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SPOT Synthesis of Peptide Arrays on Self-Assembled Monolayers and their Evaluation as Enzyme Substrates

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Functionalised self-assembled monolayers (SAMs) of alkanethiols[1] on gold surfaces are increasingly gaining in popularity for the study of biological interactions and biochemical reactions.[2] SAMs present well-defined biocompatible surfaces that are easy to prepare and are amenable to detailed physicochemical analysis of molecular structure and binding interactions, in particular through MALDI-ToF MS[3,4,5] (matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry) and SPR[6,7,8] (surface plasmon resonance). So far, a broad range of chemical and biochemical reactions have been described[5,9] that are compatible with SAMs, but in all cases the number of consecutive reaction steps conducted on the surface has been small,[10,11] in particular in array format. This raises the question of whether the platform is robust enough to withstand repeated reaction and washing cycles for long biopolymer synthesis. Here, we show that SAMs on planar gold surfaces can indeed be used to generate peptides and glycopeptides of sizes necessary for biological evaluation (up to dodecamers). Using SPOT synthesis,[12] we show that diverse arrays of peptides and glycopeptides can be generated and can subsequently be used to evaluate substrate specificity of proteases and glycosyltransferases. An important advantage of the SAM platform is that all reactions can be monitored directly on the array by using mass spectrometry, which provides quality control.

Since its introduction in the early 1990s by R. Franck,[12] the SPOT synthesis methodology has had a tremendous impact on the development of microarrays.[13] Parallel synthesis can be carried out simultaneously on a single array to generate complex libraries of compounds in a time and cost-efficient manner. Although not limited to the field of peptide synthesis,[14] proteomics has mostly benefited from the SPOT methodology and arrays of thousands of peptides have been prepared by using conventional Fmoc-based peptide chemistry.[15] Nowadays, peptide arrays prepared by SPOT synthesis are routinely used for the screening of antibody binding.[16] Furthermore, peptide–receptor, peptide–microbe and protein–protein interactions, enzyme substrate specificity and peptide–DNA interactions have been studied by using peptide arrays generated by the SPOT methodology.[17,18] However, despite the great benefits offered by this method, coupling yields can vary from one amino acid to the other, and therefore the amount and purity of peptides can also vary. Monitoring reactions on the support relies on the use of a coloured dye, and quality control of the peptide requires its cleavage from the support after completion of synthesis for subsequent solution-phase analysis by HPLC or MALDI-ToF MS.[19] Consequently, assay results obtained from an array prepared by SPOT synthesis sometimes need to be confirmed by using purified samples of peptides in a solution-phase assay.[15]

By combining the robustness and versatility of SAM-coated gold platforms and the easy-to-handle SPOT peptide synthesis methodology, an efficient method for preparing peptide arrays on gold surfaces is described herein. The method allows on-chip monitoring of the synthesis and subsequent evaluation of enzymatic modifications by MALDI-ToF mass spectrometry (Figure 1).

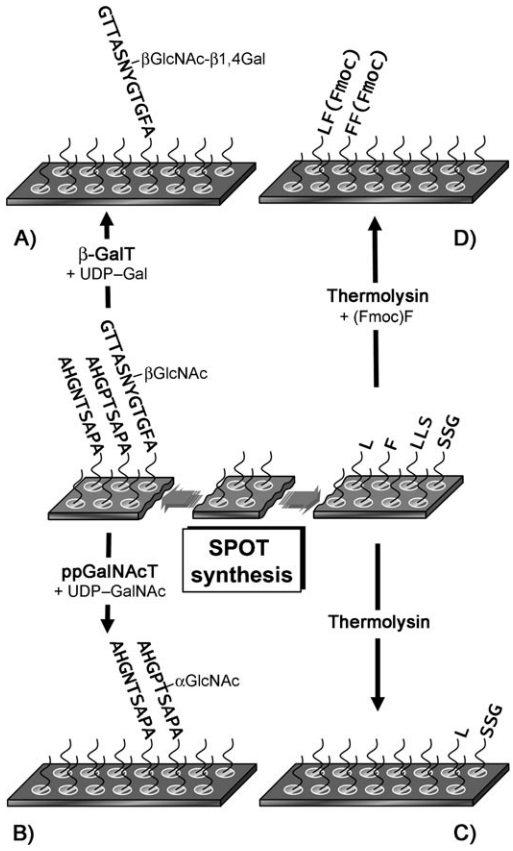

Figure 1. SPOT synthesis and biological evaluation of peptide arrays on SAM-coated gold surfaces; the single-letter code is used for amino acids. The array was probed with A) β-1,4-galactosyltransferase (β-GalT), B) polypeptide *N*-acetylgalactosaminyl transferase (ppGalNAcT), C) thermolysin, D) thermolysin in the presence of saturating amounts of Fmoc–phenylalanine.

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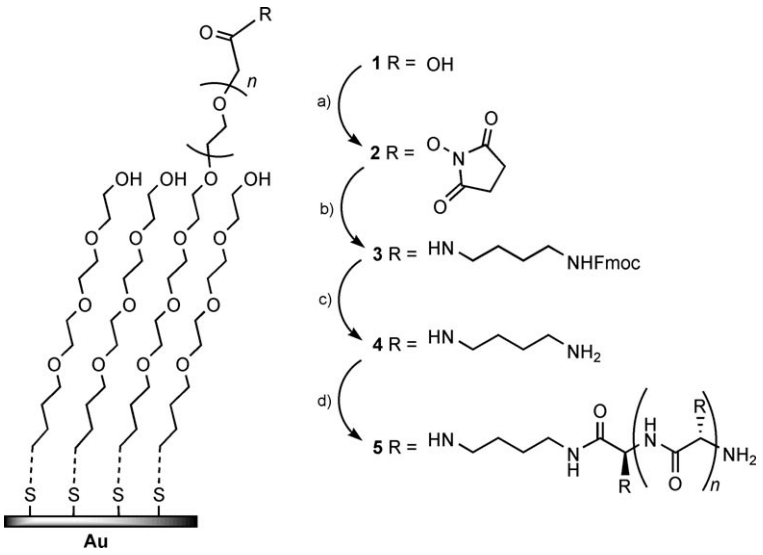

Scheme 1. Strategy for SPOT synthesis of peptide arrays on SAM-coated gold surface. a) EDC, NHS, DMF, 1 h, room temperature; b) $H_2N(CH_2)_4NHFmoc$, DMF, 16 h, room temperature; c) 20% piperidine, DMF, 10 min, room temperature; d) i) SPOT synthesis (Fmoc–amino acid, PyBOP, DIPEA, DMF then 20% piperidine, DMF); ii) 50% TFA, DCM.

Details of the SPOT synthesis of peptides on functionalised SAM surfaces are shown in Scheme 1. A disposable 64-well gold slide (Applied Biosystems) was coated with a mixed SAM terminated either by a carboxylic acid or hydroxyl group; this provided control over the surface density by mixing the two at different ratios. After activation of carboxylic acid **1** as an *N*-hydroxysuccinimide ester **2**, treatment with *N*-Fmoc diaminobutane (to give **3**) followed by removal of the Fmoc provided the amine functionality **4**, which was suitable for coupling with Fmoc-protected amino acids. Under standard peptide synthesis conditions, repeating cycles of amino acid coupling, Fmoc cleavage and final TFA-mediated side-chain deprotection afforded the peptide library **5**. Analysis of each step by MALDI-ToF MS ensured the careful control of the reactions and circumvented the need for release of the peptide from the support for solution-phase analysis.

Initial studies were conducted on a SAM composed of a 1:1 mixture of the two alkanethiols. To establish the optimal SPOT conditions, a model tripeptide, GlyLeuSer, was first synthesised (Figure 2). After preparation of the surface, FmocSer(O*t*Bu)OH was coupled by spotting an amino acid solution (100 mM; 0.4 μL per spot) in DMF, preactivated with a mixture of PyBOP (1 equiv) and DIPEA (2 equiv) for 5 min. After 1 h incubation at 37 °C, quantitative coupling (as judged by MALDI-ToF MS) was obtained (Figure 2A) with strong signals at *m/z* 1068 and 1487, which corresponded to the sodium adducts of the Fmoc–amino acid-terminated alkanethiol and the mixed disulfide formed with the tri(ethyleneglycol)-terminated alkanethiol, respectively, and complete disappearance of the starting material. Following removal of the Fmoc with a solution of piperidine (20%) in DMF, the second amino acid was introduced by coupling of FmocLeuOH under the same conditions (Figure 2B, *m/z* 1181 and 1600). Another cycle of Fmoc removal and coupling with FmocGlyOH afforded the tripeptide (Figure 2C, *m/z* 1238 and 1657), which was subsequently deprotected with piperidine (20%) and finally treated with TFA solution (50%) in DCM to ensure cleavage of the *tert*-butyl protecting group of the serine (Figure 2D, *m/z* 960 and 1379). Following these encouraging results, peptide libraries were generated and tested against a number of enzymes (Figure 1). The use of MALDI-ToF MS allowed the enzyme activity to be monitored in a label-free manner by direct analysis of the mass of the immobilised substrates.

In the first example, the substrate specificity of a protease was studied. Proteases are a large class of

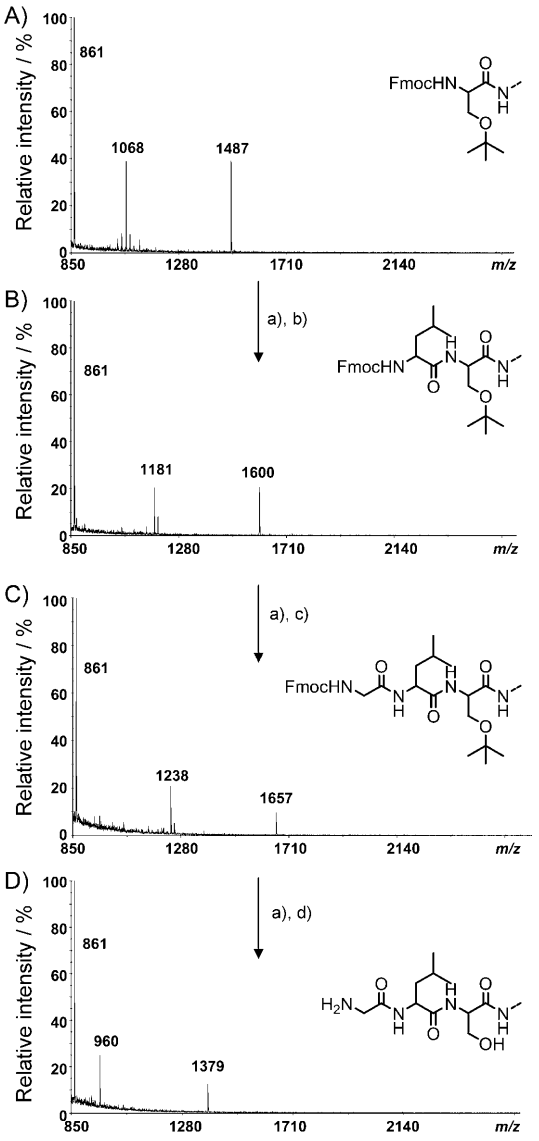

Figure 2. SPOT synthesis of GlyLeuSer on SAM as monitored by direct MALDI-ToF mass spectrometry. A) Coupling of FmocSer(O*t*Bu)OH to the surface; B) coupling of the second amino acid; C) coupling of the third amino acid; D) cleavage of the *tert*-butyl protecting group of the serine; *m/z* 861 corresponds to the sodium adduct of the symmetrical disulfide formed by the triethylene glycol-terminated alkanethiol. a) 20% piperidine/DMF, 10 min, room temperature; b) 100 mM FmocLeuOH, 1 equiv PyBOP, 2 equiv DIPEA, 1 h, 37 °C; c) 100 mM FmocGlyOH, 1 equiv PyBOP, 2 equiv DIPEA, 1 h, 37 °C; d) 50% TFA/DCM, 4 h, room temperature.

enzymes involved in various physiological functions and are important therapeutic targets[20] as well as being used as catalysts for biotechnological applications.[21] Substrate specificity of proteases has previously been studied by using fluorescent peptide derivatives to obtain "fingerprints" of the enzymes.[22] The present SAM platform provided the unique opportunity to observe peptide bond cleavage directly by using mass spectrometry. To demonstrate this approach, an array of 56 tripeptides was generated by SPOT synthesis and the substrate specificity of the industrially important protease, thermolysin, was explored (Figure 1C). Following library construction, the slide was incubated with a solution of enzyme (2 mg mL^{-1}) in potassium phosphate buffer (pH 7.5) at 37 °C, overnight, and each spot on the array was analysed by MALDI-ToF MS. The peptide library and the result after thermolysin treatment are shown in Table 1. A clear preference of thermolysin for hydrophobic and/or aromatic residues, such as leucine, isoleucine, valine, phenylalanine or tyrosine at the P1′ position (for P,P′ nomenclature, see ref. [21]) was thus demonstrated; this is in agreement with the known specificity of the enzyme as derived from solution studies.[20]

Table 1. Substrate specificity of thermolysin against a 56 tripeptide array. Unmarked sequence: not cleaved; italicised sequence: terminal amino acid was cleaved (e.g., GFS was cleaved to FS); underlined sequence: the dipeptide was cleaved (e.g., SAL was cleaved to L). Peptides were attached to the SAM surface through the C-terminal amino acid (N→C).

GAS	*GFS*	*GLS*	GPS	*GIS*	GGS	GSS
LAQ	*LFQ*	*LLQ*	LPQ	*LIQ*	LGQ	LSQ
FAR	*FFR*	*FLR*	FPR	*FIR*	*FGR*	*FSR*
SAL	SFL	SLL	SPL	SIL	SGL	SSL
AAG	*AFG*	*ALG*	APG	*AIG*	AGG	ASG
TAD	TFD	TLD	TPD	TID	TGD	TSD
VAA	VFA	*VLA*	VPA	*VIA*	VGA	VSA
YAF	YFF	YLF	YFP	YIF	YGF	YSF

In previous studies, we have shown that proteases can also be used for the synthesis of peptide bonds on polymer surfaces, such as PEGA (poly(ethyleneglycol) grafted onto polyacrylamide).[23,24] Detailed analysis of such reactions was troublesome because of the need for cleavable linkers, which were in some cases also labile in the presence of proteases.[25] The SAM platform proved to be much more successful (Figure 1D). Thus, phenylalanine and leucine were immobilised on the SAM array as described before, and were incubated with a mixture of thermolysin and a saturated solution of either FmocGly, FmocPhe, FmocLeu or FmocAla. MALDI-ToF MS analysis of the array showed that thermolysin was able to achieve complete coupling (as judged by MS) of FmocLeu to an immobilised Phe or Leu. In other cases, mixtures of Fmoc–dipeptides and unreacted starting materials were detected, except for FmocGly, which the enzyme was not able to transfer (details of the experimental data are given in the Supporting Information).

The second class of enzymes that we tested were glycosyltransferases. We have recently described enzymatic glycosylation of immobilised peptides on gold surfaces using these enzymes.[5] In our previous work, each individual peptide on such arrays was synthesised before immobilisation onto the gold surface, which limited the throughput of the method for screening substrate specificities. Conversely, SPOT synthesis allowed for a fast, parallel synthesis of peptide arrays suitable to be probed for glycosyltransferase activities (Figure 1B). An array of peptides derived from the sequence of the mucin Muc1 tandem-repeat AHGVTSAPA[26] was prepared, and the amino acids were varied at the +1 and −1 position relative to the threonine glycosylation site (underlined). Previous work had shown that the Muc1 peptide is a moderate substrate for the UDP-*N*-acetyl-D-galactosamine:polypeptide-*N*-acetyl-D-galactosaminyl transferase (ppGalNAcT2), and partial glycosylation is obtained after overnight incubation with the enzyme and UDP–GalNAc donor.[5] MALDI-ToF MS analysis of the Muc1-derived array obtained by SPOT synthesis showed successful glycosylation after overnight incubation with ppGalNAcT2, and further revealed an influence of the neighbouring amino acid on the extent of glycosylation (Figure 3). Although proper quantification is the most challenging issue when dealing with mass spectrometry, some semiquantitative data were obtained by comparing the ratios of the signals of the glycopeptide product and peptide substrate. A similar approach has recently been described[10,27] for obtaining relative yields of reaction. As each set of peptide/glycopeptide differs only by a GalNAc moiety, relative intensities can be compared by using the formula $r = \Sigma I_{(x)}/(\Sigma I_{(x)} + \Sigma I_{(y)})$, where r is the relative intensity, $\Sigma I_{(x)}$ the sum of the intensities of the glycopeptide signals and $\Sigma I_{(y)}$ the sum of the intensities of the parent peptide substrate (all proton, sodium and potassium adducts of alkanethiol and disulfide species are taken into account when detected).[27] Figure 3 shows the relative intensities for each peptide/glycopeptide set, with values normalised with respect to 1 (i.e., no effect) for the unmodified Muc1 peptide, which has a Ser at the +1 position and a Val at the −1 position. Overall, we found that glycosylation of the Muc1-derived peptides by

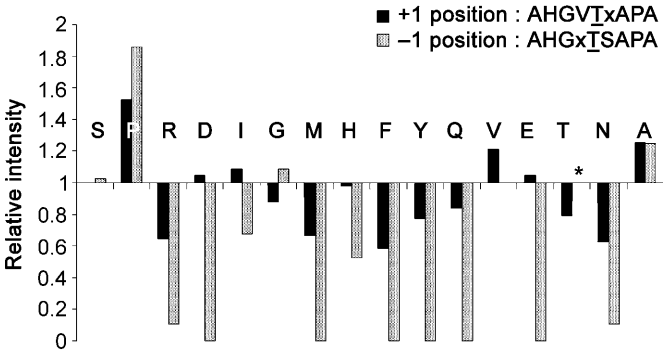

Figure 3. Effect of the amino acid at the +1 and −1 positions on the glycosylation of Muc1-derived peptides by ppGalNAcT2. The bars represent the ratio glycopeptide:peptide signal intensities as determined by MALDI-ToF MS for each peptide. Values have been normalised to 1 (no effect) for Ser at the +1 position and Val at −1 position (i.e., the unmodified Muc1 peptide AHGVTSAPA); *: threonine at the −1 position was also glycosylated (see discussion).

ppGalNAcT2 was more dependent on the amino acid at the −1 position than the amino acid at the +1 position. Significant enhancement of glycosylation was observed when the threonine glycosylation site was next to a proline residue, especially at the −1 position, and to a lesser extent, with the alanine substituted peptides. On the contrary, many of the changes at the −1 position resulted in no detectable level of glycosylation. A significant decrease in glycosylation was also observed when the serine at the +1 position was substituted for a threonine; this is consistent with the observation that bulky amino acids, such as phenylalanine and tyrosine, all had a negative impact, regardless of their position. Noteworthy, the new findings with the natural substrate of the enzyme are consistent with other reported studies in solution with unnatural synthetic peptide substrates,[28] and therefore confirm the use of this SAM platform as a valuable tool for comparing glycosylation patterns. Furthermore, MALDI-ToF MS analysis showed that the Muc1-derived peptide AHGTTSAPA, in which the −1 valine has been changed to threonine, resulted in a mixture of mono- and diglycosylated peptides. On the contrary, the peptides AHGVTTAPA (with threonine at the +1 position) and AHGSTSAPA, which has three potential glycosylation sites, both gave a single monoglycosylated peptide after treatment with the enzyme. Such detailed analysis would not have been possible with previously reported lectin-based analytical techniques.

Finally, to expand the scope of the SPOT synthesis on gold, a range of di-, tri- and glycosyl amino acids were tested as building blocks; all gave satisfactory results, and thereby allowed rapid access to longer peptide and glycopeptide arrays. For example, the glycopeptide AcAAPT(αMan)PVAAP (Figure 4A) was synthesized by using a per-acetylated mannosyl serine building block and subsequently acetylated at its N terminus by treatment with neat acetic anhydride at 37 °C for 15 min. Despite the three proline residues that have sometimes proved troublesome in SPOT synthesis,[19] clean couplings were obtained as the desired glycopeptide was the only product detected after completion of synthesis. Interestingly, the acetylation step proved to be selective for the primary amine and left the hydroxyl-terminated alkanethiol unchanged (Figure 4A, *m/z* 2101 corresponds to the sodium adduct of the mixed disulfide formed between the glycopeptide-terminated and the tri(ethyleneglycol)-terminated alkanethiols). The mannosyl residue could then be successfully O-deacetylated by using a solution of sodium methoxide in methanol.[10] The use of glycosyl amino acids with unprotected sugar side chains[29] was also explored for the synthesis of a 12-mer glycopeptide GTTASN-(βGlcNAc)YGTGFA, although the less reactive GlcNAcAsn[29d] required a longer reaction time (2 h). Furthermore, by incubating the surface-bound peptide with the bovine enzyme β1,4-GalT in the presence of UDP–Gal and $MnCl_2$, overnight, at 37 °C, enzymatic elongation of the carbohydrate moiety was achieved and yielded the corresponding LacNAc–glycopeptide (Figure 1A and Figure 4B, *m/z* 2197 and 2616).

In conclusion, we have developed an efficient and reliable method for construction of peptide arrays on gold surfaces using conventional SPOT synthesis conditions. Notably, the platform based on SAMs of alkanethiols on gold proved compatible with all required chemical steps, such as repeat cycles of amino acid coupling, *N*-Fmoc deprotection, N-acetylation, O-deacetylation of glycosyl moieties and final TFA-mediated cleavage of the side-chain protecting groups. Efficiency of all reactions was easily monitored directly on the array by using MALDI-ToF MS, which avoided the need for release of the peptide from the support for solution-phase analysis. Furthermore, we demonstrated that (glyco)peptides of sizes up to dodecamers could be synthesised by a chemical and/or chemoenzymatic approach. The usefulness of this methodology for the rapid screening of enzyme substrate specificity in a parallel, label-free manner was also illustrated herein with a protease and glycosyltransferases.

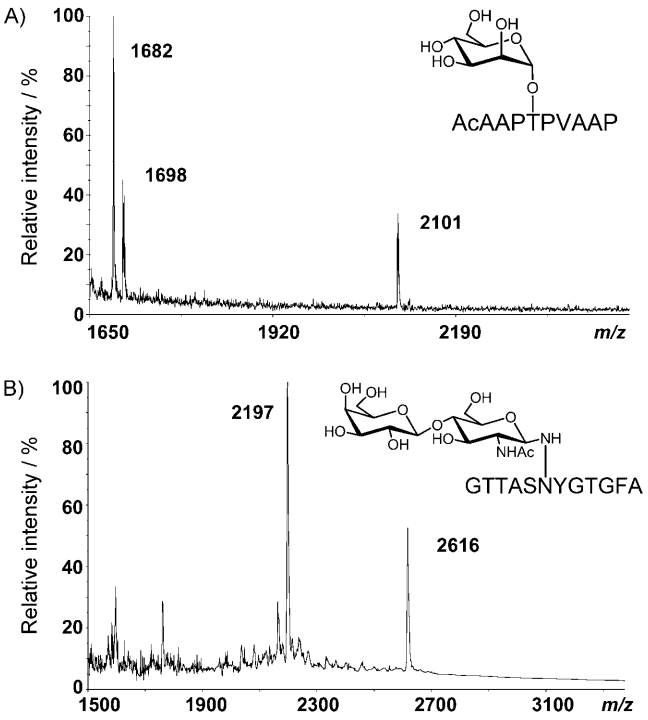

Figure 4. MALDI-ToF MS spectra of: A) a *N*-acetylated mannopeptide (*m/z* 1682 and 1698: sodium and potassium adducts of the linked glycopeptide, respectively; and *m/z* 2101: sodium adduct of the mixed disulfide respectively); B) 12-mer *N*-LacNAc (βGal(1,4)βGlcNAc)-peptide obtained by enzymatic glycosylation of its parent GlcNAc–peptide (*m/z* 2197 and 2616: sodium adducts of the linked glycopeptides and of the mixed disulfide, respectively).

Acknowledgements

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Keywords: glycosyltransferase · mass spectrometry · peptide arrays · self-assembled monolayers · SPOT synthesis

[1] D. Witt, R. Klajn, P. Barski, B. A. Grzybowski, *Curr. Org. Chem.* **2004**, *8*, 1763–1797.

[2] For some recent elegant examples see a) B. T. Houseman, J. H. Huh, S. J. Kron, M. Mrksich, *Nat. Biotechnol.* **2002**, *20*, 270–274; b) M. Mrksich, *ACS Nano* **2008**, *2*, 7–18.

[3] J. Su, M. Mrksich, *Angew. Chem.* **2002**, *114*, 4909–4912; *Angew. Chem. Int. Ed.* **2002**, *41*, 4715–4718.
[4] D.-H. Min, J. Su, M. Mrksich, *Angew. Chem.* **2004**, *116*, 6099–6103; *Angew. Chem. Int. Ed.* **2004**, *43*, 5973–5977.
[5] N. Laurent, J. Voglmeir, A. Wright, J. Blackburn, N. T. Pham, S. C. C. Wong, S. J. Gaskell, S. L. Flitsch, *ChemBioChem* **2008**, *9*, 883–887.
[6] B. T. Houseman, M. Mrksich, *Chem. Biol.* **2002**, *9*, 443–447.
[7] Z.-l. Zhi, N. Laurent, A. K. Powell, R. Karamanska, M. Fais, J. Voglmeir, A. Wright, J. M. Blackburn, P. R. Crocker, D. A. Russell, S. Flitsch, R. A. Field, J. E. Turnbull, *ChemBioChem* **2008**, *9*, 1568–1575.
[8] R. Karamanska, J. Clarke, O. Blixt, J. I. MacRae, J. Q. Zhang, P. R. Crocker, N. Laurent, A. Wright, S. L. Flitsch, D. A. Russell, R. A. Field, *Glycoconjugate J.* **2008**, *25*, 69–74.
[9] J. Li, P. S. Tiara, M. Mrksich, *Langmuir* **2007**, *23*, 11826–11835.
[10] L. Ban, M. Mrksich, *Angew. Chem.* **2008**, *120*, 3444–3447; *Angew. Chem. Int. Ed.* **2008**, *47*, 3396–3399.
[11] W. R. Yang, D. B. Hibbert, R. Zhang, G. D. Willett, J. J. Gooding, *Langmuir* **2005**, *21*, 260–265.
[12] R. Frank, *Tetrahedron* **1992**, *48*, 9217–9232.
[13] R. Frank, *J. Immunol. Methods* **2002**, *267*, 13–26.
[14] See for example: H. E. Blackwell, *Curr. Opin. Chem. Biol.* **2006**, *10*, 203–212.
[15] K. Hilpert, D. F. H. Winkler, R. E. W. Hancock, *Nat. Protocols* **2007**, *2*, 1333–1349.
[16] U. Reineke, A. Kramer, J. Schneider-Mergener, *Curr. Top. Microbiol. Immunol.* **1999**, *243*, 23–36.
[17] U. Reineke, R. Volkmer-Engert, J. Schneider-Mergener, *Curr. Opin. Biotechnol.* **2001**, *12*, 59–64.
[18] K. Hilpert, D. F. H. Winkler, R. E. W. Hancock, *Biotechnol. Genet. Eng. Rev.* **2007**, *24*, 31–106.
[19] M. Beyer, T. Felgenhauer, F. R. Bischoff, F. Breitling, V. Stadler, *Biomaterials* **2006**, *27*, 3505–3514.
[20] D. J. Maly, L. Huang, J. A. Ellman, *ChemBioChem* **2002**, *3*, 16–37.
[21] F. Bordusa, *Chem. Rev.* **2002**, *102*, 4817–4867.
[22] a) M. Meldal, I. Svendsen, K. Breddam, F.-I. Auzanneau, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3314–3318; b) J. E. Sheppeck, II, H. Kar, L. Gosink, J. B. Wheatley, E. Gjerstad, S. M. Loftus, A. R. Zubiria, J. W. Janc, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2639–2642; c) C. M. Salisbury, D. J. Maly, J. A. Ellman, *J. Am. Chem. Soc.* **2002**, *124*, 14868–14870; d) R. H. P. Doezé, B. A. Maltman, C. L. Egan, R. V. Ulijn, S. L. Flitsch, *Angew. Chem.* **2004**, *116*, 3200–3203; *Angew. Chem. Int. Ed.* **2004**, *43*, 3138–3141; e) J. J. Díaz-Mochón, L. Bialy, M. Bradley, *Chem. Commun.* **2006**, 3984–3986.
[23] R. V. Ulijn, B. Baragana, P. J. Halling, S. L. Flitsch, *J. Am. Chem. Soc.* **2002**, *124*, 10988–10989.
[24] R. V. Ulijn, N. Bisek, P. J. Halling, S. L. Flitsch, *Org. Biomol. Chem.* **2003**, *1*, 1277–1281.
[25] B. A. Maltman, M. Bejugam, S. L. Flitsch, *Org. Biomol. Chem.* **2005**, *3*, 2505–2507.
[26] H. H. Wandall, H. Hassan, E. Mirgorodskaya, A. K. Kristensen, P. Roepstorff, E. P. Bennet, *J. Biol. Chem.* **1997**, *272*, 23503–23514.
[27] Z. A. Gurard-Levin, M. Mrksich, *Biochemistry* **2008**, *47*, 6242–6250.
[28] T. A. Gerken, J. Raman, T. A. Fritz, O. Jamison, *J. Biol. Chem.* **2006**, *281*, 32403–32416.
[29] For example of glycopeptide synthesis with Fmoc amino acids carrying unprotected carbohydrates, see a) N. Yamamoto, Y. Ohmori, T. Sakakibara, K. Sasaki, L. R. Juneja, Y. Kajihara, *Angew. Chem.* **2003**, *115*, 2641–2644; *Angew. Chem. Int. Ed.* **2003**, *42*, 2537–2540; b) K. B. Reimer, M. Meldal, S. Kusumoto, K. Fusake, K. Bock, *J. Chem. Soc. Perkin Trans. 1* **1993**, 925–932; c) O. Seitz, C.-H. Wong, *J. Am. Chem. Soc.* **1997**, *119*, 8766–8776; d) M. Bejugam, S. L. Flitsch, *Org. Lett.* **2004**, *6*, 4001–4004.

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