Photogenerated acid (PGA) was used as the acid to remove the protection group from amino acids or peptide oligomers. Comparative study of the deprotection using a PGA, trisaryl sulfonium antimony hexafluoride (SSb), and trifluoroacetic acid (TFA) was performed on glass microscope slides. The results showed that PGA can replace TFA in the deprotection step of oligopeptide synthesis with comparable efficiencies. Acids needed for the deprotection step were generated in situ by light activation of the precursor molecule on the microwell substrate. A maskless laser light illumination system was used to activate the precursor. The accuracy of the amino acid sequence of the synthesized oligopeptide and the location of the synthesis was illustrated by the specific recognition binding of two different models: lead(II) ion–peptide biosensor for lead(II) and human protein p53 (residue 20–25)–mouse MAB DO1. After parallel synthesis of the target peptide models and their analogues based on the predetermined pattern, specific binding treatment, and fluorescence labeling, the fluorescence emission images of the oligopeptide microarray showed fluorescence intensity as a result of specific binding at the correct locations of the array. The stepwise synthesis efficiencies of pentapeptide synthesis on the microwell substrate range are ~96–100% and do not decrease with respect to the chain length of the peptide.

**Introduction**

Miniaturization of conventional experiments to a microarray platform reduces reagent consumption, minimizes reaction volumes, increases the sample concentration, and accelerates the reaction kinetics (Schena et al., 1998). The microarray platform allows parallel synthesis of molecular libraries, massively parallel assaying, and data acquisition. Of the various libraries of bioorganic compounds, those of peptides have increasingly been used as useful tools in all areas of biomedical research. They have been successfully employed to study antibody–antigen interactions, to develop enzyme inhibitors and antimicrobials, and to engineer novel properties in antibodies (Houghten, 1995; Rodda et al., 1996). There are two main methods of making peptide microarrays.

The first method is based on presynthesizing the oligopeptides. The presynthesized oligopeptides are then transferred to the surface of the array substrate either by mechanical microspotting or by piezoelectric delivery adapted from inkjet technology. This technique allows rapid implementation with high versatility and low cost. However, very large numbers of oligopeptides must be synthesized, purified, and stored before peptide microarray fabrication (Schena et al., 1998). The second method relies on the combination of photolithography technology and light-labile protected amino acid chemistry. Different peptide oligomers are simultaneously synthesized directly on the array substrate. The first generation of the peptide microarray of this type was developed by the Affymetrix group (Fodor et al., 1991). The locations of the synthesis for different peptides are controlled by a series of photolithographic masks. This technique has the advantage of generating addressable peptide array. However, the need for many photomasks, which are expensive and time-consuming to build and design, limits the use of this method.

It is clear that an alternative high yield and low cost method to fabricate peptide microarrays should be investigated. Pellois et al. (2000) used BOC chemistry and a micromirror projection system to synthesize oligopeptides. In this manuscript we describe another similar alternative. Our method is based on two main concepts: First, we use the chemistry of conventional liquid phase synthesis using acid deprotection of amino acids; second, we use spatially directed generation of acids with a laser scanner for parallel synthesis of peptides. The conventional acid deprotection strategy was selected because it has been widely shown that efficiency of the peptide synthesis is very high. Photogenerated acid could be obtained by irradiating the precursor with UV light at its absorption wavelength. Therefore, the deprotection occurred only in the designated area. PGA has been successfully used to efficiently remove the protection group in DNA synthesis (Leproust et al., 2000). As a result, many different peptides could be simultaneously synthesized at predetermined locations on the array substrate.

To test the concept, comparative synthesis of pentapeptide models was performed on glass microscope slides using conventional acid, trifluoroacetic acid, and PGA to remove the protection group from the peptide. The stepwise synthesis efficiency of the two chemical
methods was quantified and was found to be identical within measurement accuracy. Then the light-directed parallel synthesis of oligopeptide models was performed on microarray substrates. The accuracy of the amino acid sequence in the oligopeptide models was tested by specific recognition binding.

**Materials and Methods**

Dimethylformamide (DMF), dichloromethane (DCM), diisopropylcarbodiimide (DIC), diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were peptide synthesis grade and obtained from Fisher Scientific. Aminopropyltriethoxysilane, propyltriethoxysilane, and (heptadecafluoro-1,2,2-trihydroxydecyl)triethoxysilane were from Gelest, Inc. All BOC-amino acids were from Calbiochem-Novabiochem. Acetic anhydride, acetic acid, dansyl chloride, ethanol, H2O2, hydroxybenzotriazole, isooctane, methanol, and piperidine were from Sigma Chemical. Mixed trisarylsulphonium antimony-hexafluoride (50% SSb stabilized in propylene carbonate) was from Secant Chemical, Inc., Winchendon, MA. Mouse anti-human p53 clone DO-1 was from Serotec, Inc. 5,6-Carboxyfluorescein (FAM) was from Molecular Probes. Borofloat glass wafers were from Schott. Photosist and developer solutions were from Hoechst Celanese and Clarion. Chromium etchant solution was from Cyantek, Inc., and photoresist stripper was from J. T. Baker, Inc. All other chemicals were obtained from Fisher Scientific.

**Patterned Microwell Microarray Substrate.** Microarray glass plates (22 mm × 75 mm) containing 144 cylindrical wells with diameter of 600 μm and depth of 30 μm confined in a 1.4 × 1.4 cm² area were prepared by semiconductor manufacturing techniques. Details of preparation are given elsewhere (Srivannavit, 2002). The surface outside the well was derivatized with a solution of (heptadecafluoro-1,2,2-trihydroxydecyl) triethoxysilane in isooctane (10 mM, 50 mL) overnight at room temperature under gentle shaking.

**Derivatization of Substrate Surface.** Glass microscope slides or microarrays platforms were cleaned with a mixture of H2SO4 and H2O2 (6:4, v/v) for 30 min at room temperature. After thoroughly washing with deionized water and 95% ethanol, the substrates were treated with a mixture of 0.1% aminopropyltriethoxysilane and 2.5% triethoxysilane in 95% ethanol for 30 min. The substrates were then washed with ethanol and cured at 110 °C for 20 min. The substrates were kept in a dry, N2-filled chamber until used.

**Peptide Synthesis on Glass Microscope Slide.** Derivatized substrates were treated with BOC-protected amino acids (0.5 mmol) in 20 mL of DCM/DMF (1:1, v/v) in the presence of DIC (0.5 mmol) and HOBt (0.5 mmol) for 2–4 h at room temperature. The samples were then washed thoroughly with DCM/DMF (1:1, v/v), DMF, DCM, and DMF, respectively. To permanently block the remaining free amino terminal on the surface, the samples were treated with 50% acetic anhydride in DMF for 30 min, followed by washing with solvents.

To remove the BOC protection group with the conventional method, the samples were treated with 50% TFA in DCM for 1 h at room temperature followed by a thorough washing. When the photogenerated acid was used, the samples were treated with 10% SSb in DCM and illuminated with UV light. The deprotection was activated by irradiation with the UV light for 20 min. The reaction was allowed to go on for an additional 20 min. The samples were then washed with solvents. After deprotection by acids, the samples were neutralized by 5% DIEA in DMF for 5 min, followed by solvent washing.

The coupling, capping, and deprotection cycle was repeated until the oligopeptide of the desired length was obtained. The removal of the protection group on the side chain of amino acids was done following the instructions of the vendor (Calbiochem-Novabiochem).

**Peptide Synthesis on Microwell Microarray Substrate.** The microwell substrate was placed into the reactor cartridge. The coupling and capping steps were achieved by injection of the reaction solutions, as used in the synthesis on the glass slide, into the reactor. The deprotection at the desired locations was performed by using 2.5% SSb (mixed with perylene, 1:0.1) activated by illumination of those locations with the laser beam. Perylene was included as a sensitizer for SSb, because the light adsorption peak for SSb precursor is centered at 365 nm whereas the wavelength of our light source is 405 nm. The exact mechanism of sensitization by perylene is not understood but is believed to be primarily due to electron transfer to SSb precursor (Crivello et al., 1979). The optimized illumination time was 30 s for each location. The reaction was allowed to continue after illumination for an additional 4 min, after which a solution of 5% DIEA was injected to neutralize the acid generated. The reactor was flushed with a series of washing solvents. The deprotection and neutralization steps were repeated five times at the same location to obtain a high level of deprotection based on the preliminary studies.

**UV Light Illumination System.** The UV illumination system used in this project to carry out parallel synthesis of oligopeptides is shown in Figure 1. The system consisted of a laser diode (405 nm, 25 mW) as the light source, pinhole and focusing lens to refine the size of the laser beam, laser scanner to project the laser beam to different locations, a beam splitter, and a video camera to assist in the alignment of the laser beam to microwells, inside the reactor cartridge. The illumination of laser beam and pattern of projection were synchronized and controlled by Laser Designer and Showtime software.

**Fluorescence Labeling and Detection.** Two types of fluorophores, 5,6-carboxyfluorescein (FAM) and dansyl chloride (dns) were used in this study. Both fluorophores are capable of binding with the amino functional group. FAM was used to detect free amino terminal presence on the surface of the substrate, and dns was used for the signaling of the metal binding peptide.
To detect the free amino group, the samples were treated with a solution of FAM ester containing 5 μM FAM, HOBt (0.0338 g), and DIC (100 μL) in DMF for 4 h at room temperature. After thorough washing with DMF and ethanol, the samples were then covered with Vectashield mounting medium (H-1000) to improve the signal/noise ratio for 1 h. The prepared sample was placed under a cooled CCD camera and illuminated with light at 488 nm, and the emission was detected at 520 nm. The fluorescence images of the samples were acquired, processed, and analyzed using PMIS Image Processing Software program.

In the case of 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride, dns), the samples were treated with a mixture of dns (1.25 mmol) and triethylamine (0.125 mmol) in DMF for 4 h at room temperature. The fluorescence detection was done in the same manner as previously described for FAM with the exception that the exciting wavelength was 365 nm.

**Lead(II) Ion and Arsenic(III) Ion Binding Assay.**

For the Pb(II) ion assay, sample substrates were washed with buffer solution containing 100 mM Hepes (pH 7.1) and 10% methanol. Samples were then treated with 0.1 mM lead chloride in buffer overnight at room temperature. After washing with buffer solution and methanol, samples were left to dry in an inert atmosphere in the dark.

For the As(III) ion assay, the assay was performed by the same procedure as the Pb(II) assay but with 10 mM sodium acetate (pH 4.0) containing 100 mM NaCl as the buffer and 0.1 mM sodium arsenite (NaAsO₂) as the metal ion solution.

**Immunostaining of Human p53 (Residue 20–25).**

Samples were washed with PBS (phosphate-buffered saline, 25 mM NaH₂PO₄, 125 mM NaCl, pH 7.4), PBST (PBS, 0.2% (v/v) Tween 20), and PBS, respectively. Samples were then incubated with mouse MAb DO-1, which recognizes amino acid residue 20–25 of human tumor suppressor protein p53, in PBS at room temperature for 2 h. After washing with PBS and methanol, samples were treated with FITC-tagged secondary rabbit anti-mouse immunoglobulin (IgG) overnight at 4 °C. Following washing with PBST and PBS, fluorescence of the samples was measured.

**Results and Discussions**

**Comparative Peptide Synthesis using TFA and SSb.**

Different amino acid monomers were separately attached to the surface of derivatized glass microscope slides. After the coupling reaction, samples were labeled with FAM to detect the completion of the reaction. The same procedure was then divided into two groups, which were treated with either 50% TFA or 10% SSb in DCM to remove the protection group, followed by FAM labeling. The fluorescence intensity of a clean glass microscope slide was used as the positive control for the coupling reaction of amino acid monomers and as the negative control for the deprotection reaction. There was no free amino terminal on the surface, the slide was not labeled by FAM. The fluorescence intensity detected was treated as the background due to nonspecific binding of FAM to the glass surface. The derivatized glass slide was used as the negative control for coupling reaction and positive control for the deprotection reaction. In the derivatization, the linker molecule is attached to the surface using its siloxane groups, thus giving a free amino terminal on the surface of the substrate. The number of these free amino terminals represents the maximum number of attachment sites for amino acid monomers or FAM. The FEIs showed that the fluorescence intensity of the samples after amino acid coupling was at the same level as that of clean glass. This indicated that the coupling was more than 98% and there were no free amino terminals of the linker molecules left on the surface. The fluorescence intensity values of the samples were measured and compared to the fluorescence intensity of the negative control. The results show that the efficiencies of the synthesis using SSb were at the same level (within a standard deviation) as those using TFA for all steps of the synthesis, thus indicating that SSb could be as efficient as TFA in the deprotection step of the peptide synthesis. The stepwise efficiencies range between 80% and 100%. The results did not show any dependence of stepwise efficiency on the chain length of the peptide. This is in agreement with the study of the synthesis efficiency in solid-phase peptide synthesis as a function of chain length using aminomethyl copoly(styrene-1%-divinylbenzene) resin as the substrate (Sarlin et al., 1984).

**Efficiency of Stepwise Peptide Synthesis on Glass Microscope Slide.**

To avoid the variations due to different types of amino acids on the fluorescence intensity, pentapeptide of glycine was used as the model to obtain and compare the stepwise efficiency. The pentapeptide model was synthesized by using either SSb or TFA in the deprotection step. The fluorescence intensity of the protected amino terminal at the end of each deprotection step of the synthesis cycle was compared to that of the previous cycle to calculate the stepwise efficiency. The efficiency was total efficiency including the effect of both coupling and deprotection of each cycle. However, the coupling step was performed under the same conditions for both acid methods, and therefore the difference in the efficiency was treated as being the result of the difference in the deprotection. The results (Figure 2) show that the efficiencies of the synthesis using SSb were at the same level (within a standard deviation) as those using TFA for all steps of the synthesis, thus indicating that SSb could be as efficient as TFA in the deprotection step of the peptide synthesis. The stepwise efficiencies range between 80% and 100%. The results did not show any dependence of stepwise efficiency on the chain length of the peptide. This is in agreement with the study of the synthesis efficiency in solid-phase peptide synthesis as a function of chain length using aminomethyl copoly(styrene-1%-divinylbenzene) resin as the substrate (Sarlin et al., 1984).

**Light-Directed Parallel Synthesis of Metal Binding Peptide and Its Analogues.**

In this experiment, PGA chemistry was combined with a controllable light illumination system to perform parallel synthesis of different oligopeptides on a microarray platform. Four oligopeptides, dns-Glu-Cys-Glu-Glu, H₂N-Glu-Glu-Glu-Glu, dns-Cys-Cys-Cys-Cys, and dns-Gly-Gly-Gly-Gly were simultaneously synthesized on the microwell platform. The first peptide analogue was found to have specific Pb(II) binding capability detected by the increase in fluorescence intensity of dns after binding (Deo and Godwin, 2000). Other analogues were selected to represent the incorrect peptide sequence and should not be able to bind with Pb²⁺. All peptide analogues were simultaneously synthesized using PGA chemistry and were then treated with FAM to detect the completion of the reaction. The fluorescence intensity values of the samples were measured and compared to the fluorescence intensity of the negative control. The results show that the efficiencies of the synthesis using SSb were at the same level (within a standard deviation) as those using TFA for all steps of the synthesis, thus indicating that SSb could be as efficient as TFA in the deprotection step of the peptide synthesis. The stepwise efficiencies range between 80% and 100%. The results did not show any dependence of stepwise efficiency on the chain length of the peptide. This is in agreement with the study of the synthesis efficiency in solid-phase peptide synthesis as a function of chain length using aminomethyl copoly(styrene-1%-divinylbenzene) resin as the substrate (Sarlin et al., 1984).
synthesized on the microwell substrate by the procedure described in Materials and Methods. The synthesis locations of each analogue are shown in Figure 3a. The microwell was then treated with 1 mM PbCl₂ in buffer solution, and the fluorescence emission intensities were detected. The FEI in Figure 3b displays the difference in fluorescence intensity increase upon binding with the Pb(II) ion at different well locations. A significant increase in fluorescence intensity was observed only at the predetermined well locations of oligopeptide dns-Glu-Cys-Glu-Glu; the increase was not observed for the other three analogues. The FEI indicated the fluorescence signal at the correct locations corresponding to the pattern of the tetrapeptides.

Furthermore, the microarray of the four peptide analogues was used as a screening tool to detect a new target peptide that can bind with As(III). The same array logues was used as a screening tool to detect a new target pattern of the tetrapeptides.

The oligopeptide, SDLHKL, and its two analogues (DSLGKL and SGLHKL) were synthesized simultaneously on the surface of a microwell substrate at the sites where SDLHKL peptide is located. This result revealed a new promising target peptide that can be used as a biosensor for As(III).

The results of binding for both metal ions were confirmed by separate synthesis of the peptide analogues on different glass microscope slides using conventional TFA procedure (data not shown). The increases in fluorescence intensity with respect to the peptide analogues were in agreement with the results obtained from the peptide microarray.

These results show that the peptide synthesis using the PGA chemistry and laser diode/scanner system is a promising alternative method to fabricate a peptide microarray with high accuracy in both peptide sequence and synthesis location.

**Light-Directed Parallel Synthesis of Human p53 (Residue 20–25) and Its Analogues.** One major application of a peptide library for use in immunoassays. It was shown that oligopeptide arrays made by the photolithographic mask method are a powerful tool to study antibody-peptide interactions (Fodor et al., 2000). In this study, the specific binding of hexapeptide Ser-Asp-Leu-His-Lys-Leu (SDLHKL), which is the amino acid residue 20–25 of human p53 protein, and MAb DO-1 (Stephen et al., 1995) were selected as the model for application of immunoassaying.

The oligopeptide, SDLHKL, and its two analogues (DSLGKL and SGLHKL) were synthesized simultaneously as a result of metal–peptide binding.

**Figure 3.** Microarray of tetrapeptides for Pb(II) binding: (a) Pattern of localized parallel synthesis of dansyl-labeled tetrapeptide, dns-Glu-Cys-Glu-Glu (black), dns-Glu-Glu-Glu-Glu (crosshatch), dns-Cys-Cys-Cys-Cys (gray) and dns-Gly-Gly-Gly-Gly (white). (b) Fluorescence image of dansyl-labeled tetrapeptides simultaneously synthesized on microarray using SSB as the deprotection agent and laser diode/scanner to activate the deprotection at each site based on the designed pattern. The array was treated with 1 mM PbCl₂ in buffer solution containing 100 mM Hepes, 150 mM NaCl (pH 7.1), and 10% methanol. (c) Fluorescence image of the same array after treatment with 1 mM EDTA followed by 1 mM NaAsO₂ in buffer solution containing 10 mM sodium acetate (pH 4.0) and 100 mM NaCl. The fluorescence intensity in the image is the increased intensity as a result of metal–peptide binding.

**Figure 4.** Microarray of hexapeptides for immunoassay: (a) Pattern of localized parallel synthesis of Ser-Asp-Leu-His-Lys-Leu (SDLHKL), white), Asp-Ser-Leu-His-Lys-Leu (C, light gray), and Ser-Gly-Leu-His-Lys-Leu (I, dark gray). (b) Fluorescence image of hexapeptide array simultaneously synthesized by using SSB as the deprotection agent and laser diode/scanner to activate the deprotection at each site based on the designed pattern. The array was probed with mouse monoclonal antibody DO-1 directed against amino acid residue 20–25 of p53 and followed by incubation with FITC-tagged secondary rabbit anti-mouse immunoglobulin (IgG).
A lumination system developed can be used for peptide synthesis on the microwell substrate. However, they did not illustrate the efficiency of the deprotection in the synthesis. To study the efficiency, oligomers of glycine were synthesized simultaneously at different well locations. Figure 5 illustrates the FEI after the deprotection step of each synthesis cycle. The first well indicates the fluorescence intensity after deprotection of the first glycine residue. The other wells show the fluorescence intensity after deprotection of the next glycine residue up to the fifth residue, respectively. The results show that the fluorescence intensity values of each deprotection step are approximately the same. The stepwise synthesis efficiency was calculated from the ratio of fluorescence intensity of a certain step to that of its previous step. The calculated stepwise synthesis was very high (96–100%) and relatively constant.

Conclusions

An alternative technique to fabricate peptide microarrays was developed, which relies on the chemistry of PGA and the maskless light illumination system. Using this combination, the simultaneous synthesis of different peptides at different locations was achievable. The accuracy of the amino acid sequence of the synthesized oligopeptide and the location of the synthesis was illustrated by the specific recognition binding of two different models: metal–peptide binding and antigen–antibody binding. The results from both systems confirmed the precision of the oligopeptide synthesis. The stepwise synthesis efficiency was very high and constant based on the synthesis of pentapeptide on the microwell substrate. The results presented herein indicate that the technique developed in this investigation is a promising alternative for fabricating a peptide microarray with high accuracy. The setup required for the operation is simple and convenient.

Notation

BOC tert-butoxycarbonyl
CCD charged couple device
DCM dichloromethane
DIC diisopropylcarbodiimide
DIEA diisopropylethylamine
DMAP dimethylaminopyridine
DMF dimethylformamide
dns dansyl chloride
EDTA ethylenediamine tetraacetic acid
FAM carboxyfluorescein
FEI fluorescence emission image
HF hydrofluoric acid
HOBT hydroxybenzotriazole
MAb monoclonal antibody
PGA photogenerated acid
SSb triarylsulfoniumhexafluoroantimonate
TFA trifluoroacetic acid
UV ultraviolet light

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References and Notes


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