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STRATEGIES AND TECHNIQUES IN SIMULTANEOUS SOLID PHASE SYNTHESIS BASED ON THE SEGMENTATION OF MEMBRANE TYPE SUPPORTS

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Abstract: A brief survey of current strategies in the chemical synthesis of oligonucleotide and peptide sequence libraries for systematic structure-function analysis is given. Special emphasis on techniques employing membrane type solid supports, in particular cellulose paper, established in this laboratory is detailed.

Virtually every life process is a manifestation of specific and finely tuned interactions between the complex structures of biological macromolecules. The majority of current systematic experimental approaches to study structure-activity relationships (SAR), for deciphering the basic principles of these interactions, as well as to search for new active compounds, rely on the generation by chemical synthesis and subsequent analysis of defined small fragments and mutant sequences. In recent years, several strategies have been devised and realized by appropriate techniques based on the principle of solid phase synthesis, with the aim to provide the immense diversity of sequence variations including natural and non-natural monomers to biological activity screening. The special features of synthetic routes, solid support materials, and technical performances have been advantageously combined to establish a panel of very powerful methods to present large collections of sequence variants (sequence libraries) in a suitable form as substrates in bio-assays. The synthetic sequences are applied either directly in solution or solid phase binding assays or are incorporated into larger structures by chemical or enzymatic means (recombinant DNA methods, fragment coupling etc.). Figure 1 gives a survey of different types of sequence libraries used to experimentally address particular biological questions.

The simplest way to generate a large number of different sequences is by incorporating mixtures of monomers at selected (or a stretch of successive) positions during the stepwise chemical assembly of an oligomer (Fig. 1a,b,c). Individual or groups of sequences can be identified according to their biological activity if the assay provides a suitable selection mechanism. Therefore, such random approaches have been mainly applied to oligonucleotide synthesis, as clonal propagation and segregation is a typical feature of recombinant DNA methods. The relative coupling rates of nucleotide monomers are quite similar, and almost independent of the position within the oligomer chain (phosphoramidites react with equal rates[1]; phosphodiesters react in the ratio of 1(C):1(T):0.7(A):0.6(G)[2]). Thus, the type and frequency of mutant sequences in the library can be fairly accurately predetermined by adjusting the relative concentrations of monomers in the coupling mixture[3]. The spectrum of mutant sequences can be varied from mainly point mutations up to a full representation of all possible 4^N combinations. There are numerous reports on the preparation and use of such mixed oligonucleotide libraries in oligonucleotide directed mutagenesis, hybridisation and related techniques and more recently in the generation of fusion phage libraries[4]. The mixed coupling approach, however, is problematic in the preparation of peptide libraries because of the very different coupling rates of amino acid monomers, which in addition are strongly influenced by the sequences of the growing chains they are added to. Therefore, such a type of mixed peptide library will have a strong bias of chemical origin, and this approach has only rarely been reported[5]. Recently, this drawback was circumvented by the use of a different synthesis strategy (see below), and less

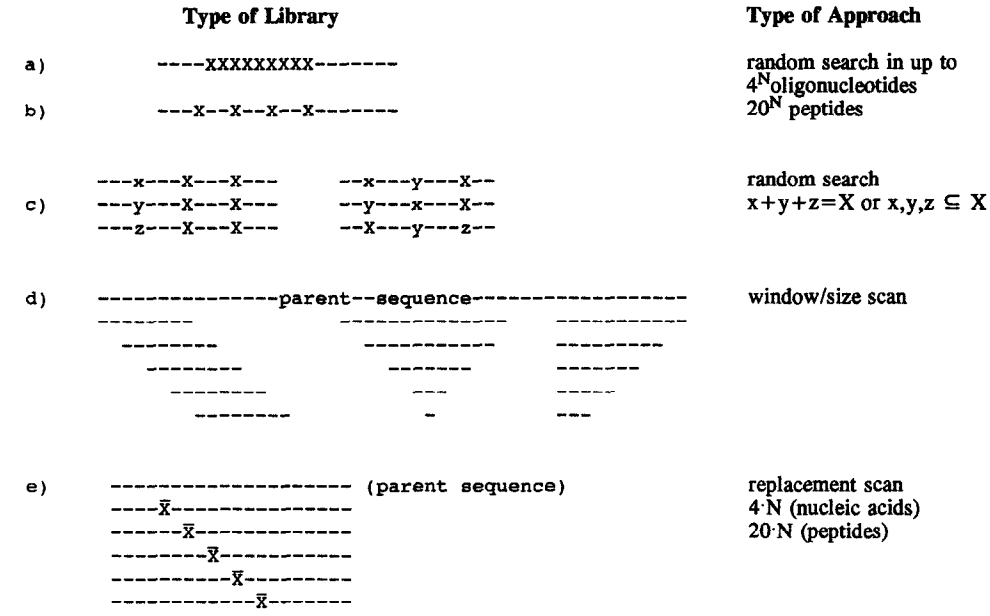


Figure 1. Schematic representation of strategies to generate different types of sequence libraries. - represents the position of a monomer, X (x,y,z) a random position (mixed coupling),and $\bar{X}$ the separate substitutions by individual monomers; N is the number of positions varied. The given complexities of libraries include only the full set of natural monomers.

biased peptide libraries have become available.

Alternatively, a stepwise chemical assembly of large numbers of distinct sequences is accomplished by appropriate techniques for simultaneous multiple synthesis, which keeps the efforts in time and workload at a reasonable level. These methods primarily provide collections of physically separated individual compounds, and no selection mechanism is required to identify the active sequences. In principle, simultaneous synthesis follows two different technical concepts (Figure 2). The parallel coupling mode is based on the use of a fixed array of chemical reactors (tubes[6], wells[7], columns[8], pins[9], cotton strips mounted in a centrifuge rotor[10], etc.) to which solvents and reactants are delivered. The number of parallel coupling reactions required is the same as the number of sequences to be prepared. In the multiple coupling mode, different support entities are combined and reacted together with the same monomer. This reduces the number of parallel coupling reactions considerably, at least down to the number of different monomers that are included. However, it is important that the support material allows individual resins to be kept separate and followed unambiguously at any step. Membranes[11], cartridges in rotating discs[12], bags[13], and porous teflon wafers[14] are being used for this purpose. Several techniques have been implemented as fully automated procedures[6,7,8,10,14].

Simultaneous synthesis techniques are primarily applied to prepare more specialized libraries such as overlapping fragments defined by length and offset, as well as series of substitution analogues (Fig. 1d,e). However, a particular variation of the multiple coupling approach was recently described for the preparation of random peptide libraries[15]. By dropping the option of following individual support entities and reducing their size down to a single resin bead (one bead - one sequence), complete libraries of penta- and hexapeptides ($3 \cdot 10^6$ to $3 \cdot 10^7$ sequences) have been prepared using a "mix and split" technique. Active peptides are identified subsequently either by labelling (as a result of the assay procedure) and physical isolation of the respective beads, followed by microsequencing, or by an iterative process of narrowing the complexity of the solution phase peptide mixture down to a single sequence. Furthermore, a combination

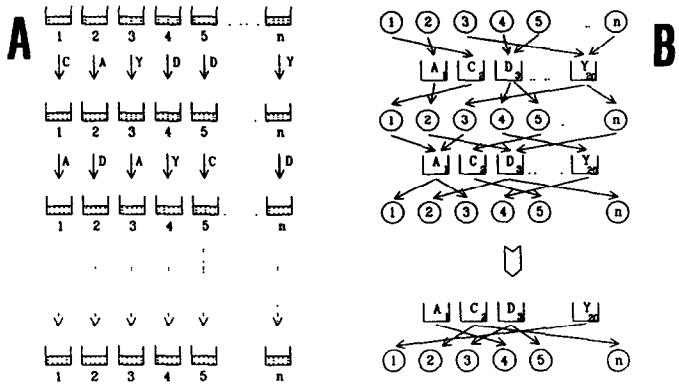


Figure 2. The two different concepts of carrying out simultaneous multiple syntheses on appropriately designed and organised supports. A: individual coupling reactions in parallel reactors. B: multiple coupling reactions to groups of individual support entities.

of the two concepts (i.e.multiple couplings to fixed arrays of reactors) has been realized using a checkerboard type arrangement of micro-squares on a suitably functionalised flat glass surface[16]. For chemical coupling with nucleotide or amino acid monomers, arrays of squares are addressed either by light induced terminal deblocking or physical separation. Because straight lines of squares are linked via a mask and reacted simultaneously with one type of monomer per coupling cycle, the spatial arrangement of the sequences is the result of a combinatorial process. In particular, light directed synthesis allows for an exceptional high density of distinct sites (> 40,000 per mm^2). This type of immobilised peptide library has been used in solid phase binding assays (epitope analysis). The corresponding oligonucleotide libraries are used in parallel hybridisation studies such as DNA mapping, fingerprinting, diagnostics, and are believed to have the potential for a conceptually new method of DNA sequence analysis (sequencing by hybridisation)[17].

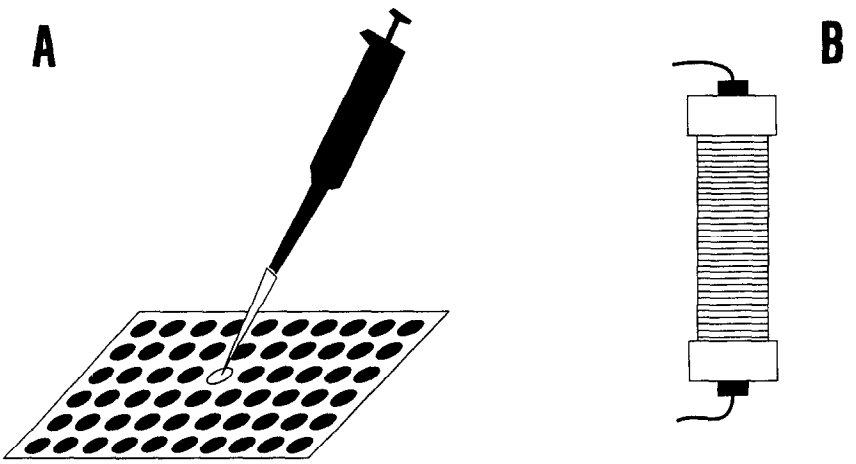


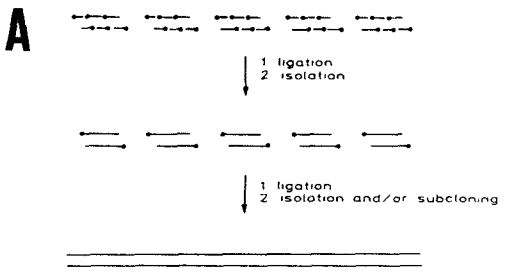
Figure 3. Two different ways of using membrane segments for simultaneous chemical synthesis. A: positionally addressable parallel reactions on an array of SPOTs on a membrane sheet. B: multiple reactions on a stack of discs in a column reactor.

Segments of Membranes as Supports in Simultaneous Solid Phase Synthesis

The primary target in the conception of a particular synthesis strategy is the choice of a suitable solid support that must fit the chemical, technical, and final assay requirements. Over the last ten years, this laboratory has been interested in the application of porous membranes, in particular cellulose paper, for simultaneous solid phase synthesis. This support material has a polar, rigid, and non-swelling structure, a high porosity, and good solvent penetration. It provides a high degree of accessible and reactive hydroxyl functions for easy chemical derivatisation, and is readily available in a large variety of qualities, thicknesses, and shapes. The chemical stability is compatible with most organic solvents and a broad range of reaction conditions. Cellulose paper is of sufficient mechanical stability, withstanding even harsh manipulations, and can be resistantly marked with a pencil. These properties have been exploited to develop techniques for simultaneous synthesis following both the multiple and the parallel mode of chain assembly (Fig. 3).

Multiple Coupling Reactions on Cellulose Discs

In the form of circular discs, the paper support is particularly suitable for carrying out multiple coupling reactions under low pressure continuous-flow conditions when stacked one on top of the other into a column type reactor, each disc labeled with a pencil marking. Such a reactor, thus, is tightly filled with support material built up from horizontal segments (segmented support, Fig. 3B). Obviously, groups of membrane discs or pieces can be used in other reaction vessels[11e,g]. On one disc (1-2 cm ϕ Whatmann 3MM paper) 3-8 μmol of oligomer can be prepared. This "filter method" was the first successful technique reported for simultaneous multiple synthesis, and as initially established, the rapid preparation of oligonucleotides by both phosphotriester[11a,b] and phosphoramidite[11c] chemistries have been demonstrated. Its primary use was for the construction of synthetic genes by enzymatic ligation of overlapping fragments (Fig. 4A) and site-directed mutagenesis of such genes by simple fragment exchange[18]. Other specific applications include the preparation of series of oligonucleotide analogues with non-natural monomers[19], and the segregation of random sequence libraries generated by mixed coupling into pools of defined reduced complexity (e.g. G/C- versus A/T-rich or other sub-



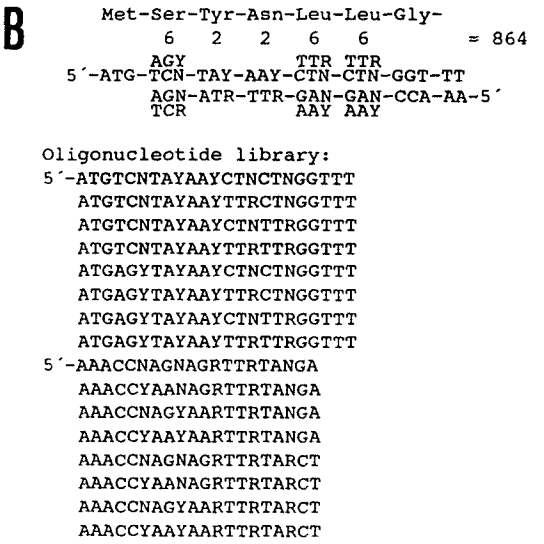


Figure 4. A: Construction of a long DNA double strand by enzymatic ligation of short (10 to 20 nucleotides long) overlapping oligonucleotides. Dots indicate 5'-phosphorylated ends. B: Construction of a mixed DNA cassette coding only for the synonymous codons. N=A,C,G,T; R=A,G; Y=C,T.

populations of hybridisation probes, etc.), or to exclude certain combinations of monomers in the library (Fig. 1c). A particular example of the latter case is given in Fig. 4B where a random DNA cassette coding for the same amino acid sequence (only synonymous codons allowed) has been constructed from a panel of selectively randomised oligonucleotides. A similar library was used to study the influence of codon choice on gene expression in E. coli[20]. The concept of the "filter method" for oligonucleotide synthesis was recently taken up employing teflon wafers as support segments with the aim to devise a fully automated process[14]. Exactly the same "filter technique", except for a different derivatisation of the paper discs, was later adapted to the synthesis of peptides following Fmoc/tBu protocols[11d].

Positionally Addressable Parallel Synthesis on Paper Membranes (SPOT-Synthesis)

An alternative way to achieve segmentation of a membrane support is shown in Fig. 3A. A droplet of liquid applied onto a porous membrane such as cellulose paper is absorbed and spreads over a restricted circular area (spot). Using a solvent of low volatility containing appropriate reactants, such a spot can form a reactor for chemical conversions involving reactive functions anchored to the membrane matrix, e.g. conventional solid phase synthesis. A great number of distinct spots can be arranged on a larger membrane sheet and each of these is individually addressable by manual or automatic delivery of the respective monomer solutions onto the spots. This principle allows for a very simple, rapid and flexible parallel assembly of different oligomer chains. Synthetic steps common to all spots are carried out by immersing the whole membrane into respective reagents and solvents. The volume dispensed and the absorptive capacity of the membrane determine the spot size. According to the specific functionality of the matrix, the spot size correlates with the particular scale of the synthesis. The spot size also controls the minimal distance between spot positions and thereby the number of spots that can be distributed over a given size of a membrane sheet. Typically, volumes of 0.5 to 1 μl are used for manual performance on a thin paper such as Whatman 540 (Fig. 5A). Miniaturisation should allow the accommodation of up to one hundred spots per 1 cm^2. Chemical and technical performance of this type of simultaneous parallel solid phase synthesis has been elaborated for the preparation of series of peptide sequences at a 50 nmol scale up to a length of 20 residues utilizing conventional Fmoc/tBu chemistry[21]. Peptides were either used immobilised on the paper in solid phase binding assays (Fig. 5B) or were cleaved from individual spots, fully characterised (FAB-MS and amino acid analysis) and used in solution phase assays. In principle, the spot technique should also be applicable to the stepwise parallel assembly of other oligomers, e.g. oligonucleotides, provided that the chemistry allows simple handling of reagents.

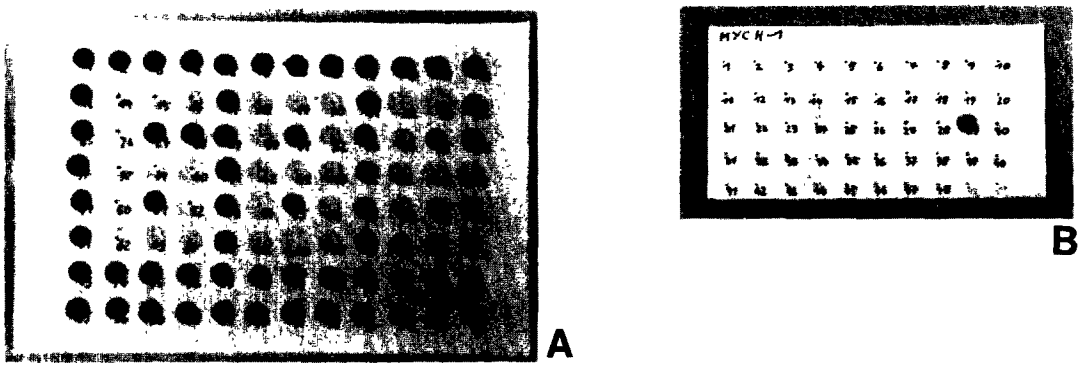


Figure 5. A: A SPOTs-membrane in an 8x12 microtiter plate adapted format. Reactive amino-anchor functions are stained with bromophenol blue. The stain indicates size and position of the spots as well as completeness of amino acid coupling reactions by color change to yellow (compare dark and light spots). B: Solid phase ELISA identifies binding of a monoclonal antibody to a single hexapeptide out of a scan over 53 residues with an offset of one residue.

This article has primarily focussed on technical aspects of multiple chemical synthesis. Because of limitation in space, it cannot cover the many facets connected with the application of the described techniques. In particular, the only briefly mentioned use of non-natural monomers is a very important tool offered by chemical synthesis in general. The systematic screening of artificial sequences utilizing the described techniques provides novel and potent approaches in drug development.

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21. a) Frank, R.; Güler, S. *Peptides: Chemistry and Biology*; Smith, J. A.; Rivier, J. E., Eds.; ESCOM: Leiden, 1992; pp.519-520; b) A SPOTs-Synthesis Kit is available from Cambridge Research Biochemicals, Northwich, UK.