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Solid-Phase Synthesis on Planar Supports

Abstract: *Planar supports represent a unique opportunity in designing novel approaches to solid-phase synthesis of peptides and small organic molecules. Published work includes assembly on cellulose paper sheets, cotton strips, or membranes, as well as ultrahigh-density synthesis on glass supports. Planar carriers allow for the synthesis to be performed without any reaction vessels (inclusion volume chemistry), construction of libraries with only one representation of each structure, or for continuous synthesis (replacing sequence in time by sequence in space).* © 1999 John Wiley & Sons, Inc. Biopoly 47: 397–404, 1998

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INTRODUCTION

Bruce Merrifield's concept for conducting peptide synthesis on a solid support[1] impacted the conventional processes and techniques of the traditional solution-phase peptide chemists during the 1960s and 1970. More importantly, Merrifield's discovery led to the development of solid-phase organic synthesis and ultimately to the birth of combinatorial chemistry. Merrifield contemplated the use of various functionalized solid supports,[2] but found, however, an optimal support in 2% cross-linked polystyrene in pellicular form. His efforts concentrated in the incorporation of this polymeric support as a feasible method to prepare both small[1] and relatively large peptide sequences as well as small proteins.[3] As a result, solid-phase synthesis of peptides and oligonucleotides became a generally accepted method for the preparation of these classes of compounds. Recently, the application of solid-phase synthesis as a technique for the preparation of small organic molecules has been employed in the drug discovery process.

Application of solid supports in a form different than the polymeric bead of various diameters (35–700 μm) is relatively unique, even though alternative shapes were shown advantageous for several applications. Polyacrylic acid grafted polypropylene "pins"[4] were used for the synthesis of the first peptide library.[5] Additionally, pins were beneficial for multiple peptide synthesis, applicable for nonpeptide syntheses, and via modification by the attachment of "crowns" were adapted for the construction of sequences at a larger scale.[6–10] Other techniques and methods for multiple peptide synthesis[11–13] as well as the preparation of libraries[14–17] have been previously reviewed and will not be discussed further.

However, there are specific applications in which the shape of the support plays a critical role in the design of the synthesis or subsequent screening of the target. Planar solid-phase supports provide an excellent surface to prepare and assay peptides and small molecules. This article will describe the merits and advantages of these supports for the assembly of peptides, small molecules, and combinatorial libraries. The synthesis on solid surfaces for the generation of "spatially addressable combinatorial libraries" has recently been reviewed.[18]

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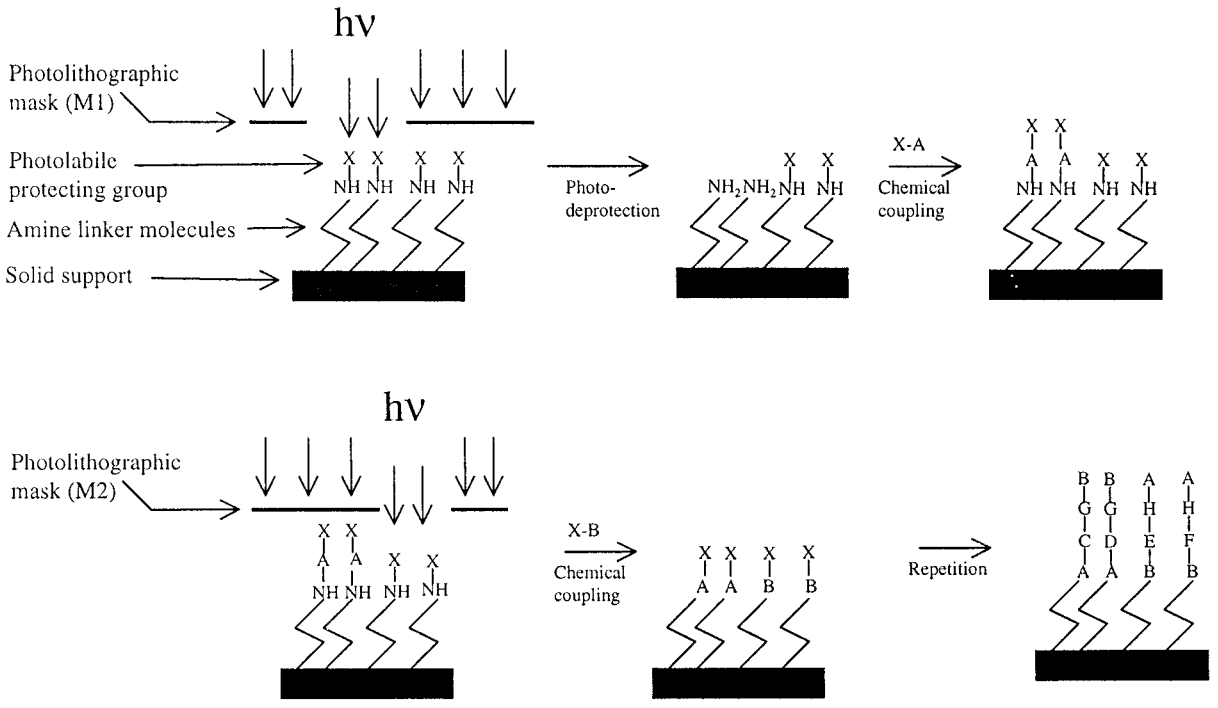

FIGURE 1 Scheme of light-directed, spatially addressable parallel chemical synthesis.

SYNTHESIS OF LIBRARIES ON GLASS SUPPORT

Glass supports provide advantages due to its dimensional stability and chemical inertness. These features were utilized by Fodor and co-workers[19] for the development of the light-directed, spatially addressable parallel chemical synthesis technology. The method was referred to as very large-scale immobilized polymer synthesis (VLSIPS) and became the foundation for the Affymax company to address specific molecular interaction between a drug and receptor. The success of Affymax initiated the avalanche for the formation of biotechnological companies (Selectide, Sphinx, Pharmacopoeia, and Houghten Pharmaceuticals) based on novel technologies for drug development.

VLSIPS is a conceptually simple synthetic technique and was incorporated for the construction of a peptide library (Figure 1). A functionalized solid (glass) surface is treated with an *N*α-photocleavable (e.g., nitroveratryloxycarbonyl) protected amino acid to coat the glass plane. In the next step, a mask is applied to the surface in order to irradiate the glass at defined locations for removal of the *N*α-protecting group. Alternative to the masking technique is the application of a laser for deprotection of a defined location on "a chip." The chip is acylated with an appropriate *N*α-photocleavable protected amino acid. The coupling reaction occurs only in previously designated irradiated areas. The process of irradiation and coupling is repeated as many times as required for the construction of a pattern of peptides on the glass surface. Following linear assembly, the peptide chains are deprotected and a binding assay may be performed. The location on the glass surface of a positively reacting ligand characterizes the target's structure, which may be determined from the synthetic algorithm. This technique allows the construction of an extremely dense pattern of peptides. In the initial experiments, typical resolution was 50 μm, but as the technique evolved, densities greater than 250,000 compounds per square centimeter were achieved. Light-directed surface synthesis may be applied to both peptide and polynucleotide arrays as well as other polymers, provided construction incorporates the same algorithm.[21] A disadvantage to this synthetic method is the requirement for relatively complex instrumentation, and thus this technique has been applied to a limited number of laboratories. The incorporation of glass supports for solid-phase binding assays (i.e., epitope analysis) has been used in various diagnostic applications. Affymetrix, a spin-off company of Affymax, immobilizes oligonucleotide librar-

ies for DNA mapping and DNA sequence analysis via hybridization.

SYNTHESIS ON FUNCTIONALIZED MEMBRANES

Merrifield's group[22,23] pioneered solid-phase assembly of peptides using sheets of functionalized polyethylene. A low density noncross-linked polyethylene sheet was γ-irradiated in the presence of a solution of styrene in methanol. A membrane consisting of 440 wt % polystyrene graft was obtained, which was aminomethylated to yield a surface with a substitution of 1 mmol/g. This support was used for the parallel synthesis of peptides following the "tea-bag" strategy in which marked pieces of support are subjected to common steps of the synthesis in one vessel (deprotection, washing, neutralization) and individual amino acids are coupled in separate reactors. Synthetic results were comparable to those obtained with classical solid supports. Millipore scientists[24] developed a polypropylene membrane coated with cross-linked polyhydroxypropylacrylate and compared its use in the synthesis of several medium-sized peptides. In these experiments, the membrane was sealed into a disposable polypropylene cartridge wrapped around the central rod, which served as a column in a flow-through synthesizer.

SYNTHESIS AND SCREENING OF LIBRARIES ON CELLULOSE PAPER SHEETS

Cellulose paper was shown by Frank to be an excellent support for the multiple synthesis of thousands of peptides[25,26] as well as the construction of libraries.[13] Synthesis on segmental paper support was originally developed for the assembly of oligonucleotides, where its application was simpler to perform since the technique requires only four building blocks.[27] The process for peptide synthesis includes resorting of labeled paper disks following each coupling step and performing the acylation reactions with all of the disks coupled to the same amino acid placed in one reaction vessel. Physical properties of the paper do not allow for shaking, which is usually applied to ensure a complete reaction. Therefore, the synthesis is performed in a flow-through arrangement; paper disks are packed in the column percolated by the appropriate activated amino acid solution.[25]

An alternative strategy for the synthesis of peptides on paper is performed by "spotting" the solution of protected amino acids onto the functionalized cellulose surface in the presence of an activating reagent ("SPOT" synthesis).[28,29] In this case, the reaction vessel is the polymeric support, liquid manipulation (shaking) during the synthesis is eliminated, and the reaction is driven to completion via the diffusion of the liquid into the paper. The principle of internal ("inclusion") volume synthesis was examined using polymeric carriers on a multiple synthesizer utilizing centrifugation for liquid elimination.[30,31] The results were comparable as well as exceeded the classical arrangement of solid-phase peptide synthesis.[32] SPOT synthesis may be applied to both the assembly of large arrays of individual sequences and arrays of partially defined peptide mixtures.[13] In the latter case, a mixture of protected amino acids is used in the coupling step rather than an individual amino acid solution. To achieve equimolar incorporation of individual components of the mixture, the concentration of amino acids is adjusted according to their reactivities.[5] Alternatively, the coupling may be performed in two steps. Initially, subequimolar amount (0.8 molar excess) of the activated mixture is used repetitively with prolonged coupling times followed by the addition of a large excess of the solution containing the mixture of residues.[33–35] This method is referred to as the multiple substoichiometric addition approach. Finally, SPOT synthesis has been automated by the addition of pipetting robots.[13]

Libraries attached to a paper support were used for the determination of antibody binding determinants,[36–39] seroreactive regions of viral proteins,[40] substrate specificity of protein kinases,[41,42] DnaK chaperone,[43] and various protein–protein interactions,[35,44–46] including the interaction between interleukin-6 and its receptor.[47] In addition to the biological applications, paper-bound libraries were incorporated for determining specific ligands for binding various metals[33,48,49] or DNA.[33] The use of cellulose paper-bound libraries has been previously reviewed.[13, 49] To better appreciate the power of screening cellulose paper- bound libraries as well as visualizing the obtained results, the publications of Tegge et al.[41] and Kramer et al.[49] are recommended.

SYNTHESIS OF LIBRARIES WITH NONSTATISTICAL REPRESENTATION OF INDIVIDUAL MEMBERS

The specific characteristic of a membrane, sheet, or thread-like type of carrier is its divisibility. This fea-

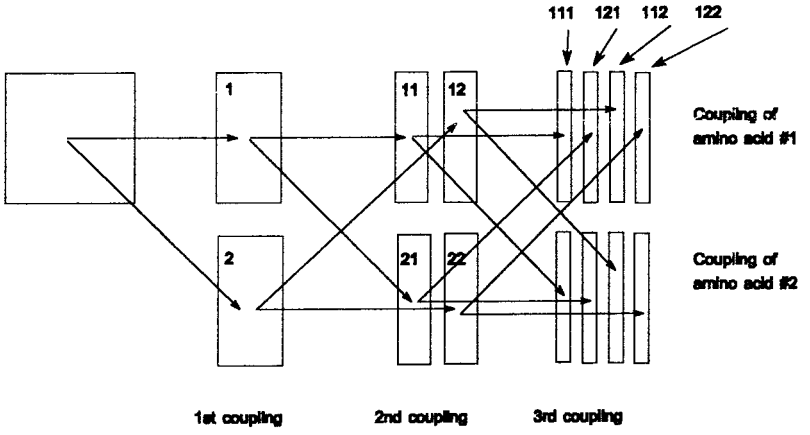

FIGURE 2 General scheme for the synthesis of nonrandom libraries.

ture may be used for the construction of libraries with a nonstatistical distribution of members.[50] Synthesis of libraries by the split and mix technique generates random mixtures of all possible structures. Therefore, there is always uncertainty about the completeness of the library. This issue is critical in the case of small libraries and situations requiring the most economical use of reagents. A technique was designed allowing the synthesis of all members of a particular library with only one representation of each structure. This technique is based on the concept of a continually dividable carrier (membrane, sheet, thread). The synthetic "fate" of a library component may be easily traced based on its size or shape in the particular stage of the synthesis. Figure 2 illustrates the principle of this technique using a library of 8 compounds generated by three steps of randomization using two building blocks in each step. In general, the synthesis of a library commences with n pieces of the carrier that are coupled with n different building blocks (this is the first randomization). Each of the n pieces is then divided into m parts and these smaller sections are distributed into m reaction vessels in which m reactions are performed (this is the second randomization). The process may be repeated as many times as required within the physical limits of handling the polymeric particles. This process produces a library of $n \times m \times \cdots = \underline{X}$ compounds on $\underline{X}$ polymeric particles in which compounds cannot be omitted and all members are represented only once.

Prior experience with cotton[51] led to the selection of cotton threads as an experimental solid support for the assembly of "directed" or "nonrandom" libraries. The mechanical limit of cotton thread is achieved at a dimension of several millimeters, which defines the practical capacity of a library at 10 to 50,000 compounds. While the structure of the compound on the individual solid support fraction (particle) is not known when utilizing this approach, the uniqueness of each compound and completeness of the library may be ascertained. As an alternative carrier to cotton threads, functionalized teflon membranes were examined. The lowest manageable area was determined to be 1 mm^2. Since the membrane is only 10 μm thick, a library of several million compounds may be feasibly constructed, although automation of this process is strongly recommended.

Two model peptide libraries were prepared to prove the concept of "nonrandom" libraries: (a) a library on divided cotton thread containing 125 peptide mixtures, and (b) a library of 2888 peptides on divided functionalized teflon membrane. For the preparation of the first library, the cotton thread (125 cm) was substituted by β-alanine and glycine. The thread was divided into five pieces and Gly, Ala, Leu, Phe, and Tyr were coupled to each section, respectively. In the next step, a mixture of the nineteen proteogenic L-amino acids (Cys omitted) was coupled to the five pieces of thread. In the two successive steps, the thread fragments were divided again and acylated with the above-mentioned amino acids in five separate reaction vessels. The synthesis produced a library of 125 tetrapeptide motifs that possessed mixtures of 19 peptides on each particle with positions 1, 2, and 4 defined. Products of the synthesis were partially detached from the support by exposure to gaseous ammonia and extracted with a buffer. Anti-β -endorphin antibodies binding assay identified one particle as containing biologically active material. Sequencing of the corresponding cotton fraction identified the sequence Tyr–Gly–Xxx–Phe in which Xxx is a mixture of the 19 proteogenic L-amino acids (Cys omitted).

This sequence corresponds to the known motif for anti-β -endorphin antibodies.

The second library was synthesized on a 16 $\times$ 16 cm sheet of functionalized teflon membrane. The membrane was acylated with β-alanine and a linker composed of a repeated sequence of β-alanine and glycine was constructed. The synthesis commenced with coupling Phe to half of the membrane and Leu to the remaining section. In the second step, the membrane was split into halves again, and Gln and Phe were coupled. The same procedure was followed in the third step for the addition of Pro and Gly. In the fourth step, each membrane piece (now 4 $\times$ 8 cm) was divided into 19 sections and one of the 19 L-amino acids was used for the coupling in 19 vessels. Each fragment of membrane (4 $\times$ 0.4 cm) was divided again into 19 pieces ($\sim$2 $\times$ 4 mm) and the coupling of the 19 L-amino acids was repeated. This procedure resulted in a library with the structure Ooo–Ooo–Pro/Gly–Gln/Phe–Phe/Leu in which Ooo is one of the 19 L-amino acids. His–Pro–Gln is the known motif for streptavidin binding, and this library contained 38 copies of Ooo–His–Pro–Gln–Phe/Leu and, more specifically, one copy of the sequence Leu–His–Pro–Gln–Phe. Additionally, four theoretical occurrences of a Tyr–Gly–__–Phe sequence, the known motif for anti-β-endorphin binding, is expected. More specifically, the library possessed one copy of Tyr–Gly–Gly–Phe–Leu. The library was screened with both streptavidin and anti-β-endorphin model targets using a solid-phase binding protocol. Screening of this library yielded 17 pieces of membrane that reacted specifically with streptavidin. The expected sequence His–Pro–Gln was found in individually sequenced pieces and was confirmed by a multiple sequencing experiment. Incubation with anti-β-endorphin provided three specifically reacting pieces containing the motif Tyr–Gly–__–Phe.

Nonrandom or directed libraries may be used as an alternative to random libraries in both solid-phase binding and solution assays. With this technique the number of individual compounds in a library is limited by the size of the carrier implemented in the synthesis. An alternative to continuously dividable carriers for the synthesis of large numbers of library compounds is the application of "tea bags,"[52] paper disks,[25] cotton pieces,[51] or pins.[4] In this case the synthesis of a nonrandom library may be performed in such a manner that the structure of a compound in each bag or piece of paper, cotton, or pin is known since every component of the library may be labeled. However, the synthesis of millions or even tens of thousands of structures is unrealistic and not economical due to the size requirements of an individual synthetic piece, which must carry the positive identification. A recently introduced technique for labeling with radio frequency tags[53,54] did not solve this problem since this method is also demanding in space (10,000 tags creates a volume of 6 L).

MULTIPLE PEPTIDE SYNTHESIS ON COTTON STRIPS

The purest form of cellulose, cotton, was found to be a convenient solid-phase support, especially for multiple peptide synthesis[51,55,56] and the generation of peptide libraries.[57,58] The cotton strips may be conveniently modified chemically and provide an inexpensive solid support. The reagent solutions may be soaked into the cotton structure and only the liquid inside of the cotton fabric is required for a complete chemical transformation. The technique, referred to as "inclusion volume synthesis,"[32] does not require a reaction vessel and serves as the basis for construction of a multiple peptide synthesizer.[30,31] In this instrument, 24 cotton segments are placed on the perimeter of a centrifuge rotor and pneumatically actuated pumps introduce individual reagents; gear pumps add common reagents. All liquids are removed from the solid support following completion of the coupling reaction, which occurs via spinning of the rotor. The advantage of cotton as a support is the near complete removal of liquids (6% remaining liquid), which simplifies the washing procedure. In comparison, after centrifugation, classical polystyrene based carriers placed in "tea bags" retain up to 38% of the liquid load. Nevertheless, "tea bags" were successfully used in the described machine and the increased residual inclusion volume did not inhibit complete coupling reactions. Finally, cotton supports offer a convenient, noninvasive monitoring of the coupling step by bromophenol blue.[59,60] The cotton segments change color from blue to yellow upon completion of the coupling.

Cotton strips may be used in multiple synthesis analogous to the technique for tea bag synthesis. Hundreds of cotton pieces, marked by pencil, are resorted for the coupling steps performed in individual vessels. Deprotection and washing steps are carried out in a common reactor. The liquids may be removed by filtration, centrifugation, or squeezing the liquid from the textile structure. The synthesis may also be conducted in a polypropylene syringe, which does not require a frit since a cotton piece cannot be removed through the syringe opening. Squeezing the syringe

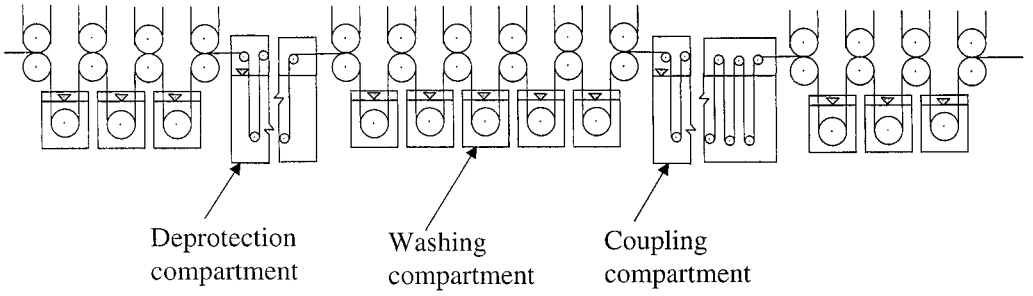

FIGURE 3 Principle of continuous peptide synthesis. Solid carrier (cotton) is being led through a series of washing to deprotection to washing to coupling compartments. Depicted segment of the instrument allows attachment of one amino acid. Arranging analogous segments into the system allows building of a peptide chain.

piston removes the liquid very efficiently. The polystyrene carrier was compared with cotton in the parallel synthesis of 50 peptides and cotton was found comparable to the classical carrier.[55] The lower preparative yields in syntheses on cotton were ascribed to mechanical losses during the synthesis.

CONTINUOUS SOLID-PHASE SYNTHESIS

"Continuous solid-phase synthesis" (CSPS) is commonly mistaken for "continuous-flow solid-phase synthesis" performed in a column loaded with a solid support and percolated with appropriate reagents. CSPS is a method in which a sequence of operations is arranged in space as opposed to time thus leading to a continuous production of peptides or other organic molecules (Figure 3). A strip of solid carrier is led through a series of bathes in which a particular operation is performed such as attachment of a protected amino acid, wash, deprotection of an amino group, and neutralization. After passing through all the operations required for the complete synthesis, the peptide is detached from the carrier and the product is collected continuously at the end of the "assembly line." The carrier may be, in principle, recycled; however, inexpensive supports such as cotton are typically disposed. The application of CSPS in the industrial production of peptides may be perceived as not economical since a machine with sufficient capacity is expected to be too large in size and not practical. However, the following simple calculation illustrates the potential of this technique. A one-inch wide strip of cotton (1 g/4 inches) moving at a speed of 1 inch per minute can provide 1 kg of decapeptide in a month (consuming 10 kg of cotton carrier). The technique of CSPS was proven on a simple manual machine with methionine enkephalin as a model peptide.[61]

CONCLUSION

Alternative solid supports are beginning to be evaluated for applications rarely contemplated in the prelibrary era. Planar supports are potentially one of the many possibilities in which solid-phase synthesis offers as a challenge to the prepared minds of synthetic chemists and biochemists.

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