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Investigation of Solid-Phase Peptide Synthesis by the Near-Infrared Multispectral Imaging Technique: A Detection Method for Combinatorial Chemistry

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A near-infrared (NIR) multispectral imaging spectrometer was used to monitor solid-phase peptide synthesis. This imaging spectrometer has fast scanning ability and high sensitivity because it is based on an acousto-optic tunable filter and a NIR InGaAs focal plane array camera. This NIR imaging instrument possesses all the advantages of conventional NIR spectrometers; namely, it can be used for noninvasive monitoring of the reactions and identification of the products during the solid-phase peptide synthesis of glycine, alanine, and valine mediated by aminomethylstyrene resin beads. The reaction was determined by monitoring either the decrease of the band at 1529 nm, which is due to the amine group on the beads, or the increase of the amide band generated at 1483 nm. The amine band at 1529 nm was also used to determine the presence of the Fmoc protecting groups and the efficiency of its removal. More importantly, this NIR imaging spectrometer has additional features that conventional NIR spectrometers cannot offer; namely, its ability to measure spectra at different positions within a sample. This feature was utilized for the first demonstration in which reactions of three different solid-phase peptide syntheses (in a three-compartment cell) were simultaneously monitored. As expected, the kinetics obtained for three reactions are similar to those obtained when the each of the reactions was individually determined. In this study, data recorded by 16 × 16 pixels were used to calculate a spectrum for each sample. However, a relatively good spectrum can be obtained by using data recorded by a single pixel. Since the NIR camera used in this camera is equipped with 240 × 320 pixels, this NIR mutispectral imaging technique is not limited to the three-compartment cell used in this study but rather can be used as the detection method for the solid-phase peptide synthesis in combinatorial chemistry.

Combinatorial synthesis has evolved, in the past few years, from a novel synthetic strategy into the key component incorporated in almost every drug discovery program.[1−4] With the use of solid-phase synthesis on polymer substrates, vast numbers of compounds can be readily obtained by the combinatorial synthesis.[1−3] The generation and screening of such immense numbers of compounds demands innovative analytical methods for the nondestructive and in situ monitoring and analysis. Conventional analytical techniques such as TLC, HPLC, GC, and MS are not suitable since they require cleavage of the compound of interest from the polymeric support.[1−3] Through magic angle spinning, the NMR technique can be used to analyze solvent-swollen resin. However, it suffers from low sensitivity and long acquisition time, specially for analysis of high sample throughput and reaction kinetics.[1−3] To date, near- and middle-infrared methods are probably the most promising technique.[4−11] Infrared

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techniques have been successfully used to monitor and to analyze products of a variety of solid-phase combinatorial reactions.[4−11] However, despite their effectiveness, the application of the IR techniques in combinatorial chemistry is still in the early stage of the development and there are limitations that restrict its general use. The main concerns are due to the speed and cost of the FT-IR instruments. An FT-IR instrument equipped with a microscope was required in almost all of these studies.[4−11] In addition to the high cost, such an instrument can measure only a single position in a sample. When measurements are made on a large number of compounds, as in combinatorial synthesis, it is slow, labor intensive, and also prone to errors. As a consequence, the FT-IR technique has not been widely used in combinatorial chemistry. An infrared method capable of rapidly and simultaneously acquiring spectral information of a large number of samples is, therefore, desirable. A near-infrared (NIR) multispectral imaging spectrometer can offer a solution for this problem.

A multispectral imaging spectrometer is an instrument that can simultaneously record the spectral and spatial information of a sample. Chemical information on a large area of a single sample or samples can be elucidated from recorded images. In these instruments, the spatial distribution of the sample is obtained by a camera, and the spectral information is gained by scanning a dispersive element.[12−16] To be used for on-line monitoring and remote sensing, the instrument must be able to sensitively and rapidly record spectral images of a sample. These requirements can be satisfactorily met with the instrument recently developed in our laboratory.[17,18] In this instrument, an acousto-optic tunable filter (AOTF) is used for spectral scanning and an InGaAs camera is used for recording NIR images.[17,18] AOTF is a electronic tunable filter that has rapid scanning ability, high diffraction efficiency, wide spectral tuning range, and high resolution.[19,20] Because of these advantages, the multispectral imaging instrument based on an AOTF is compact, has no moving parts, and has rapid scanning ability. The sensitivity of the instrument is high because of the high diffraction efficiency of the AOTF (i.e., high light thoughput) and the high sensitivity of the recently available InGaAs camera (i.e., this InGaAs camera has the highest sensitivity among available NIR cameras such as InSb and HgCdTe). Measurements that were not possible can now be made with this imaging spectrometer. These include the determination of the chemical inhomogeneity of copolymers and the authentication of stock certificates and currency.[17] The kinetic evidence of inhomogeneity of the curing of epoxy by amine can also be measured using this instrument[18]

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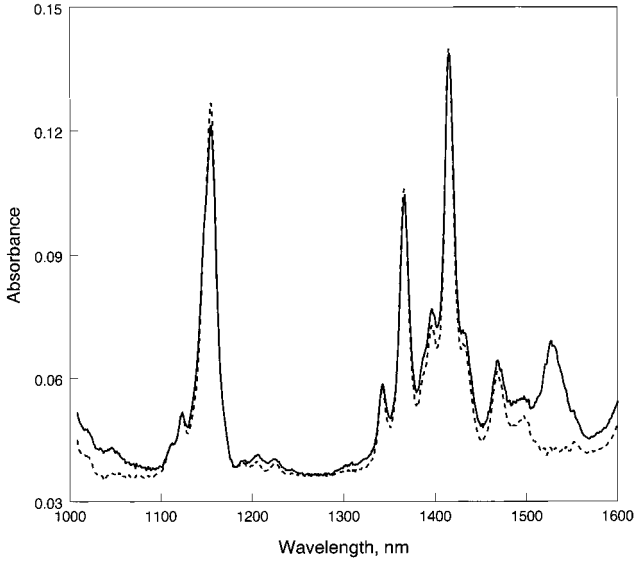

Figure 1. Absorption spectra of the aminomethylstyrene beads in methylene chloride (solid line) and methylene chloride alone (dotted line).

The information presented is indeed provocative and clearly indicates that it is possible to use the AOTF-based NIR multispectral imaging spectrometer to monitor and characterize multiple products in solid-phase combinatorial synthesis. It will be demonstrated for the first time that this imaging spectrometer can be successfully used to monitor solid-phase peptide synthesis by aminomethylstyrene resin beads.

EXPERIMENTAL SECTION

Instrumentation. The near-infrared multispectral imaging instrument used in this study is similar to that used in our previous experiments.[17,18] Essentially, light from a 250-W halogen tungsten lamp was dispersed by a noncollinear TeO_2 acousto-optic tunable filter and detected by a 12-bit InGaAs focal plane array near-infrared camera equipped with 320 × 240 pixels (Sensors Unlimited model SU 320-1.7RT-D/RS 170).

Solid-Phase Peptide Synthesis. Solid-phase peptide syntheses were performed using aminomethylstyrene resin beads which have a loading of 1.9 mmol/g and size of 400−500 μm. This resin was obtained from Polymer Laboratories (Amherst, MA) and used as received. All other chemicals were purchased from Aldrich Chemicals (Milwaukee, WI).

(1) NIR Spectrum of the Beads. The aminomethylstyrene resin was dispersed in methylene chloride and allowed to equilibrate for at least 1 h so that the beads could swell to a constant size. The NIR spectra (from 1000 to 1600 nm) of the swollen beads in CH_2Cl_2 (in a 2-mm path length cell) were measured using the multispectral imaging instrument. Because the swollen beads were at the top of the cell and CH_2Cl_2 was at the bottom layer, the recorded images provide the spectrum of the swollen beads as well as that of the methylene chloride alone. From these two spectra, the spectrum of the beads can be calculated (Figure 1). As illustrated, the only difference between two spectra is the single, well-resolved band at 1529 nm. This band is due to the amino groups attached to the beads. Since these immobilized amino groups are involved in all solid-phase syntheses, this result indicates that it is possible to use the NIR

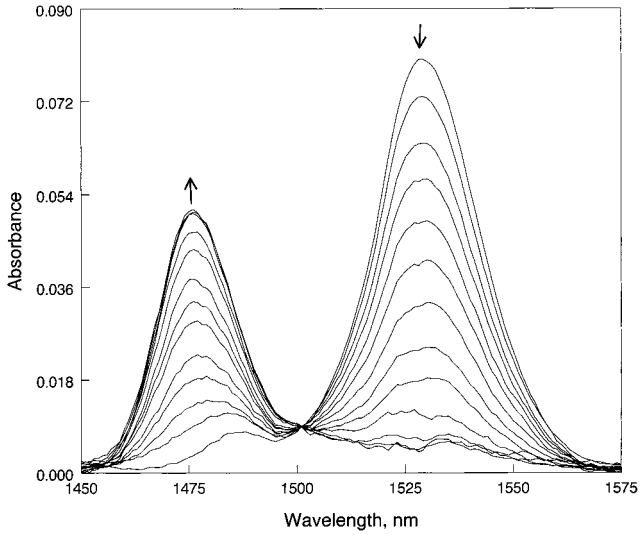

Figure 2. Changes in the absorption spectra during the coupling between the aminmethylstyrene beads and benzoyl chloride. Adding benzoyl chloride led to the decrease in the amine band at 1529 nm and the increase in the amide band at 1476 nm.

multispectral imaging technique to monitor the solid-phase reaction based on these beads.

(2) Calibration. Initially, a simple reaction between benzoyl chloride and the amino groups on the aminomethylstyrene resin was investigated in order to confirm that it is possible to use the amine band of the beads at 1529 nm to monitor the reaction. This reaction was performed in a homemade cell designed for the solid-phase synthesis. This cell was constructed from aluminum with two microscope slides as windows to provide a clear optical aperture of 4 cm × 2 cm and a path length of 7.5 mm. The cell has an inlet at the top and an outlet at the bottom. Porous glass frits were installed at its outlet to keep the beads inside and to allow the solvent to flow freely at a high flow rate through the cell. Chloroform was used as the solvent for this experiment as well as for other subsequent experiments.

A 250-mg sample of the beads was introduced into the cell filled with $CHCl_3$ and allowed to equilibrate for 1 h before the first spectrum was taken. According to the specifications provided by the manufacture (Polymer Laboratories), these beads have a loading of 1.9 mmol/g. The concentration of the amino groups on the 250-mg beads is, therefore, 0.475 mmol. A 1.6-mmol sample of triethylamine in ~2 mL of $CHCl_3$ and 0.054 mmol of benzoyl chloride in ~1 mL of $CHCl_3$ were then consecutively added to the cell. Triethylamine was also added in a large excess in order to prevent the side product (hydrogen chloride) from forming a salt with the amino groups on the beads (which, in turn, would prevent the coupling reaction to proceed). The cell was then shaken for ~5 min for the reaction to complete. The beads were then washed five times with chloroform and the second spectrum was taken. Another portions of triethylamine (1.6 mmol) and benzoyl chloride (0.054 mmol) were then added; the mixture was shaken and washed and its spectrum was measured. The addition of the triethylamine and benzoyl chloride was continued until there were no changes in the spectra of the washed product.

Figure 2 shows the set of spectra of the beads, obtained after adding different amounts of benzoyl chloride. As illustrated, the band at 1529 nm, which is due to the amino groups on the beads,

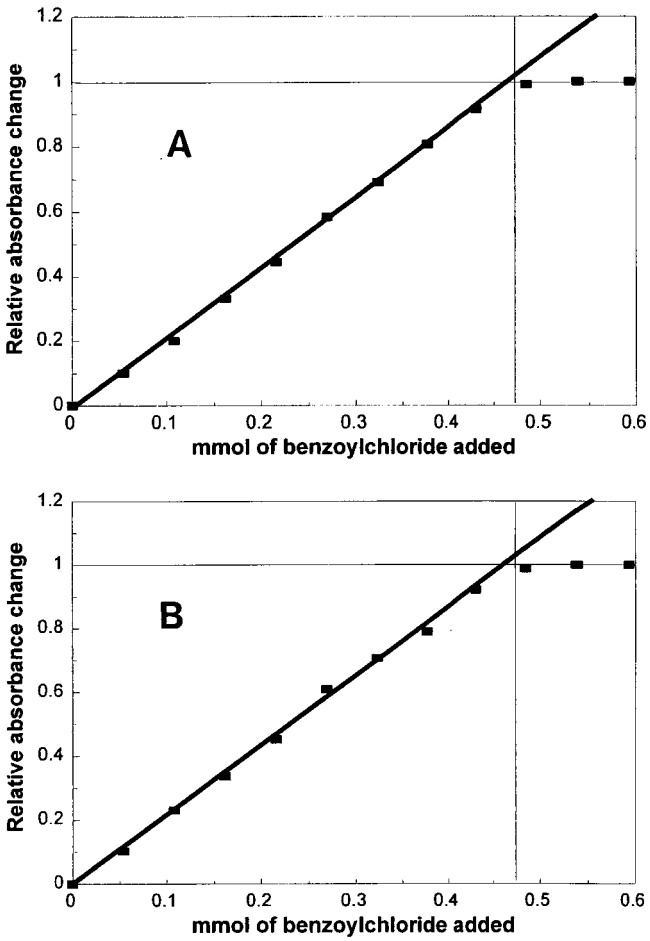

Figure 3. (A) Changes in the absorbance at 1529 nm by the amine group plotted as a function of added benzoyl chloride concentration and (B) changes in the absorbance at 1476 nm by the amide group plotted as a function of added benzoyl chloride concentration. The horizontal line represents the saturation level of the aminomethylstyrene beads, and the vertical line represents its theoretical loading amount.

decreases concomitantly with the generation and subsequently with the increase of another band at 1476 nm. As will be explained in the following section, this 1476-nm band is due to the amide bond formed between the amine group of the beads and the acid chloride group of the benzolyl chloride. An isobestic point at 1501 nm can be clearly observed. Relative changes in the absorbances of these two peaks are plotted as a function of added benzoyl chloride concentration (Figure 3). As illustrated, the changes in the absorbance for both bands are linearly proportional to the concentration of the benzoyl chloride added (it should be noted that because the relative, not absolute changes in the absorbance were plotted in these figures, both straight lines have positive slopes even though the 1529-nm band decreases while the 1476-nm band increases concomitantly with added benzoyl chloride concentration). However, the linear relationship for both bands was observed only up to 0.475 mmol of added benzoyl chloride. Thereafter, no changes in the absorption were observed for both cases. This is as expected because, as described previously, the concentration of the amino groups on the beads is 0.475 mmol. All amino groups available on the beads were reacted when 0.475 mmol of benzoyl chloride was added. Thereafter, any additional addition of the benzoyl chloride would not lead to any reaction.

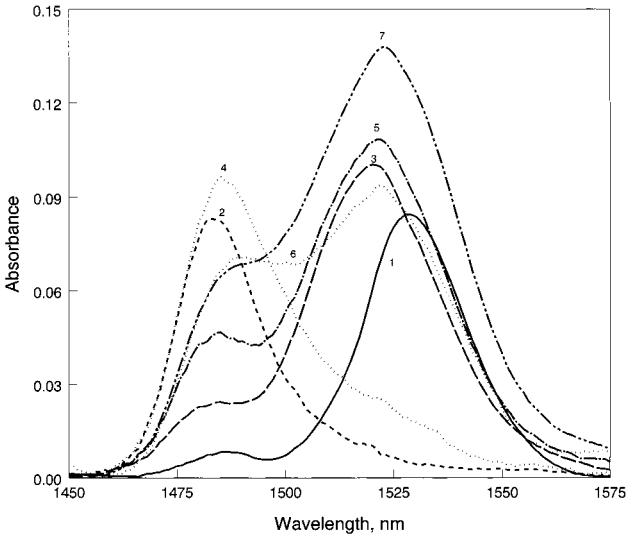

Figure 4. Absorption spectra during a tripeptide synthesis of alanine on aminomethylstyrene beads: (1) beads-NH_2, (2) beads-NH-Ala-Fmoc, (3) beads-Ala, (4) beads-Ala-Ala-Fmoc, (5) beads-Ala-Ala, (6) beads-Ala-Ala-Ala-Fmoc, and (7) beads-Ala-Ala-Ala.

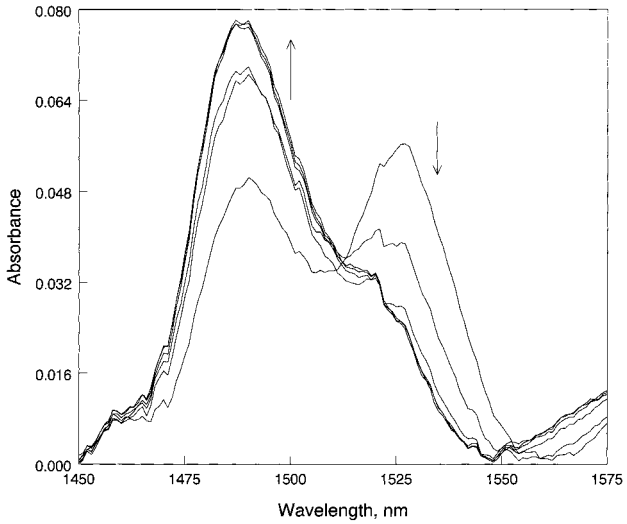

Figure 5. Changes in the absorption spectra during the first coupling reaction between Ala-Fmoc and the amino groups on the resin. The spectra were taken 12, 26, 51, 73, and 95 min after the initiation of the coupling reaction.

These results clearly demonstrate that it is possible to use these two absorption bands for the quantitative analysis of solid-phase reactions mediated by these beads.

(3) Peptide Synthesis. Peptide synthesis was initially investigated in a single-compartment cell used in the previous calibration experiments. Three different amino acids, glycine, alanine, and valine, were used in this study. The amino groups of these amino acids were protected with the *N*-fluorenylmethoxycarbonyl (Fmoc) protecting group. The peptide synthesis was carried out in three steps: activation of the amino acid, coupling reaction, and deprotection of the amino group.

(a) Activation. Three equivalents (relative to the amount of amino groups on the solid support) of amino acid was dissolved in a minimum amount of DMF (∼2 mL). Then 4 equiv of 1,3-diisopropylcarbodiimide (DIC) in 1 mL of chloroform was added, and the mixture was allowed to equilibrate for 2 min to completely activate the caboxylic acid group.

(b) Coupling. The activated amino acid was then added to the reactor cell, which contained 250 mg of resin suspended in a minimum amount of chloroform. The NIR images of the mixture were recorded by the NIR multispectral imaging instrument after this addition, and the recording continued for at least 2 h to monitor the coupling reaction. Upon completion, the beads were washed five times with chloroform and the final spectrum of the beads was recorded in pure chloroform.

(c) Deprotection. The removal of the Fmoc protecting group was accomplished by washing the beads three times (for five minutes each) with 20% piperidine in DMF, and then five times with chloroform. The NIR spectra were then recorded. The resin support was now ready to be linked with a second amino acid.

The absorption spectra were calculated by using the average intensity over 60 × 60 pixels in each pictures (a total set of 161 pictures were recorded from 1440 to 1600 nm). Each spectrum shown is an average over 5 recordings.

RESULTS AND DISCUSSION

Solid-Phase Peptide Synthesis in a Single-Compartment Cell. Figure 4 shows the spectra taken for each steps of the synthesis of a Ala-Ala-Ala tripeptide. Spectrum 1 represents the starting resin (beads-NH_2). As expected, this spectrum exhibits a peak at 1529 nm and is similar to the spectrum obtained for the same beads recorded in the previous calibration step (Figure 2). Spectrum 2 is for the product obtained after the first coupling reaction (beads-NH-Ala-Fmoc). This spectrum has a new band at 1483 nm. This band is very similar to the band at 1476 nm found for the amide group in the previous calibration step (Figure 2). It can, therefore, be attributed to the two amide groups in the coupling product. Because the absorption of the amino group on the beads at 1529 nm for this (beads-NH-Ala-Fmoc) is nearly zero, the yield for this coupling reaction is estimated to be close to 100%. Spectrum 3 was obtained after removing the Fmoc protecting group (beads-NH-Ala). In addition to the amide band at 1483 nm, this spectrum also has another band at ∼1529 nm. This is as expected because this band is due to the amine group generated in the compound after removing the Fmoc protecting group. Spectrum 4 is for the product of the second coupling reaction (beads-NH-Ala-Ala-Fmoc). This spectrum has, as expected, a large amide band at 1483 nm. However, there is also a small shoulder at ∼1529 nm. Since this shoulder is in the same wavelength region as that of the amino group observed in Figure 3, it is possible that the second coupling reaction was not completed even after 2 h. Removing the Fmoc protecting group of this compound enabled the recording of spectrum 5 for (beads-NH-Ala-Ala). This spectrum has two bands: 1483 and 1529 nm. The first band is due to the amide bond. The intensity of this band is ∼2-fold higher than that of spectrum 3 (for (beads-NH-Ala)). This is as expected because there are two amide bonds in (beads-NH-Ala-Ala) but only one in (beads-NH-Ala). The amino band at 1529 nm for this dipeptide (Figure 5) has, as expected, an intensity similar to that of the single coupling compound (Figure 3). Spectrum 6 is for the product obtained after the third coupling (beads-NH-Ala-Ala-Ala-Fmoc). The amine band at 1529 nm is still high in the spectrum. It seems to indicate that the third coupling reaction could not be brought to completion. This is probably due to a lack of reacting time and/or mixing. Spectrum 7 is the spectrum for the (beads-

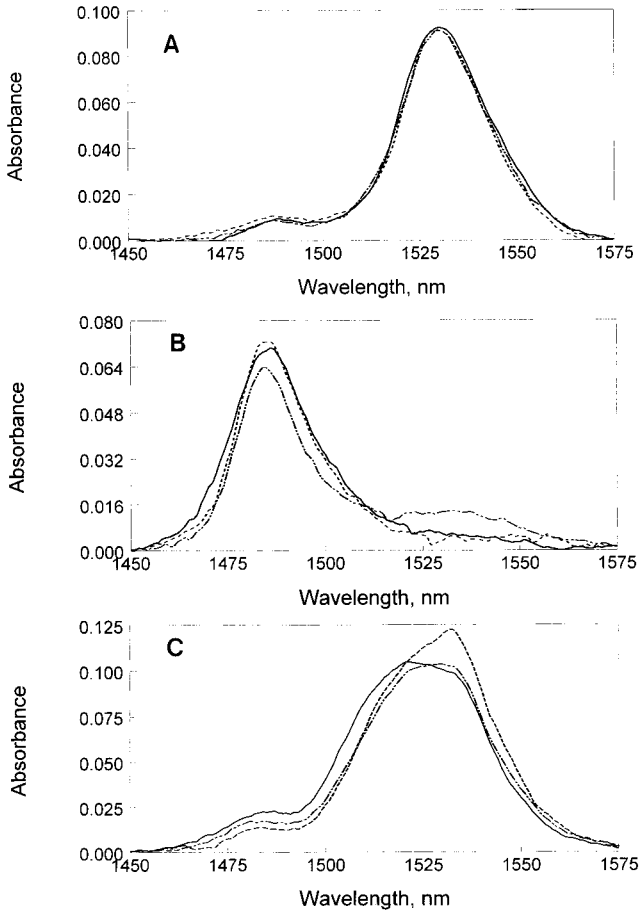

Figure 6. (A) Absorption spectra of the aminomethylstyrene beads in each compartment of the three-compartment cell; (B) absorption spectra of the coupling products between the beads and valine (-···-), glycine (—), and alanine (- - -); (C) absorption spectra of the coupling products after removing the Fmoc protecting groups for valine (-···-), glycine (—) and alanine (- - -).

NH-Ala-Ala-Ala), obtained after removing the Fmoc protecting group. As illustrated, the amine band at 1529 nm is still high in this spectrum.

Multispectra images were recorded during the first and second coupling reaction. Spectra were then calculated from these images. In these spectra, only the baseline was corrected. Correction for solvent was not performed because the amount of DMF present was not known. Spectra obtained during the first coupling reaction are shown in Figure 5. As illustrated, the intensity of the amine band at 1529 nm in the first spectrum taken 25 min after the initiation of the first coupling reaction is about half of that of the starting material (Figure 1). This seems to indicate that the first coupling reaction proceeded relatively fast at the beginning. The changes in the intensity of this band became smaller as the reaction proceeded, and reached a constant value when the reaction was finished. These results clearly indicate that it is possible to use the NIR multispectral imaging technique for in situ investigation of the solid-phase peptide synthesis.

Solid-Phase Peptide Synthesis in a Multicompartment Cell. Experiments in this section were designed to demonstrate that the NIR imaging technique can be used to monitor not only a single reaction as presented in the previous section but rather many different reactions simultaneously. A new multicompartment cell was constructed for this purpose. This cell has three different compartments. A rubber frame made from Vyton was placed between the aluminum frame and the two-microscope glass slide windows to prevent flowing of the sample from one compartment to the others. Each compartment has a clear optical aperture of 2 mm × 40 mm. Similar to the single compartment cell used in the previous section, this multicompartment cell also has an inlet and outlet for each of its compartments, and glass frits were installed at the outlet to keep the beads inside the cell and to allow the solvent to flow through. The cell path length was reduced from 7.5 to 5 mm.

The solid, dotted, and dot-and-point lines in Figure 6A denote the NIR spectra of the beads in each of the three compartments. These spectra were calculated from the same set of NIR images, but at different positions correspond to each compartment of the cell. The amount of pixels used to calculate the spectra in this case was not 60 × 60 pixels as in the previous single-compartment cell but was only 16 × 16 pixels. Correction was made to compensate for a small difference between the wavelength in the center compartment (i.e., center of the image) and the edges of the image due to the horizontal wavelength gradient induced by the dispersive optical components in the NIR multispectral imaging instrument. As expected, the spectra of the beads are the same in all three compartments of the cell.

Because of the smaller cell volume, the amount of beads in each compartment was estimated to be 12-fold smaller than that used in the single-compartment cell. Relatively lower concentrations of the reactants were, therefore, used for the coupling reactions. However, it was found that reducing their concentrations by 12-fold led to incomplete coupling reactions (spectra not shown). As a consequence, the concentrations of the reactants were reduced by 6-fold. Three different amino acids, alanine, glycine, and valine, were used as reactant for each compartment. Figure 6B shows the spectra obtained after the first coupling for each compartment. It is evident that reaction occurred between the amino acids and the amine groups on the beads because a pronounced amide band at 14776 nm was observed in all three spectra (Figure 6B). In fact, the spectra for glycine (solid line) and alanine (dashed line) are very similar to the spectra obtained after the first coupling in a single-compartment cell (spectrum 2 in Figure 4). These results indicate that the coupling reactions were completed for alanine and glycine. In the case of valine, the band due to the unreacted amine group at 1529 nm is relatively higher, and the amide band at 1483 nm is lower than those for alanine and glycine. This seems to imply that the efficiency of the coupling reaction for valine is much lower than those for alanine and glycine. Expectedly, removing the Fmoc protecting groups of the coupling products leads to the regeneration of the amine band at 1529 nm (Figure 6C).

To facilitate the evaluation, the spectra of each step of the reaction were plotted for each individual amino acid. Figure 7A shows the spectrum of the beads before the reaction (beads-NH_2), after the coupling (beads-NH-amino acid-Fmoc), and after removing the Fmoc protecting group (beads-NH-amino acid) for valine. Similarly, those for glycine and alanine were plotted in Figure 7B and C, respectively. As illustrated in all three figures, valine, glycine, and alanine were successfully coupled to the amine groups on the beads. It is interesting to observe that the amine band at

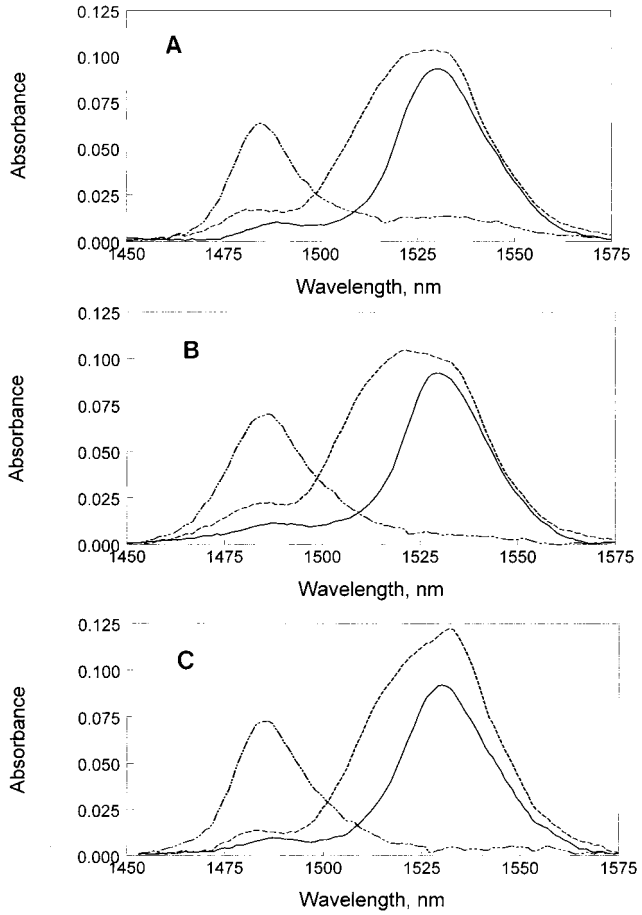

Figure 7. Changes in the absorption spectra of the starting resin beads, the FMOC coupling product, and the detected coupling product measured in a three-compartment cell for valine (A), glycine (B), and alanine (C). Solid lines are the spectra of the starting resin (—), (-···-) are the spectra of the coupling products, and (- - -) are the spectra of the products after removing the Fmoc protecting groups.

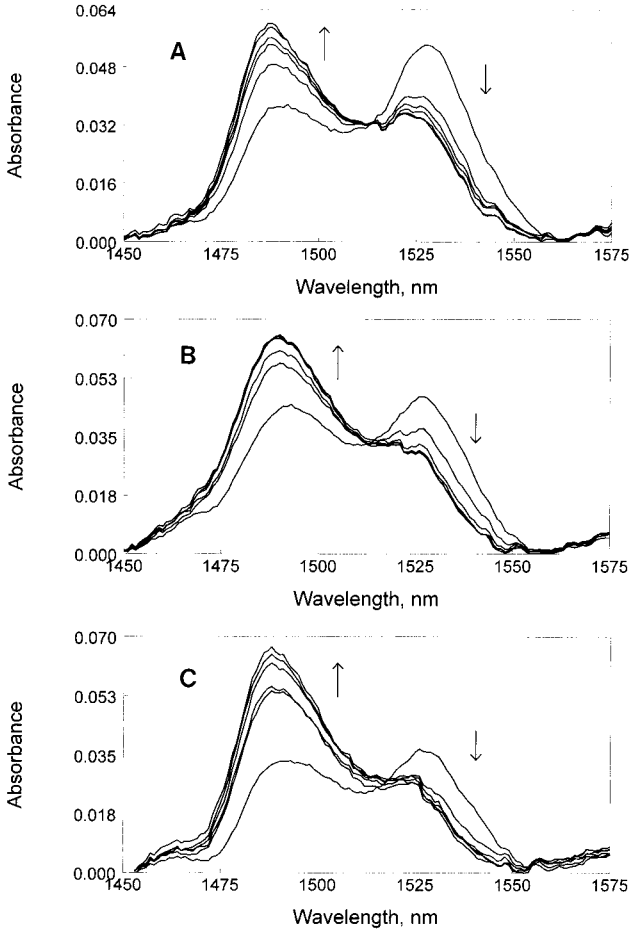

Figure 8. Absorption spectra taken every 20 min during the first 2 h of the coupling reactions in three-compartment cell for valine (A), glycine (B), and alanine (C).

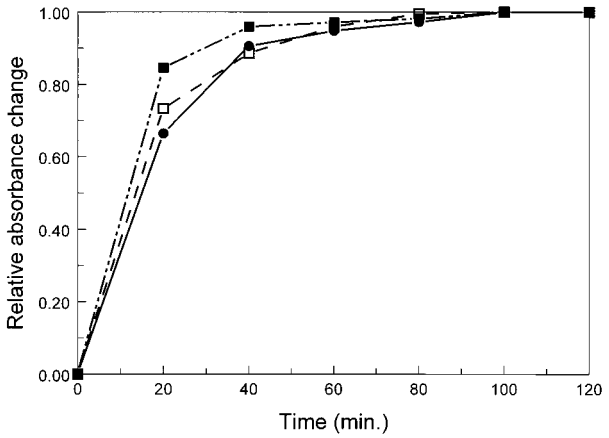

Figure 9. Relative changes in absorbance at 1529 nm during the coupling reaction for (●) valine, (□) glycine, and (■) alanine.

1529 nm for the final product is higher than that in the starting resin for all three amino acids. This is rather unexpected because, after removing the Fmoc protecting group, the number of NH_2 groups on the final product should be the same as that on the starting beads. The higher absorbance at 1529 nm observed in the final deprotected products may be due to the differences in the absorption coefficients of the amine groups on the beads and those on the amino acid final products. This difference was also observed for the experiments in the single-compartment cell (in Figure 4, the absorbance at 1529 nm is higher for spectrum 1 than for spectrum 3).

The NIR multispectral imaging spectrometer can also be used to measure the relative reaction rates of each amino acids. Panels A−C of Figure 8 show the changes in the absorption spectra during the coupling reactions for valine, glycine, and alanine, respectively. These spectra were taken every 25 min during the first 2 h of the coupling reaction. Each spectrum is an average of five images. After each measurement, the cell was shaken for 1 min. It is clear from the spectra that a large amount of amino acid had already reacted when the first spectrum was measured. Nevertheless, by comparing three figures (for three different amino acids), differences in the relative absorbance changes can be observed. The relative reaction rates for all three amino acids can be estimated based on the use of the absorbance at 1529 nm of Figure 7A as a reference. Results obtained are illustrated in Figure 9. It is evident from this figure that the coupling reaction was fastest for alanine and slowest for valine. These results are in good agreement with those presented in Figure 6B. As illustrated in Figure 6B, all coupling reactions were nearly complete except for valine because, in this case, the unreacted amine was present as the small remaining peak at 1529 nm.

In summary, it has been demonstrated that solid-phase peptide synthesis can be successfully monitored by using the newly developed NIR multispectral imaging spectrometer. This imaging spectrometer has fast scanning ability and high sensitivity because it is based on an AOTF and a field-deployable, room-temperature InGaAs focal plane array NIR camera. As expected, this NIR imaging instrument possesses all the advantages of conventional NIR spectrometers; namely, it can be used for the noninvasive monitoring of the reactions and identification of the products during the solid-phase peptide syntheses of glycine, alanine, and valine mediated by aminomethylstyrene resin beads. The reaction was determined by monitoring either the decrease of the band at 1529 nm, which is due to the amine group on the beads, or the increase of the amide band at 1483 nm. The amine band at 1529 nm was also used to determine the presence of the Fmoc protecting groups and the efficiency of their removal. More importantly, this NIR imaging spectrometer has additional features that conventional NIR spectrometers cannot offer, namely, its ability to measure spectra at different positions within a sample. This feature was utilized for the first demonstration in which reactions of three different solid-phase peptide syntheses (in a three-compartment cell) were simultaneously monitored. As expected, the kinetics obtained for three reactions are similar to those obtained when the each of the reactions was individually determined. It is important to realize that the number of compartments of the cell used for this study is not limited to three. Multicompartment cells including those used in combinatorial chemistry (e.g., cell with 96 × 96 compartment) can be used. Furthermore, in this study, up to 16 × 16 pixels were used to calculate a spectrum for each sample. However, as demonstrated in our earlier study,[18] a relatively good spectrum can be obtained by using data recorded by a single pixel. Since the NIR camera used in this camera is equipped with 240 × 320 pixels, it is evident that this NIR mutispectral imaging technique can be effectively used as the detection method for the solid-phase peptide synthesis in combinatorial chemistry. That is, this NIR imaging spectrometer should be able to simultaneously monitor solid-phase peptide syntheses in a multicompartment cell equipped with 96 × 96 compartments or more. This possibility is the subject of our intense study.

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