Light-directed maskless synthesis of peptide arrays using photolabile amino acid monomers†

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Novel photolabile amino acid monomers for photolithographic solid-phase peptide synthesis has been developed and a method for the maskless synthesis of individually addressable peptide microarrays using new building blocks has been described; these peptide microarrays are suitable for repetitive epitope-binding assays.

The use of photolabile protecting groups in peptide[1] chemistry has been well established. Useful photolabile protecting groups must be stable to mild chemical treatments, but photolytically cleaved in high yield by irradiation at wavelengths which do not damage the protected molecule. Peptide chips[2] are an emerging technology that could replace many of the bioanalytical methods currently used in drug discovery, diagnostics, and cell biology. The array format for analyzing peptide and protein function offers an attractive experimental alternative to traditional library screens. Several strategies[3] incorporating arrayed peptides and proteins have emerged from studies using combinatorial synthesis of peptides and peptide like molecules. The number of peptides synthesized per unit area can be greatly increased by combining solid-phase synthesis with photolithographic techniques.

In 1991, Fodor *et al.*[4] demonstrated for the first time that addressable arrays (*e.g.*, peptides) could be synthesized on glass surfaces using photomasks and photolabile building blocks. However, soon after this report synthetic efforts tended to shift to oligonucleotide arrays,[5] because of interest in genomics analysis, the relative ease of oligonucleotide synthesis and the fact that oligonucleotide synthesis requires only four building blocks, whereas peptide synthesis requires twenty. Now, however, with the burgeoning growth of proteomics,[6] attention is returning to peptide arrays.

Singh-Gasson *et al.*[7] in 1999 developed a maskless technology to direct light to specific locations. This technology uses a Digital Micromirror Device (DMD), which replaces the physical masks and can be conveniently controlled by a computer. This cut down the cost of making physical masks and does not involve the tedious procedure of aligning masks.

Until now *o*-nitroveratryloxycarbonyl[8] (NVOC), α-methyl-*o*-nitropiperonyloxycarbonyl[9] (MeNPOC) and 2-(2-nitrophenyl)-propyloxycarbonyl[10] (NPPOC) have been used as protecting groups of amino acids for light-directed peptide synthesis. However, these groups are rather slowly removed. A good photolabile group[11] may be assessed by combining in the same structure two major features, *i.e.* the methylene dioxy group onto the benzene ring which greatly facilitates the photolytic cleavage above 350 nm wavelength and, the elongation of the side-chain with a single methylene moiety and addition of a methyl substituent to the α-carbon which enhances the cleavage kinetics.[12] More recently Berroy *et al.*[13] have demonstrated that the efficiency of photolytic cleavage of 2-(3,4-methylenedioxy-6-nitrophenyl)-propyloxycarbonyl (MNPPOC) protected nucleotides is significantly better than that for NVOC or MeNPOC (Fig. 1) protected nucleotides. We describe here the synthesis of MNPPOC-protected amino acids, and the maskless synthesis of individually addressable peptide microarrays using novel photolabile monomers. We demonstrate that these peptide microarrays are suitable for repetitive epitope-binding assays.

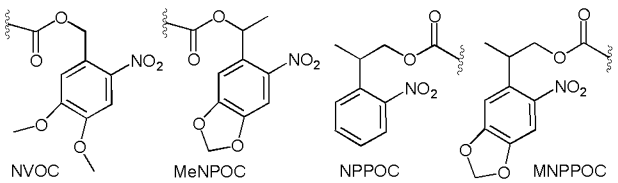
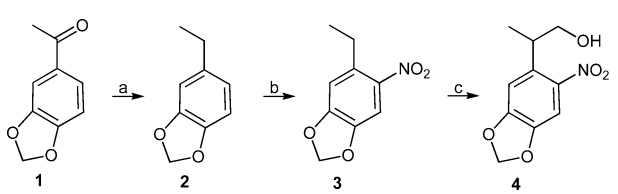


Fig. 1 Photolabile protecting groups.

To obtain MNPPOC-protected amino acids (**7**), we first devised an improved synthesis of 2-(3,4-methylenedioxy-6-nitrophenyl)-propanol (MNPPOH)[13] (**4**) (Scheme 1). In our methods, 3,4-(methylenedioxy)-acetophenone was reduced to 3,4-(methylenedioxy)-ethylbenzene (**2**) in the presence of hydrazine hydrate followed by treatment of **2** with 40% HNO_3 to obtain 3,4-(methylenedioxy)-6-nitrophenylethane (**3**). Compound **3** was converted to MNPPOH (**4**) by treatment with paraformaldehyde and benzyltrimethylammonium hydroxide.[14] The alcohol **4** was treated with phosgene to give MNPPOC chloride (**5**). Reaction of **5** with various L-amino acids (**6**) in the presence of sodium carbonate (pH 9.5–10) generated **7** (Scheme 2).



Scheme 1 *Reagents and conditions*: (a) $NH_2NH_2{\cdot}H_2O$, KOH, $HOCH_2$-CH_2OH, 160–180 °C, 2 h; (b) 40% HNO_3, CH_2Cl_2, 1.5 h; (c) $(HCHO)_n$, Triton B (40% in MeOH), reflux, 6 h.

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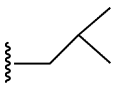
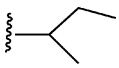
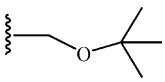
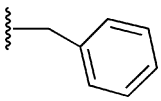
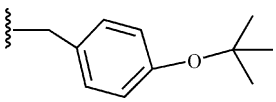
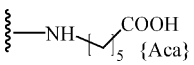
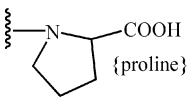
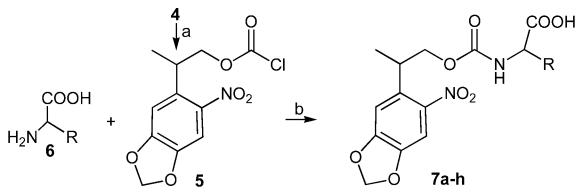
Table 1 Reaction times, yields and masses of products **7**

Entry	R	Time/h	Yield[a] (%)	Calculated [M⁺]	Found[b] [M + H⁺]	[M + Na⁺]	[M + K⁺]
7a	H	15	89	326.1	327.1	349.1	365.1
7b		18	87	340.1	341.1	363.0	379.0
7c		18	86	382.1	383.2	405.2	421.1
7d		17	84	382.1	383.2	405.2	421.1
7e		16	84	412.2	413.3	435.2	451.1
7f		16	85	416.1	417.1	439.1	455.0
7g		17	88	488.2	489.3	511.3	527.2
7h		18	83	382.1	383.2	405.1	421.1
7i		15	81	366.1	367.1	389.1	405.1

[a] Isolated yield. [b] LC-MS (API-ES⁺).



Scheme 2 *Reagents and conditions*: (a) $COCl_2$, THF, 0 °C, 3 h; (b) Na_2CO_3, 1,4-dioxane–water (1 : 1), rt, 15–18 h. (Table 1).

For the light-directed maskless peptide arrays synthesis, it was worthwhile to evaluate the efficacy of the critical photomediated deprotection in an array format, MNPPOC-Ala was coupled to an amino-glass slide. Specific areas (pixels) of slides were irradiated with DMD (365 nm, 20 mW cm^{-2}) for different times and then treated with fluorescent dye (BODIPY) followed by scanning on a GenPix Scanner. It was found that the optimum MNPPOC-Ala coupling time was 20 min, and the photodeprotection time was about 2 min in acetonitrile. A similar experiment was repeated with NPPOC-Ala and the photodeprotection time was found to be more than 2 min in acetonitrile.

Per-step coupling efficiency was determined basically as outlined in Fig. 2, in which step 1 and 2 were repeated with MNPPOC-Ala by synthesizing one to three layers of Ala. The fluorescent

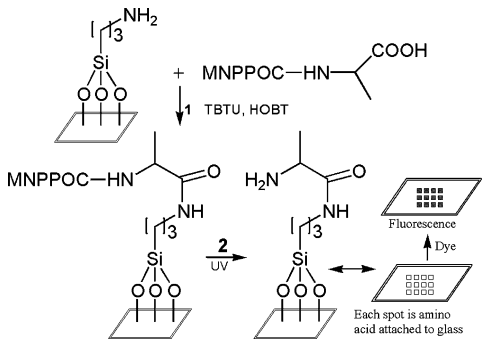


Fig. 2 Attachment of MNPPOC-Ala to the amine coated glass slide followed by UV deprotection and fluorescence detection.

dye binding and scanning shows about 98% of per-step coupling yield.

As a prelude to making complex more arrays, we sought to demonstrate the ability to make biologically active peptides on glass surfaces, by synthesizing Leu-enkephalin,[15] Tyr-Gly-Gly-Phe-Leu-Aca (YGGFL), where Aca is the peptide-to-glass spacer, 6-aminocaproic acid, for which an antibody is commercially available (anti-Leu-enkephalin polyclonal antibody from Chemicon International). On the same slide we also synthesized, as a control,

a false peptide, PGGFL, where the N-terminal Tyr has been substituted by Pro. After synthesis, the slide was treated with ovalbumin to reduce non-specific binding, followed by the anti-Leu-enkephalin. After being washed, the slide was treated with protein-A (labeled with fluorescent BODIPY dye), which binds to antibodies, and finally the slide was scanned. Fig. 3A shows replicate bindings to YGGFL (top row of three), Leu only (next line down), and PGGFL (second line down). In this experiment, only the "true" antigen, YGGFL, fluoresces; neither Leu nor the false peptide, PGGFL fluoresces.

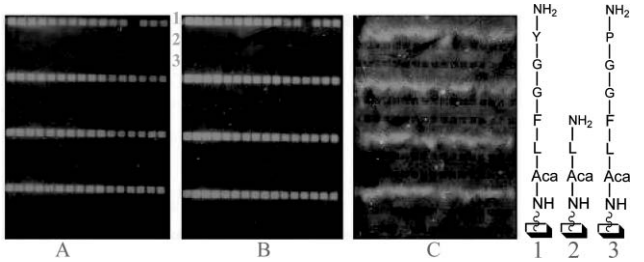


Fig. 3 Binding of anti-Leu-enkephalin antibody to YGGFL (pixel size ~100 μm). In all figures, the top row is YGGFL (1), below that is Leu only (2), and below that is PGGFL (3). These sets of rows are duplicated four times vertically; the gap in the top row is a blank left to orient the patterns. A, original scan after treatment with antibody and fluorescent protein-A; B, scan after stripping slide and retreating with antibody and protein-A; C, scan after denaturation of protein.

To further demonstrate the nature of the binding, the slide was treated with trifluoroacetic acid which disrupts antigen–antibody binding and strips all proteins from the slide. When the slide was scanned again, no fluorescence was seen (not shown in Fig. 3). The same slide was treated with anti-Leu-enkephalin antibody and fluorescent protein-A, washed and scanned again. As seen in Fig. 3B precisely the same pattern is observed. The slide was kept in the presence of moisture at room temperature for 24 h and was finally scanned (Fig. 3C), the expected denaturation of proteins was observed.

In order to verify the presence of actual peptide on glass surface, Tyr-Gly-Gly-Phe-Leu-Aca was synthesized by derivatizing the entire surface with Fmoc-Rink Linker followed by the above procedure of maskless photolithography on area 1 cm × 1 cm. The peptide was cleaved off at Rink Linker in acid from the glass slide and was finally analyzed by API-ES mass spectrometry.

In conclusion, these experiments demonstrate that: (a) a biologically active peptide can be synthesized on a glass surface in high cycle yields using novel photolabile building blocks and "maskless" photolithography; (b) an antibody (a biological protein) can bind to the *specific* peptide antigen and can be detected using conventional immunological techniques; (c) the antigen–antibody complex can be stripped off and the peptide antigen reprobed. In principle, a single peptide array could be probed sequentially.

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