Segmented solid supports: My personal addiction to Merrifield's solid phase synthesis

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Abstract

The Merrifield solid phase synthesis turned out to be the key method to put into practice many of the concepts I conceived during my scientific work concerned with the systematic empirical search for biologically active synthetic compounds. This is true for the first combinatorial “filter discs” method invented for the simultaneous synthesis of oligonucleotides, as well as for all following methods, then dedicated to peptide and later to small molecule synthesis, such as the “cut and combine” method, the “SPOT synthesis” method and the recent “SC^2” method for the generation of compound repertoires in the micro-array format.

Introduction

For the final phase of my studies in organic chemistry at the University of Hamburg, fortunate circumstances led me to join a young group of chemists headed by Hubert Köster at the Institute of Organic Chemistry and Biochemistry for my diploma and doctoral research from 1974 to 1979. It was here that I was introduced to Merrifield´s solid phase synthesis concept because some colleagues were involved in adopting it to the multistep synthesis of oligodeoxynucleotides. Hubert Köster himself worked already before in Göttingen with Friedrich Cramer at the Max Planck Institute for Experimental Medicine on the development of novel support materials for solid phase oligonucleotide synthesis. However, oligonucleotide chemistry was not yet advanced enough to really profit from the beauty and efficiency of the concept.

The Köster group integrated me into an exciting project on the chemical synthesis of a small gene coding for the octapeptide hormone Angiotensin II, a DNA duplex with a defined sequence of 33 nucleotide base pairs and an impressive molecular weight of 20,260 d, (Köster at al., 1975). As a chemist, I was fascinated by the power of the emerging techniques of genetic engineering with new methods for DNA manipulation allowing the clonal selection and amplification of virtually single molecules from synthetic mixtures. I was particularly intrigued by the input from organic synthesis on these developments, the contribution of Khorana´s group at the MIT to the elucidation of the genetic code being the most exciting example, honoured with the Nobel prize in 1968. For our Angiotensin II-gene, a concept developed by Khorana´s group for the construction of an alanine tRNA gene was adopted. It involved the synthesis of small (6- to 11mer) fragments covering head to tail both strands of the double helix and each overlapping in their sequences with two fragments from the opposite strand (Fig. 1). These fragments (all were prepared by solution phase synthesis) were finally covalently linked (ligated) with the help of an enzyme, a DNA ligase, upon forming the double strand by hybridization. I worked partially on the gene synthesis project but focussed more on general strategic aspects for the assembly of double stranded DNA with the aim of optimizing the design and use of the synthetic fragments as well as their efficient enzymatic ligation (Frank, 1979). The uniform structure of DNA suggested to me a more rational assembly strategy.

At that time, I began speculating on the option of having a complete universal set of oligonucleotide fragments of unitary length L (an oligonucleotide library in modern terms) that could be used to assemble any DNA sequence at will and, in particular, series of variants (mutants) for systematic functional screening through simple fragment exchange. This universal set of oligonucleotides had to be in a format that allows immediate retrieving of the required fragments, mixing them together and enzymatically forming the DNA ready for cloning. My own experiments indicated that at least 6 nucleotides for pairing with the opposite fragment are required for reliable selective enzymatic ligation. Thus, the theoretical number of fragments for an anticipated universal set would amount to 4^{2xLH} where L_H is 6, or a reduced set for only protein coding genes utilizing 24 triplets where two triplets then give the required overlap L_H for hybridization. These numbers, however, were far larger than several ten- even hundred-thousands and, thus, exceeded what was reasonable at that time (Figure 2). Oligonucleotide synthesis was still a formidable task and it took months to prepare a relatively short oligo by solution phase chemistry. For my diploma thesis I had to synthesize an oligonucleotide fragment of only six nucleotide units in length, and I spent six months mainly in the cold room for labour intensive work-up of coupling reactions by ion exchange chromatography and ended with a thin non-visible film of product on the walls of my pear shaped flask. Colleagues from the institute made jokes on our “chemistry in empty flasks”, but this yield of a few micromoles was sufficient for thousands of biological experiments.

The requests for oligonucleotides from biologists, however, increased tremendously with the explosive developments in molecular biology and genetic engineering. Although already disclosed in 1963 by Letsinger and Kornet, practical and reliable solid phase synthesis methods became available only in about 1978 through the work of K. Itakura´s and M.J. Gait´s groups (for a review see Winnacker, 1987). Novel support materials were developed because the classical Merrifield resin was not well suited for oligo synthesis. Oligonucleotide chemistry was revolutionized with the introduction of the phosphoramidite method by Beaucage and Caruthers (1981) and the first fully automated synthesizers (gene machines) came onto the market soon after. But even these machines were not really effective (fast and parallel) enough for my goal. This motivated me to conceive a new method for the chemical synthesis of more than one or just a few distinct compounds at a time.

Multiple chemical synthesis on cellulose discs (the filter method)

It took two more years in which I, together with my colleague, Helmut Blöcker, moved to the Genetics Department at the GBF (the German National Research Centre for Biotechnology). At this new venue we received strong support from a group headed by John Collins (co-inventor of cosmid cloning), already involved in gene cloning and sharing our vision of using gene synthesis for molecular biological studies and particular the production of mutated proteins which required large number of oligos for site specific mutagenesis, a technique still in its infancy. It was here that I found a practical solution for the numbers problem (Frank et al., 1983). This approach was different from those used by other groups working at that time on either the parallel synthesis in reactor arrays (Fig. 3A) or the stochastic synthesis with mixtures of nucleotide monomers (mixed primers or probes). My central idea was to utilize the support material itself to keep individual compounds separated during the series of assembly steps. This allows one to combine many support segments carrying different unique nascent molecules into one common reactor, coupling these simultaneously with one type of building block (e.g. extension with a monomer or mixture of monomers). After each elongation cycle, the segments are simply reorganized for the next monomer addition (Fig. 3B). This procedure effectively reduces the number (N) of separate chemical reactions required for the assembly of n compounds with m assembly steps incorporating r different monomers per step down to $N = r \cdot m$ compared to $N = n \cdot m$ for any parallel approach. The larger n is compared to r, the more significant is the benefit. This is particularly evident with nucleic acids where r equals only 4. The resulting process is a truly combinatorial one and in this respect conceptually identical to the "tea bag" method of Richard Houghten (1985) as well as the "mix and split" method of Furka et al. (1988).

The experimental realization of this approach was very much determined by the chemistry and application of oligonucleotides. This required that (i) each segment should be quite small, as only nanomole amounts of the product were needed; (ii) these could be combined to give only a minimal void volume, since the excesses of monomers were quite precious materials; (iii) the chemical reactions could be carried out under appropriate conditions with exclusion of moisture and air, and (iv) each individual product could be immediately identified and retrieved from the collection. Particularly the latter prerequisite led to the most difficult problem of indexing the support segments. We investigated numerous materials based on resins used in oligonucleotide synthesis, such as glass fibre membranes, silica gel in porous plastic bags, and PTFE porous discs amongst others. However, ordinary chromatography paper made from pure cellulose (cotton linters) largely fulfilled all the criteria named previously. The texture of this material was particularly useful: It is easily chemically derivatized and small pieces of any form can be cut from paper sheets. These can easily be indexed with simple black carbon markings, e.g. by pencil, and can be tightly stacked into a column type reactor (Fig. 4 A,B) connected to a solvent delivery system (Fig. 4C). With this method a group of participants at a two week EMBO practical course held in our lab in 1984 was able to synthesize all sixty 15mer fragments for an γ-interferon gene, purify and ligate these to give a 457 base pairs long DNA duplex.

The nucleic acid community recognized this method quite readily and several papers appeared in the following years which improved chemical and technical details of this "filter disc" method. This included the development of better supports and steps towards automation. Although DNA synthesizers were advanced and perfected, impressively allowing the rapid assembly of oligonucleotides more than 200 bases long with cycle times of less than 5 min per base adition, a leading custom-synthesis house is utilized a type of segmental support process, the “Abacus”, to manufacture several thousand oligos per day (http://www.sigmaaldrich.com/Brands/Sigma_Genosys.html). Genome covering oligonucleotide repertoires (banks) and synthetic genes have already become affordable commercial products in the 1990s.

Cellulose is a problematic support for oligonucleotide synthesis because it is not fully inert, residual hydroxyl groups being accessible for phosphorylation, thereby initiating new chains. This drawback is less relevant with amide/peptide synthesis. Merrifield evaluated cellulose as support for peptide synthesis and discarded it because it was not compatible with the chemistry he used. The situation changed with the establishment of milder Fmoc/tBu-chemitry by the Sheppard and the Meienhofer labs. With the aim of adopting the combinatorial filter disc methodology, I was given the opportunity to establish a peptide synthesis group at the GBF, primarily to support the growing need of immunologists at the institute. The following years were much stimulated by the exciting work of M.Z. Atassi and H. M. Geysen and colleagues on the systematic analyses of epitopes using large sets of synthetic peptide fragments derived from protein antigens. February 1985 I spent in the lab of Bernd Gutte, a former co-worker of Bruce Merrifield, to learn the Merrifield Boc-synthesis, and immediately began experiments with the cellulose support in Zürich (Frank and Döring, 1988). About at the same time, Jutta Eichler in the lab of Michael Bienert in Berlin worked in the same direction (Eichler et al. 1988). They, however, favoured a different cellulose form, namely the cotton tissue (Eichler et al., 1991). At the GBF we used cellulose discs and an 8 column manually operated solvent delivery system for about 5 years to synthesise thousands of peptides for in-house research and service.

Parallel synthesis on continuous cellulose membranes: the array format

Solid phase synthesis on cellulose paper only became a more widely used support in the context of another type of multiple synthesis technique, the SPOT synthesis method (Frank, 1992). Most prominently the lack of a robust and affordable concept for fully automation of the disc sorting process that will allow working with twenty and even more building blocks enforced the need of alternative methods in order to increase capacities for peptide screening. Inspired by the multi-pin method (Geysen at al., 1984) and the first automated parallel synthesizers based on pipetting robots (Schnorrenberg and Gerhardt, 1989), the idea of a planar array of synthesis sites arose. A relevant patent application by E. Southern from 1988 on the generation of oligonucleotide arrays only came to my knowledge when the method was published in 1992 (Southern et al., 1992). Again the texture of cellulose paper suggested the most easy and flexible way of generating spatially separated sites for simultaneous synthesis on a continuous membrane surface. Upon dispensing a small droplet of liquid, the droplet is absorbed and forms a circular spot. Using a solvent of low volatility containing appropriate reagents, such a spot forms an open reactor for chemical conversions involving reactive functions anchored to the membrane support. This principle was later named “inclusion volume solid phase synthesis" (Eichler et al., 1996). A great number of separate spots can be arranged as an array on a larger membrane sheet and each of the spots is individually addressable by manual or automated delivery of the corresponding reagent solutions (Fig. 5A and B). Synthetic steps common to all spot reactors are carried out by washing the whole membrane with respective reagents and solvents.

The SPOT-synthesis concept is particularly attractive because of its ease, flexibility, robust and simple performance, yet allowing full control on the chemistry by online monitoring of chemical steps. This became possible through the application of the bromophenol blue for monitoring free amino functions, as developed by Viktor Krchnak and colleagues in Prague (Krchnák et al.,1988). This also makes peptide SPOT-synthesis a very colorful experience. The following illustrates just how straightforward the idea had become reality: a biochemistry student from Berlin, Sinan Güler, actually set up the whole manual SPOT synthesis process including the bioassay for an antibody epitope mapping experiment, during a six week practical course in my lab. The collaboration with Heinrich Gausepohl at ABIMED Analysen-Technik (now Intavis Bioanalytic Instruments, Cologne) on the automation of the spotting process (Fig. 5B) then opened up the full potential of the technique by assessing many thousands of syntheses in a high density array format reducing reaction volumes down to the nanoliter scale (Fig. 5C). The SPOT-patent application was filed in August 1990 (Frank and Güler, 1990) shortly after Affymax´s disclosure of the photolithographic peptide synthesis (Pirrung et al. 1990). Although not as impressively miniaturized as the Affymax system, SPOT synthesis clearly fulfils similar demands but with ease of experimental performance, flexibility of array and library formatting, and the affordability of the equipment required (sometimes SPOT synthesis is called the "poor man´s Affymax!). Consequently, SPOT-synthesis became a rapidly accepted method. The comfortable nanomolar scale of individual products, as well as the options to isolate individual products as well as bound ligands from excised spots offers experimental opportunities not available with the Affymax glass chip arrays of that time (Fodor et al., 1991) including the more advanced follow-up technologies (Pellois et al., 2002). Meanwhile, peptide and other synthetic repertoires (peptoids, PNA, small molecules) are being prepared with this method and are applied to numerous different types of assays and library approaches that are developed in labs all around the world, most notably by the group of Jens Schneider-Mergener at the Charité in Berlin (Frank and Schneider-Mergener, 2002). His company Jerini AG acquired the patent family in 1999 and uses the method for its peptide drug development program. Obviously, cellulose proved to be an excellent support for in situ bioassays with immobilised probe molecules as it shows no high affinity for proteins and nucleic acids; many type of assays have been adopted to peptide arrays on cellulose membranes (Frank, 2002).

SC²-microarrays

A membrane-bound peptide and small molecule array as manufactured by SPOT synthesis is ideally suited for a single user and can be re-probed only a limited number of times. Furthermore, SPOT synthesis on porous membranes has its limitations when reducing the spot size below 1 mm and becomes costly and tedious when large numbers of copies of an identical array are required. We have therefore developed a special novel process for manufacturing and application of synthetic peptide/compound libraries in the form of chemical mini- or microarrays, thus maintaining the advantageous features of cellulose-bound probe molecules while allowing further miniaturization and large scale production of identical arrays (Dikmans et al. 2006).

The assembly of the peptide/compound collection follows essentially standard SPOT synthesis protocols with an array format adapted from the 384-well microtiter plates, except that a special, acid sensitive amino-cellulose membrane is used. Individual spots are separated post-assembly with the help of a 384-compatible punching device. This delivers the cellulose-compound conjugate disc segments of 3 mm diameter into the wells of four 96-deepwell plates. The discs are treated with a TFA cocktail, containing >80% TFA plus scavengers, as used in routine solid-phase peptide synthesis. This treatment solubilizes the support itself, along with the compounds still covalently attached, and simultaneously cleaves acid sensitive side chain protecting groups. Precipitation with ether removes the bulk of acid together with the cleavage chemistry and the dried precipitate is then dissolved in DMSO. After appropriate dilution with DMSO, these solutions of compound-support conjugates are printed and adsorbed onto the target planar surfaces, usually glass microscope slides. We therefore call this process "spotting compound-support conjugates": SC^2. One standard cellulose spot yields 0.5 ml of DMSO stock solution from which nl to pl aliquots can be used to print up to 10^6 to 10^8 mini- to microarray copies.

This methodology has delivered novel tools for studies of host-pathogen interactions. It has become possible to systematically test serum collections for humoral immune responses to pathogens on a larger scale. One approach involving the generation of peptide maps of almost all potential antigens of a pathogen (e.g. a viral or bacterial genome) is a typical example of an experimental approach that requires hundreds of array copies. For example, we have synthesized and printed about 3 000 overlapping 15mer peptide fragments covering the major virulence proteins of Yersinia enterocolitica for the immune-profiling of pathogen challenged mice, over time and in various genetically altered mouse strains.

Conclusion

Although the publication titled "A new general approach for the simultaneous chemical synthesis of large numbers of oligonucleotides: segmental solid supports" which appeared in July 1983 in Nucleic Acids Research did not become my personal citation classic, I consider it my most innovative contribution to research. I conclude this, not only from the continuing impact it had on all my following work, but also from the irritated and devastating comments the reviewers had for our manuscript. As a blood-young researcher, this shocked me deeply and made the group very cautious on further generalizing statements. However, this was soon put into more favourable perspective once other multiple and parallel synthesis methods were subsequently published, supporting the value of our concept. Novel technologies now have made the old filter disc method obsolete, but the use of segmental solid supports has continued in various alternative forms in modern parallel chemical synthesis and biological screening. With the Irory bar-coded reactors (cans) even our dream of a fully automated process has been put into practice.

Acknowledgements

This is a very personal report on a technology driven research work which was very much inspired by Merrifield´s solid phase synthesis concept. I invite colleagues to comment on this text in order to correct my potential one-sided view. I am deeply grateful to Hubert Köster for giving me the opportunity to join his group, as well as Helmut Blöcker and John Collins for their supporting my scientific career at the GBF in Braunschweig. I am thankful to Johannes Meienhofer for his encouraging discussion on the world of peptide synthesis, as well as Bernd Gutte and his group in Zurich for giving me a hand in making my first peptide on solid phase. My cordial thanks go to the co-workers and collaborators who shared with me during all these years the exciting journey through millions of synthetic molecules. Finally, I close with my admiration to Bruce Merrifield for laying the ground to all of the work described.

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Legends to Figures

Figure 1. Original figure from reference 1 showing the construction plan for the DNA duplex coding the angiotensin II peptide including start and stop codons.

Figure 2. Original figure from my PhD thesis on "Rational strategies for the construction of double stranded DNA". A: rational plan for the same angiotensin II gene as in Fig. 1 but using in frame coding hexanucleotide fragments (up: X = variable codon positions; down: selected from a reduced set of complementary codes). B: Diversity of fully random oligonucleotide repertoires with increasing hybridisation lengths (L_H = hybridisation length between to opposite fragments). C: Diversity of oligonucleotide repertoires with increasing L_H made from the reduced set of 24 complementary codons.

Figure 3. Concepts for simultaneous solid phase synthesis. A) Parallel synthesis in an array of separate reactors. B) Multiple synthesis by sorting support segments into common reactors.

Figure 4. Simultaneous multiple synthesis of oligonucleotides with pencil-labelled cellulose discs as segmental supports. A) Array of labelled discs sorted into four groups for coupling with either A-, C-, G- or T-nucleotide building block. B) A column reactor with a stack of ca. 100 discs during the detritylation step; a ring of red methoxytrityl cations is running through the column. C) Home-made manually operated 4-column solvent delivery system.

Figure 5. Simultaneous parallel synthesis with the SPOT method. A) Manual distribution of microliter aliquots of activated amino acid building blocks to the preformed array of amino functionalized spots; pipetting follows a computer-generated scheme and utilizes pencil

numbers written next to the bromophenol blue stained spots which turn to yellow upon complete coupling. B) The PC-controlled ASP 222 spotting robot having a reservoir block holding up to 48 building block solutions, a platform holding six membranes with each 425 spots and a stainless steel needle for reagent delivery. C) An array of 2500 peptide spots on a 8 x 12 cm cellulose membrane probed with an antibody and developed with an alkaline phosphatase conjugated secondary antibody. Dark spots indicate antibody binding through the formation of a MTT/BCIP dye precipitate.

Figure 6. Three sets of each 1000 peptide fragments (15mers) plus two lines of each 25 reference spots were printed from SC^2 -stock solutions in DMSO on a glass microscope slide, then probed with a mixture of reference antibody and mouse serum; bound antibodies were detected with a Cy3-labelled anti-mouse antibody.