Enzymatic synthesis of peptides on a solid support

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We have previously shown that dipeptides can be synthesised in high yields from amino acids using protease catalysis in aqueous media, if the amino component is immobilised on porous PEGA resin (a copolymer of polyethylene glycol and polyacrylamide). Here we explore the scope of this methodology for using protected and glycosylated amino acids as well as the synthesis of longer peptides on resin and show that such a method can also be applied on non-porous surfaces, in particular on gold.

1. Introduction

Proteases represent a large class of enzymes involved in many physiological functions and are important therapeutic targets[1] for various diseases as well as being used as catalysts for biotechnological applications.[2] In water, the equilibrium of amide bond formation and hydrolysis (Fig. 1) is generally shifted towards hydrolysis, and thus proteases naturally hydrolyse amide bonds. However, under low water conditions the equilibrium can shift towards amide synthesis and proteases have shown to be effective catalysts for peptide bond formation in a highly stereospecific manner under mild conditions and without need for activation.[3] We have shown previously that such equilibrium shift is also observed when using a resin-bound amino substrate and a soluble acyl donor even if the reaction is conducted in bulk aqueous solution (Fig. 1).[4]

Thermolysin is a protease widely employed for peptide synthesis and is well-known for the industrial synthesis of aspartame.[5] While the specificities and selectivities of the proteases usually limit their synthetic application, thermolysin with its broad specificity for carboxylic acid substrate can be employed to catalyse a variety of peptide synthesis reactions from acyl donors with different functionalities.[6] Thus, thermolysin was found to catalyse peptide-bond formation between hydrophobic Fmoc-protected amino acids and a phenylalanine immobilised on $PEGA_{1900}$ (poly-(acrylamide)-ethylene glycol) resin *via* the Wang (HMPA, hydroxymethylphenoxyacetyl) linker in bulk aqueous media.[4a] The aim of the present study was to apply these findings to the synthesis of larger peptides and to investigate which components can be used as soluble substrates to create a library of peptides with varying size and functionalities.

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2. Results and discussion

Enzymatic synthesis on porous support

Our initial studies reported the formation of dipeptide sequences using the protease thermolysin from *Bacillus thermoproteolyticus Rokko.*[4a,b] Yields for hydrophobic amino acid couplings (R_1 in Fig. 1) were generally high, whereas lower yields were observed for polar and charged amino acids. From these studies the question arose whether this enzymatic method would be limited to hydrophobic dipeptides, or whether it could be applied to a wider range of amino acids and to the synthesis of longer peptides. We sought to overcome the low yields obtained with polar and charged amino acids by using hydrophobic protecting groups,

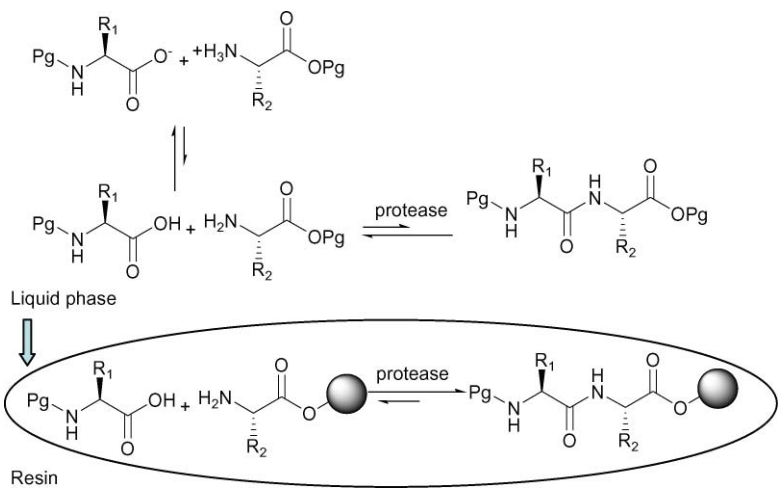

Fig. 1 Protease-catalysed synthesis/hydrolysis of dipeptides in solution and on resin. Whereas in aqueous solution hydrolysis is favoured, the reaction equilibrium on PEGA resin favours dipeptide synthesis (Pg: protecting group).

Table 1 Yields of dipeptides obtained by thermolysin catalysed bond formation on Leu-HMPA-PEGA as shown in Fig. 2

Entry	Acyl donor	% Fmoc-AA-Leu[a]
1	Fmoc-Gly	95
2	Fmoc-Leu	96
3	Fmoc-Phe	94
4	Fmoc-Trp	96
5	Fmoc-Ala	52
6	Fmoc-Pro	13
7	Fmoc-Met	22
8	Fmoc-Cys(Trt)	8
9	Fmoc-Cys(tBu)	38
10	Fmoc-Tyr	95
11	Fmoc-Tyr(tBu)	98
12	Fmoc-Ser	4
13	Fmoc-Ser(tBu)	71
14	Fmoc-Ser(Bn)	65
15	Fmoc-Thr	29
16	Fmoc-Thr(Bn)	14
17	Fmoc-Asp	22
18	Fmoc-Asp(tBu)	91
19	Fmoc-Asp(Bn)	85
20	Fmoc-Glu	35
21	Fmoc-Glu(tBu)	76
22	Fmoc-Asn	33
23	Fmoc-Asn(Trt)	25
24	Fmoc-Gln	43
25	Fmoc-Arg	<1
26	Fmoc-Arg(Pbf)	49
27	Fmoc-His	5
28	Fmoc-His(Trt)	33
29	Fmoc-His(Bn)	41
30	Fmoc-Lys	0
31	Fmoc-Lys(Boc)	96
32	Fmoc-Lys(Mtt)	4
33	Fmoc-Ser(α-D-Man(OH)$_4$)	0
34	Fmoc-Ser(α-D-Man(OAc)$_4$)	78
35	Fmoc-Ser(β-D-Gal(OAc)$_4$)	70

[a] Yields determined by LC-MS.

Table 2 Thermolysin-catalysed coupling of N-protected di and tripeptides to Leu-HMPA-PEGA

Entry	Acyl donor	Product (ratio)
1	FmocPhePhe	FmocPhePheLeu (4.5) FmocPheLeu (95.5)
2	FmocTyrAla	FmocTyrAlaLeu (25) FmocTyrLeu (75)
3	FmocPheAsp	FmocPheAspLeu (21) FmocPheLeu (79)
4	FmocPheAsp(tBu)	FmocPheAsp(tBu)Leu (18) FmocPheLeu (82)
5	FmocPheSer	FmocPheSerLeu
6	FmocPheSer(Bn)	FmocPheSer(Bn)Leu (21) FmocPheLeu (79)
7	FmocPheCys(Bn)	FmocPheCys(Bn)Leu (77) FmocPheLeu (23)
8	FmocPheArg	FmocPheLeu (traces)
9	FmocGlyGly	FmocGlyGlyLeu (90) FmocGlyLeu (10)
10	FmocPheGly	FmocPheGlyLeu (37) FmocPheLeu (63)
11	ZAlaTyr	ZAlaTyrLeu
12	ZGluTyr	ZGluTyrLeu
13	ZLeuTyr	ZLeuTyrLeu
14	ZPhePhe	Library
15	ZLeuLeu	Library
16	ZTrpLeu	Library
17	FmocGlyGlyGly	FmocGlyGlyGlyLeu (22) FmocGlyLeu (88)
18	FmocPhePhePhe	FmocPhePhePheLeu (traces) FmocPheLeu
19	FmocLeuTyrPhe	FmocLeuTyrPheLeu (58) FmocLeuPheLeu (20) FmocLeuTyrLeu (11) FmocLeuTyrPhePheLeu (11)
20	FmocPheLeuPhe	FmocPheLeuOH (92) FmocPhePheLeuOH (8)

similar to those employed in chemical solid phase peptide synthesis (SPPS). To evaluate the effect of protecting groups, experiments were performed by adding a wide range of Fmoc-protected amino acids having either a protected or non-protected side-chain residue (Fig. 2) to leucine anchored *via* a Wang linker to PEGA resin (Leu-HMPA-PEGA). The reactions were carried out in a suspension of 0.025 mmol Fmoc-amino acid in 1 mL potassium phosphate buffer (KPi) 0.1 M at pH 7.5 with 2 mg of thermolysin and 5 mg of resin-bound leucine (0.5 μmol). Yields were determined using LC-MS after cleavage from the linker by integrating the UV traces at 301 nm.[4a,4b] As expected, the best yields were obtained with hydrophobic amino acids, which gave nearly quantitative coupling in agreement with previous results[4a] (Table 1, entries 1–6, 10). Hydrophilic amino acids gave very low yields (Table 1, entries 12, 15, 17, 20, 22, 24, 25, 27, 30), whereas their side-chain protected counterparts resulted in increased product formation. Comparison of the yields obtained with Fmoc-Lys(Boc) (entry 31) and Fmoc-Lys(Mtt) (entry 32) suggested that apart from the hydrophobicity, the size of the protecting group may also have a considerable effect on the coupling efficiency. Comparable results to those presented in Table 1 were obtained using an immobilised phenylalanine instead of leucine as the *C*-terminal amino acid (data not shown).

Our group has a strong interest in the synthesis of glycopeptides.[7] To explore the breadth of the present methodology, we therefore tested *N*-Fmoc protected glycosylated amino acids, such as galactosylated and mannosylated serine. Interestingly, glycosyl amino acid with an unprotected glycosyl moiety (entry 33) resulted in no detectable amount of coupling product. However, using *per*-acetylated mannosyl or galactosyl serine, high yields were obtained (entries 34–35), therefore showing that thermolysin was able to tolerate highly diverse structural modifications of the acyl donors.

Given that thermolysin is an endopeptidase, the amide coupling reaction described here was tested for the coupling of peptide fragments, adding di- and tripeptides instead of single amino acids as acyl donors to the reaction (Fig. 3). For example when

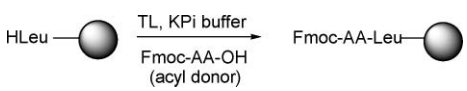

Fig. 2 Thermolysin catalysed formation of dipeptides on resin.

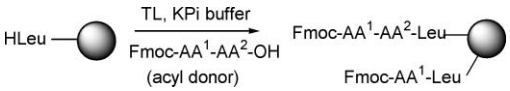

Fig. 3 Thermolysin catalysed coupling of dipeptides.

FmocPheSer was mixed with thermolysin and Leu-HMPA-PEGA, LC-MS analysis of the product showed that the enzyme was able to achieve coupling to yield the corresponding tripeptide FmocPheSerLeu (Table 2, entry 5). However, when Fmoc-protected dipeptides that are potential substrates for thermolysin were chosen as acyl donors, mixtures of di- and tripeptides were formed (Table 2). These dipeptide side-products appeared to be formed from Fmoc-protected amino acids released from the dipeptide substrate in solution followed by coupling to the solid phase substrate. Overall, the yields of coupling were moderate (30–40%). Similarly, Z-protected dipeptides **11–13** yielded pure tripeptides, whereas **14–16** were more prompt to hydrolysis and thus, libraries of various size peptides were obtained. When even longer peptides were investigated as acyl donors, mixtures of peptides were obtained, which appeared to be the results of a series of hydrolysis/synthesis steps leading to some degree of scrambling of peptide sequence (Table 2, entries 17–20). The best result of tetrapeptide was obtained when using FmocLeuTyrPhe as substrate (Table 2, entry 19).

An alternative way of generating larger peptides on the solid phase using this biocatalytic method is by stepwise elongation using a series of repeated coupling and Fmoc-deprotection steps similar to a chemical SPPS protocol.[8] The advantage of such a method is that coupling is mediated by biocatalysis and thus no activation of amino acid is needed. Furthermore, when using a racemic mixture of D/L amino acid, selective formation of L/L dipeptide can be achieved by thermolysin.[8] This approach might be particularly useful for non-natural L-amino acids that are not readily available because the resolution of enantiomers from racemic mixtures and the coupling is achieved in one step. First, the most simple case of synthesis of polyleucine peptides was studied by repeated cycles of enzymatic coupling at 30 °C and chemical Fmoc-deprotection (Fig. 4). Each step was monitored by LC-MS and it was found that clean coupling could be achieved up to the tetramer Fmoc-Leu_4-HMPA-PEGA (Fig. 4A) without any visible side-products. Beyond tetraleucine, hydrolysis of peptide started to compete with synthesis and a mixture of products were obtained. The maximal achievable length was Fmoc-Leu_6-HMPA-PEGA, which was part of a mixture comprising 3 to 6 Leu residues (Fig. 4B). For other amino acids such as poly-Phe and poly-Tyr, formation of mixtures occurred already during the second coupling step towards Fmoc-Phe_3-HMPA-PEGA and Fmoc-Tyr_3-HMPA-PEGA respectively. The Fmoc tetrapeptides Phe_4 and Tyr_4 were the maximal achievable length. It was also found that the library distribution was dependent on the temperature of enzymatic reaction, as mixtures of poly-Leu peptides were obtained already during the third coupling step when performed at higher temperatures of 37 °C. On the contrary, synthesis of a PheLeuPheLeu tetrapeptide (with alternating Phe and Leu residues) gave only traces of shorter peptides. Therefore, it is likely that the smaller peptides arise from hydrolysis of the resin bound peptide by thermolysin, and that hydrolysis appears to be dependent on the amino acid sequence of the substrate. These results also suggest that the enzyme-catalysed peptide synthesis and hydrolysis reactions on the solid phase are reversible and distribution of the peptide libraries depends on reaction conditions. Further studies probing this reversibility are currently under way.

Enzymatic synthesis on gold surfaces

A disadvantage of using porous polymer beads such as PEGA or Tentagel is poor enzyme diffusion into the bead which limits their application to proteins of lower than average molecular weight.[9]

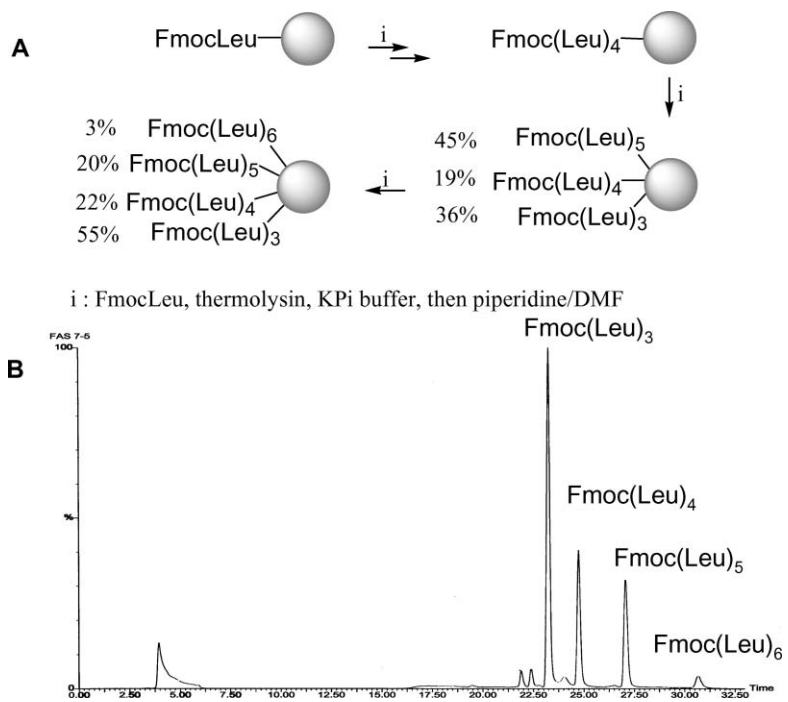

Fig. 4 **A:** Stepwise elongation of peptides on solid phase. **B**: LC trace of a poly-leucine library.

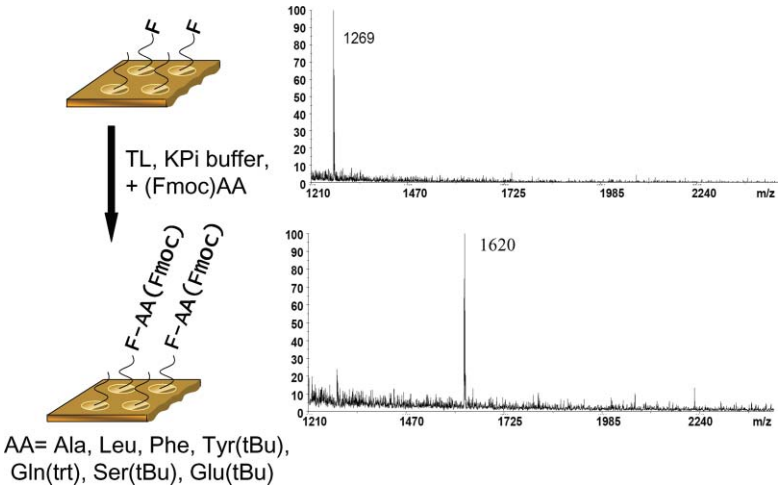

Fig. 5 Thermolysin catalysed amino acid transfer on a gold surface. Example of MALDI-ToF MS spectra before (top) and after (bottom) incubation of an immobilised Phe with thermolysin and FmocLeu. *m/z* 1269 is the sodium adduct of the mixed disulfide formed between the hydroxyl-terminated and the Phe-terminated alkanethiols. *m/z* 1620 is the potassium adduct of the product detected as a mixed disulfide.

Very recently, we have investigated the use of self-assembled monolayers (SAMs) on gold surfaces and have generally found excellent enzyme accessibility.[10] These surfaces are easy to prepare and monitoring of enzymatic reactions can be achieved using label-free analytical techniques such as MALDI-ToF MS (matrix-assisted laser desorption ionization/time-of flight mass spectrometry).[11] Noteworthy, analysis can be performed on-chip, therefore ruling out the need for cleavage from the support. Therefore, the ability of thermolysin to catalyse amino acid transfer on gold surfaces was investigated (Fig. 5).

To this end, Fmoc phenylalanine was immobilised onto a disposable 64-well gold slide (Applied Biosystems) coated with a 1 : 1 mixture of alkanethiols terminated either by a primary amine or by a non-reactive hydroxyl group (providing a control of the surface density by mixing the two linkers at different ratios).[10b] After Fmoc-deprotection, the array was incubated with a mixture of thermolysin and a saturated solution of Fmoc-amino acid in KPi buffer at 37 °C. After 16 h, MALDI-ToF MS interrogation of the array revealed that thermolysin was able to catalyse the coupling of these amino acids to the immobilised Phe (Table 3). For example, coupling of Fmoc-Leu (Fig. 5) resulted in the complete disappearance of the starting material and formation of a single species corresponding to the addition of the Fmoc-Leu. Other Fmoc amino acids were tested, and MS data are shown in Table 3.

Overall, these results were in good agreement with those obtained using PEGA-anchored peptides and demonstrated that themolysin was able to react on a planar surface where surface density can be tailored in order to maximize the enzyme's accessibility. Furthermore, parallel screening can be performed on a single chip, and the use of MALDI-ToF MS analysis provided a straightforward insight into the determination of thermolysin activity.

Table 3 *m/z* detected after incubation of immobilized Phe with a mixture of thermolysin and Fmoc amino acid (for clarity only masses of the sodium and potassium adducts of the mixed disulfide formed between the peptide and the tri(ethyleneglycol) alkanethiols are shown)

Entry	Fmoc amino acid added	Product	*m/z* detected
1	FmocPhe	FmocPhePhe	1654 (M + K)
2	FmocLeu	FmocLeuPhe	1620 (M + K)
3	FmocAla	Phe	1285 (M + K)
		FmocAlaPhe	1575 (M + K)
4	FmocGly	Phe	1269 (M + Na)
5	FmocSer(tBu)	Phe	1269 (M + Na)
		FmocSer(tBu)Phe	1634 (M + Na)
6	FmocLys(Boc)	Phe	1285 (M + K)
		FmocLys(Boc)Phe	1735 (M + K)
7	FmocAsp	Phe	1269 (M + Na)
8	FmocGlu(tBu)	Phe	1269 (M + Na)
		FmocGlu(tBu)Phe	1676 (M + Na)
9	FmocTyr(tBu)	Phe	1269 (M + Na)
		FmocTyr(tBu)Phe	1710 (M + Na)
10	FmocTyr	Phe	1285 (M + K)
11	FmocGln(trt)	Phe	1285 (M + K)
		FmocGln(trt)Phe	1861 (M + Na)
			1877 (M + K)

3 Conclusions

In summary, these results have shown that the initial coupling reaction of two natural amino acids can be broadened to generate a wide range of peptides particularly by using side-chain protected amino acids. The structure of such peptides is currently limited by thermolysin specificity, and studies on using complementary proteases are on the way. In a number of examples studied here, mixtures of products were obtained, which appeared to be the results of consecutive hydrolysis and synthesis steps, suggesting a reversibility under the reaction conditions. Finally, we have shown that the shift of equilibrium observed initially only for PEGA resin, can also occur on other surfaces, such as SAMs on gold. Such surfaces are increasingly used in solid phase biocatalysis and should broaden further the scope of this methodology.

4 Experimental details

General methods

Liquid chromatography/mass spectrometry was performed on an Agilent 1100 Series LC system, equipped with a 1100 series MSD with a multimode ion source. Samples were analysed with a Phenomenex Luna® 5 μ C18 column (250 × 2 mm) and a 0–85% MeCN (0.1% TFA)/H_2O (0.1% TFA) gradient with flow rate of 0.5 ml/min. The UV trace was recorded at 210 nm, 254 nm, 280 nm and 301 nm. Data were processed with the Agilent Chemstation Software.

Attachment of the first amino acid to HMPA-PEGA

Fmoc protected amino acid (10 equivalents to resin) was dissolved in dry DCM, DIC (diisopropylcarbodimide) (5 equivalents to resin) was added dropwise and the solution was stirred under nitrogen atmosphere at RT for 20 min. DCM was evaporated and a small volume of dry DMF was added. The resulting solution was added to HMPA-derivatized PEGA resin to form a gel. DMPA (dimethylaminopyridine) (0.1 eq) was dissolved in a minimum volume of dry DMF and added to the reaction mixture, which was then agitated at RT for 2 h. The resin was then filtered and extensively washed. The loading densities were determined by Fmoc cleavage and were typically 0.06–0.07 mmol/g.

Thermolysin-catalysed peptide-bond formation on PEGA

5 mg Leu-HMPA-PEGA were treated with 0.025 mmol of the acyl donor in the presence of 2 mg thermolysin in 1 mL 0.1 M KPi buffer with pH = 7.5. After 16 h the resin was washed and the reaction products were cleaved from the resin with 95% TFA/H_2O for 2 h. Analysis and quantification of reaction products were achieved by LC-MS.

Preparation of the amine-terminated self-assembled monolayers on gold and immobilization of phenylalanine

A disposable 64-well gold surface (Applied Biosystems) was cleaned with a 5 : 1 solution of concentrated sulfuric acid and hydrogen peroxide 35% (caution: very oxidizing agent) and thoroughly rinsed with deionised water, ethanol and dried under a stream of nitrogen. Self-assembled monolayers on gold surfaces were prepared by spotting a DMSO solution (final concentration 0.1 mg/mL, 0.4 μL per spot) of a 1 : 1 mixture of carboxylic acid-terminated and tri(ethyleneglycol)-terminated alkanethiols (ProChimia, chemical formulas $HS(CH_2)_{17}(OCH_2CH_2)_6CH_2CO_2H$ and $HS(CH_2)_{17}(OCH_2CH_2)_3OH$ respectively) into each well and left overnight to react before rinsing with DMSO, ethanol and drying as above. The terminal carboxylic acid was then activated for 1 h at room temperature by spotting 0.4 μL per spot of a freshly prepared DMF solution of EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 0.2 M) and NHS (*N*-hydroxysuccinimide, 0.05 M). After rinsing and drying as above, a DMF solution of *N*-Fmoc diaminobutane (Aldrich, 100 mM) was spotted (0.4 μL per spot) and left to react overnight at room temperature. Following washing steps as above, the gold slide was dipped into a 20% solution of piperidine in DMF for 10 min to ensure Fmoc removal, then rinsed and dried as above. 10 mg of Fmoc phenylalanine were dissolved in 0.25 mL of a solution containing 52 mg/mL of PyBOP (benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) and 36 μL/mL of DIPEA (diisopropylethylamine) in dry DMF and allowed to react for 5 min at room temperature. 0.4 μL of the above solution was then spotted in each well of the gold slide, and the slide was then placed at 37 °C in a sealed container. After 1 h, the surface was rinsed with DMF, ethanol and dried under a stream of nitrogen. Terminal Fmoc removal was carried out as above.

Thermolysin-catalysed peptide-bond formation on gold

Each well of the slide was incubated overnight at 37 °C with a saturated solution of Fmoc amino acid (about 10 mg/mL) and thermolysin (2 mg/mL) in KPi buffer 0.1 M with a pH 7.5. The slide was then thoroughly rinsed with deionised water, DMF, and ethanol and dried under a stream of nitrogen.

MALDI-ToF MS analysis

Each well of the surface was coated with a solution of THAP (2,4,6-trihydroxyacetophenone, 10 mg/mL in acetone), and the target was loaded into a Voyager-DE STR Biospectrometry MALDI-ToF mass spectrometer (PerSeptive Biosystems) operating with a 337 nm nitrogen laser. Mass spectra were acquired using reflector ToF, positive ion mode using an accelerating voltage of 20 kV and an extraction delay of 200 ns.

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