Published in final edited form as:

Surf Interface Anal. 2006 November ; 38(11): 1505–1511.

Controlling protein retention on enzyme-responsive surfaces

Rachel E. Rawsterne¹, Julie E. Gough¹, Frank J. M. Rutten³, Nhan T. Pham⁴, Wilson C. K. Poon⁴, Sabine L. Flitsch^{2,5}, Beatrice Maltman⁵, Morgan R. Alexander³, and Rein V. Ulijn^{1,2,*}

¹ School of Materials, The University of Manchester, Grosvenor Street, Manchester, M1 7HS, UK

² Manchester Interdisciplinary Biocentre (MIB), The University of Manchester, 131 Princess Street, Manchester, M1 7ND, UK

³ Laboratory of Biophysics and Surface Analysis, School of Pharmacy, The University of Nottingham, NG7 2RD, UK

⁴ School of Physics, The University of Edinburgh, James Clerk Maxwell Building, The King's Buildings, Mayfield Road, Edinburgh, EH9 3JZ, UK

⁵ School of Chemistry, The University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh, EH9 3JJ, UK

Abstract

The ability to change the properties of solid surfaces on demand is a key component of a multitude of established and emerging technologies. Stimuli that have previously been used to trigger changes in surface properties include changes in solvent, light, pH, ionic strength, temperature and magnetic or electric fields. We are interested in developing surfaces that can be triggered by the catalytic action of enzymes. We demonstrate the selective protease (α -chymotrypsin and thermolysin) catalysed peptide hydrolysis of surface-tethered fluorenylmethoxycarbonyl-dipeptides. We highlight some of the challenges evident from surface analysis in overcoming enzyme retention to the surface addressed by physical adsorption of soluble PEG₂₀₀ to the surface prior to enzyme exposure. Analysis by ToF-SIMS and XPS shows that α -chymotrypsin is deposited and retained on the surfaces and that thermolysin, a much more stable enzyme, selectively cleaves the tethered peptides as intended, and is removed from the surface by washing.

Keywords

biomaterials; peptide; responsive surface; enzyme

INTRODUCTION

Responsive surfaces that can change properties upon application of external stimuli have applications in dynamic regulation of surface properties such as wettability, roughness and chemical composition. Stimuli that have previously been used to trigger surface modifications include changes in solvent,¹⁻⁴ light,^{5,6} pH,⁷⁻⁹ ionic strength,⁷⁻⁹ temperature¹⁰ and magnetic or electric fields.¹¹⁻¹⁴ For applications in biomedical contexts, these stimuli may not be ideal since they are all non-selective and/or disruptive to biological interactions. An alternative approach is to utilise enzymes as biological stimuli to trigger surface responses.¹⁵ Use of enzymes is attractive because they are uniquely chemo-, regio-

and enantio-selective; they work naturally under mild conditions (aqueous solutions, pH 5–8); and they commonly catalyse reactions near surfaces *in vivo* and are therefore well equipped for reactions at interfaces.¹⁶ Future applications of enzyme-responsive surfaces are therefore anticipated in biological settings, for example, in dynamically controlling the interactions of cells with synthetic materials.¹⁷

Earlier work by Turner *et al.* demonstrated that enzymatic modification of surface-tethered molecules is possible, in principle, by the cleavage of surface-bound phosphate esters by a lipase; however, it was observed that non-specific adsorption of protein molecules to the surface limited the effectiveness of this system.¹⁸ This problem was solved by using enzymes (chymotrypsin) covalently bound to silica beads,¹⁹ a method that both eliminated enzyme adsorption to the surface and allowed for enzyme recovery. Yeo and Mrksich have shown that polyethylene glycol (PEG) surfaces modified with 4-hydroxyphenyl valerate could be used to enzymatically switch a redox-inactive surface to a redox-active surface by de-acylation.²⁰ More recently, cells have been genetically engineered that express this enzyme at the membrane surface, thereby allowing for biospecific communication between cells and electrodes.²¹ Surface patterning by an enzymatic reaction was shown by Hyun *et al.* who demonstrated the use of an AFM tip that was dipped in a solution of a DNA-hydrolysing enzyme to pattern DNA surfaces.²² In another study, an AFM tip was used to pattern a supported gel-phase lipid bilayer by creating local deformations in the bilayer to provide access to a phospholipase.²³ Takeda *et al.* used an AFM to tether proteases capable of specific digestion of surface-bound peptides.²⁴ Recent work by Riemenschneider *et al.* also demonstrated the use of an AFM tip dipped in enzyme, in this case alkaline phosphatase molecules, to accurately position and therefore control enzyme activity on a surface.²⁵ Enzyme-cleavable groups on the surface of nanoparticles have been used to trigger their assembly/disassembly in enzyme biosensing applications. Kanaras *et al.* used gold nanoparticles coupled with thiol-modified DNA strands to manipulate nanostructures via selective enzymatic cleavage.²⁶

In this paper we investigate the use of α -chymotrypsin (from bovine pancreas) and thermolysin (from *Thermoproteolyticus rokko*), two enzymes with complementary but opposite specificities for amino acids flanking the cleavable bond, to selectively cleave two different surface-immobilised dipeptides modified with hydrophobic capping groups (*N*-fluorenylmethoxycarbonyl, Fmoc) (Table 1). By choosing a peptide linker that is recognised selectively, it may be possible to develop surfaces that can be programmed to respond exclusively to target enzymes. For example, an Fmoc-Phe-Gly-modified surface is expected to be responsive mainly to chymotrypsin, whereas an Fmoc-Gly-Phe surface would respond preferentially to thermolysin. In this paper we focus on characterisation of these enzyme-responsive surfaces using ToF-SIMS and XPS, and specifically consider the control of non-specific enzyme adsorption at the surface and approaches to reduce adsorption.

EXPERIMENTAL

Sample preparation

All dipeptide functionalised surfaces were prepared on glass coverslips that were cleaned for 30 min in Piranha solution (30 : 70 30% H₂O₂ : conc H₂SO₄), rinsed thoroughly with deionised water and dried overnight at 100 °C.

To introduce primary amines at the surface for subsequent dipeptide coupling, the coverslips were rinsed with ethanol, dried in a stream of oxygen-free nitrogen, immersed in a 1% (3-aminopropyl)triethoxysilane (APS) solution in water/ethanol for 1 h, rinsed with ethanol, dried in a stream of oxygen-free nitrogen, baked at 50 °C for 10 min, rinsed with ethanol and finally dried in a stream of oxygen-free nitrogen (Fig. 1, step a).

Three different experimental methods developed to control non-specific enzyme adsorption are compared in this paper, each of which will be described in detail and the differences between them highlighted. Briefly, Method 2 (extra wash) incorporated extra rinsing steps compared to Method 1 (wash), while Method 3 (PEG₂₀₀) involved pre-treating the surfaces with soluble PEG₂₀₀ before carrying out enzyme treatment.

Method 1 (wash)—Following silane functionalisation of the glass coverslips, *N*-fluorenylmethoxycarbonyl (Fmoc)-protected dipeptides [either Fmoc-Glycine-Phenylalanine (Fmoc-Gly-Phe) or Fmoc-Phenylalanine-Glycine (Fmoc-Phe-Gly)] were coupled to the surface (Fig. 1, step **b**) by immersing the substrates in a solution comprising 0.2 mmol Fmoc-dipeptide, 0.4 mmol 1-hydroxybenzotriazole (HOBt) and 0.4 mmol *N,N'*-diisopropylcarbodiimide (DIC) in 10 ml dry *N,N*-dimethylformamide (DMF) for 2 1/2 h. Each sample underwent the following rinsing scheme: dry DMF, ethanol, 25 : 75 ethanol : deionised water, deionised water, ethanol, and then dried in a stream of oxygen-free nitrogen. To maximise surface immobilisation of the Fmoc-protected dipeptides, each sample was immersed again overnight in a fresh solution as before (known as 'double coupling'), followed by the same rinsing scheme.

Incubation with enzymes (Fig. 1, step **c**) was performed by immersing the Fmoc-protected dipeptide surfaces **C** in a 4 μM aqueous solution of either α -chymotrypsin (from bovine pancreas) or thermolysin (from *Thermoproteolyticus rokko*) with a control of deionised water (Fig. 1, step **c**), for 1 h at 37 °C and 5% CO₂. Each sample then underwent the following rinsing scheme: buffer, 45 : 45 : 10 acetonitrile (ACN) : deionised water : formic acid, 25 : 75 ethanol : deionised water, deionised water, ethanol, and were then dried in a stream of oxygen-free nitrogen prior to analysis by water contact angle measurement, ToF-SIMS and XPS.

Method 2 (extra wash)—Fmoc-protected dipeptides were coupled to the aminefunctionalised surfaces as described in Method 1. However, this second method differed slightly from the first in that two extra rinsing steps were introduced: 1% trifluoroacetic acid (TFA) in ACN, dry DMF, 1% TFA in ACN, ethanol, 25 : 75 ethanol : deionised water, deionised water, and ethanol. The samples were again dried in a stream of oxygen-free nitrogen. Enzyme treatment and subsequent rinsing schemes were carried out in the same way as described in Method 1.

Method 3 (PEG₂₀₀)—Fmoc-protected dipeptides were again coupled to the aminefunctionalised surfaces as described in Method 1, and the new rinsing scheme used in Method 2 was also employed here. This method differed from the previous two by the addition of an extra step: prior to immersing the samples in the enzyme solutions, all samples were first incubated at 37 °C and 5% CO₂ in an aqueous 5% by volume solution of soluble PEG₂₀₀ for 18 h. This procedure has been taken from a previously established protocol.²⁷ Subsequent incubation with enzymes was then carried out as described in Method 1. The rinsing procedure following exposure to enzymes was modified further using the extra rinsing steps developed in Method 2 as follows: 1% TFA in ACN, buffer, 45 : 45 : 10 ACN : deionised water : formic acid, 25 : 75 ethanol : deionised water, deionised water, and ethanol. The samples were again dried in a stream of oxygen-free nitrogen prior to analysis.

Water contact angle analysis

Water droplet contact angle measurements were performed on dipeptide surfaces both prior and subsequent to enzyme hydrolysis. These were done using a CAM200 Optical Contact Meter (KSV Instruments Ltd, Helsinki, Finland) and the sessile drop method was used

throughout: a droplet of deionised water (3 μl , measured using a precision syringe) was formed at the end of the needle and lowered onto the surface, and the needle withdrawn until the drop detached itself. The contact angle was measured after the drop had come to rest (approximately 2 s) between the intersection of a fit of the drop shape to the Young–Laplace equation and a manually positioned substrate baseline. Both sides of three drops on different areas of the sample were measured, and the average is quoted.

ToF-SIMS analysis

Secondary ion mass spectrometric analysis was carried out using a ToF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany) equipped with a gallium liquid metal ion gun and a single-stage reflectron analyser. Typical operating conditions were: a primary ion energy of 15 kV, a pulsed target current of approximately 1.3 pA and post-acceleration of 10 kV. Low-energy electrons (20 eV) were used to compensate for charging due to the positive primary ion beam. Large-scale images were acquired by rastering the stage under the pulsed primary ion beam, using a raster of 64×64 pixels. All doses were kept well below the static limit, with a maximum dose of 10^{12} ions per cm^2 for both polarities combined. Acquisition of full raw datasets allowed for the retrospective construction of spectra from the imaged areas. Positive spectra were normalised to the intensity of the common C_2H_3^+ fragment for comparison.

XPS analysis

XPS was carried out on a Kratos Axis Ultra (Kratos Analytical Ltd, Manchester, UK) using a monochromated beam from an aluminium source run at 150 W. A take-off angle of 90° was used, and all samples were analysed with the charge neutraliser on. The survey spectra were collected using a pass energy of 80 eV and the C 1s, N 1s and O 1s core level spectra were collected at a pass energy of 20 eV. Since charge neutralisation was used, the spectra were corrected to position the C–C within the C 1s core level at a binding energy of 285.0 eV. Symmetrical sum Gaussian/Lorentzian 30% peak shapes were used for all components and shifts are quoted relative to the C–C component at 285.0 eV.

RESULTS AND DISCUSSION

A uniform monolayer of primary amine groups was successfully introduced onto glass slides using APS (Fig. 1, step **a**). The nitrogen 1s core level (not shown) indicated that in addition to this molecular orientation a significant proportion of protonated amines were present, interpreted as interaction of the amine end with surface hydroxyl groups.²⁸ Next, 9-fluorenylmethoxycarbonyl (Fmoc) protected dipeptides (either Fmoc-Gly-Phe or Fmoc-Phe-Gly) were immobilised directly onto the surface primary amines using DIC/HOBt coupling methods in DMF (Fig. 1, step **b**). The Fmoc capping group (Fig. 1, surface **C**) is particularly useful for ToF-SIMS analysis of surfaces since it gives rise to two distinct high-intensity positive ion fragments at $m/z = 165$ D and 179 D.^{29,30} Figure 2(A) shows 5×5 mm intensity maps of these fragments, and that of Si, for each surface during the modification process, and clearly shows a homogeneous distribution at the millimetre scale. At higher magnification, a similar uniform distribution was detected on the micrometre scale (not shown). The intensity of the Fmoc fragments indicates that the Fmoc functionalities are present only on peptide-modified surfaces (Fig. 1, surface **C**). This is further confirmed by the spectra shown in Fig. 2(B), where peaks at $m/z = 165$ D and 179 D are present only on the Fmoc-Gly-Phe and Fmoc-Phe-Gly-modified surfaces. In addition, the intensity maps for Si (Fig. 2(A)) decreased, indicating coverage of the Si by the Fmoc-dipeptides.

XPS was carried out to provide a complementary quantitative analysis and Fig. 2(C) shows the C 1s core levels from glass, silanated glass (APS) and both Fmoc-protected dipeptides. It

is clear that after APS surface modification (Fig. 1, steps **b** and **c**) a greater variety of carbon species is formed, i.e. on glass (Fig. 1, surface **A**) there is only hydrocarbon C–C; the silanated glass surface (Fig. 1, surface **B**) has C–N (at a shift of 1.6 eV) and C=O or O–C–O (at a shift of 3.3 eV) in addition to the C–C; and the Fmoc-dipeptide surfaces (Fig. 1, surface **C**) also have a peak (at a shift of 4.5 eV) corresponding to the carbamate group, O–C(=O)–N, that links the fluorenyl to the peptide (Fig. 1, surface **C**).

Having produced and characterised homogeneous surfaces, the next step was to investigate enzyme hydrolysis of these surfaces using α -chymotrypsin and thermolysin. Since the ultimate aim of this work is to produce enzyme-responsive surfaces, i.e. those that change their properties and functions upon selective enzymatic cleavage, changes in surface wettability were initially used as an indicator of surface switching. Removal of the hydrophobic Fmoc-protecting group to reveal the amino acid was expected to reduce the water contact angle following enzyme treatment. Fmoc-dipeptide surfaces (Fig. 1, surface **C**) all had water contact angles of approximately 72°, independent of the underlying amino acids, indicating good coverage of the surface with the Fmoc moiety. This is in agreement with values given by Frutos *et al.* measured on a gold-thiolate Fmoc self-assembled monolayer system.³¹ Table 2 shows that the expected water contact angles for chemically synthesised Phe and Gly are 61° and 48°, respectively. Therefore, these values are what would be expected upon enzymatic cleavage for Fmoc-Gly-Phe (with thermolysin) and Fmoc-Phe-Gly (with α -chymotrypsin).

The water contact angles measured following enzyme treatment are lower than the value for chemically synthesised Phe (the product if cleavage occurred), suggesting that enzyme has adsorbed on to the surface lowering the water contact angle. This was corroborated by ToF-SIMS results, which showed extensive tryptophan (C₉H₈N) retention (Fig. 3). The tryptophan ion is a good marker for enzyme retention since it gives rise to a distinct (positive) ion at $m/z = 130$ D, and both enzymes contain this amino acid (Table 1) – chymotrypsin contains eight tryptophan units per molecule of enzyme (254 amino acid residues in total); thermolysin contains three tryptophan units per molecule of enzyme (315 amino acid residues in total). Similarly, thermolysin exposure also lowered the water contact angles, with Fmoc-Gly-Phe to a greater extent than Fmoc-Phe-Gly. ToF-SIMS results (Fig. 3) again showed enzyme retention, suggesting that the water contact angles are affected by this layer of enzyme.

In order to further reduce enzyme adsorption to the surface, extra rinsing steps were introduced (Method 2), namely, 1% TFA in ACN before and after rinsing with DMF. Again, water contact angles (Table 2) showed a reduction for both dipeptide surfaces with both enzymes although to a lesser extent than using Method 1. The relative intensity of tryptophan revealed by ToF-SIMS in Fig. 3 shows that chymotrypsin adsorption was reduced, while thermolysin adsorption remained at a similar level.

Since enzyme retention at the surface still appeared to be a significant problem, we developed Method 3, wherein all Fmoc-dipeptide surfaces were immersed in a soluble PEG₂₀₀ solution for 18 h prior to exposure to enzymes.^{32,33} Since the PEG₂₀₀ is physisorbed to the surface, it is thought to sterically shield the surface to prevent interaction with the protein and therefore prevent protein adsorption. Using this method, the measured water contact angles (Table 2) exhibit larger variance as indicated by the standard deviations, suggesting uneven PEG₂₀₀ retention at the surface. This was confirmed by ToF-SIMS, where the (positive) ions at $m/z = 31$ D and $m/z = 45$ D, corresponding to CH₃O⁺ and C₂H₅O⁺ PEG fragments, respectively, were present and exhibited similar intensities on all surfaces (not shown). ToF-SIMS shows that enzyme retention is significantly decreased

compared to Methods 1 and 2, since the intensity of the tryptophan residue is the lowest for both dipeptides following exposure to both enzymes for this method (Fig. 3).

Having succeeded in significantly reducing enzyme retention, we then analysed surfaces after enzyme hydrolysis of Fmoc-dipeptides (Fig. 1, step c). While ToF-SIMS is a useful tool for identification of molecular species, it is often unreliable for quantification; therefore XPS analysis was undertaken to follow the selective enzymatic cleavage of Fmoc-protected dipeptides. Figure 4 shows the XPS C1s core level spectra from the surfaces produced following exposure to enzymes in Method 3. All surfaces contain C–C (at 285 eV), C–N (at a shift of 1.6 eV) and C=O/O–C–O (at a shift of 3.3 eV) that form part of the Fmoc-dipeptide and APS structures immobilised to the glass substrates. The O–C(=O)–N (carbamate) group links the fluorenyl to the peptide, and examination of Fig. 4 indicates Fmoc is always present (at a shift of 4.5 eV) to some extent even after enzyme treatment. However, the proportion of carbamate present after enzyme treatment suggested that in all cases enzyme treatment has resulted in enzymatic hydrolysis, with the trend expected on the basis of enzyme selectivity (Fmoc-Gly-Phe preferentially cleaved by thermolysin and Fmoc-Phe-Gly preferentially cleaved by chymotrypsin). For Fmoc-Gly-Phe following enzyme treatment compared to the water incubated control, chymotrypsin reduces the carbamate content by 13% and thermolysin reduces it by 19%. The reduction for chymotrypsin suggests that the enzyme may have cleaved the bond on the carboxylic acid side of Phe, an observation previously reported by Turner *et al.* using SIMS and XPS in a similar system, although this work used chymotrypsin covalently bound to silica beads.¹⁹ For Fmoc-Phe-Gly, chymotrypsin has lowered the carbamate proportion more than thermolysin, by 24% and only 4%, respectively. Therefore, while hydrolysis was incomplete, the expected selectivity was observed.

The overlayer thicknesses of the peptide surfaces were calculated from the XPS carbon intensities using the Seah and Spencer method,³⁴ the results of which are shown in Fig. 5. The water control for the Fmoc-Phe-Gly layer is thicker than that of Fmoc-Gly-Phe and this can be attributed to the greater flexibility of the Fmoc-Gly-Phe molecule. The thickness of both layers is consistent with approximately a monolayer of Fmoc-protected dipeptide. An increase in carbon-containing overlayer thickness was found for Fmoc-Phe-Gly exposed to chymotrypsin, while the thickness of Fmoc-Gly-Phe remained unchanged, observations that can be explained in terms of the retention of enzyme at the surface. The overlayer thicknesses for the samples exposed to thermolysin did not show an increase in overlayer thickness. In this case a significant reduction in thickness of 2–3 Å was observed exclusively for Fmoc-Gly-Phe, suggesting that thermolysin can be used to selectively hydrolyse peptide bonds at the solid/liquid interface, in agreement with the observed reduction of the carbamate peak. This observation agrees with the documented selectivity³⁵ of this enzyme and also with results found using polymer hydrogels.³⁶ The reduced adsorption of thermolysin on the surface may be explained by its well-documented stability in harsh conditions, including organic solvents,³⁷ high salt concentrations³⁸ and high temperatures. Enzyme engineering approaches may be employed to evolve enzymes with improved activities and stabilities at the solid/liquid interface for applications that involve enzyme reactions at the solid/liquid interface.

CONCLUSIONS

We have demonstrated selective protease-catalysed peptide hydrolysis of surface-tethered dipeptides and highlighted some of the challenges in overcoming enzyme retention at the surface. Retention has been significantly decreased by first introducing extra rinsing steps and by adsorbing soluble PEG₂₀₀ to the Fmoc-dipeptide surfaces prior to exposure to enzymes. Analysis by ToF-SIMS and XPS shows that chymotrypsin is retained on the

Fmoc-dipeptide surfaces. Thermolysin selectively cleaves the peptides as expected and less is retained on the surface. The results for thermolysin may be related to its well-documented stability and the fact that it is less readily denatured on surfaces. This study suggests that improved enzyme and surface engineering strategies are required to tackle enzyme adsorption at the solid/liquid interface if enzyme-switchable surfaces are to be of general use.

Acknowledgments

We would like to thank The Leverhulme Trust for financial support of RER, and the Wellcome Trust for financial support of WCKP, NTP and SLF; the EPSRC; and Miss Emily Smith, University of Nottingham, for assistance with XPS.

REFERENCES

1. Minko S, Müller M, Motornov M, Nitschke M, Grundke K, Stamm M. J. Am. Chem. Soc. 2003; 125:3896. [PubMed: 12656624]
2. Peng J, Xuan Y, Wang HF, Yang YM, Li BY, Han YC. J. Chem. Phys. 2004; 120:11163. [PubMed: 15268146]
3. Harnish B, Robinson JT, Pei Z, Ramström O, Yan M. Chem. Mater. 2005; 17:4092.
4. Xu H, Norsten TB, Uzun O, Jeoung E, Rotello VM. Chem. Commun. 2005; 41:5157. [PubMed: 16228020]
5. Abbot S, Ralston J, Reynolds G, Hayes R. Langmuir. 1999; 15:8923.
6. Ichimura K, Oh SK, Nakagawa M. Science. 2000; 288:1624. [PubMed: 10834837]
7. Matthews JR, Tuncel D, Jacobs RMJ, Bain CD, Anderson HL. J. Am. Chem. Soc. 2003; 125:6428. [PubMed: 12785782]
8. Ionov L, Houbenov N, Sidorenko A, Stamm M, Luzinov I, Minko S. Langmuir. 2004; 20:9916. [PubMed: 15518474]
9. Burke SE, Barrett CJ. Biomacromolecules. 2003; 4:1773. [PubMed: 14606908]
10. Hyun J, Lee W-K, Nath N, Chilkoti A, Zauscher S. J. Am. Chem. Soc. 2004; 126:7330. [PubMed: 15186170]
11. Lahann J, Mitragotri S, Tran T-N, Kaido H, Sundaram J, Choi IS, Hoffer S, Somorjai GA, Langer R. Science. 2003; 299:371. [PubMed: 12532011]
12. Chrisey LA, Lee GU, O'Ferrall CE. Nucleic Acids Res. 1996; 24:3031. [PubMed: 8760890]
13. Charles PT, Vora GJ, Andreadis JD, Fortney AJ, Meador CE, Dulcey CS, Stenger DA. Langmuir. 2003; 19:1586.
14. Ren S, Yang S, Zhao Y. Langmuir. 2003; 19:2763.
15. Ulijn RV. J. Mater. Chem. 2006; 16:2217.
16. Halling PJ, Ulijn RV, Flitsch SL. Curr. Opin. Biotechnol. 2005; 16:385. [PubMed: 16005203]
17. Mrksich M. Curr. Opin. Chem. Biol. 2002; 6:794. [PubMed: 12470733]
18. Turner DC, Peek BM, Wertz TE, Archibald DD, Geer RE, Gaber BP. Langmuir. 1996; 12:4411.
19. Turner DC, Testoff MA, Conrad DW, Gaber BP. Langmuir. 1997; 13:4855.
20. Yeo WS, Mrksich M. Angew. Chem., Int. Ed. Engl. 2003; 42:3121. [PubMed: 12866096]
21. Collier JH, Mrksich M. Proc. Natl. Acad. Sci. U.S.A. 2006; 103:2021. [PubMed: 16461913]
22. Hyun J, Kim J, Craig SL, Chilkoti A. J. Am. Chem. Soc. 2004; 126:4770. [PubMed: 15080668]
23. Clausen-Schaumann H, Grandbois M, Gaub HE. Adv. Mater. 1998; 10:949.
24. Takeda S, Nakamura C, Miyamoto C, Nakamura N, Kageshima M, Tokumoto H, Miyake J. Nano Lett. 2003; 3:1471.
25. Riemenschneider L, Blank S, Radmacher M. Nano Lett. 2005; 5:1643. [PubMed: 16159199]
26. Kanaras AG, Wang Z, Bates AD, Cosstick R, Brust M. Angew. Chem., Int. Ed. Engl. 2003; 42:191. [PubMed: 12532347]

27. Deere, J. Personal Communication. Department of Pure and Applied Chemistry, University of Strathclyde; 2005.
28. Kowalczyk D, Slomkowski S, Chehimi MM, Delamar M. *Int. J. Adhes. Adhesives*. 1996; 16:227.
29. Drouot C, Enjalbal C, Fulcrand P, Martinez J, Aubagnac J-L, Combarieu R, de Puydt Y. *Tetrahedron Lett.* 1997; 38:2455.
30. Enjalbal C, Maux D, Subra G, Martinez J, Combarieu R, Aubagnac J-L. *Tetrahedron Lett.* 1999; 40:6217.
31. Frutos AG, Brockman JM, Corn RM. *Langmuir*. 2000; 16:2192.
32. Lee S-W, Laibinis PE. *Biomaterials*. 1998; 19:1669. [PubMed: 9840002]
33. Krsko P, Libera M. *Mater. Today*. 2005; 8:36.
34. Seah MP, Spencer SJ. *Surf. Interface Anal.* 2002; 33:640.
35. Henrikson RL. *Methods Enzymol.* 1977; 47:175. [PubMed: 927174]
36. Thornton PD, McConnell G, Ulijn RV. *Chem. Commun.* 2005; 47:5913. [PubMed: 16317473]
37. Ulijn RV, Janssen AEM, Moore BD, Halling PJ, Kelly SM, Price NC. *ChemBioChem*. 2002; 3:1112. [PubMed: 12404637]
38. Erbeldinger M, Ni X, Halling PJ. *Biotechnol. Bioeng.* 2001; 72:69. [PubMed: 11084596]

Figure 1.

Procedure to make homogeneous chemically functionalised surfaces: **A** is the glass coverslip; **(a)** is the silanisation step using APS; **B** is the surface presenting free amines; **(b)** is the standard DIC/HOBt coupling steps; **C** is the surface modified with either Fmoc-Gly-Phe or Fmoc-Phe-Gly; **(c)** is the enzyme hydrolysis step; and **D** is the hydrolysed surface presenting either Gly or Phe.

Figure 2.

Surface functionalisation followed by ToF-SIMS and XPS. (a) comprises 5×5 mm intensity maps of various positive ions from each surface during each stage of Fmoc-dipeptide preparation, i.e. glass, silanated glass (APS), Fmoc-Gly-Phe and Fmoc-Phe-Gly. The fragments are Si at $m/z = 28$ D, Fmoc at $m/z = 165$ D (C₁₃H₉) and 179 D (C₁₄H₁₁). (b) SIMS spectra for the Fmoc fragments $m/z = 165$ D (C₁₃H₉) and 179 D (C₁₄H₁₁) normalised to the C₂H₃ ion for glass, silanated glass (APS), Fmoc-Gly-Phe and Fmoc-Phe-Gly. (c) XPS C 1s core level spectra for glass, silanated glass (APS) and both Fmoc-protected dipeptides.

Figure 3. Histogram showing the relative intensity of tryptophan (i.e. enzyme retention) for each stage of each method. Values are means where $n = 3$, $\pm$ SD.

Figure 4.

XPS C 1s core level spectra for both Fmoc-dipeptide surfaces following incubation with both enzymes and the water controls using Method 3. Note that the C-N peaks from both dipeptide surfaces following enzyme exposure appear to be more developed than those on the as-prepared surfaces shown in Fig. 2(C), which can be attributed to a small contribution from PEG in the form of C-O, since it appears at approximately the same chemical shift (1.6 eV).

Figure 5. Overlayer thickness of both Fmoc-dipeptide surfaces following incubation with both enzymes and the water controls calculated using the Seah and Spencer method.³⁴ Values are means where $n = 2$, $\pm$ maximum/minimum.

Primary substrate specificities and molecular weights of the different proteases used in this work. Note the difference in number of tryptophan units in each enzyme. P1 and P1' refer to the amino acids directly adjacent to the cleaved amide bond

Table 2

Average water contact angles of each type of surface using Method 1 (wash), Method 2 (extra wash) and Method 3 (PEG₂₀₀). All dipeptide surfaces in this table had a water contact angle of 72° prior to enzyme exposure. Values are means where $n = 6$, $\pm$ SD

Sample	Method 1 (wash)	Method 2 (extra wash)	Method 3 (PEG ₂₀₀)
Phe		61° $\pm$ 1.4	
Fmoc-Gly-Phe		72° $\pm$ 2.2	
Fmoc-Gly-Phe, Chymotrypsin	57° $\pm$ 1.5	64° $\pm$ 2.9	62° $\pm$ 4.6
Fmoc-Gly-Phe, Thermolysin	55° $\pm$ 1.0	71° $\pm$ 0.8	66° $\pm$ 0.5
Fmoc-Gly-Phe, water control	67° $\pm$ 0.8	74° $\pm$ 1.2	68° $\pm$ 0.9
Gly		48° $\pm$ 3.0	
Fmoc-Phe-Gly		72° $\pm$ 2.4	
Fmoc-Phe-Gly, Chymotrypsin	56° $\pm$ 0.6	70° $\pm$ 0.9	62° $\pm$ 2.2
Fmoc-Phe-Gly, Thermolysin	68° $\pm$ 0.6	58° $\pm$ 2.5	68° $\pm$ 2.1
Fmoc-Phe-Gly, Water control	70° $\pm$ 0.5	72° $\pm$ 0.1	65° $\pm$ 2.4