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COMMUNICATION

Oxo-ester mediated native chemical ligation on microarrays: an efficient and chemoselective coupling methodology†

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We report a highly efficient and selective method for the coupling of peptides and glycoconjugates bearing *N*-terminal cysteines to activated surfaces. This chemoselective method generates stable amide linkages without using any thiol additives.

Peptide and protein microarrays are well established in the field of proteomics and provide platform technologies for drug discovery and clinical diagnosis.[1] Such arrays are mostly generated by covalent coupling of pre-prepared ligands to solid surfaces.[2] The conjugation chemistry needs to be highly chemoselective, site-specific and efficient to ensure correct presentation of the ligand on the surface. Next to rather classical chemical acylation methods, more specific bioorthogonal coupling methods[3] have been developed such as Staudinger ligation,[4] "click chemistry",[5] and native chemical ligation (NCL).[6,7]

NCL is particularly attractive as it can be carried out under physiological conditions.[8] NCL generally involves the reaction of a thioester and a cysteine (Cys) derivative, such as an *N*-terminal Cys-peptide. If the NCL is performed on solid phase, either the Cys- or the thioester-peptide needs to be immobilised.[6,9] Starting with immobilised Cys requires thioester-peptides, which are incompatible with Fmoc protecting groups in solid phase peptide synthesis.[10] The reverse coupling method proceeds from thioester-functionalised surfaces **1** by coupling of the Cys derivative *via* a reversible transthioesterification to give thioester **2** and the native peptide **3** after irreversible rearrangement (Fig. 1). The formation of **1** has been shown first by Lesaicherre *et al.*[11] albeit in only 1/10 of monolayer coverage.[8]

As an alternative, we have therefore investigated NCL coupling protocols starting from readily available activated oxo-ester **5** on surfaces (Fig. 2) instead of using thioester surface **1**.[12,13] A concern using such oxo-esters would be higher reactivity and it was therefore important to investigate if selectivity of cysteines over other amines could be retained in the coupling reaction.

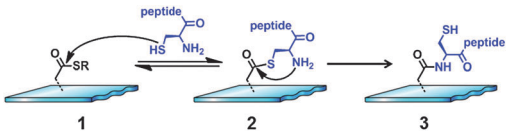

Fig. 1 The native chemical ligation (NCL) performed on thioester-functionalised surfaces.

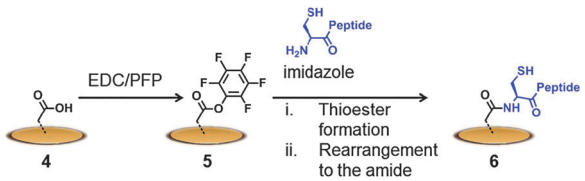

Fig. 2 The chemoselective coupling of Cys derivatives on PFP-activated gold plates is a powerful method for oxo-ester mediated NCL on surfaces.

All our experiments were conducted on gold arrays covered with self-assembled monolayers (SAMs) formed by alkane thiols,[14] which are omega-functionalised with hexaethylene glycol (OEG_6) carboxylic acid, thus providing points for chemical modification (**4**, Fig. 3). Any reaction on this surface can be monitored directly and label-free using matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-ToF MS).[15]

In the first instance, a range of activated oxo-esters such as *para*-nitrophenyl (*p*NP), *N*-hydroxysuccinimidoyl (NHS) and 2,3,4,5,6-pentafluorophenyl (PFP) esters were generated from carboxy-functionalised surfaces and were reacted with Cys. Analysis of the reactions by MALDI-ToF mass spectrometry suggested that the coupling reactions were most efficient using PFP activation and this activation method was therefore chosen for subsequent studies.

The coupling of Cys itself was then further investigated for efficiency of coupling and selectivity over other amino acids.[16,17] The coupling was tested at different Cys concentrations using the standard NCL conditions (7.5 eq. tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 5 M guanidine buffer, 75 mM Na_2HPO_4, pH 7)[18] and it was found that coupling was observed even at a concentration of 2 mM of Cys, which compared favourably to the 50 mM previously necessary for

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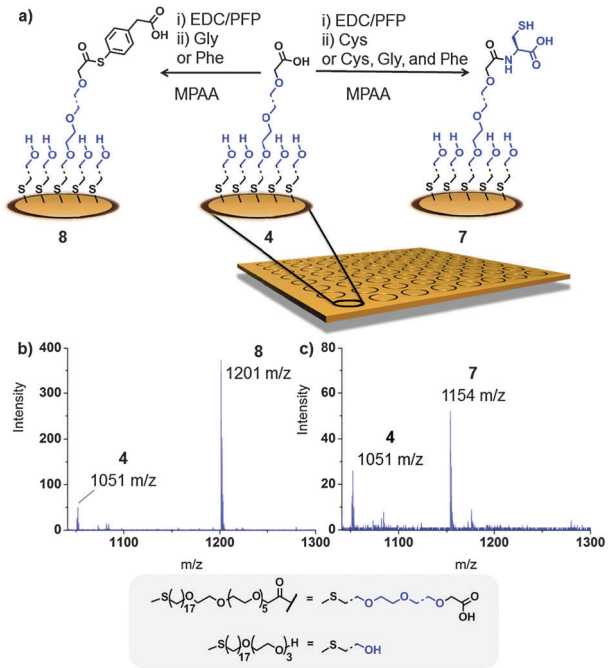


Fig. 3 (a) Coupling experiments with 4-mercaptophenylacetic acid (MPAA). (b) MALDI-ToF MS analysis of 7. (c) MALDI-ToF MS analysis of **7**.

peptide coupling. By addition of imidazole[12,17] we were able to further lower the Cys concentration necessary to 0.25 mM. Thiophenol and 4-mercaptophenylacetic acid (MPAA) were less effective.

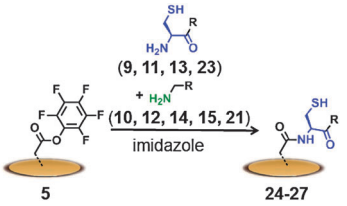
With a robust coupling protocol in hand, we investigated the selectivity of Cys coupling over other amino acids. Interestingly, when the activated surfaces **5** were reacted with glycine (Gly) or phenylalanine (Phe) in the presence of thiol MPAA as additive, only thioester **8** was formed and no conversion to the respective amide was observed (Fig. 3). The same reaction with Cys and MPAA, however, showed the Cys amide **7** as the sole product. These experiments suggest that on the surface *O*- to *S*-transesterification (**5** to **8**) is fast. The thioester **8** consequently undergoes a reaction with Cys *via* transthioesterification which rearranges to the amide **7**. This difference in acylation rates was also observed in competition experiments. When Gly, Phe and Cys were added to the reaction mixture in equimolar amount in the presence of MPAA only the Cys-product **7** was formed.

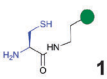
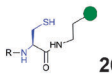
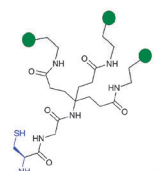
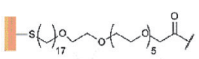
Interestingly, similar selectivities were observed in the presence of imidazole (instead of the thiol MPAA).[12] Competitive studies with mixtures of β-alanine (β-Ala), Phe, Gly and Cys (1 mM each) also showed exclusively the Cys product even at 1 : 25 ratio of Cys : Gly (Fig. S17, ESI†). All subsequent reactions were therefore performed with imidazole as additive.

Similar selectivities were observed with longer peptides containing either cysteine or glycine at their *N*-termini (Table 1). When mixtures of peptides **9** and **10**, **11** and **10**, and **11** and **12**, respectively, were applied to the surface, only the products **24** or **25** were observed.

As an alternative and more sensitive method of analysis, surface plasmon resonance (SPR) was employed. **13** and **14** were applied in a competitive assay at a concentration of

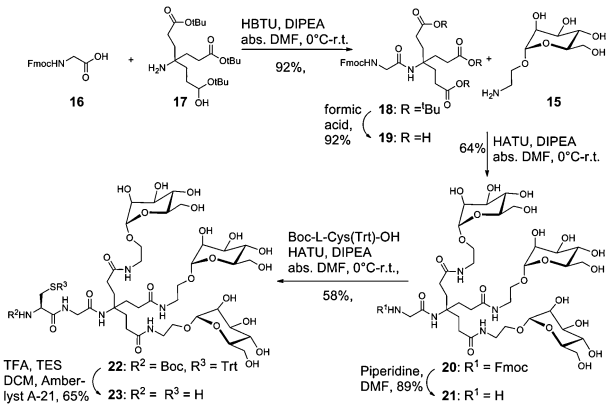
Table 1 Competitive assay employing *N*-Cys and *N*-amino peptides and glycoamino acids



Entry	Starting material	Product
1	CQDSETRTFY **9** + GALGVTSAPAG **10**	R'-CQDSETRTFY **24**
2	CALGVTSAPAG **11** + GALGVTSAPAG **10** or GAPGPTPGPAGK **12**	R'-CALGVTSAPAG **25**
3	 **13** + 15 or  **14**	 **26**
4	**23** + **21**	 **27**
R* = 		*N*-Acetyl-β-D-glucosamine α-D-Mannose

2 mM each on PFP activated SAMs. The SPR slide was subsequently interrogated with the mannose binding lectin concanavalin A (ConA) followed by the *N*-acetylglucosamine (GlcNAc) binding lectin wheat germ agglutinin (WGA).[19] There was strong ConA and no WGA binding observed which showed that exclusively the Cys-Man **13** had coupled to the surface, but not **14**.

After these promising results with small amino acids and peptides, the coupling method was tested on more complex bioconjugates. Of particular interest was the coupling of glycoconjugates, which are used in glycoarrays[20] and which can often only be obtained in small quantities. Efficient coupling strategies are therefore of great importance to the field. The cysteine derivatives such as **13** are easily obtained from aminoethyl glycosides **15**. In competition with **15**, cysteine derivative **13** was exclusively coupled to surfaces giving the product **26** as shown in MALDI-ToF MS analysis. To test this coupling strategy further, we investigated multivalent glycoconjugates, which have become important tools to probe multivalent binding events on cell surfaces.[21] The required trivalent glycoclusters[22] were synthesised as shown in Scheme 1. Trivalent glycoclusters **21** and **23** were synthesized



Scheme 1 Synthesis of trivalent glycoclusters **21** and **23**.

starting from the known building block **17**.[23] For the preparation of the trivalent wedge **19** Fmoc-glycine (**16**) was coupled in a HBTU/DIPEA-mediated reaction with **17** followed by deprotection of the tBu esters with formic acid yielding compound **19** in 85% over two steps. Mannosides were introduced *via* peptide coupling following published chemistry[24] giving **20** in a satisfying yield of 64%. Fmoc deprotection with piperidine in DMF gave the trivalent cluster mannoside **21** in 89% yield. An amount of **21** was further functionalized with Boc-L-Cys(Trt)-OH in another peptide coupling reaction yielding **22** in 58%. This step was followed by one step removal of the Boc and Trityl protecting groups using TFA in DCM under addition of TES followed by a treatment with basic ion exchange resin Amberlyst A-21 following a literature protocol.[25] After size exclusion and reversed phase chromatography the Cys-terminated ligand **23** was obtained in 65% yield.

The trivalent cluster mannosides manno-Cys **23** and manno-Gly **21** were then compared in coupling experiments to the gold array surfaces. From reaction mixtures containing 4 mM each of **21** and **23** the manno-Cys product **27** was formed exclusively (Table 1).

Complex bioconjugates containing *N*-terminal cysteines can be efficiently coupled to surfaces through amide bond formation *via* native chemical ligation starting with activated oxo-esters on the surface. These surface oxo-esters are easily prepared from their carboxylic acids. Our studies have shown that the ligation in the presence of imidazole is highly selective for cysteine derivatives over competing amines.

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