Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 625–633
Published online 13 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.884

JOURNAL OF PEPTIDE SCIENCE

Spot arrays on modified glass surfaces for efficient SPOT synthesis and on-chip bioassay of peptides

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Received 19 March 2007; Revised 8 May 2007; Accepted 14 May 2007

Abstract: To make SPOT synthesis of peptides and their assays on glass surfaces more convenient, a simple method for making spot arrays on a slide glass was designed through patterning with a photoresist and perfluorination followed by amination with various silane compounds and polymers. With these spot-arrayed glass surfaces, we could measure the coupling completion of each Fmoc amino acid on the glass surface by direct fluorescence analysis after fluorescence-labeling the amino groups on the surface of each spot. Then we synthesized several types of decapeptides and HPQ-pentapeptides on the spot-arrayed glasses and identified the optimal surface condition for stepwise peptide coupling and on-chip bioassay. After optimizing the surface conditions, we synthesized a model library of biotin-Gly-Ala-P_1-Gly (P_1: one of 19 amino acids) and successfully replicated the well-known α-chymotrypsin subsite specificities through Cy5-streptavidin binding to the remaining biotin on the surface after the enzymatic digestion. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: SPOT synthesis; perfluorination; coupling completion; stepwise coupling yields; streptavidin binding; α-chymotrypsin substrate

INTRODUCTION

Owing to the findings of new drug targets based on the knowledge of complete human genome sequences, the demand for highly effective and simple methods for peptide library synthesis and protein identification is increasing. Recent advances in the microfabrication processes and surface modification technologies enable the peptide microarray format to be common tools for high-throughput screening.

In terms of the preparation methods, the peptide microarrays are mainly classified into two types: immobilization of presynthesized peptide derivatives on a solid surface [1–6] and *in situ* parallel synthesis directly on the array surface. Of these two methods, *in situ* parallel synthesis provides a miniaturized and spatially addressed peptide array more rapidly and economically and with higher density. Therefore, despite a sacrifice in the quality of the resulting surface-bound peptides, this method has been widely used. *In situ* microarray synthesis has been exploited in two ways according to their synthetic strategies: SPOT synthesis [7] and the photolithographic methods [8,9]. While the resulting integrity of the peptide arrays is not as good as in photolithographic methods, SPOT synthesis is more popular for the construction of peptide arrays and peptidomimetic libraries [10–12] because of its several advantages such as relatively simple experimental procedures, inexpensive equipments and facile automation. In addition, combined with the recent developments of many new synthetic methods accompanied by high-throughput solid- and solution-phase screening methods, the SPOT protocols have been successfully applied to a wide range of biochemical research [13,14]. Over the past years, SPOT synthesis has been performed on various planar solid supports [15], such as ester-derivatized cellulose sheets [7], cellulose-amino-hydroxypropyl ether (CAPE) membranes [11,16], amino-functionalized polypropylene membranes [15] and even gold surfaces [17], for subsequent bioassays and analyses [15–20]. To date, however, most membrane supports suffer from difficulties in fluorescence analysis because of their intrinsic low transparency and high background fluorescence. Moreover, the resulting microarray patterns are often inconsistent in shape and/or size, which are caused by blurring of the spots.

Therefore, we have designed a simple method to prepattern spot arrays on a slide glass, which makes SPOT synthesis of peptides and their assays more convenient. Spot arrays on a glass surface (1.8 $mm^2 \times 40$ spots) were prepared through patterning with a photoresist and subsequent chemical blocking with perfluorosilanization. The patterned photoresist acts as temporary protection against perfluorosilanization and the covalently perfluorinated layer forms the outer wall of each spot, which prevents spot blurring by the reaction solution used, thereby leading to consistent shape and size of each spot. The photoresist is then extensively washed out and the spot surfaces are then aminated with γ-aminopropyltriethoxysilane (γ-APTS), with a hydrophilic polymer such as diamino-polyethyleneglycol (diamino PEG) or with chitosan after the epoxide groups were introduced through silanization (Figure 1). With these spot-arrayed glass surfaces, the coupling completions of each Fmoc-amino

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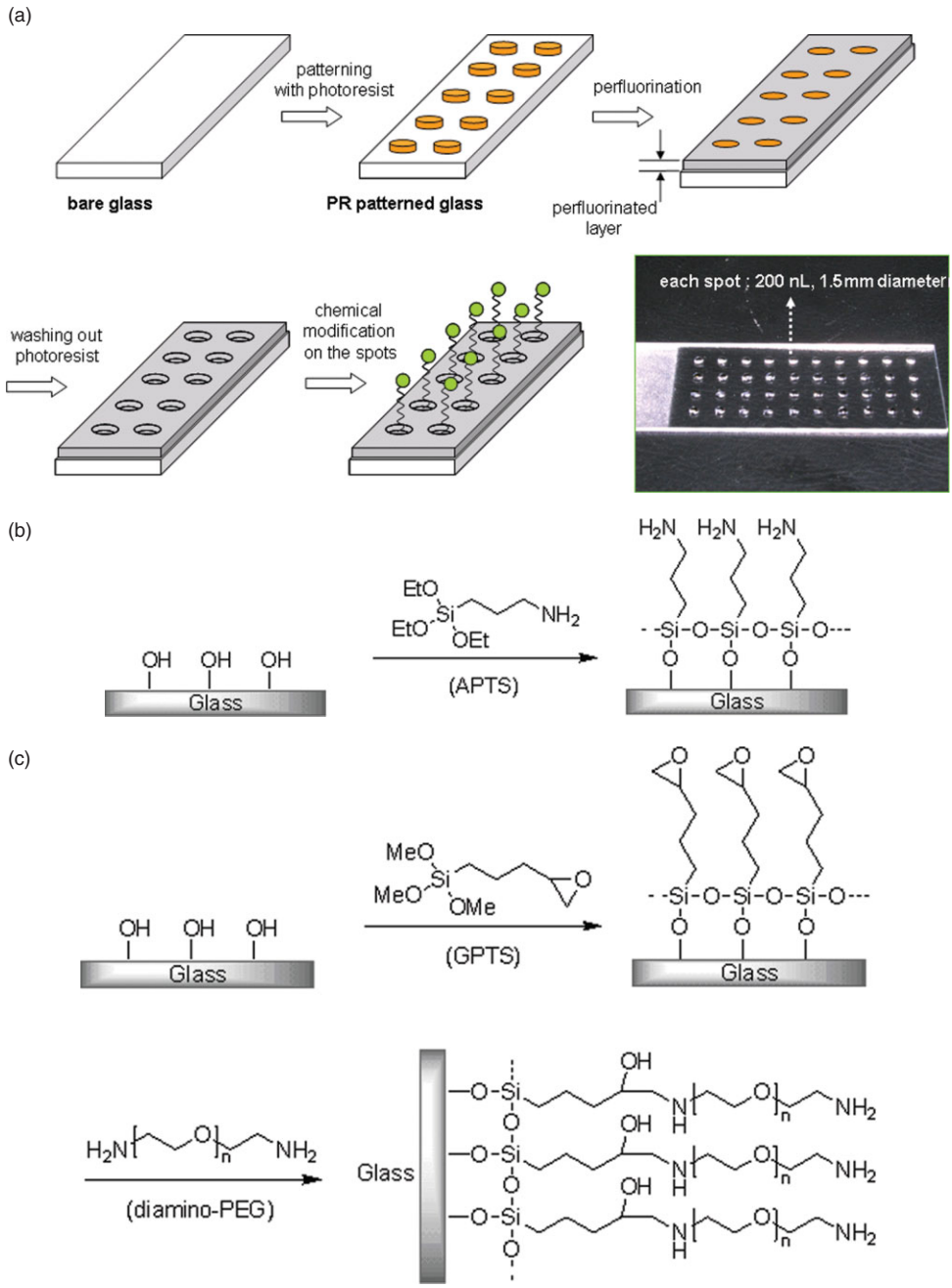

Figure 1 Preparation of amine-patterned glass surfaces with perfluorination: (a) amino patterning steps through photoresist patterning and perfluorination; (b) hydrophobic amination with γ-APTS; (c) hydrophilic amination with diamino PEG; and (d) with chitosan. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

acid on the glass surface were measured and the optimal surface condition for stepwise peptide couplings and bioassays was identified.

MATERIALS AND METHODS

Materials

γ-APTS, 3-glycidoxypropyltrimethoxysilane (GPTS), chitosan (75–85% deacetylated chitin, MW = 50 000–190 000), 4,7,10-trioxa-1,13-tridecanediamine (diamino TEG), 1-hydroxybenzotriazole (HOBt), benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP), diisopropylethylamine (DIPEA), pyridine, acetic anhydride, thioanisole and 1,2-ethanedithiol were purchased from Aldrich (Milwaukee, USA); trifluoroacetic acid (TFA) from Acros Organics (Geel, Belgium); and 1*H*, 1*H*, 2*H*, 2*H*-perfluorodecylmethyldichlorosilane from Lancaster Synthesis (Heysham, UK). Photoresist AZ 4330 was purchased from Clariant Corporation (Somerville, USA). Fluorescein isothiocyanate (FITC), D-biotin, bovine serum albumin (BSA), Cy3-streptavidin, and polyoxyethylene sorbitan monolaurate (Tween 20) were purchased from Sigma (St Louis, USA)

DOI: 10.1002/psc

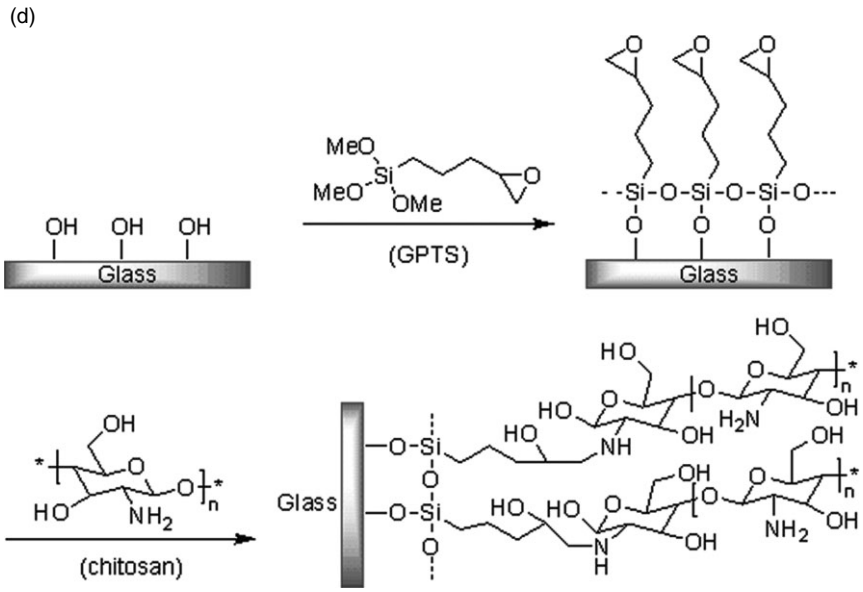

Figure 1 (*Continued*).

and the *O*, *O*′-bis-(2-aminopropyl)polypropylene glycol-*block*-polyethylene glycol-*block*-polypropylene glycol 700, 1500, 1900 (Jeffamine ED-800, Jeffamine ED-1600, Jeffamine ED-2000, respectively) from Fluka (Buchs, Switzerland). All Fmoc-amino acids were purchased from BeadTech (Seoul, Korea) except Fmoc-6-aminocaproic acid (Fmoc-ε-ACA) and Fmoc-β-alanine (Fmoc-β-Ala) (from Bachem AG; Budendorf, Switzerland). *N*-Methylpyrrolidinone (NMP) was purchased from Junsei (Tokyo, Japan) and 2,2,4-trimethylpentane from Duksan Pure Chemicals (Seoul, Korea). All other chemicals and solvents were purchased from DC Chemicals (Seoul, Korea).

The glass slides (75 mm × 25 mm, Micro Slides #2948) were purchased from Corning Glass Works (New York, USA). Fluorescence data were acquired with the GenePix 4000B chip scanner and GenePix Pro 5.0 software, both from Molecular Devices Corporation (Sunnyvale, USA).

Preparation of Spot-arrayed Glass Surface

Glass slides were cleaned with piranha solution (H_2SO_4/H_2O_2: 4/1) for 30 min and washed with acetone (×3) and chloroform (×3). Photoresist AZ 4330 was then roll-coated over a prepatterned silk screen to initiate surface modification of the glass slide. The photoresist-patterned glass was then treated with 1% (v/v) 1*H*, 1*H*, 2*H*, 2*H*-perfluorodecylmethyldichlorosilane in 2,2,4-trimethylpentane solution for 5 min to form a perfluorinated layer on the glass surface. After washing with *n*-hexane (×5) and chloroform (×3), the glass slide was thoroughly washed with acetone to remove the patterned photoresist and air-dried for subsequent processing. The spot-arrayed glass was silanized with 5% (v/v) γ-APTS/chloroform at 50 °C for 1 h for direct amination, or with 5% (v/v) GPTS/chloroform at 50 °C for 24 h to prepare an epoxydated surface. The silanized glass slides were then washed with chloroform (×5) and dried in vacuum for 1 h.

Grafting Hydrophilic Polymers onto the Epoxydated Glass Surface

To prepare a PEG-grafted surface, the epoxydated spot-arrayed glass was reacted with 5% (v/v) diamino TEG, Jeffamine ED-800, Jeffamine ED-1600 or Jeffamine ED-2000/chloroform for 24 h at 50 °C.

To prepare a chitosan-grafted surface, the epoxydated spot-arrayed glass was reacted with 1% (w/v) chitosan/1% acetic acid (aq.) for 24 h at 50 °C and sequentially washed with 1% acetic acid (aq.) (×10), DI water (×5), acetone (×3) and chloroform (×3).

Measuring the Coupling Completion of Each Fmoc-Amino Acid on the Spot-arrayed Glass Surface

Preactivated solution (6.7 mM, 200 nl) of each Fmoc-amino acid (with 1 equiv. of BOP, HOBt, and DIPEA) in NMP was spotted on the aminated glass surfaces with different reaction times (from 5 to 120 min). The remaining amine groups were capped using 10% (v/v) acetic anhydride and pyridine (1 : 1) in NMP for 30 min at 25 °C. After Fmoc-deprotection with 20% piperidine in NMP for 30 min at 25 °C, the liberated free amine groups were reacted with a 5.0 mM solution of FITC in NMP (200 nl) for 2 h at 25 °C to enable FITC labeling to quantify the coupled amino acid quantities (Figure 2). By analyzing the fluorescence intensities at 532 nm *versus* the reaction time, the coupling completion of each Fmoc-amino acid was determined when the fluorescence intensities reached their maxima (Figure 3).

Peptide Syntheses on the Spot-arrayed Glass Surface

All peptide coupling reactions were carried out following the Fmoc strategy. The detailed conditions for the coupling of each Fmoc-amino acid, capping and Fmoc-deprotection were the same as for the above procedure except for fixing the

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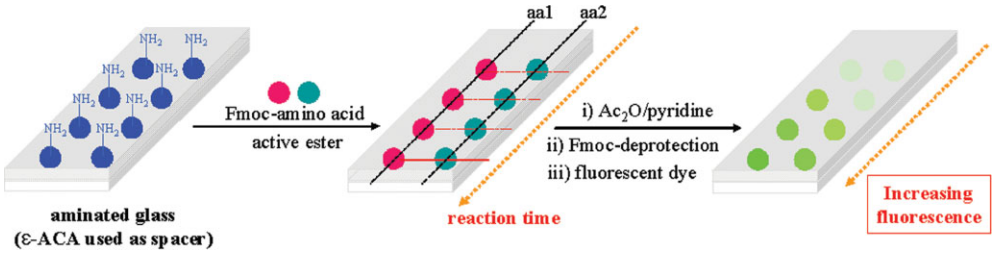

Figure 2 Measurement of the coupling completion for each Fmoc-amino acid. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

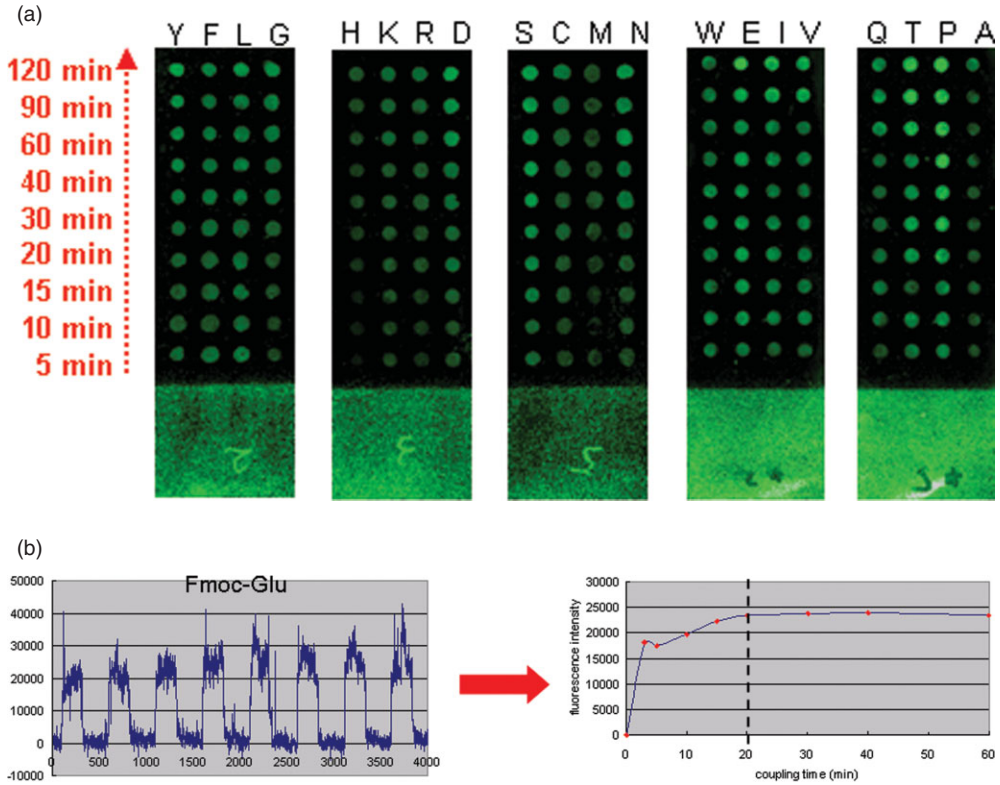

Figure 3 Determining the coupling completion of Fmoc-amino acids (Fmoc-Glu) on a glass surface: (a) fluorescence scanning images of each spot according to the reaction time; (b) quantification of fluorescence data for coupling completion determination. All Fmoc-amino acids have been abbreviated by a single letter; each spot (1.5 mm diameter) contains 200 nl of stock solution. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

coupling time at 1 h. Side-chain deprotection was carried out with reagent K (TFA/phenol/water/thioanisole/ethanedithiol; 82.5/5/5/5/2.5) for 1 h at 25 °C. For the labeling of biotin, 6.7 mM preactivated solution of D-biotin with 1 equiv. of HBTU, HOBt, and DIPEA in NMP was spotted on its appropriate amine-terminated spot and incubated for 4 h at 25 °C.

Measurement of Stepwise Coupling Efficiencies for Peptide Syntheses on the Spot-arrayed Glass Surface

After each coupling step and subsequent capping by acetylation on the spot arrays, Fmoc groups were deprotected and then a labeling spot site was reacted with FITC to form a fluorescence-labeled peptide fragment; other remaining spot sites were subjected to the next coupling. All the coupling/labeling steps were repeated to form FITC-labeled peptide-ladder fragments on each spot, and their fluorescence intensities at 532 nm were measured to quantify the stepwise peptide coupling efficiency by the following equation:

$$\%\ \textit{Yield}\ (\text{step}\ n) = (I_n/I_{n-1}) \times 100$$

where I_n = fluorescence intensity for a peptide oligomer of length n.

Streptavidin Binding Assay on Glass Surface-bound Substrates

The peptide-arrayed glass was submerged in 0.5% (w/v) BSA in 50 mM phosphate buffered saline (PBS, pH 7.5) solution and incubated for 1 h at 30 °C for the blocking of nonspecific

DOI: 10.1002/psc

Table 1 Coupling completion time of each Fmoc-amino acid[a] on the glass surface

Amino acid	Coupling time (min)	Amino acid	Coupling time (min)
Fmoc-Gly	30	Fmoc-Ala	30
Fmoc-Val	30	Fmoc-Leu	30
Fmoc-Ile	30	Fmoc-Pro	40
Fmoc-Phe	30	Fmoc-Met	30
Fmoc-His(Trt)	30	Fmoc-Lys(Boc)	40
Fmoc-Arg(Pbf)	60	Fmoc-Trp	40
Fmoc-Asn(Trt)	60	Fmoc-Gln(Trt)	30
Fmoc-Asp(*t*Bu)	60	Fmoc-Glu(*t*Bu)	20
Fmoc-Ser(*t*Bu)	30	Fmoc-Thr(*t*Bu)	40
Fmoc-Tyr(*t*Bu)	40	Fmoc-Cys(Trt)	20

[a] The concentration of Fmoc-amino acids was fixed as 6.7 mM in NMP with 1 equiv. of BOP, HOBt and DIPEA.

protein binding. The glass slides were then sonicated using DI water (×3) for 5 min and air-dried. After BSA blocking, the glass slides were submerged in a 1 μg/ml solution of Cy3/Cy5-streptavidin in 50 mM PBS (pH 7.5) and incubated for 2 h at 30 °C. The glass slide was then sonicated using 0.1% (v/v) tween 20 in 50 mM PBS (pH 7.5) (×3) and with DI water (×3) for 5 min followed by drying with N_2 purging for subsequent fluorescence measurements at 532/635 nm.

α-Chymotrypsin Substrate Screening on the Spot-arrayed Glass Surface

After building a biotin-Gly-Ala-P_1-Gly library on a chitosan-grafted spot-arrayed chip with an ε-ACA-β-Ala-ε-ACA-β-Ala-ε-ACA-β-Ala-ε-ACA-β-Ala (BEBEBEBE) spacer, the glass slides were submerged in a 1 mg/ml α-chymotrypsin solution in 100 mM tris-HCl buffer (pH = 7.0, with 150 mM NaCl) and incubated for 2 h at 30 °C. The glass slides were then treated with Cy-5 streptavidin as previously described for the quantification of remaining D-biotin after enzymatic digestion.

RESULTS AND DISCUSSION

To determine the optimal condition for peptide synthesis on the spot-arrayed glass surface, coupling completion of Fmoc-amino acids to the glass surface was investigated using spot-arrayed glass slides. The solution of each preactivated Fmoc-amino acid in NMP, at a fixed concentration, was spotted on the γ-APTS-modified glass surface for different reaction times. The amounts of coupled Fmoc-amino acids were then quantified using the fluorescence intensity of each spot *versus* the reaction time after replacing the Fmoc group with FITC. It was determined that all Fmoc-amino acids were completely coupled within 1 h (20–60 min) to the glass surface when using a fixed concentration of 6.7 mM in NMP (Table 1, see the experimental section for details). Thus, the coupling condition of Fmoc-amino acids in subsequent experiments was fixed as 6.7 mM of preactivated Fmoc-amino acid in NMP for 1 h.

We then proceeded to synthesize some arbitrary peptide sequences, e.g. $(AGG)_n$, $(EGG)_n$, $(KGG)_n$, and $(FGG)_n$, in parallel on the γ-APTS-modified glass surface via the Fmoc strategy and measured their coupling yields in each coupling step to verify that peptide synthesis on the perfluorosilane-patterned glass surfaces occurred efficiently. At the end of each coupling step (1 h reaction) and subsequent capping by acetylation, the *N*-terminal of each peptide fragment was labeled with FITC to quantify the amount of elongated peptide according to fluorescence intensity. Unfortunately, we observed significant yield drops at the 3rd, 6th and 7th coupling steps in all the 9-mer peptide sequences, which resulted in very low overall yields (5 ~ 7%). This result is believed to be caused

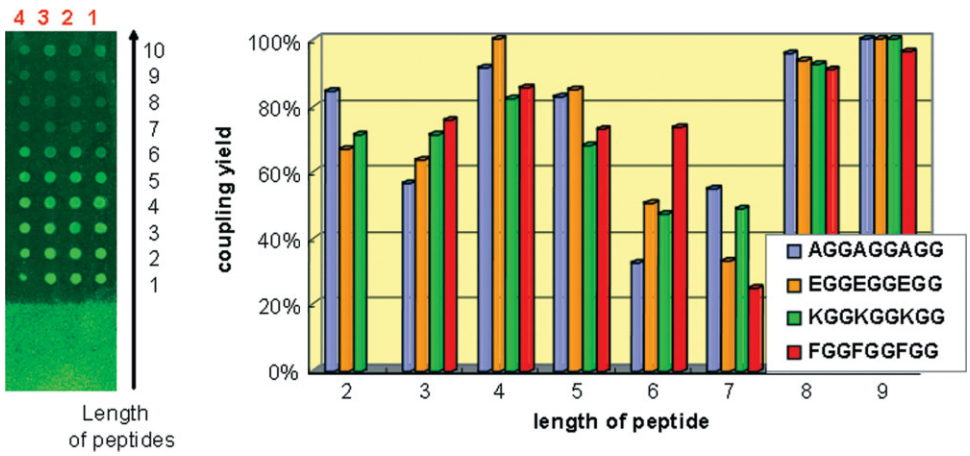

Figure 4 Measurement of stepwise coupling yields on the γ-APTS-aminated glass surface. The left side shows the fluorescence images (Lane 1: (AGG)*n*; Lane 2: (EGG)*n*; Lane 3: (KGG)*n*; Lane 4: (FGG)*n*). The right side graph indicates the coupling yields at each coupling step.

DOI: 10.1002/psc

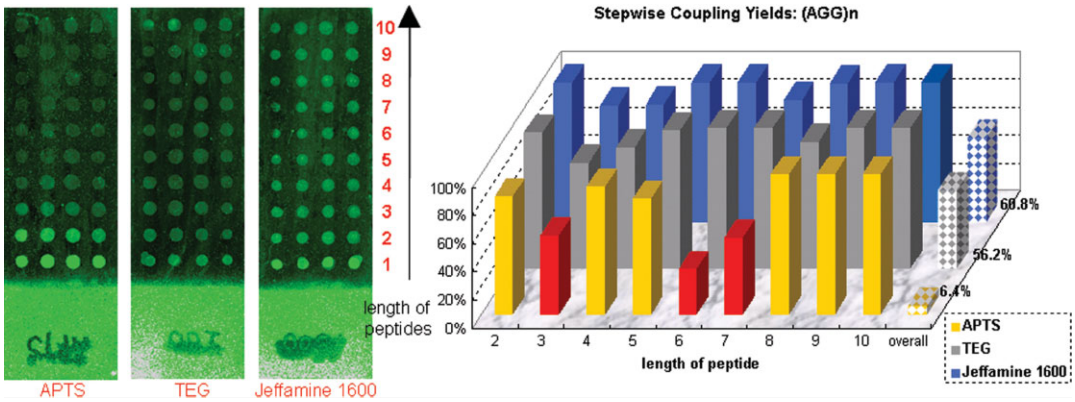

Figure 5 Measurement of stepwise coupling yields of GAGGAGGAGG on variously aminated glass surfaces. The left side shows the fluorescence images of each spot while the right side graph is a comparison of yields by each coupling step according to the aminated surface.

by the intermolecular interactions between the peptide chains on the spots (Figure 4).

We then introduced hydrophilic PEG chains on the glass surfaces with the intent to reduce the functional group densities on a flat glass surface and also steric hindrance between peptide chains, thereby enhancing peptide coupling efficiencies. Thus, we synthesized the GAGGAGGAGG sequence via Fmoc strategy on three types of glass surfaces: diamino TEG-grafted glass, Jeffamine ED-1600-grafted glass and γ-APTS-modified glass, and compared their stepwise coupling yields by fluorescence intensity measurement. As shown in Figure 5, a dramatic enhancement of the overall coupling yields was observed on hydrophilic PEG-chain-grafted glass surfaces (γ-APTS-modified surface, 6.4%; TEG-grafted surface, 56.2%; Jeffamine ED-1600-grafted surface, 60.8%). We confirmed, therefore, that hydrophilic polymer-grafted surfaces are more favorable for peptide synthesis than the γ-APTS-modified surface.

Thereafter, we compared the effects of variously modified glass surfaces on bioassay results by changing the spacer length between the surface-bound peptides and the glass surface. We synthesized HPQ/HPM-containing model pentapeptides known to have affinity to streptavidin [21] on each specific spot site. Firstly, we coupled ε-aminocaproic acid (ε-ACA; E) and β-alanine (β-Ala; B) in turn to the glass surface via the Fmoc strategy to introduce various spacers such as BE, BEBE, BEBEBE and BEBEBEBE on the diamino TEG-, Jeffamine ED-800-, Jeffamine ED-2000- and chitosan-grafted surfaces. Then seven pentapeptides (IHPQG, IGHPQ, VHPMA, REHPQ, FHPQG and IQHPQ as binding sequences for streptavidin and HPQIG as a negative control) were synthesized in parallel on the spot-arrayed glass via the Fmoc strategy, and all the glass slides were blocked with BSA and incubated with Cy3-labeled-streptavidin for binding. From the fluorescence data, we were able to compare the specific/nonspecific binding affinities (S/N ratio) of surface-bound biotin and HPQIG by changing the spacer length over the various polymer-grafted glass surfaces. We determined that the chitosan-grafted surface exhibited the highest S/N ratio (>3.5) and characteristic signal enhancement with a $(BE)_4$ (~40 Å) spacer (Figure 6). Thus, we have identified the chitosan-grafted surface to be most favorable for bioassays of surface-bound substrates.

We also compared the binding affinities of the HPQ-pentapeptides on a chitosan-grafted glass chip along with various spacer lengths. Three types of pentapeptide ligands were selected [22] (IGHPQ, IHPQG and HPQIG) and their binding affinities toward Cy3-streptavidin were then measured. We clearly observed that the specific affinities of IHPQG and IGHPQ increased in keeping with spacer length and became saturated when the spacer was $(BE)_4$ (~40 Å); the non-specific affinity of HPQIG, however, remained constant (Figure 7). From these results, we have concluded that the optimal surface conditions for effective bioassays of chip-surface-bound peptide ligands to be a chitosan-grafted glass surface with spacer lengths exceeding

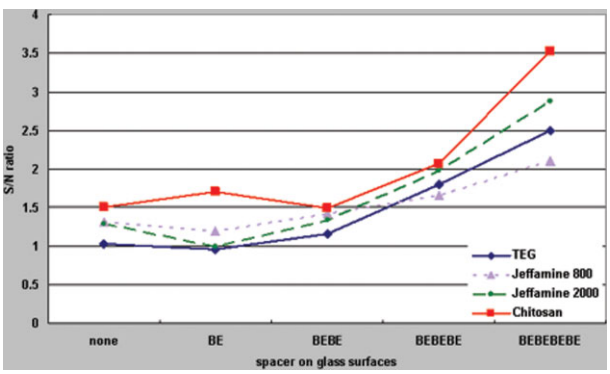

Figure 6 Binding affinities of surface-bound biotin toward streptavidin according to spacer length. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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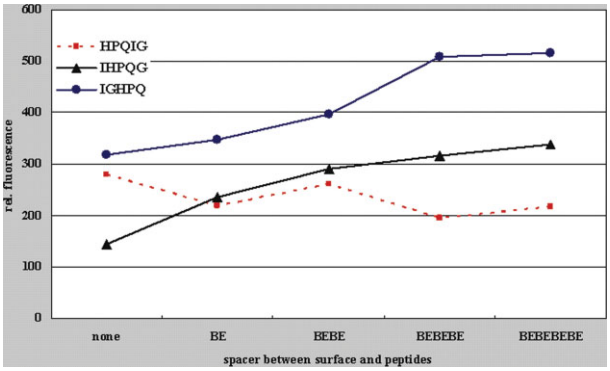

Figure 7 Binding affinities of surface-bound HPQ-pentapeptides according to spacer length on the chitosan-grafted surface. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

40 Å. Moreover, we clearly observed the different affinities of chip-surface-bound pentapeptides on a $(BE)_4$-chitosan-grafted surface and found that the binding affinity of a peptide ligand toward Cy3-streptavidin decreased in the order of biotin > IQHPQ > IGHPQ > FHPQG > REHPQ > VHPMA > IHPQG > HPQIG (Figure 8).

Finally, we synthesized D-biotin-labeled Gly-Ala-P_1-Gly peptides on the $(BE)_4$-chitosan-grafted glass surface via the Fmoc strategy and applied them as α-chymotrypsin substrates. After the enzymatic digestion by α-chymotrypsin, the glass slides were blocked with

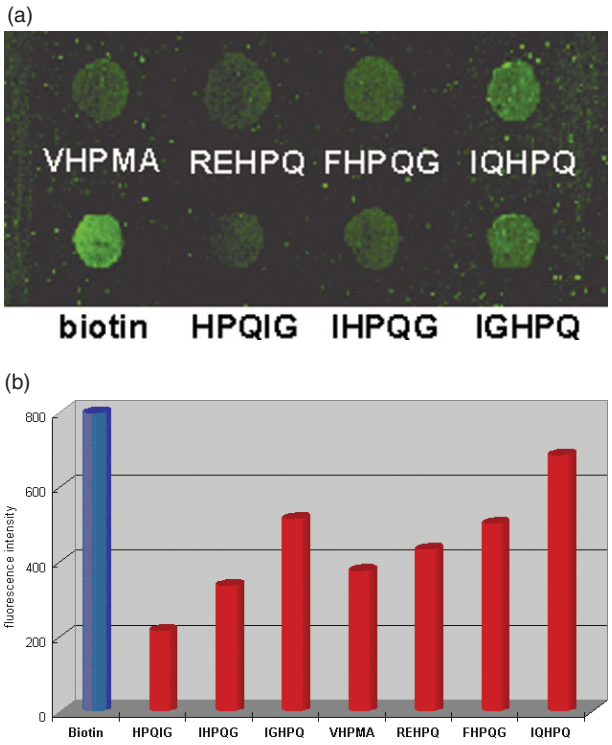

Figure 8 Binding affinities of surface-bound HPQ-pentapeptides and biotin toward streptavidin on a BEBE-BEBE-chitosan grafted glass surface: (a) fluorescence image at 532 nm after binding with Cy3-streptavidin; (b) comparison of fluorescence intensities of chip-surface-bound substrates. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

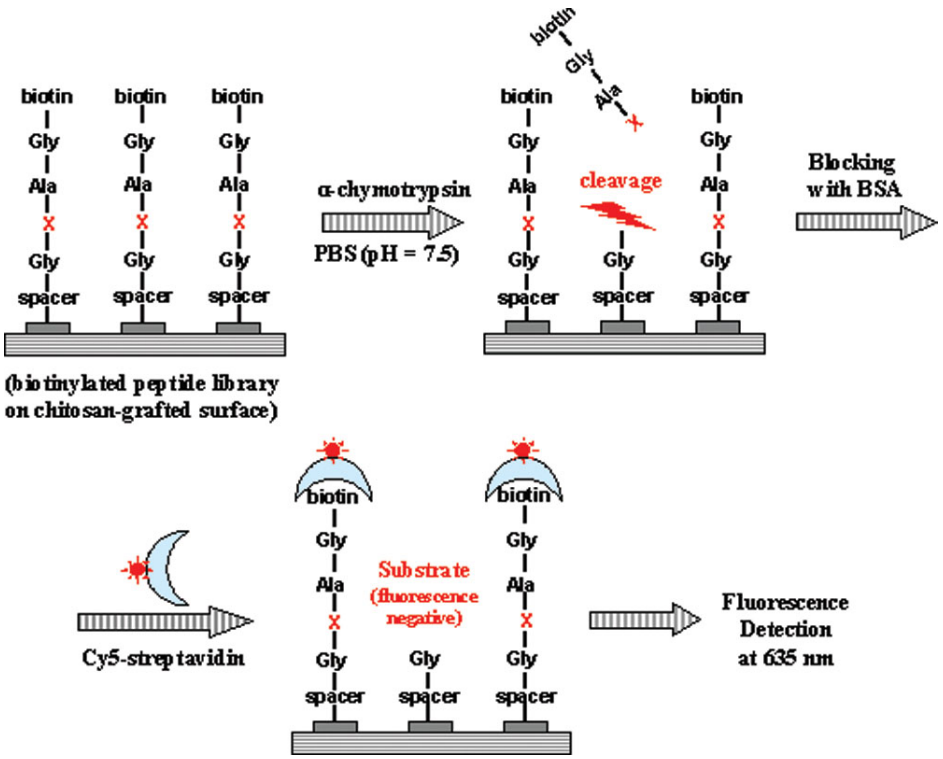

Figure 9 α-Chymotrypsin substrate screening using a biotinylated peptide microarray on a chitosan-grafted glass chip. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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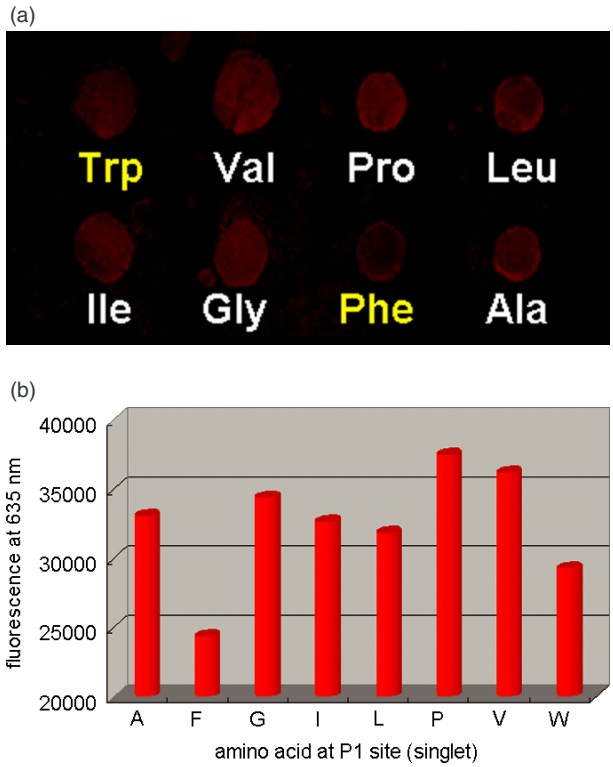

Figure 10 Screening of α-chymotrypsin substrates on a spot-arrayed glass chip: (a) fluorescence images (635 nm) of biotin-Gly-Ala-P1-Gly (varied P1) after incubation with Cy5-streptavidin; (b) the amount of remaining biotinylated peptide, biotin-Gly-Ala-P1-Gly, after the enzymatic digestion by α-chymotrypsin. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

BSA and incubated with Cy5-streptavidin to obtain different fluorescence intensities at 635 nm, which corresponded to the remaining surface-bound biotin after enzymatic digestion (Figure 9). We could easily identify that only biotin-Gly-Ala-Phe-Gly and biotin-Ala-Phe-Trp-Gly, which included the known substrate sequence for α-chymotrypsin, were specifically cleaved from the chip surface resulting in readily distinguishable reduced fluorescent intensities (Figure 10). On the basis of these results, we are confident that the outlined method for SPOT synthesis of peptides on glass surfaces can be applied for high-throughput screening of protease subsite specificities.

CONCLUSION

In conclusion, we have developed a simple method to prepare glass surfaces suitable for SPOT synthesis of peptides by prepatterning spot arrays with a photoresist and perfluorosilanization. After removal of the photoresist, we aminated the patterned glass surfaces by various chemicals such as γ-APTS, diaminoPEG, and chitosan, and finally obtained glass surfaces which were suitable for the spotting process during peptide synthesis and bioassay with respect to consistency of both spot shape and spot size.

The results of stepwise coupling of the model peptide sequences demonstrated that hydrophilic polymer-grafted surfaces are more favorable in peptide synthesis than the γ-APTS-modified surface. Then, we could identify the effective spacer length (BEBEBEBE; ~40 Å) on the glass chip surfaces for on-chip bioassay. With these results, we could successfully synthesize some model peptides with SPOT synthesis on the glass surface and could screen their various bioactivities via an on-chip screening method.

Our surface modification method with photoresist patterning and perfluorination enabled SPOT synthesis on variously modified glass surfaces, and finally led to good performance in peptide synthesis and bioassay on a glass surface. Owing to its simplicity and inherent flexibility, we expect that our surface modification method will offer a breakthrough in SPOT synthesis of peptides and their bioassay on a glass surface.

Acknowledgements

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A050432), and by the ERC program of MOST/KOSEF (R11-2000-075-03001-0).

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