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Emerging chemical and biological approaches for the preparation of discovery libraries

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The term of combinatorial chemistry has come to embrace all types of small-molecule library strategies. In both academic and industrial settings, the development of many combinatorial techniques has been driven by a desire to generate diverse libraries of molecules. Recently, chemical approaches have been reported that range from alternative library design concepts to new synthetic procedures that allow for selective product formation in extremely short reaction times. Concurrently, biological techniques have made great strides by adapting naturally occurring strategies for library preparation and screening. With proper understanding of library design, combinatorial endeavors and molecular libraries will continue to impact on the development of the next generation of tools for modern medicine.

Small molecules, that is compounds of a molecular weight <1500 Da, are powerful tools for studying biological systems. In particular, the ability of small molecules to modulate biological processes through their interaction with larger macromolecules, such as proteins and DNA, provides an opportunity for potentially useful therapeutics and for tools to probe biological systems. Despite the desirable properties of these compounds, identifying potent, selective molecules for biological targets remains a daunting challenge for the chemical biology community [1]. Nature's solution for identifying small molecules is to 'mix and match', hence a combinatorial approach that uses selection procedures to identify a potential lead, which is further refined by mutation and survival of the fittest. Using this example as a guide, chemists and biochemists have developed technologies that assemble building blocks in a plethora of combinations to construct chemical libraries. In parallel, biologists have harnessed combinatorial strategies to generate biopolymer libraries (>10^9 members) by employing technologies such as phage display [2]. Despite the many technological advances in library preparation in the last two decades, the rate at which drugs reach the marketplace has not increased [3]. Consequently, to accelerate the drug-discovery and lead-identification processes, further development is necessary in the preparation of synthetic and biochemical libraries.

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Initially, synthetic combinatorial experiments focused on the development of large libraries that were derived from a core structure and expanded by introducing various appendages onto the core. Using this strategy, it was believed that the chances of discovering a potent, selective molecule by accessing a range of chemical space would increase. However, recently it has been shown that this approach only evaluates a small amount of chemical space and is better suited to the refinement of lead candidates [4].

When describing the ability of a library to explore different parts of chemical space, it is common to use the term diversity. Indeed, the diversity of compound libraries is as important to the value of a library as the number of members. Over the last two decades, two major philosophies for generating chemical libraries have been developed: (1) traditional synthetic organic synthesis; and (2) the use of a biochemical approach to select for suitable activity. In both cases, the contents of a library are screened using an *in vitro* assay to evaluate the biological activity of each library member. Recently, lead-likeness, oral absorption and Lipinski's rules have played crucial roles in the consideration of library design [5]. Conversely, virtual screening, which examines chemical libraries *in silico*, has been shown to reduce lead-discovery time by sifting through large virtual libraries using molecular modeling. This topic, although of great interest, has been reviewed recently and is beyond the scope of this article [6]. Finally, successfully identifying potent, selective small molecules for selected biological targets requires a thorough understanding of library design and planning. Moreover,

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crafting elegant library design with an understanding and execution of novel technologies can accelerate the lead-discovery process.

High-throughput synthesis techniques

The foundation of synthetic combinatorial chemistry can be traced to Merrifield's development of solid-phase peptide synthesis in the early 1960s [7]. For the first time, this technology provided a method for rapidly separating reagents and byproducts from solid-support-bound reaction products by washing the support with various solvents. Merrifield's groundbreaking techniques have been extended to small molecules including natural and synthetic compounds [8]. The use of solid supports allow chemists to systematically mix and match various building blocks to generate libraries of molecules without purifying intermediates, and has given rise to what has been termed combinatorial chemistry [9]. However, using solid-phase organic synthesis (SPOS) to prepare small-molecule libraries also poses new challenges to organic chemists. For example, reactions must proceed with high efficiency because of the lack of purification of synthetic intermediates. In addition, the reactions must be compatible with up to thousands of different substrates to utilize the reaction sequence in a combinatorial format. Furthermore, the heterogeneous nature of the solid support impacts the reaction kinetics significantly and limits potential reaction conditions [10].

To aid in the problems with compatibility of reaction partners and the heterogeneous nature of solid-phase reactions, several phase separation or tagging strategies have been developed. In synthetic schemes employing precipitation tags, also known as liquid-phase organic synthesis (LPOS), the starting material is attached to a tag that is soluble in most organic solvents, which allows intermediates to be purified by adding a solvent that induces precipitation of the tag and the attached substrate [11,12]. The excess reagents are rinsed away and the tag resolubilized for the subsequent reaction. In this technique, all intermediates can be characterized by conventional techniques, such as NMR and mass spectrometry, to assist in intermediate identification. Complementary to LPOS, fluorous tags have also been used in a combinatorial format by tagging the starting material with a polyfluorocarbon chain [13]. Fluorous tags are soluble in both organic and fluorocarbon solvents, which allows for homogeneous reaction conditions followed by isolation of the tagged intermediates by extraction of the reaction mixture with immiscible fluorocarbon solvents, for example either aqueous extraction or passage through a column of fluorinated silica gel.

Phase-tagging technologies and solid supports have been employed successfully numerous times in traditional library synthesis techniques, such as parallel and split-pool synthesis [14–16]. However, it should be noted that most combinatorial formats are specific to reaction conditions in addition to the type of structural motif being synthesized. Great strides have been made in polymer design and in the development of new polymeric supports for organic synthesis, and this remains an active area of research.

Diversity: crucial aspects

Traditionally, there are two classes of compound libraries: natural product and synthetic compounds. Generally, natural products display greater structural diversity and complexity than chemist-derived synthetic molecules; however, the preparation, purification and characterization of these complex molecules is much less straightforward. By contrast, synthetic compound libraries have the advantage of high-purity and well-characterized structures. Ideally, it is desirable to have the elements of complexity from natural products in a well-characterized combinatorial format to benefit from both aspects of natural product and synthetic compound libraries. To accomplish this, Schreiber has developed an approach to library synthesis called diversity-oriented synthesis (DOS) [17]. The foundation of DOS is related intimately to combinatorial chemistry, however, the order in which diversity in introduced is reversed. For example, in a typical combinatorial-chemical format, diversification comes through a common core structure by altering the appendages (Figure 1a). Conversely, DOS retains similar appendages but alters the core structure (Figure 1b). Reversing the introduction of diversity increases the molecular complexity and the libraries are said to have 'natural product-like' complexity. The increased complexity is a result of the core structure accessing greater chemical space for the placement of the conserved appendages.

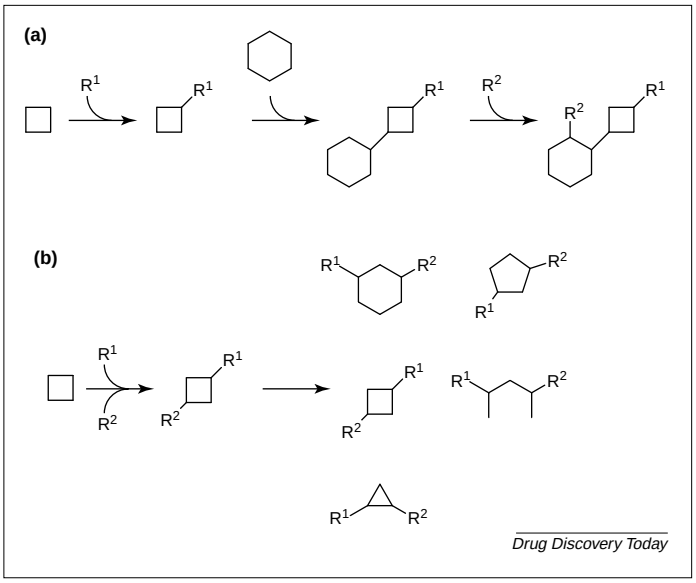

FIGURE 1

Approaches for the synthesis of small-molecule libraries. (a) Traditional organic synthesis. Starting with a core scaffold, diversity elements are appended and built on until the desired target molecule is attained. **(b)** Diversity-oriented synthesis. Although the appended functionalities are the same, the core scaffold changes to access different parts of chemical space.

To maximize library diversity using DOS, an alternative analysis is necessary. Specifically, target-oriented synthesis is generally examined by using retro-synthetic analysis wherein a complex molecule is broken down in a series of steps to simple starting materials. By contrast, using DOS to maximize diversity in a chemical library requires planning in the forward direction to increase the diversity and skeletal complexity. Starting from an array of similar structures and converting them to various, complex molecules maximizes the diversity of a library. The adjustment in analysis when employing DOS is necessary because the skeletal core changes, whereas in conventional combinatorial chemistry the core is conserved. Therefore, when carrying out DOS, several conventional retro-synthetic analyses are required to achieve the same goal that is achieved by performing one analysis in the forward

direction. As a result of the increase in diversity, DOS libraries can be used to identify lead compounds for several unrelated biological targets, which has practical advantages compared with conventional target-oriented libraries that, generally, are refinements of a lead compound [17,18].

It is important to note that there is no method to determine absolute diversity because the diversity of a given library is inherently a comparison of that library with some reference set. However, methods have been developed to assist in measuring relative diversity. To quantify the relative diversity of a library, a statistical method called principal component analysis (PCA) can be performed [18]. In this process, each compound in a library is assigned a set of *n* descriptors. The descriptors can include several physical properties, such as molecular weight and artificial membrane permeability, and biological binding constants. The compounds are then represented as a vector in *n*-dimensional space. The entire dataset is then analyzed using PCA and results in a new, unitless axis – called either principal components or eigenvectors. Each new axis is a linear combination of the original descriptors, calculated to represent as much of the variance in the dataset as possible in each successive principal component, based on correlations between the original descriptors. After replotting each compound as a vector in one-, two-, or three-dimensional space using its coordinates, or eigenvalues, the ability of a given library to access different parts of chemical space can be measured. This technique helps to assess library quality as well as to quantify relative diversity.

Chemical technologies: new starting points for library design

Microwave-assisted organic synthesis

Several technologies that have developed in the last decade facilitate the rapid generation of compound libraries. For example, microwave-assisted organic synthesis (MAOS) [19] has recently become a useful technology for the rapid optimization of reactions in addition to uncovering new chemical reactivity. Microwave-enhanced chemistry is based on the efficient heating of materials by microwave dielectric heating effects. This phenomenon depends on the ability of a specific material, solvent or reagent to absorb microwave energy and convert it into heat. Traditionally, organic reactions are heated thermally using conductive heating, such as an oil bath. However, compared with microwave heating, thermal heating is a comparatively slow and inefficient method for transferring energy into a reaction system. This lack of efficiency can be attributed to differences in thermal conductivity of the various materials being penetrated, which results in the temperature of the reaction vessel being higher than the reaction mixture. By contrast, microwave irradiation produces efficient internal heating by directly coupling microwave energy with the molecules that are present in the reaction mixture.

Rate accelerations and altered product distributions using microwave energy, as opposed to traditional thermal heating methods, have been observed recently in reaction profiles. This has led to speculation about so-called 'specific' or 'non-thermal' microwave effects [20–23]. However, in most cases, scientists agree the reason for the observed rate enhancements is purely a kinetic effect as a consequence of the high reaction temperatures that are attained rapidly when irradiating polar materials in a microwave field. Moreover, by applying the Arrhenius equation, $k = Ae^{-E_a/RT}$, Baghurst and Mingos have shown that a transformation that requires 68 days to reach 90% conversion at 27°C achieves the same degree of conversion in 1.61 s at 227°C, which demonstrates a purely kinetic effect for the observed rate enhancement [24].

Based upon this dramatic kinetic acceleration MAOS provides a unique opportunity to address some of the above problems with SPOS, such as heterogeneous reaction conditions, nonlinear kinetic behavior, slow reactions, solvation problems and degradation of the polymer support because of long reaction times. The use of cross-linked, microporous and macroporous polystyrene resins are used most often as SPOS supports for MAOS [25–28]. Although it is commonly believed that temperatures >130°C result in degradation of polystyrene-based resins [29], it has been demonstrated recently in microwave-assisted SPOS that these resins can withstand microwave irradiation for short periods of time even at >200°C [30]. Moreover, other commonly used liquid-phase supports, such as polyethylene glycol and fluorous-tagged molecules, also have been used successfully in MAOS [31–35].

A plethora of reaction types have been demonstrated using microwave-assisted SPOS and, recently, the technology has been adapted to multi-well plate format [36] for traditional combinatorial chemistry. In particular, combinatorial chemists have shown renewed interest in transition-metal-assisted reactions because of the short reaction times and the diversity that can be employed using these reactions [37]. Before MAOS, transition-metal reactions have had limited use in high-throughput synthesis because the long time needed at high temperatures commonly produces an unacceptable array of side-products. Additionally, the growing concerns about the quality of compound libraries [38] make MAOS an attractive technology for a high-throughput synthesis laboratory because of the high conversions observed for several reactions.

Continuous-flow organic synthesis

Despite the rapid development of synthetic methodology during the last decade in fields such as catalysis, asymmetric synthesis and combinatorial chemistry, organic synthesis is still carried out in a batch-wise manner. For over a century, synthetic organic chemistry experiments have been executed in standardized glassware. Consequently, compounds have been synthesized batch-wise, regardless of the kind of chemistry performed. Today, most high-throughput synthesis is performed in batches, whereas flow-through processes are restricted to production processes. Interestingly, because the main advantages of the flow-through approach are facile automation, reproducibility, safety and process reliability, constant reaction parameters (e.g. temperature, time, amount of reagents and solvents) are assured and the conversion of high-throughput synthesis to continuous-flow technologies should be extremely advantageous.

The switch from a batch-wise synthetic protocol to a flow-through concept has its advantages [39]. Specifically, it reveals opportunities that are rarely achieved with similar simplicity in batch reactors. For example, by assembling a line of reactors, multi-step syntheses can be accomplished with minimum purification between two given reaction steps (Figure 2). In addition, using either a divergent or a convergent multi-step synthesis strategy to create libraries of complex target molecules is feasible with flow systems. By incorporating separation and analytical techniques into a flow system, such as employing standard HPLC equipment in conjunction

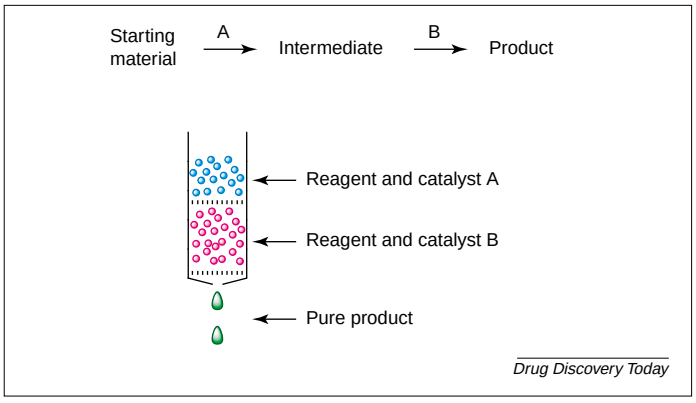

FIGURE 2

Continuous-flow synthesis. An example of a two-step reaction sequence is shown. Given the appropriate conditions, the entire scheme can be performed by loading the starting material at the top of the column and collecting the pure product as it exits the column.

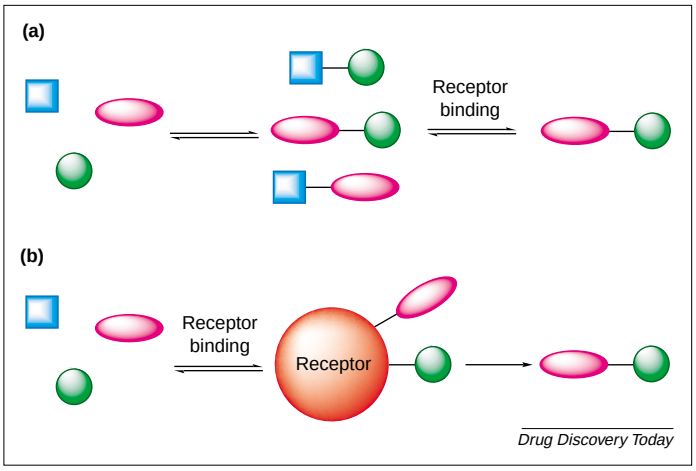

FIGURE 3

Receptor-assisted combinatorial chemistry methods. (a) Dynamic combinatorial chemistry. In this strategy, the synthesis of library members is reversible thermodynamically, thus, the binding of a given library member to the receptor alters the equilibrium of the reaction mixture, which allows its identification. **(b)** Receptor-accelerated synthesis (RAS). Two building blocks associate with a given receptor in proximity to each other, thus increasing their effective molarity. This leads to formation of a covalent bond between the two blocks and generates the inhibitor molecule.

with detector devices like mass spectrometry and diode-array detection, the concept can be exploited further.

Despite the promise of continuous-flow organic synthesis, several problems are still to be overcome. Namely, the limitations in the maximum number of sequential reaction steps, inert properties of all materials in the flow-through system to many different organic solvents, the need to switch the solvent for selected reaction steps, efficient regeneration of reaction columns, and facilities to purify intermediates, makes the conversion from traditional synthetic techniques to continuous flow problematic. Nevertheless, although the number of examples of flow-through processes performed in the laboratory is small [40], a bright future for this approach to chemical synthesis is expected, which will further advance chemical-library preparation.

Biochemical technologies: using nature's code

Receptor-assisted combinatorial chemistry

Most combinatorial adventures in drug discovery have relied on focused libraries that utilize either templates or functional groups to provide affinity to a receptor. However, an emerging method in combinatorial chemistry, termed receptor-assisted combinatorial chemistry (RCC) [41,42], adds stoichiometric amounts of the receptor during library synthesis. Adding the receptor to the starting materials biases the union of two complementary building blocks by altering either the kinetics or the thermodynamics of the chemical-coupling reaction, thereby combining synthesis and screening procedures in one step. Furthermore, instead of specific receptor assays, this method detects increased amounts of the best binding compounds with established analytical methods such as HPLC, mass spectrometry, NMR spectroscopy and X-ray crystallography. In the last decade, two major RCC methods have emerged, dynamic combinatorial libraries and receptor-accelerated synthesis (RAS).

In dynamic combinatorial chemistry, secondary reactions that shift covalent bond-formation equilibria are exploited to discover tight binding molecules for a given target (Figure 3a). In this technique, all of the building blocks are present in a reaction mixture at one time and they rapidly equilibrate between all possible combinations of product. The addition of a receptor causes the tightest-binding products to be sequestered from the reaction mixture, thereby shifting the synthetic equilibrium towards increasing amounts of this compound, according to LeChatelier's principle. A classic example of dynamic combinatorial chemistry is demonstrated by Huc and Lehn [43] who identified imine-derived inhibitors of carbonic anhydrase by mixing a collection of amines and aldehydes. The equilibrium was locked-in by irreversible reduction of the imines to the corresponding amines with $NaBH_3CN$. To detect the active inhibitor, the library components are separated and analyzed by HPLC. It is important to note that detection methods that rely on separation procedures (e.g. HPLC) require a lock-in step to identify lead inhibitors, whereas this reaction is unnecessary in techniques that analyze library composition in real time (e.g. NMR). Although this method is suitable in this context, detection using HPLC reduces the potential library size significantly because of the inherent resolution limit. Furthermore, large libraries increase the likelihood of finding several library members that have similar binding constants and, thus, similar changes in concentration, which leads to several hits. One possible solution to the detection problem currently being evaluated involves LC–MS–MS as a sensitive detection method [44].

In RAS, a library of building blocks with complementary reactive functionalities competitively binds to a receptor in close proximity. Subsequent irreversible cross-coupling of two building blocks results in a tight-binding ligand (Figure 3b). The affinity of the receptor for the input building blocks enables the kinetic acceleration of the coupling reaction by means of an increase in the effective molarity of each reactant, which results in enrichment and selectivity of product formation. For enrichment to occur, the receptor must bind tightly to the two building blocks simultaneously, eliminating cases where two weak-binding species associate to form a strong-binding ligand. Thus, additional refinement of a tight-binding core structure is difficult with RAS if the appendages themselves do not adhere well. Consequently, an appropriate application of RAS is to optimize linkers between two molecules that bind well at adjacent sites. One of the early examples

of RAS identified inhibitors of carbonic anhydrase by accelerating the coupling of alkyl chlorides to α-mercaptotosylamides that contain a sulfonamide group that binds to the zinc atom at the active site [45]. This acceleration gave a two-fold increase in product formation for molecules that had a nine-fold difference in affinity constants. This proof-of-concept experiment demonstrates that by bringing coupling partners close in proximity the receptor biases the chemistry to select for tight-binding compounds. More recently, click chemistry has also been implemented in RAS strategies to discover enzyme inhibitors [46].

Small-molecule phage display

Phage display is a powerful technology that rapidly evaluates large libraries using a specific selection criterion [47]. In the past decade, work with large libraries of antibodies displayed on bacteriophage (>10^9 members) has demonstrated that the probability of isolating high-affinity proteins and/or peptides to target antigens increases with library size [48], provided that a methodology is available for biopanning and facile identification of 'winning' biomolecules. In principle, it should be possible to use similar strategies to isolate small molecules if large libraries of compounds can be synthesized, screened and deconvoluted. Several strategies have been developed to encode synthetic molecules with either DNA or RNA tags so that the whole library can be selected for target-binding in one pool, followed by sequencing of the DNA or RNA tags to quickly identify the structures of the selected compounds [49–53]. Alternatively, the small molecules are displayed on the surface of the phage and the DNA 'barcode' is embedded in the genomic DNA of the phage, which is encapsulated by the phage capsid proteins [54,55]. After the phage-displayed small-molecule library has been selected for target binding, amplification and sequencing of the corresponding phage identifies the selected molecule. Using a phage platform for small-molecule encoding has the advantage that the DNA 'barcode' is shielded by the phage capsid proteins. This eliminates interference by DNA secondary structure with small-molecule–target-protein interactions and also protects the DNA 'barcode' from degradation. As a result, the phage-displayed small-molecule libraries can be selected easily for binding to *in vitro* targets. Moreover, because amplification of the phage is highly efficient, enhancement of the selected phages provides a sensitive method to evaluate a library screen. Generally, a single phage particle displays tens to hundreds of molecules, mostly because of the nonspecific nature of the conjugation reaction. Thus, the high density of small-molecule ligands on a single phage particle might be problematic and lead to avid binding with the target protein.

To circumvent the problems of multiple copies of a molecule on a single phage particle, a strategy has been developed recently [56] that displays one copy of the small molecule of choice per phage particle. In this, a small-molecule site is attached to the peptidyl carrier protein (PCP), a 10 kDa, stably folded domain, that is displayed monovalently on phage. For this study, Sfp phosphopantetheinyl transferase was used to covalently link small molecules to a specific serine residue of PCP, with various coenzyme A (CoA)–small-molecule conjugates as substrates. It was shown that displaying small molecules on phage is highly efficient; typically, a single round of selection gave >2000-fold enrichment of the ligands with nanomolar dissociation constants for the target protein. In addition, to accelerate the selection process, the authors used DNA microarrays to decode the identities of the selected small molecules. In this proof-of-principle experiment, biotin, fluorescein, *N*-methylmesoporphyrin, glutathione and D-galactose were conjugated individually to CoA by either disulfide or thioether formation.

For the first time, this provides an efficient method to monovalently display small molecules on the phage surface through a Sfp-catalyzed, site-specific modification that utilizes phage-displayed PCP. Using this method it should be possible to generate libraries of small molecules that are conjugated monovalently to the phage surface, enabling an easy selection process to identify potent small-molecule ligands.

Concluding remarks

Synthetic and biochemical libraries hold tremendous promise for the future of chemical biology and drug discovery. The design and evaluation of synthetic and biochemical libraries that are being developed currently underpin the successful advancement of modern medicine. The recent Molecular Library Initiative (MLI) from the National Institutes of Health is designed to accommodate high-throughput synthesis and screening, which demonstrates the increasing need for quality synthetic libraries to successfully develop biological probes and, eventually, lead to new drugs. In conjunction with the MLI, academic laboratories across the world have been challenged to help foster the development of new tools and targets that will lead to new insights in chemical biology. In this respect, one application of small molecules in biology is to modulate protein–protein interactions [57]. The ability of small molecules to regulate these complex macromolecular interactions will, ultimately, challenge the design of current libraries and technologies. Gratifyingly, successful advancement of any new target will open the door to exciting new biological targets and further advance modern medicine.

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