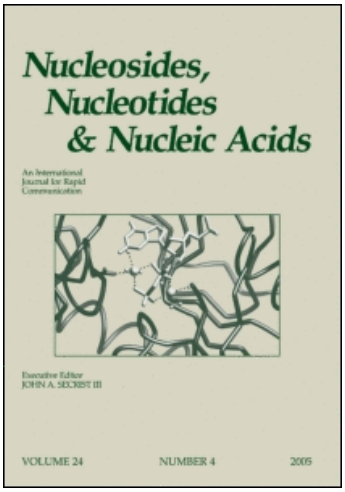

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SYNTHETIC OLIGONUCLEOTIDE COMBINATORIAL LIBRARIES - TOOLS FOR STUDYING NUCLEIC ACID INTERACTIONS

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ABSTRACT. This paper gives an overview of methods available for, and difficulties encountered in the preparation of synthetic oligonucleotide combinatorial libraries. The application of libraries immobilized to high-load beads to the screening of specific two-component (nucleic acid / nucleic acid) and three-component (ds nucleic acid/protein) interactions is described. The effector oligonucleotides immobilized to a single bead are identified by direct Maxam-Gilbert sequencing.

INTRODUCTION

Some General Considerations on Synthetic Oligonucleotide Combinatorial Libraries

Synthetic oligonucleotide combinatorial libraries (SOCL's) are spatial arrays of all (complete library) or a defined fraction of all (partial library) sequences of a given length[1]. SOCL's can come in two different formats, namely continuous or discontinuous. Libraries in continuous format contain the different structural elements, i.e. oligonucleotide sequences, juxtaposed on the surface of suitable carriers. Glass plates[2] or silicium wafers[3] have initially been used for this purpose, and the different sequences arranged side-by-side in a two-dimensional fashion.

The necessity to generate continuous libraries in a two-dimensional arrangement of elements through spatially addressed synthesis has revived the interest in rational methods of parallel oligonucleotide preparations[4]. New techniques have been evolved, which achieve chain elongation of a selected portion of the surface elements either by mechanically controlled exposure[2] or by partial photochemical deblocking[5] of sequences to be included into the condensation. In both cases good results for the parallel preparation of large numbers of sequences require either sophisticated apparatus or excellent chemical methods of „light-

directed" synthesis. Further, the size and resolution of areas containing different individual sequences limit the size of two-dimensional libraries and the length / number of sequential variants.

In a more recent line of development, polypropylene films have been functionalized with amino groups by plasma discharge and thus used for generating SOCL's[6]. Through chemical functionalization[7,8] we obtained polypropylene tapes with relatively high capacity useful for oligonucleotide microsynthesis[9] as well as for the preparation of „one-dimensional" oligonucleotide libraries[7,9]. The one-dimensional positioning of library elements on polypropylene film has the advantage, that such „tapes" can be generated in any length and easily handled by rolling or folding. A rational method for the simple mechanized preparation of such libraries of sequentially overlapping oligonucleotides by combining a meander-shaped modular unit with a commercial synthesizer is described in an accompanying paper[9] from our laboratory.

The term „discontinuous" applies to libraries composed of particle-bound elements. Ideally, each bead is the carrier for an individual macromolecular species. The preparation of such libraries has been first described for immobilized peptides by K.S. Lam et al.[10] and is commonly known as the „split-couple-combine" technique. While large members of individually loaded beads can thus be prepared with a minimum of effort, the identity of the library element bound to a single bead is a priori unknown. „Flagging" or „tagging" methods have been proposed, that allow to follow the fate of an individual bead through different cycles of chain elongation using either chemical[11,12] or radio frequency identification[13,14] tags. In spite of their elegance, such „tagging" methods will generally increase the synthetic effort and cost involved in library preparation. Beaded oligonucleotide libraries, although mentioned in an earlier paper[11], were, to our knowledge previously not available!

Recently, we have described the preparation of SOCL's by the „split-couple-combine" technique and, through proper selection of the supports, were able to directly identify the load of a single bead by Maxam-Gilbert sequence analysis[15,16] following hybridisation with the respective opposite strand. Here, we give further examples of the application of such beaded SOCL's in two-component (nucleic acid vs. nucleic acid) interactions and a first example for three-component (nucleic acid / nucleic acid / protein) molecular recognition.

Materials and Methods:

Preparation of beaded oligonucleotide libraries:

For the preparation of a beaded oligonucleotide library we used Tentagel^{R} - NH_2 particles (32 μm diameter; Rapp Polymers, Tübingen). The 3'-terminal nucleoside was directly coupled to the amino groups of the carrier generating a phosphoramidate linkage, which is labile to stronger acid[17], but stable under usual conditions of usage of the library. Oligonucleotide synthesis was done running standard phosphoramidite cycles on a Gene Assembler Plus synthesizer (Pharmacia). After each set of four cycles the synthesis was stopped, the beads combined and, again, partaged into four columns. Monitoring the release of the trityl group, the chain elongation proceeded with ca. 99 % average yield.

Application for two-component nucleic acid interaction (hybridisation):

For hybridisation to the beaded library oligonucleotides were prepared containing either a ^{32}P-radiolabel at the 5'-end or labelled by the introduction of a fluorescent dye either at the 5'-end or at the 3'-terminal base[18]. Hybridisation of labelled oligonucleotides to the beaded library and stringent washing was done as described previously[7]. For identification beads showing most intense fluorescence were picked under a microscope with a micromanipulator. After dehybridisation, the oligonucleotide load bound to single beads was released by acid[17] and sequenced according to a variant of the Maxam-Gilbert procedure[7] or, alternatively, by combination with the „single reaction sequencing" described by Negri et al.[19].

Application to three-component interaction of biomolecules:

A partial 30^{mer} library of the composition $(dT)_4$ $(dN)_6$ $(dT)_4$ (N = A, C, G, T) was prepared as described above, using the split-couple-combine technique for the six middle positions. The product of this synthesis was applied as immobilized library. The corresponding 30^{mer} library of the composition $(dA)_4$ $(dN)_6$ $(dA)_4$ (N = A, C, G, T) was prepared in parallel by routine synthesis and worked up in solution after release from the support.

15 mg of the beaded library and 1 $O.D._{260}$ of the soluble library were mixed in 500 μl restriction buffer (devoid of MgO^{++}), heated to 70°, then cooled to ambient temperature within 30 min. with agitation. After two washes with 500 μl each of buffer the beads were incubated with 50 u restriction enzyme (labelled with sulforhodamine-3-acid chloride) in

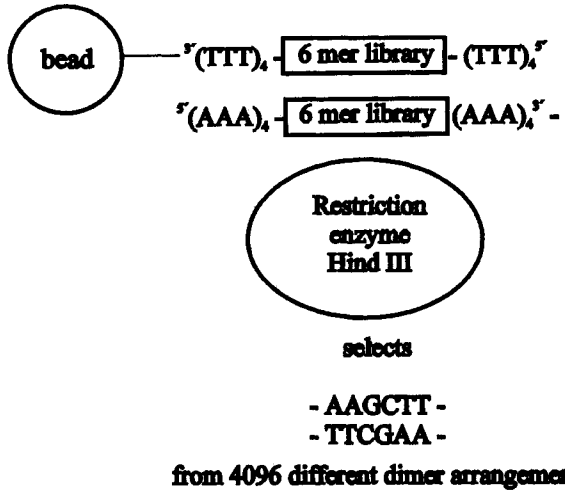
200 µl restriction buffer for 1 h at 37°. After extensive washing several highly fluorescent beads were picked, enzyme and non-immobilized oligonucleotide were removed by washing with water at 70°. The immobilized oligonucleotide loading was detached from the support and sequenced as described above.

RESULTS AND DISCUSSION

Synthetic oligonucleotide combinatorial libraries have been prepared by adapting the „split-couple-combine" method initially described by Lam et al.[10]. As a consequence from previous studies[7] we have selected relatively highly loaded beads as carriers for these libraries. Thus, most experiments have been done with polyoxyethylene-grafted polystyrene beads (Tentagel^R, Rapp Polymers), which are functionalized with primary amino groups. Therefore, oligonucleotides can be linked via phosphoramidate bonds stable to conditions of multiple reuse in neutral buffers, but labile to stronger acid treatment[17]. This permits to use standard phosphoramidite cycles for chain elongation, where the high yields ensure >90% structural integrity of the immobilisate. A special benefit from highly loaded bead systems is the possibility of directly analyzing the oligonucleotides released from a single bead by Maxam-Gilbert sequencing or a related technique[7]. This takes away the requirement for „tagging" or other deconvolution procedures.

The hybridisation between two complementary oligonucleotide strands, in these initial studies, served mainly to demonstrate the specificity of molecular recognition. For simplicity, we used as a standard test system, a three-component library containing the immobilized sequences 5' dATCGAGAC, 5' dATCGTGAC and 5' dTTCGAGAG, which were screened with a fluorescein-labelled probe sequence 5' dGTCTCGAT. Beads containing oligonucleotides with 0, 1 and 2 mismatches were easily distinguished under a fluorescence microscope. Those exhibiting highest fluorescence intensity were picked with a micromanipulator and the sequence of the immobilized oligonucleotide was analyzed as described previously[7]. This proved to be, in all cases, the zero-mismatch sequence. While this procedure served well for studying the performance of different bead systems[7], automated sorting in a FACS apparatus will be desirable for the screening of complete libraries of medium chain length.

In the next step, we were interested to check the specificity of our beaded library in the molecular recognition of a three-component system composed of two complementary



Scheme 1 :
3 - component interaction of beaded ds - oligonucleotide library with Hind III.
The enzyme selects 5'- AAGCTT - from 4096 different dimer arrangements
3'- TTCGAA-

oligonucleotide strands and a restriction enzyme. For this purpose a beaded 30^{mer} partial library containing a complete set of hexamer variants flanked by dodecathymidylate on both sides was treated with a similar library in solution composed of a randomized hexamer segment flanked by 2 x 12 adenylate units (scheme 1).

Hybridisation of these two libraries gave a double-stranded SOCL. Excess soluble 30^{mer} and eventual hybrids formed herefrom were removed by washing. Then the ds library was incubated with fluorescently labelled restriction enzyme Hind III. By depleting the restriction buffer of Mg^{2+} complex formation will result in binding of the enzyme without cleavage of the substrate. In fact, we could select, by visual inspection, several beads, which, through intense fluorescence, indicated the presence of bound restriction enzyme (Fig. 1).

Dissocation of the ternary complex, followed by sequence analysis, demonstrated that, indeed, these beads were loaded with the recognition sequence 5'....dAAGCTT.... Similar experiments were done with Eco RI, leading to selection and identification of beads containing the unique recognition sequence.

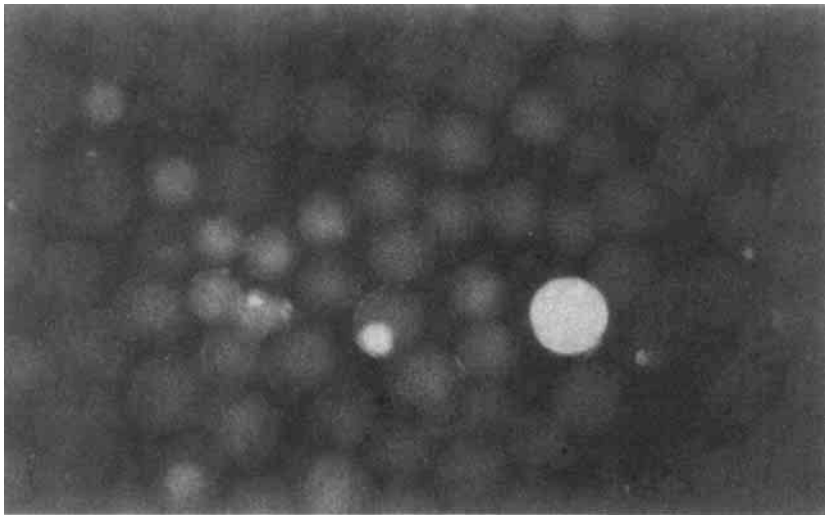


Figure 1

CONCLUSION

Beaded SOCL's can be prepared by the „split-couple-combine" method with high yield of oligonucleotide chain elongation. By selecting a high-capacity polymer carrier it is possible to identify the oligonucleotides immobilized to a single bead by direct sequence analysis. Improved methods for screening (FACS) and sequence identification (MALDI spectroscopy) are under investigation. The specificity of molecular recognition of these beaded SOCL's is demonstrated for the case of two-component (nucleic acid double strand formation) and three-component (ds nucleic acid plus protein) interactions.

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