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Techniques for high-throughput characterization of peptides, oligonucleotides and catalysis efficiency

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Combinatorial processes have been widely applied to many disciplines in chemistry and biology. The vast numbers of unique entities generated by combinatorial synthesis have led to the development of high-throughput methods for characterizing samples, to avoid bottlenecks created by the application of conventional, serial-based analytical techniques. In recent years, high-throughput and novel methods utilizing mass spectrometry, multiplexed capillary electrophoresis, various forms of optical detection, and even sound waves have been investigated for a variety of applications.

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Abbreviations

ACC	7-amino-4-carbamoylmethyl coumarin
CCD	charge-coupled device
CGE	capillary gel electrophoresis
ESI	electrospray ionization
FPA	focal plane array
IP	ionization potential
MALDI	matrix-assisted laser desorption/ionization
PAGE	polyacrylamide gel electrophoresis
PID	photoionization detection
SNP	single nucleotide polymorphism
TOF	time-of-flight

Introduction

The widespread growth of combinatorial synthesis in chemistry, biology and materials science has generated millions of new, unique entities. While the impact of combinatorial chemistry on the pharmaceutical industry has been well documented, many other fields have experienced rapid growth through the development of high-throughput, parallel methods. For example, the sequencing of the human genome has directed attention towards proteome research and the discovery of peptide-related disease markers. The extensive application of

DNA chips for genetic analysis and the development of antisense therapeutics have increased demand for high quality synthetic oligonucleotides. To increase reaction efficiencies in chemical and biochemical synthesis, new homogenous and heterogenous catalysts need to be discovered and optimized. To enable the timely and efficient characterization of these numerous and diverse products, high-throughput analytical methods are required.

This review covers recent progress regarding the development of rapid analytical tools in three emerging areas: peptide analysis, synthetic oligonucleotide characterization and catalyst discovery. It is our hope that the following discussion will give the reader an overview of some of the new and unique technologies available for increasing analytical throughput. More detailed discussions of methods can be found within the works cited herein.

High-throughput peptide analysis

The growth of proteomic research has fueled the development of techniques for performing high-throughput peptide-related analysis (see also Update). Applications requiring peptide analysis include the profiling of peptides in biological matrices for disease diagnosis, the epitope mapping of proteins, enzyme and inhibitor screening assays, and protein identification via enzymatic digestion and peptide mapping. Complex sample matrices and low sample concentrations are often encountered in peptide analysis, requiring highly sensitive and specific methods. The following section briefly highlights progress in the above-mentioned application areas.

Peptidomics refers to the qualitative and quantitative analysis of global peptide content in a biological system. Several reviews of this emerging field have recently appeared [1–3]. Peptides are present in human blood, urine, saliva, cerebrospinal, synovial (joint) and other body fluids, and play an important role in many physiological processes. From the post-translational and proteolytic processing of the roughly 100 000 proteins in the human proteome, several hundred thousand up to millions of unique peptides may exist in humans [1]. Many of these peptides are anticipated to play a significant role in disease diagnosis, progression or therapy.

The lower molecular weight and hydrophobicity of peptides relative to proteins preclude the use of traditional 2D gel electrophoresis-MS for characterization. As a result, multi-dimensional chromatographic or ultrafiltration

purification techniques are instead commonly performed before MS analysis. One recent example involves the isolation and desalting/concentration of serum peptides from matrix components using ultrafiltration followed by solid phase extraction in a 96-well plate format [4]. The concentrated and purified serum peptides are then readily available for rapid MS and MS/MS analysis.

Using highly sensitive on-line capillary liquid chromatography electrospray ionization time-of-flight mass spectrometry (LC-ESI-TOFMS), more than 1500 neuropeptides were detected in a single analysis from rat brain tissue [5]. A combination of peptide separation and fractionation by reversed-phase HPLC (RP-HPLC), matrix-assisted laser desorption ionization (MALDI)-TOFMS, and data mining form the basis of differential peptide display [6**]. Using this method, fingerprints of human central nervous system samples provided more than 6000 different signals, some of which varied for patients of a diseased state.

Synthesized peptide arrays [7] or peptide libraries constructed by phage display [8] provide a parallel approach for the epitope mapping of proteins. An epitope is the small region of a protein molecule that elicits an immune response. For elucidation of the epitope specificity of anti-FVIII antibodies, the entire amino acid sequence of human blood clotting factor VIII (FVIII) was synthesized on a cellulose membrane as small oligopeptides [7]. The sequence was split into decapeptides possessing an amino acid overlap of four. Purified anti-FVIII antibodies labeled with biotin were incubated on the membrane followed by peroxidase-labeled streptavidin. Chemiluminescence detection was utilized for identification of sequences responsible for antibody recognition.

Peptides can be used as substrates for or act as inhibitors of enzymes such as proteases, kinases or phosphorylases. The full characterization of substrate specificities of enzymes requires the synthesis and screening of large numbers of peptides. Novel readout strategies for screening solid-phase-bound peptides have been introduced. In one example, fluorogenic 7-amino-4-carbamoylmethyl coumarin (ACC)-modified peptides were bound to bovine serum albumin-aldehyde modified glass slides in a microarray format [9]. An 800-member peptide library was printed in an area less than 1.7 cm² and screened for the substrate specificities of several different protease enzymes. Proteolytic cleavage by the enzyme at the peptide N-terminus resulted in detectable fluorescence at the specific address from the remaining unacylated ACC, indicating enzyme specificity.

High-throughput peptide analysis is also utilized in the identification and characterization of proteins. Individual proteins or even whole cell extracts are enzymatically cleaved into smaller peptides for peptide mapping or peptide mass fingerprinting. Analysis has been performed

using several different methods including 2D-gel electrophoresis followed by MALDI-MS [10,11] or multiplexed capillary electrophoresis with UV absorbance detection [12*]. Recently, Tabb *et al.* [13] developed a non-gel-based technique for comprehensive proteomic analysis of cells. A protease was used to digest whole cell extracts composed of numerous proteins. The resultant complex peptide mixtures were subjected to separation by multi-dimensional chromatography and MS and/or MS/MS analysis.

High-throughput synthetic oligonucleotide characterization

Synthetic oligonucleotides are used throughout molecular biology in a variety of applications including PCR, single-nucleotide polymorphism (SNP) assays, site-directed mutagenesis, mutation detection and genotyping. Recently, oligonucleotide use has expanded widely with the introduction of microarray-based chip technology and the growth of clinical antisense research.

It is necessary to characterize and often purify synthetic oligonucleotides before use to ensure adequate performance. When used as primers in DNA sequencing and PCR work, often no purification is needed [14]. For more demanding applications such as hybridization assays, SNP analysis and antisense research, oligonucleotide probe purification is critical to success. It has been demonstrated that oligonucleotide microarray performance is highly dependent on spot concentration and uniformity, both of which are directly related to probe purity [15–17].

The high demand for quality oligonucleotides has motivated the development of higher-throughput methods for their synthesis, purification and characterization. This section of the review covers recent progress in high-throughput oligonucleotide characterization.

Synthetic oligonucleotide production is a highly automated, combinatorial process. Today's manufacturers are capable of producing tens of thousands of unique sequences per day. Oligonucleotides are usually customized to a user's application and produced base-by-base on commercial multiplex nucleic acid synthesizers employing solid-phase phosphoramidite chemistry. There are four steps to each synthesis cycle: detritylation, coupling, capping and oxidation. The trityl group is cleaved before coupling of the next nucleotide. The coupling efficiency is typically 98–99%, meaning that 1–2% of support-bound sequences fail to undergo addition with each cycle [18,19]. These failed sequences are capped to ensure they do not participate in the rest of the synthesis. The fraction of full-length product present after synthesis is equal to $(E_{ff})^{n-1}$, where E_{ff} is the reaction efficiency and 'n' is equal to the number of bases in the oligonucleotide product. Table 1 shows the percent of full-length product present for varying sequence lengths and reaction efficiencies. It is apparent that a

Table 1**Percent full-length product present after oligonucleotide synthesis.**

Oligo length	Coupling efficiency			
	0.995	0.990	0.985	0.980
20mer	91%	83%	75%	68%
50mer	78%	61%	48%	37%
75mer	69%	48%	33%	22%
100mer	61%	37%	22%	14%

small variance in reaction efficiency can have a significant impact, especially when synthesizing longer sequences.

Polyacrylamide gel electrophoresis (PAGE) and HPLC have been traditionally employed for oligonucleotide purification and characterization. For purification, PAGE offers superior resolution. Preparative scale HPLC offers higher automation and mass recovery, but does not remove $n-1$ product as effectively and is generally limited to < 50 mers. Recently, high-throughput strategies employing 'trityl-on purification' (TOP) have been introduced. Oligonucleotides are synthesized with the final 5'-terminus trityl protecting group remaining, and solid-phase extraction in a 96-well plate is used to isolate full-length product. Purity levels $\geq 90\%$ can be achieved for up to 50mer lengths [18,19].

Neither PAGE nor HPLC can provide the level of throughput necessary to characterize large numbers of samples. Therefore, higher-throughput alternative techniques have been developed for quality control of oligonucleotide identity and purity. The two predominant methods are MS and capillary gel electrophoresis (CGE).

Mass spectrometry

For rapid quality control of oligonucleotide identity, MS is clearly the method of choice [20]. Verification of full-length product is performed by comparison of the measured sample mass to the theoretically calculated mass based on the sequence. The two most popular MS techniques for oligonucleotide analysis are MALDI-TOFMS and ESI-MS. The preferred method for mass analysis of oligos up to 15 000 Da or approximately 50mers is MALDI-TOFMS [20–22]. Longer oligos do not ionize or fly well and are generally outside of the optimal resolution of MALDI-TOFMS instrumentation [23]. Multispot platforms provide high-throughput sampling. Improved sample preparation methods to remove non-volatile alkali metal ions before MS analysis can increase resolution and detection sensitivity [22].

Although more salt sensitive and slower than MALDI, ESI-MS is capable of analyzing longer oligonucleotides. As ESI-MS is a 'softer' ionization technique, less fragmentation is observed. However, ion suppression effects

and charge adduct formation often require desalting of samples before analysis. Direct sample injection in conjunction with 2-propanol organic cosolvent and spermidine modifier permitted the analysis of up to 51mer oligonucleotides at a throughput of 3–4 min per sample [23]. Recently, an improved method was introduced employing automated on-line sample preparation/desalting combined with ESI-MS [24**]. Mass confirmation analysis of oligonucleotides up to 120mers was demonstrated with a mass accuracy of 100 ppm and a throughput of approximately 1000 samples/day. A low-throughput LC/MS mode provided sample purity information at a rate of 20 min/sample.

Capillary gel electrophoresis

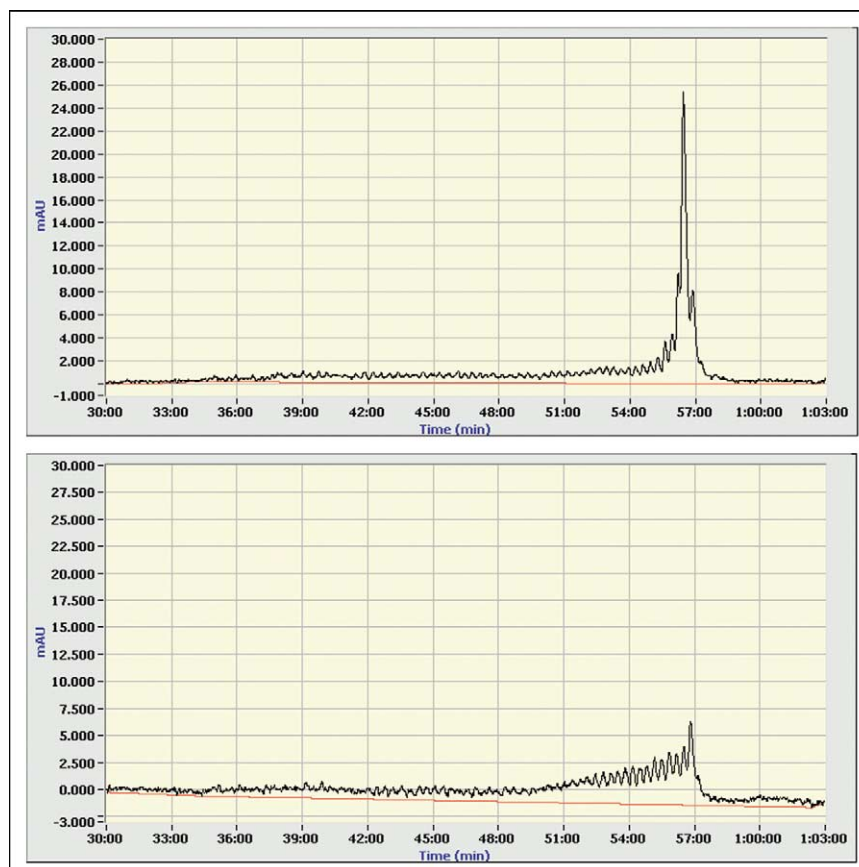
Although indispensable for identity confirmation, MS is not adequate for quantitative purity assessments of oligonucleotides, as mass peak heights are not necessarily directly proportional to sample concentration due to variations in ionization efficiencies between sample components [25]. To analyze oligonucleotide purity and concentration [14], CGE employing UV absorption detection has been extensively used [14,25,26,27**,28]. Separation is performed at basic pH values where oligonucleotide mass-to-charge ratios are nearly constant. Gel-based sieving matrices provide size-based separations capable of resolving $n-1$ impurities. Advantages of CGE include superior resolution, nanoliter sampling volumes and ease of automation.

Single capillary CGE methods provide analysis times of 15–110 min per sample, depending on the capillary length and gel matrix [25]. Only a statistical sampling of products is possible with this throughput. The use of a sequential injection before analysis (SIBA) method was recently explored to increase the analytical throughput of CGE [28]. Up to five separate samples were injected sequentially before performing the separation. Using this approach, automated analysis of 96 different 25mers could be achieved in 20 h.

The recent introduction of 96-capillary multiplexed CE-UV instrumentation provides a significant increase in analytical throughput for the quality control of oligonucleotide purity [27**]. Resolution of $n-1$ product was demonstrated up to 80mer lengths with a throughput of 96 samples per hour. Figure 1 shows an example of two different unpurified 70mers analyzed simultaneously in a 96-capillary array. The top electropherogram reflects the expected product, while the bottom electropherogram shows a failed synthesis. The throughput of multiplexed CGE provides the capability to comprehensively screen oligonucleotides for purity before use in critical applications.

The recent interfacing of CGE to ESI-MS to provide both purity and mass identification of oligonucleotides

Figure 1



Electropherograms of unpurified 70mer oligonucleotides obtained simultaneously in different capillaries of a 96-capillary array instrument. The purity of 39.1% was obtained for the top sample. The bottom sample failed to produce significant product. Reprinted with permission from [27**].

is also of note [29*]. It is anticipated that the growth of oligonucleotide-based therapeutics will further promote the development of high-throughput methods of analysis.

High-throughput characterization of catalysis efficacy

The first reported application of combinatorial principles to catalyst discovery by Menger appeared less than a decade ago [30]. Considerable savings in development costs, personnel and time can be realized by applying combinatorial strategies to select only highly active catalysts for further optimization and testing. Several reviews have appeared in previous years discussing the use of combinatorial chemistry in catalysis research [31,32]. No universally applicable analytical technique exists for high-throughput catalyst screening. However, many unique and elegant approaches have been investigated (see also Update). This section of our review is intended to highlight recent progress in high-throughput monitoring of catalytic processes. Table 2 provides a comparison of techniques in terms of their chemical specificity and analytical throughput.

MS continues to find widespread use in heterogeneous catalysis monitoring because of its high sensitivity and near universal detection capabilities. Claus and co-workers implemented a scanning MS for continuous analysis of parallel gas-phase reactions [33]. The configuration provided time resolved and spatially resolved sampling of complex gaseous mixtures at a throughput of 1 min/catalyst. Nayar *et al.* detailed a laser-activated membrane introduction MS system for heterogeneous catalyst evaluation [34]. At the heart of the apparatus is an interfacial semipermeable membrane separating the gaseous or liquid analyte stream and MS vacuum. An array of 12 catalysts was evaluated in less than one hour with no moving parts.

A high-throughput GC-based system for screening enantioselective catalysts has been described [35]. Two GCs containing the same optimized chiral stationary phase were connected and judiciously interfaced with robotics and software to provide a throughput of 600–800 samples/day.

Yeung and co-workers applied 96-capillary multiplexed CE-UV for the screening and optimization of a homogeneously catalyzed synthetic reaction [36]. Eight different

Table 2

Comparison of high throughput methods investigated for catalytic monitoring.

Method	Detailed chemical information?	Parallel analysis?	Estimated throughput	References
Mass spectrometry	Yes	No	1 min/sample	[33,34,44**]
Gas chromatography	Depends on detector	No	5–30 min/sample	[35]
Multiplexed capillary electrophoresis-UV absorbance detection	No	Yes	96 samples/30 min	[36]
Fluorescence emission	No	Yes	< 1 min/library	[37,45]
Photoionization detection	No	Yes	< 15 min/library	[38]
Photoacoustic effect	No	Yes	< 1 min/library	[39]
Infrared thermography	No	Yes	< 1 min/library	[44**,45]
Infrared spectroscopy	Yes	Yes	< 1 min/library	[40–43]
Colorimetric detection	No	Yes	3000 samples/day	[43,46**]

palladium catalysts and 11 different bases (88 combinations) were simultaneously tested in a 96-well plate format. Total reaction yields, isomeric selectivities, and reaction kinetics were obtained from nanoliter injection volumes without perturbing the reactions.

Potyralo and co-workers presented a non-destructive fluorescence-based ratiometric method for evaluating polymerization catalysts in 96-well plates [37]. The native fluorescence emission of polymerized bisphenol A polycarbonate was correlated to the number-average molecular weight. The selectivity of linear versus branched product was correlated to the fluorescence ratio at two different wavelengths. A charge-coupled device (CCD) camera provided simultaneous illumination of the entire 96-well plate for rapid catalyst assessment.

The heterogenous oxidative dehydrogenation of ethane and propane was monitored with photoionization detection (PID) [38]. In the PID technique, monoenergetic photons produced by a direct current discharge lamp ionize molecules with lower ionization potentials (IP). When product IPs are lower than those of reactants or side products, extremely sensitive detection at ppb levels is achievable. An array microchannel reactor permitted the screening of 66 different ternary catalyst compositions in a single experiment.

Two real-time parallel detection systems for heterogenous catalysis based on the photoacoustic effect have been developed [39]. This technique involves the excitation of a selected molecule by a laser, from which a pressure pulse is generated and detected by a specialized microphone. If reaction products are known and they absorb with little interference, this approach can provide a fast alternative to GC or MS-based methods.

IR spectroscopy offers chemically sensitive, non-invasive, real-time monitoring of heterogenous catalytic processes. Mid-IR spectroscopy employing a low volume flow cell

was applied to the rapid on-line analysis of gaseous effluents from a heterogenous reaction [40]. Quantification was possible in 2 min, as compared with 30 min detection by GC. An array-based silicon support for high-throughput IR transmission measurements of polymer composition was constructed and tested [41]. Polymers prepared from low-octene incorporating catalysts were loaded and characterized in the 56-element support at a throughput of >300 samples/day. IR spectral imaging employing focal plane array (FPA) detectors was described first by Snively [42] and later by Schuth [43]. Multi-element gas analysis cells can be interrogated simultaneously using FPA detectors to provide spatially resolved chemical information.

Optically based detection methods are generally adaptable to high-throughput but lack specific chemical information. Information-rich techniques such as MS are traditionally low throughput. To compromise, Klein and co-workers described an intelligent combination of imaging IR thermography and positionally controlled MS sampling of a 96-fold gas-phase catalytic reactor system [44**]. All candidates are prescreened for temperature changes indicative of activity, after which only the most active catalysts are analyzed using MS. Another hybrid system combined laser-induced fluorescence imaging and IR thermography for screening heterogenous catalytic selectivity and overall activity, respectively, with high spatial and temporal resolution [45]. Screening throughputs of 15 seconds per library could be achieved.

Researchers at Symyx described a high-throughput primary screen for the heterogeneously catalyzed gas-phase oxidation of ethane to acetic acid [46**]. Libraries were spotted on quartz wafers as 16 × 16 arrays and screened in parallel using a proprietary 256-channel microfluidic reactor. Product identification was achieved via CCD imaging of the colorimetric reaction of acetic acid with methyl red dye on a thin layer chromatography plate.

More than 10 000 catalysts could be screened in a two-week period using this approach.

Conclusion

The unraveling of biological function and the discovery of new materials is progressing at unprecedented speeds. The development of new technologies and the modification of traditional serial-based methods for performing high-throughput analysis must continue to keep pace to fully realize the potential of combinatorial approaches. Peptide analysis plays a key and increasing role in the biological, pharmaceutical, and clinical sciences. The discovery of clinically relevant peptides and the development of peptidomimetic drugs will help to promote improved, higher-throughput methods for peptide analysis. Similar trends can be observed for the use of synthetic oligonucleotides. As combinatorial methods evolve in catalysis research, information obtained should enable the development of new generation high-performance catalysts.

Update

We note that an upcoming issue of *Journal of Chromatography B* will be entirely devoted to peptide separation and analysis. In this thematic issue, articles and reviews concerning pharmaceutically relevant natural peptides, quantification of peptides, and other technical progress in peptide characterization and analysis will be presented.

A fast, inexpensive, and relatively generic method for separating and quantifying the overall composition of catalytic mixtures by a combination of thin-layer chromatography separation and image analysis was recently described [47]. Colored reaction products could be directly imaged, while colorless products could be identified under UV irradiation. Less than 1 h was required for analysis of 96 different sample mixtures.

Scientists from Bayer [48] and Dow [49] companies recently reported on the benefits and issues involved in performing high-throughput catalysis research and testing. Even when achieving a screening workflow of 10 000 heterogeneous catalysts per day [48], it was necessary to employ a combination of evolutionary experimental design with data mining to effectively identify interesting mixed metal oxide catalysts for alkene epoxidation. The design of high-throughput research (HTR) programs for catalyst discovery at Dow was discussed [49], giving several specific examples of how HTR has had an impact on the development of new novel catalyst materials.

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