmoc-peptide-PEGA layers on quartz slides after 24 h incubation with enzyme solutions

Enzyme	Fmoc-Phe-Phe-Lys-PEGA	Fmoc-Phe-Ala-Ala-PEGA
Trypsin	49 ± 5	11 ± 8
Elastase	6 ± 4	56 ± 8
Thermolysin	75 ± 1	87 ± 1

Attempts to use HPLC to identify the products resulting from the proteolytic digestion of Fmoc-peptide-PEGA-coated slides were unsuccessful due to the low concentrations of Fmoc-peptide produced (<0.01 mM). Therefore to obtain higher concentrations of the product Fmoc-peptides, the same sequences were synthesised using larger quantities of PEGA-hydrogel beads. The Fmoc-peptide functionalised beads (50 mg) were then treated with enzyme solutions (1 mg enzyme in 1 mL) for 2 h and the resulting digest solutions analysed by HPLC and LCMS. The expected Fmoc-Phe-Phe-Lys (29%) or Fmoc-Phe (95%) fragments were observed after treatment of Fmoc-Phe-Phe-Lys-PEGA with trypsin or thermolysin respectively, while treatment of the same sequence with elastase gave only a small amount of Fmoc-Phe (~2%).¹⁸ Similarly, when Fmoc-Phe-Ala-Ala-PEGA was treated with trypsin only very low quantities of Fmoc-Phe, Fmoc-Phe-Ala and Fmoc-Phe-Ala-Ala (<4% combined) were observed. Incubation of Fmoc-Phe-Ala-Ala-PEGA with thermolysin gave Fmoc-Phe as expected (82%), while Fmoc-Phe-Ala-Ala was the predominant fragment observed after incubation with elastase (39%); interestingly the anticipated Fmoc-Phe-Ala product was only present in small quantities. However, inspection of the structure of PEGA suggests the CH₂CH(CH₃)NH- termini are structurally analogous to Gly or Ala, and would provide an alternative cleavage site; elastase is known to be specific for both Ala-Ala and Ala-Gly peptide links.²

To build on these proof-of-concept studies, the next challenge is to improve response times and sensitivity. Sensitivity seems to be comparable with UV-Visible spectroscopic detection, but the strong LC response to the zwitterionic Fmoc-Phe-Phe-Lys fragment suggests that increasing fragment amphiphilicity should improve sensitivity. We believe the slow response of the sensor (~8 h) is due to retarded diffusion of the proteases and peptide fragments in the PEGA gel; externally added Fmoc-Phe gave an LC response within 2 h. Such retarded diffusion could be due to hydrophobic or electrostatic interactions with the PEGA matrix^{15b} and may be solved by using other SPS supports.

This is the first combined SPS-LCD system that can detect and discriminate between proteases. Fmoc-peptide sequences can be rapidly synthesised by SPS and screened against proteases, with a positive response indicated by an optical change in the LCD. Positive responses correlated with the loss of Fmoc from the SPS surface and the expected peptide fragments were identified by HPLC and LCMS. Spatial

separation between the analyte (the peptide on the hydrogel SPS surface) and the detector (the LCD) increases the potential of LCD detection for non-interfacial enzymes and should allow the expansion of this SPS-LCD system to other enzyme classes. This combination of rapid and low-cost SPS with low-cost, portable and potentially naked-eye LCD detection should enable the development of versatile sensors for proteases and other enzymes.

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18. Please see the Supplementary Information for further details.
19. Low sodium dodecyl sulfate concentrations are known to cause similar brushstroke patterns in 5CB wells. Please see ref. 16.
20. All of the LC surface in each well was analysed by pixel counting to give the total change in brightness. No data were excluded.
21. These quartz slides also allowed Fmoc-peptide loading on the PEGA to be quantified after each coupling and deprotection stage.