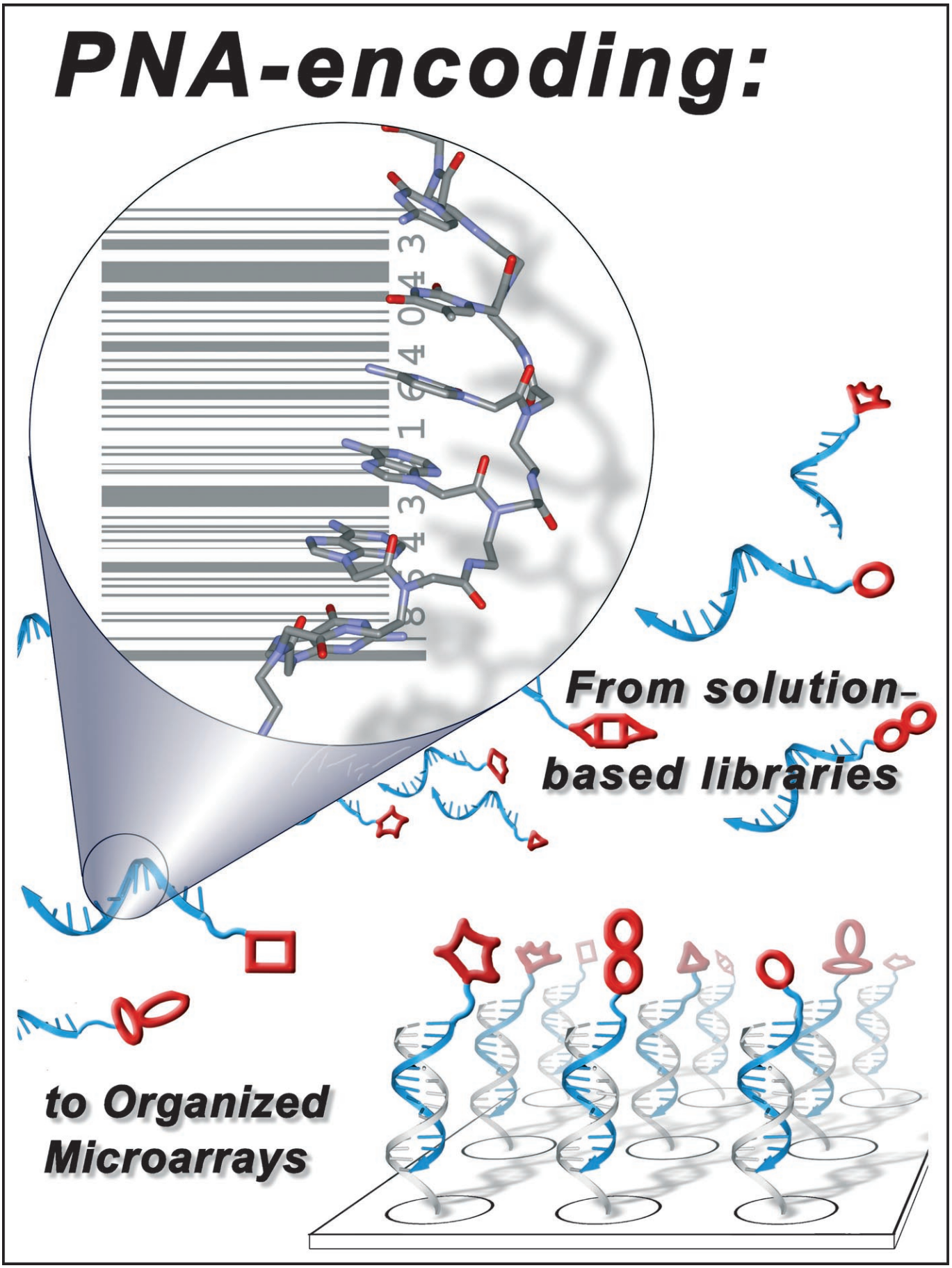

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PNA Encoding (PNA = peptide nucleic acid): From Solution-Based Libraries to Organized Microarrays

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Abstract: Microarray-based technologies have attracted attention in chemical biology by virtue of their miniaturized format, which is well suited to probe ligand–protein interactions or investigate enzymatic activity in complex biological mixtures. A number of research groups have reported the preparation of surfaces on microarrays with specific functional groups to chemoselectively attach small molecules from libraries. We have developed an alternative method whereby libraries are encoded with peptide nucleic acid (PNA), such that libraries which exist as mixtures in solution self-assemble into an organized microarray through hybridization to produce readily available DNA arrays. This allows libraries synthesized by split and mix methods to be decoded in a single step. An asset of this method compared to direct spotting is that libraries can be used in solution for bioassays prior to self-assembly into the microarray format.

Keywords: combinatorial chemistry · microarrays · PNA · self-assembly

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Introduction

One could provocatively argue that significant breakthroughs in science do not come from hypothesis-driven experiments, but rather through the astute observation of events that challenge our intuition. Considering this, much effort has been devoted to increase our capability for discovery-driven research and investigate the millions of interactions that make up a biological organism. The use of small molecules has been an effective means of selectively perturbing biological signaling and processing pathways. With the advent of combinatorial chemistry, small teams of chemists are now able to rapidly access large libraries of compounds. This ability to generate large numbers of synthetic compounds necessarily requires high throughput and robust screening methods to identify small molecules responsible for the function or phenotype of interest. The success of microarrays in screening mRNA expression profiles of thousands of genes simultaneously[1–3] has prompted researchers in bioorganic chemistry to explore this format for screening small molecules. From an analytical perspective, the prospect of screening thousand of analytes in a few microlitres is attractive.

Microarrays can be prepared by several techniques including photolithography, contact printing, and inkjet to generate arrays with densities ranging from 1000 to 500000 features per square centimeter.[4] To date, a number of chemistries (Figure 1) have been developed to derivatize glass surfaces to immobilize proteins,[5–9] oligosaccharides,[10–13] and small molecules[14–23] in the microarray format. Many of these methods require the attachment of the protein or small molecule to the glass slide and likewise require that screening with the biological sample also be performed on the surface. We have opted for a supramolecular attachment based on sequence specific hybridization of peptide nucleic acid (PNA); this allows the use of the libraries as a mixture in solution that can then be converted to an organized microarray in one step by a self-sorting process.[24–27]

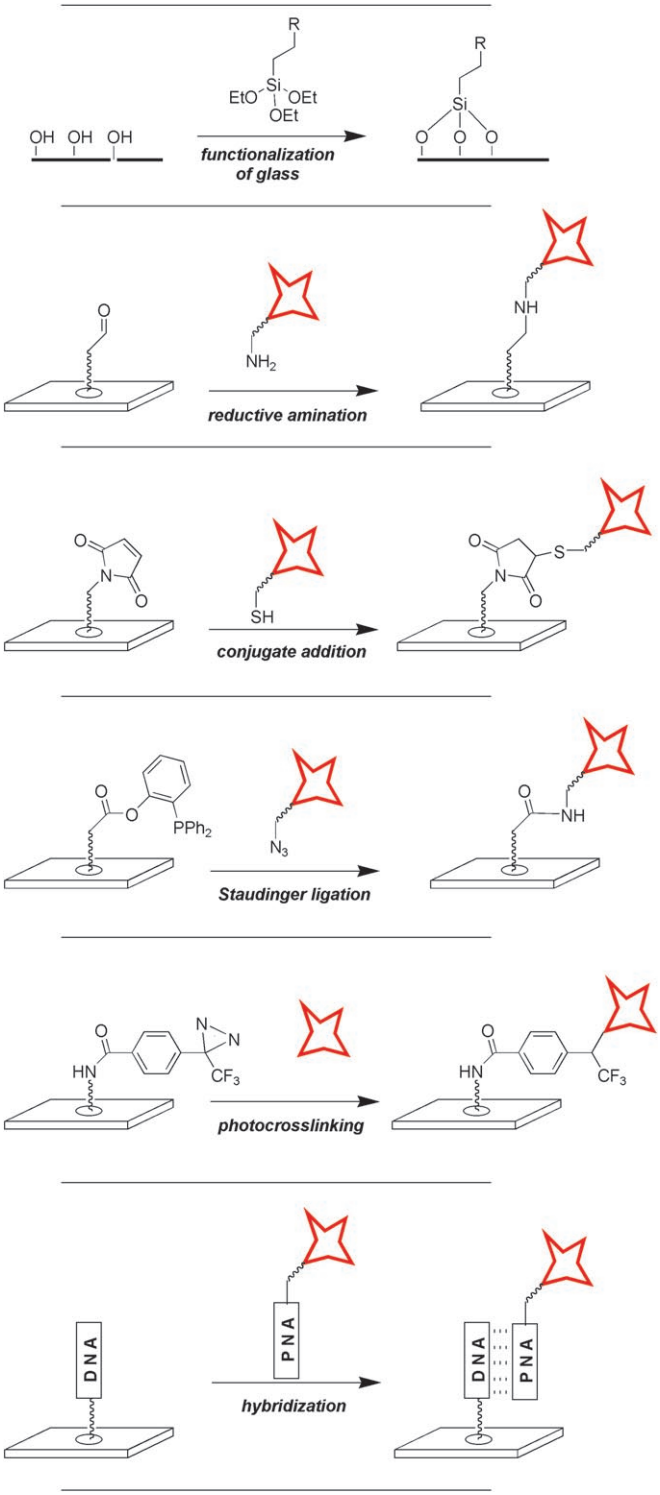

Figure 1. Selected examples of chemistries to immobilize molecules to glass surface.

Oligonucleotides as Amplifiable Tags and Supramolecular Barcodes

The sequence specificity of oligonucleotide hybridization and the fact that it can be amplified have inspired researchers to explore applications beyond its primary biological function of encoding protein sequences. An elegant example of DNA as an amplifiable tag for proteins was reported by Sano and co-workers, who tagged antibodies with DNA for immunoassays.[28] Rather than detect the antibody with luminescence or fluorescence as in traditional ELISA (ELISA = enzyme linked immunosorbent assay), they detected the antibody by amplifying its DNA tag by PCR (PCR = polymerase chain reaction). This amplification afforded an improvement of detection that was five orders of magnitude better than with traditional methods. The potential of using oligonucleotide tags as barcodes for the identification of proteins of interest has long been recognized in applications such as phage and ribosome display.[29] The application of DNA microarrays as a means of detecting or organizing oligonucleotide tagged proteins was first demonstrated by Niemeyer and co-workers; they immobilized antibodies tagged with DNA in an array format by hybridization.[30] This concept was later demonstrated with PNA tags that were chemoselectively introduced on the antibody by native chemical ligation.[31] In an elegant application of this concept, Kuimelis and co-workers exploited the puromycin-mediated reaction between *m*RNA and the growing peptide chain to generate in vitro several *m*RNA-peptide epitope fusion molecules that demonstrated an unamplified detection limit at the sub-attomole level on DNA microarrays (Figure 2A).[32] In a similar fashion, Yao and co-workers used ribosome display of a yeast cDNA library to tether mRNA to its encoded protein sequence en masse with subsequent amplification of the *m*RNA through reverse-transcription PCR and detection on a DNA microarray.[33]

The authors further employed the use of an activity probe that targets protein tyrosine phosphatase to selectively isolate proteins from the library based on enzymatic activity rather than on transcript or protein abundance (Figure 2B). The concept of DNA "barcode" has also been used to tag mutant strains of yeast.[34] The unnatural barcode sequence could be amplified and hybridized to an oligonucleotide microarray to identify which mutants strains were selected or survived under different conditions. Mirkin and co-workers used antibodies attached to gold particles that were "barcoded" with DNA tags and showed that they could detect multiple analytes in a single solution.[35] The advantage of using gold nanoparticles was that a large stoichiometric excess of oligonucleotide relatively to the antibody could be archived thereby bypassing the need of amplifying the DNA tag.

From a small molecule perspective, tagging beads from combinatorial synthesis with DNA was proposed 1992 by Brenner and Lerner,[36] who suggested that the identity of the molecule from a positively selected bead could be decoded by amplifying the DNA tag. Phagemid covalently tagged with small molecules have also been used such that the identity of the small molecule displayed on the phage could be derived by hybridization of its DNA tag.[37] On a more preparative scale, Harbury and Halpin showed that mixtures of molecules tagged with DNA could be separated

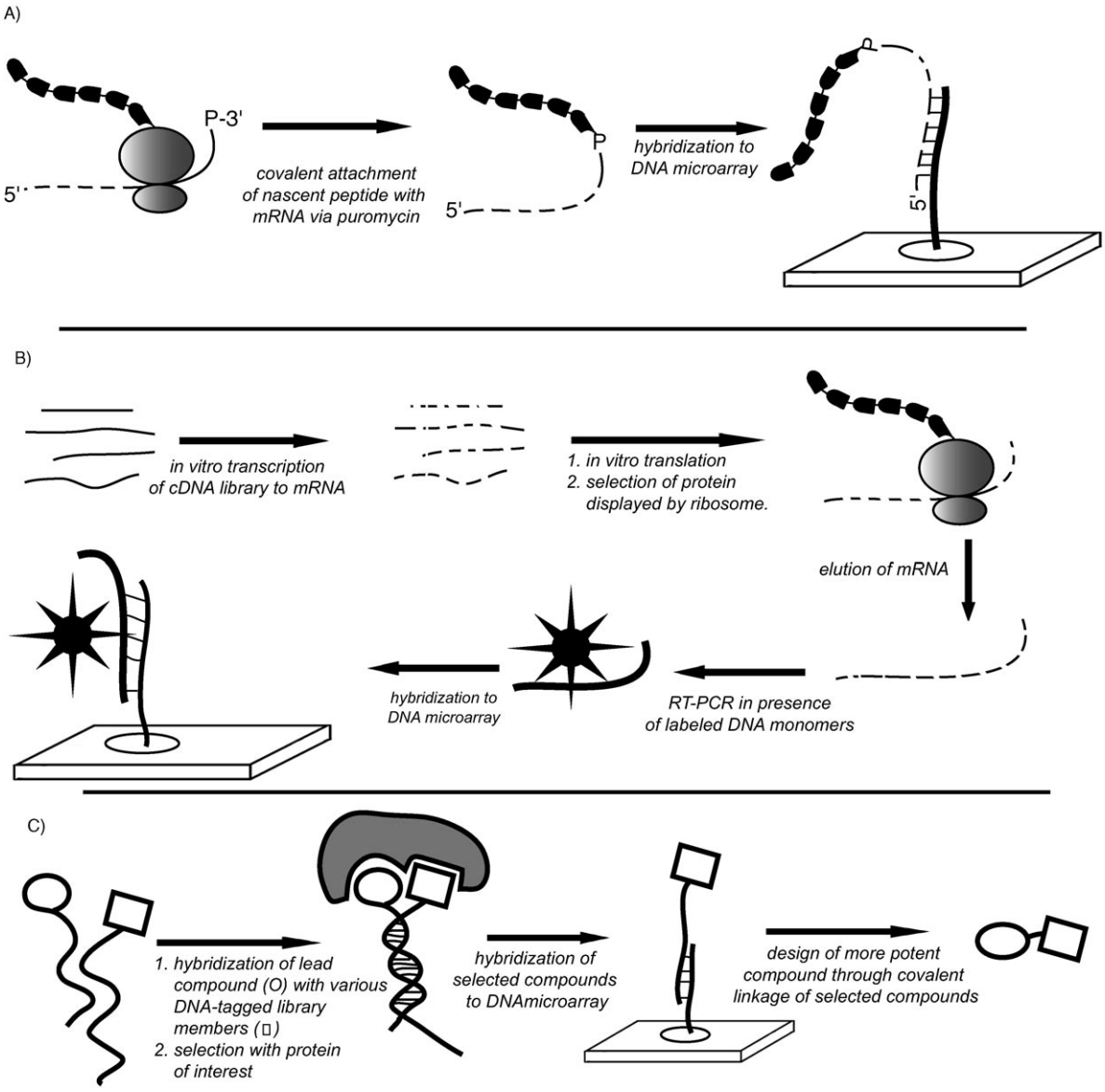

Figure 2. Selected examples of oligonucleotide encoded peptides and small molecules. A) Puromycin-mediated *m*RNA-protein fusion, B) ribosome display, C) affinity maturation of small molecules.

by using columns derivatized with complementary oligonucleotides, thereby allowing complex mixtures to be separated and routed through different reactions paths.[38]

In an other application, DNA templates have been used to direct chemical reactions by bringing reactive species tagged with DNA in close proximity through their hybridization to the template.[39,40] An attractive feature of this method is that the product is tagged with its synthetic instructions, which can be amplified after a selection process. Last but not least, affinity maturation of lead compounds has been achieved through the use of DNA-encoded small molecules that allow for the DNA-directed self assembly of multiple combinations of polyvalent compounds (Figure 2C).[41]

The predictability of the hybridization pattern has also been exploited to organize nanoparticles that were tagged with DNA to build nanoscale assemblies in a bottom-up approach.[42,43]

Library Preparation—Split and Mix Combinatorial Chemistry

Combinatorial synthesis has opened the door for small research teams to build chemical libraries in search of selective small molecule inhibitors as probes for investigating biological phenomena. To date, several thousand libraries have been reported in the literature.[44]

The two main strategies for the synthesis of libraries are parallel synthesis and split and mix combinatorial synthesis.[45] The latter is particularly powerful as the library size increases exponentially with the number of steps used to in-

troduce diversity. As illustrated in Figure 3, the synthesis of the library with five elements of diversity used in two steps yields 25 products, which are obtained in ten chemical trans-

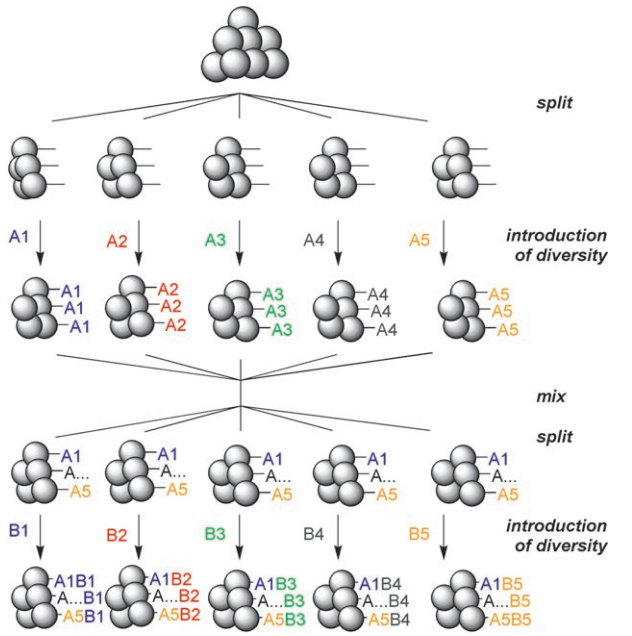

Figure 3. Schematic representation of split and mix synthesis. A batch of resin is split into five pools and the first element of diversity (A_1–A_5) is introduced. The pools are then mixed and split into five new pools such that a member of each first five pools should be present in the second pools. Then, the second element of diversity (B_1–B_5) is added to yield a library containing all permutations of $A_{1-5}B_{1-5}$. The power of this method comes from the fact that the number of product formed is exponentially related to the number of steps (in this example 5^2 products in 5+5 steps).

formations. If this library was prepared with 20 elements of diversity for four steps, as would be the case in a library containing all the permutations of a tetrapeptide, the library would have a size of $(20)^4$ compounds (i.e., 160000), but would require only 80 chemical transformation (omitting protecting group manipulations)! While emphasis is shifting away from large libraries, this method is still attractive as it is quick and does not require automation or specialized instruments. One of the major drawbacks of the split and mix synthesis is that the libraries are obtained as a mixture. This has stimulated the development of a number of encoding methods to track the synthetic path of the bead through the split and mixing processes.[46] The two most used methods are the haloaromatic tags developed by Still and co-workers[47] and the directed sorting developed by Nicolaou et al.[48] We reasoned that PNA[49,50] could be used to encode library synthesis. As shown in Figure 4, a bifunctional linker such as a lysine with mutually orthogonal Fmoc and Alloc protecting groups can be used to elaborate the PNA tag and the small library, respectively.

The resin is split into pools in which the diverse building blocks are introduced followed by their respective PNA

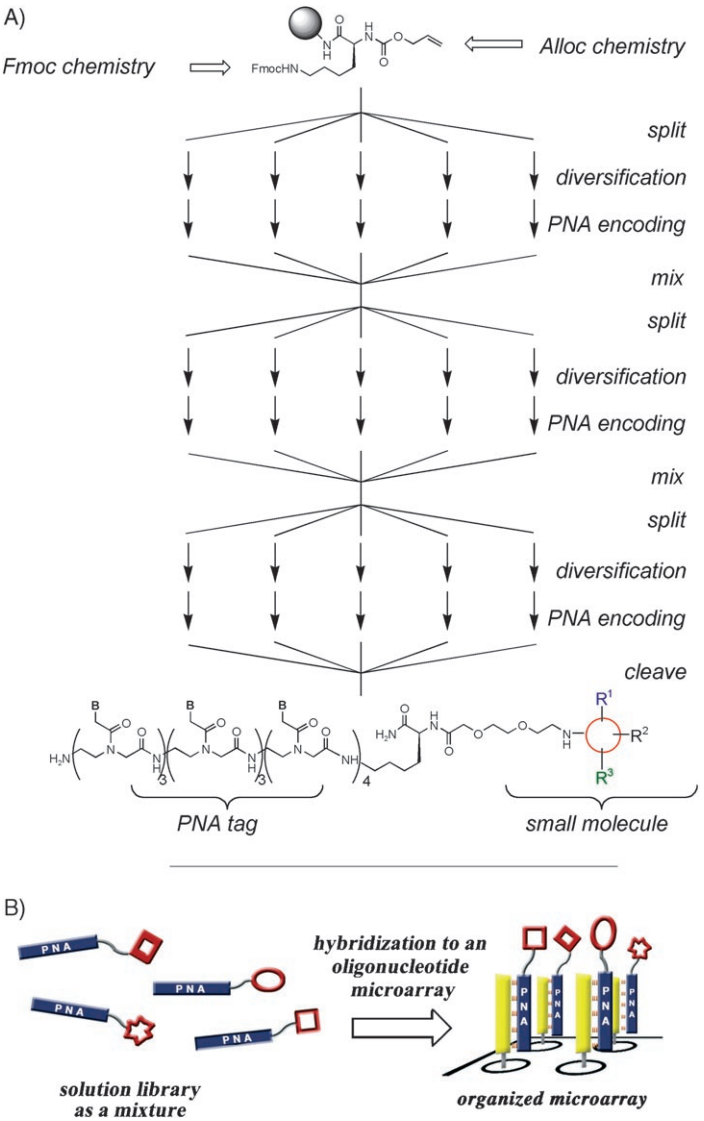

Figure 4. Schematic representation of PNA-encoded split and mix combinatorial synthesis [B = nucleotide base] (A) and conversion to an organized microarray (B).

codons and the resin is then remixed. Reiteration of this cycle affords a library wherein every molecule is tagged with its own synthetic history and thus its unique structure. Final cleavage off the solid support yields the library as a mixture in solution that can be converted to an organized format by hybridization to the DNA microarray.

The choice of PNA was primarily based on its chemical robustness and the fact that its oligomerization involves peptide coupling that is more compatible with organic synthesis than phosphoamidate chemistry of DNA oligomers. Furthermore, PNA is stable to strong acid and does not undergo a depurination reaction, as is the case with ribose nucleotides. From a screening perspective, they are not substrates for nucleic acid enzymes or proteases, and thus should not interfere with biochemical assays. As for the length of the PNA tag, considering that four combinatorial steps are sufficient

for most libraries, codons with three bases would yield a 12-mer PNA tag. As shown in Figure 5, three 12-mer PNA sequence labeled with a fluoroscein were prepared and hybridized to a GenFlex microarray (Affymetrix) containing 10000 features. This experiment showed that the 12-mer PNA was sufficient to give desirable hybridization properties (T_m and sequence specificity) and can theoretically encode millions of compounds.

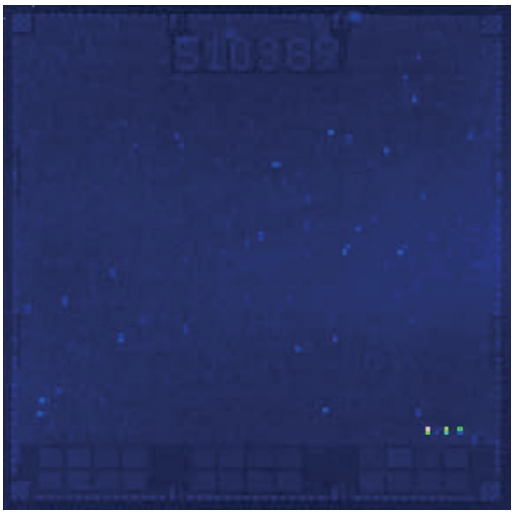

Figure 5. Hybridization of three 12-mer PNA to a GenFlex microarray (100×100 microarray of 50 μm feature).

PNA Encoding—Scope and Limitation of the Chemistry

While any encoding method necessarily imposes restrictions on the kind of chemistry that can be carried out to build a library, the chemical stability of PNA coupled with the fact that a large arsenal of nitrogen protecting groups and coupling reagents are known should minimize these constraints. Several protecting groups have already been reported for the PNA backbone nitrogen atoms,[51] the Fmoc- or Boc-protected PNA monomers being both commercially available (Fmoc = (9*H*-fluoren-9-ylmethoxy)carbonyl, Boc = *tert*-butyloxycarbonyl). The use of azide,[26] Alloc,[52] and Dde[53] have also been recently reported (Alloc = allyloxycarbonyl, Dde = 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl).
The protecting groups for the nucleotide's exocyclic nitrogen atom include TFA-cleavable groups (Bhoc, Boc, Tr), HF-cleavable groups (Cbz), and base labile groups (TFA = trifluroacetic acid, Bhoc = benzhydroxycarbonyl, Tr = trityl, Cbz = carbobenzoxy). While a number of permutations of orthogonal linkers and PNA protecting groups are conceivable, we have thus far used acid labile Rink resin with acid-labile protecting groups on the nucleotides and three different protecting groups for the terminal nitrogen atom (Figure 6). With this set of protecting groups, we have shown that an important repertoire of reactions used in combinatorial chemistry can be used with PNA-encoded chemistry, including palladium-catalyzed couplings, metathesis reactions, Mitsunobu reactions, reductive aminations, and of course amide and ester bond formations. The reactions which are unlikely to work due to the presence of amide proton in the PNA backbone are reactions such as enolate chemistry or organometallic reactions, which require strongly basic conditions.

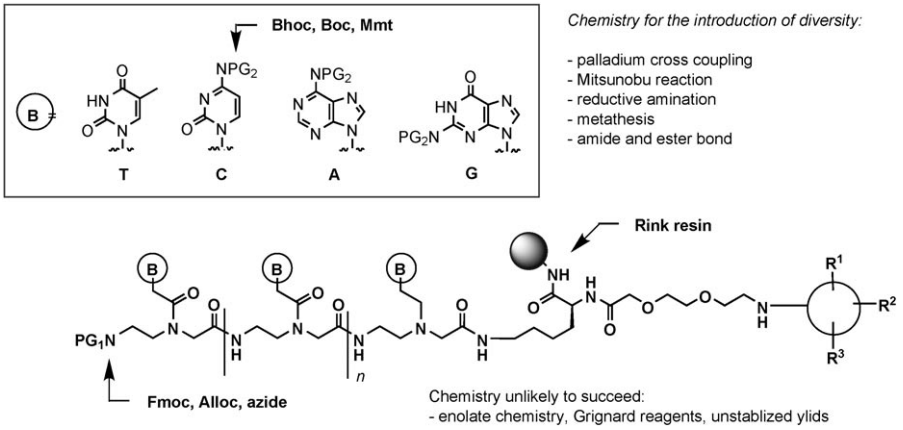

Figure 6. Current scope of PNA-encoded synthesis.

Screening and Profiling

There is a strong interest in methods to quantify enzymatic activity from complex proteomes such as crude cell lysates.[54] The microarray format offers a tangible opportunity to address this challenge. As shown in Figure 7, PNA-encoded libraries offer two possibilities to achieve this goal. In the direct-profiling example, PNA-encoded libraries of substrates can be incubated with a sample of interest and then hybridized to the array to quantify the modifications. The second possibility is to use PNA-encoded inhibitors that are all individually labeled with a fluorophore. The library is incubated with the sample of interest and the inhibitors that are bound to their target are separated from the unbound ones by size-exclusion methods. Hybridization of the selected compounds will reveal the structure of inhibitors that bound to a protein in the sample. In this latter case, the ability to use the library in solution prior to hybridization is essential, as the selection step based on molecular mass would not be possible otherwise. A considerable asset of this approach is that the proteomic sample being profiled does not

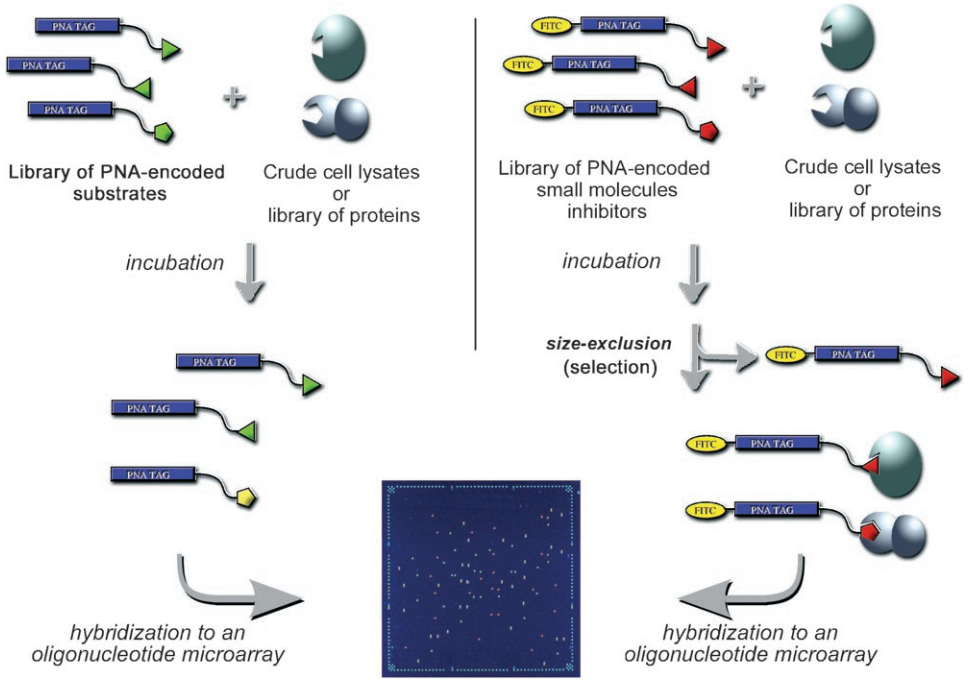

Figure 7. Profiling or screening with PNA-encoded libraries. The library of PNA-encoded substrates (left) present as a mixture in solution is incubated with the sample of interest leading to some of the subtracted being converted (green to yellow). The library is then hybridized to the microarray to identify the substrates that have been converted. Conversely, a library of fluorescently labeled PNA-encoded inhibitors (right) present as a mixture in solution is incubated with the sample of interest leading to some inhibitor binding to proteins. The bound inhibitors are isolated by size exclusion filtration and hybridized to the microarray.

need to be labeled. Considering that the average molecular weight of PNA-encoded small molecule is approximately 4000 g mol^{-1}, there is a large window of opportunity to separate the unbound PNA-encoded inhibitors from those bound to macromolecules, with typical molecular masses between 10 thousand and several hundred thousand g mol^{-1}. Selection of bound compounds by size exclusion has the advantage that it does not require any knowledge about the target of the small molecules, nevertheless more focused selection may be preferable under some circumstances. Such selection could involve immunoprecipitation or gel purification. Alternatively, proteins expressed with epitope or affinity tags could be screened as the crudely expressed protein and then selected by the affinity tag. While this type of selection step is not necessary for the substrate library, it may be used to enrich solutions in which substrate conversion was too low for direct detection. Another advantage of using the substrate libraries in solution prior to hybridization is that the interaction between the enzyme and a substrate in solution is closer to physiological conditions and, therefore, is not plagued by potential artifacts due to proteins interacting with the immobilized surface. We have demonstrated the potential of substrate libraries to profile proteases and kinases. As shown in Figure 8, by using fluorogenic substrates we showed that we could profile the substrate specificity of single proteases as well as measure proteolytic activity in complex systems such as lysates from apoptotic cells (Figure 9).[25] Furthermore, using specific peptide substrates, we should we could detect the activity of kinases. In this latter case, the phosphorylated substrates were detected using a specific antibody against phosphotyrosine (Figure 10). Importantly, we showed that we could assess the selectivity of an inhibitor against a panel of kinases. For inhibitors, we have prepared PNA-encoded libraries of mechanism-based inhibitors, such as the acrylates (Figure 11) for targeting cysteine protease,

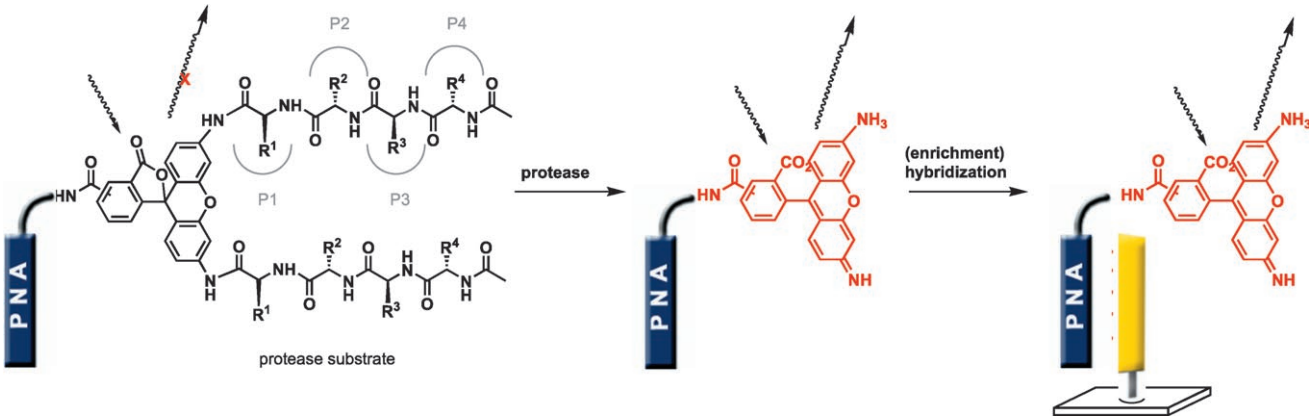

Figure 8. Profiling proteolytic activity using PNA-encoded fluorogenic substrates. The rhodamine fluorophore has low fluorescence as long as the anilines are acylated with the peptide substrates. If a protease cleaves the substrate at P1, the fluorescence increases. Hybridization of a library treated with a protease will reveal which substrate has been cleaved.

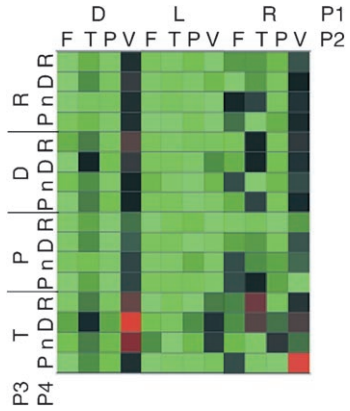

Figure 9. Difference in proteolytic activity between apoptotic cells and normal cells. Proteolysis of DTVD substrate corresponds to caspase-3 activity, an essential protease in the apoptotic cascade.

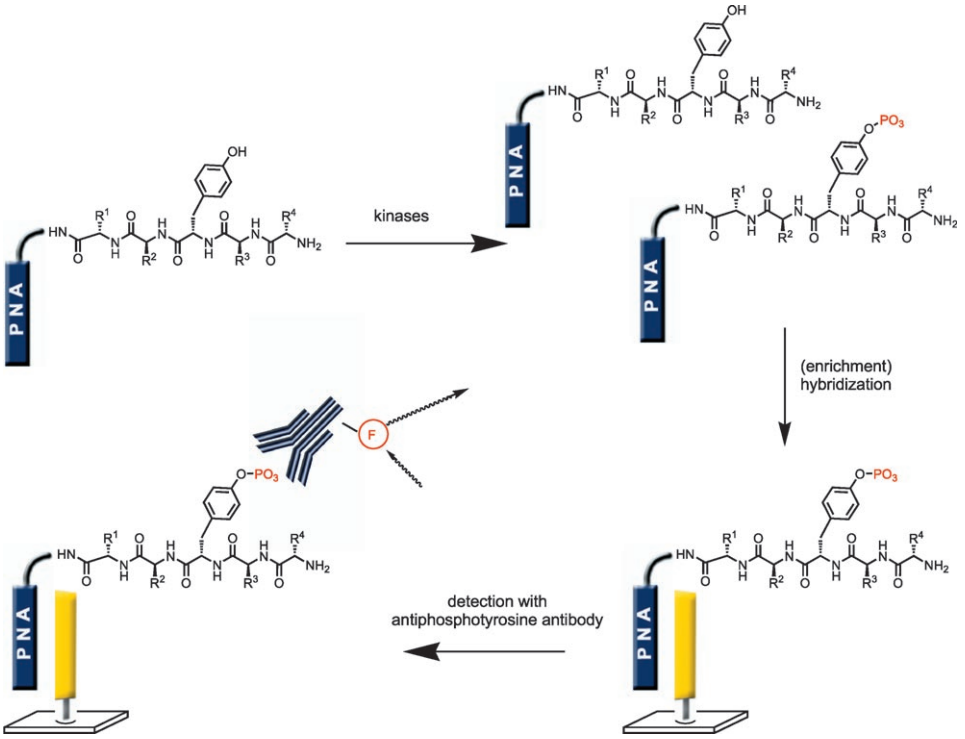

Figure 10. Profiling kinase activity by using PNA-encoded substrates. Phosphorylated substrates are detected by using a labeled antibody that specifically recognizes phosphotyrosine.

and we have shown that selected inhibitors could be isolated by size-exclusion filtration.[24,27] This led to the identification of multiple protease activities in house dust mite feces and the characterization of the Derp1 cysteine protease activity (Figure 12). Comparing the use of substrate libraries versus inhibitor libraries to profile enzymatic activity, the substrates have the potential of being more sensitive, since the enzyme can catalytically convert multiple substrates. However, from a chemical genetics perspective, to identify an enzymatic activity with an inhibitor should allow us to prove the correlation between the enzymatic activity and the phenotype.

The application of PNA-encoded small molecule probes is not limited to proteases and kinases. Indeed, several mechanism-based probes have already been demonstrated for many important enzyme classes including hydrolases,[55,56] glycosidases,[57] phosphatases,[58] sulfatransferases,[59] and methyltransferases.[60] Collections of compounds based around these and other probe scaffolds could be applied to PNA encoding, thus expanding the functional diversity of this proteomic approach. Furthermore, non-directed PNA-encoded libraries could be used to screen single or complex biological samples for the discovery of new enzymatic or binding activities.

Concluding Remarks and Future Prospects

The PNA-encoded methodology allows for combinatorial libraries synthesized in a split and mix format to be organized into microarrays by a self-sorting assembly. The PNA encoding is robust and tolerant of diverse chemistries for the synthesis of small-molecule probes. The application of this technology to profile proteases and kinases has already been demonstrated and could readily be extended to other important biochemical and regulatory transformations, such as methylation, acetylation, glycosylation, dephosphorylation, and so forth. While peptidic libraries have been used for profiling the function of enzymes, the technique is not limited to peptides, and the syntheses of small-molecule libraries are currently underway. An important asset of this technique is that library members can be selected prior to hybridization on the microarray. This provides a method of detecting protein/small-molecule interaction without labeling the protein. Likewise it should allow to enrich modified substrates that have been processed by an enzyme.

It is clear that sequence specificity of oligonucleotide hybridization has many applications as a method to encode or "barcode" as well as organize and build supramolecular assemblies. The concept and related work described herein adds precedence for the use of unnatural oligonucleotides coupled to oligonucleotide microarrays as a facile and flexible encoding and detection strategy.[61]

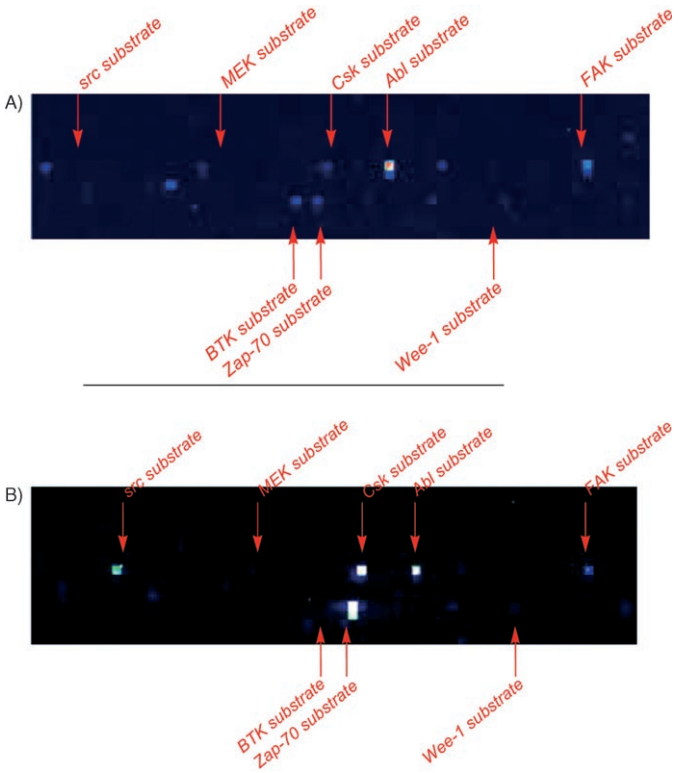

Figure 11. Detection of kinase activity using PNA-encoded kinase substrate (Src: EIYGEF; Mek: LTPYVA; Csk: AIYMFF; Abl: AIYAAP; Fak: DDYAEI; Btk: SDYMNM; Zap70:EEYFFI; Jak2: APYLKT; Wee1: GTYGVV). A) Incubation of substrates with Abl (100 nM, 60 min) and hybridization, followed by treatment with labeled antiphosphotyrosine antibody. B) Incubation of substrates with Abl, Src, Zap70 (100 nM, 60 min) and hybridization followed by treatment with labeled antiphosphotyrosine antibody.

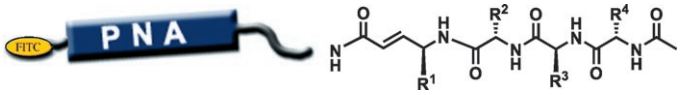

Figure 12. Structure of PNA-encoded cysteine protease inhibitor.

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[61] Note added in proof (June 10, 2005)**:** The PNA-encoded technology described herein is being embraced by other researchers as evidenced by the publication of M. Bradley and co-workers (J. J. Diaz-Mochon, L. Bialy, L. Keinicke, M. Bradley, *Chem. Communn*. **2005**, 1384–1386) at the time of the submission of the article.

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