Protein-Directed Dynamic Combinatorial Chemistry: An Efficient Strategy in Drug Design

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ABSTRACT: Protein-directed dynamic combinatorial chemistry (P-D DCC) is considered a powerful strategy to identify ligands to pharmacologically relevant protein targets. The protein selects its affinity ligands in situ through a thermodynamic templated effect in which the library composition shifts to the formation of specific library members at the expense of other (nonbinding) species. The increase in concentration of the selected species is known as amplification and leads to the discovery of new hit compounds for protein targets. This Mini-Review contains an updated overview of the protein-directed DCC applications and the fundamental aspects to take into account when designing a P-D DCC experiment such as the most biocompatible reversible reactions and the methodology used to analyze the experiments.

INTRODUCTION

Protein-directed dynamic combinatorial chemistry (P-D DCC) has evolved as a powerful and efficient hit-identification tool to find ligands for proteins of therapeutic interest. Its potential relies on the in situ selection and synthesis of the best ligands avoiding the unnecessary synthesis of the nonbinding ones. The molecular recognition process takes place in a thermodynamic controlled chemical system (dynamic combinatorial library, DCL) that is able to adapt and self-correct the bonds between the different components in the presence of a protein template. If one or more molecules present in the mixture bind to it giving a more stable complex, the equilibrium will be displaced according to Le Châtelier's principle, to amplify the amount of this compound at the expense of other nonbinding constituents (Figure 1).

Contrary to kinetic target-guided synthesis KTGS (e.g., in situ click chemistry), where the protein speeds up an irreversible reaction between a pair of building blocks by stabilizing the ternary complex, P-D DCC enables the thermodynamic selection, avoiding a possible library biased toward the products that form faster. In a P-D DCC system, weak binders will be lost, whereas the stronger ones will persist regardless of how fast they were to form. Both KTGS and P-D DCC allow exploring unexpected protein conformations since they do not hinder the protein’s flexibility. One of the drawbacks of KTGS is the need of near-stoichiometric amounts of protein, whereas in P-D DCC a substoichiometric amount of the protein guarantees the competition for the best binder.

In this Mini-Review, we present the last 10 years of achievements in P-D DCC, focusing on its experimental set up and applications. Early reviews on particular aspects of protein-directed DCC can be found in references 1–3.

PROTEIN-DIRECTED DCC DESIGN

A protein-directed DCC experiment requires a protein template, building blocks to participate in the library, a biocompatible reversible chemistry, and the analytical method(s).

PROTEIN TEMPLATE

Preserving the native protein state is critical to obtain significant results from the P-D DCC experiment. It is important to take into account that pH, temperature, buffer, ionic strength, and ions influence the stability of a protein and the reversible chemistry. An ideal DCL is performed in aqueous buffer. However, in most cases, water solubility is an issue for the building blocks that are not fully water-soluble at optimal pH range. Therefore, the necessity of a cosolvent (e.g., MeOH, DMSO) emerges, and its amount is usually less than 20% according to the protein tolerance. The protein stability under the DCL conditions should be tested before conducting the experiment. Thermal-shift assay (TSA) or fluorescence techniques can be used to determine the possible protein degradation under the DCL conditions. One of the most common methods for TSA is ThermoFluor assay in which a compound with low fluorescence signal in an aqueous solvent but high fluorescence signal in a nonpolar environment is added to the protein solution. A signal increase is detected if the protein chain unfolds and the hydrophobic core is exposed, followed by a decrease in fluorescence as protein begins to aggregate. Moreover, it is possible to follow denaturation...
The emission maximum of tryptophan residues buried in nonpolar regions of a protein fluorescence is 330 nm. As a protein sample unfolds and these residues become exposed to a polar solvent, their emission maximum suffers from a bathochromic shift usually to 350 nm.

In general, the DCL reaches the equilibrium within the first few days, depending on the reversible reaction and conditions used (Table 1). Precipitation or degradation of the protein will lead to unreliable results. Precipitation of the protein will remove its template effect from the solution, whereas its degradation leads to binders of a “different” protein.

In most P-D DCLs, room temperature is chosen to set up the DCL as long as the protein stability is assured under these conditions. In general, at room temperature, the reversible exchange is more efficient reducing the equilibration time of the DCL, although examples have been reported where low temperatures were applied to preserve the protein stability.

A substoichiometric amount of protein is recommended to ensure the competition between the DCL species. Besides, the limited availability and in some cases solubility of some protein targets make this requirement an advantage over other KTGS strategies.

DCL formation can be conducted in the presence (adaptive DCC) or absence (pre-equilibrated DCC) of the protein when the protein is unstable under the DCL conditions. Most of the examples compiled in this Review correspond to “adaptive DCLs”, as it provides the system with true adaptability.

![Figure 1. Schematic representation of protein-directed DCC.](image)

**Table 1. Protein-Directed DCC Reported**

| reversible exchange | protein target | analytical method | DCL BBs | DCL conditions | references |
|---------------------|----------------|-------------------|---------|----------------|------------|
| boronate ester      | α-CT           | 1H-B-NMR          | 6       | pH 5.8         | Leung et al.6 |
|                     | PHD2           | DC-MS             | 8 x 11  | pH 7.5, r.t.   | Demetriades et al.7 |
| imines              | HEWL           | SEC-MS            | 12      | 3 h, r.t.      | Fang et al.5  |
| hydrazone           | MPO            | activity assay    | 1 x 30  | pH 7, 25 °C    | Soubhie et al.10 |
|                     | mGAT1          | MS                | 9 x 28  | pH 7.1, 4 h, r.t. | Hauke et al.11 |
| acyl hydrazone      | GST            | LC-MS             | 11      | pH 6.2, 6 h, r.t. | Bhat et al.12 |
|                     | endothiapepsin | LC-MS             | 10      | pH 6, 24 h, r.t. | Mondal et al.19 |
|                     | FimH           | HPLC              | 2 x 5   | pH 7, 3 d      | Frei et al.13 |
|                     | UGM            | HPLC              | 7 x 14  | pH 6, 14 h, 25 °C | Fu et al.14 |
|                     | FabH           | 1H-F-NMR          | 6       | pH 6.2, 12 h, r.t. | Ekstrom et al.15 |
|                     | ALKBH3         | DSS, HPLC         | 2 x 10  | pH 6, 5 h, r.t. | Das et al.16 |
|                     | TcBDF3         | LC-MS             | 10      | pH 6.5, 10 h, r.t. | Garcia et al.17 |
|                     | NCS1           | LC-MS             | 6       | pH 7.8, 5 h, 4 °C | Canal-Martin et al.5 |
| bisacyl hydrazone   | GST            | HPLC              | 7       | pH 6.4, 25 °C  | Clipson et al.18 |
|                     | endotheiapssin | LC-MS             | 1 x 4   | pH 4.6, 20 h, r.t. | Mondal et al.19 |
| oximes              | GAT1           | MS                | 22 x 5  | pH 7.1, 4 h, 37 °C | Kern et al.22 |
| thiol-disulfide     | TGR            | LC-MS             | 6       | pH 8.8, 24 h, r.t. | Saiz et al.23 |
| hemithio-acetal     | β-galactosidase| STD-NMR           | 7       | pH 7.5, r.t.   | Caraballo et al.24 |

“The table has been adapted from Hartman et al.3 and complemented. Copyright 2019 John Wiley & Sons.

Building blocks (BBs) must contain the functional groups required for the reversible exchange of choice, they have to be geometrically and functionally diverse as well as fully soluble under the DCL conditions. If they precipitate, the DCL will enter in a kinetic trap biasing the experiment. The concentration of the building blocks results from the compromise between solubility and reversible exchange efficiency. Relatively high concentration of BBs favors the exchange, whereas an extremely low BBs concentration would slow the exchange leading to an inefficient P-D DCC system. Usually the DCL may begin with a building block known as “war-head”, which is a building block designed to direct the other building blocks to a specific pocket of the protein (e.g., active site). This war-head is therefore equipped as well with a functional group for reversible reaction with other components of the DCL. This strategy helps targeting additional protein pockets close to the “war-head” binding pocket. The “war-head” building block may be similar to an existing binder or designed by molecular modeling based on structural analysis of the protein.

**COMPOSITION OF THE DYNAMIC COMBINATORIAL LIBRARY**

Examples
In general, building blocks should be isoenergetic to avoid reaction mixtures that are strongly predisposed toward the formation of certain products, but there are examples where a predisposed DCL toward certain products was still effective.4,14

### REVERSIBLE COVALENT CHEMISTRIES IN PROTEIN-DIRECTED DCC

The essential feature of dynamic combinatorial chemistry is the reversible reaction that facilitates the exchange of the building blocks between the different library members (Table 2).

| Reversible Exchange | Reaction Scheme | General Conditions |
|---------------------|-----------------|--------------------|
| Alkene cross metathesis | \( \text{R}_1^1\text{C}==\text{R}_2^1\) \(\xrightarrow{\text{Hoveyda-Grubbs catalyst}}\) \(\text{R}_1^1\text{C}==\text{R}_2^1\) | Requires low biocompatibility. |
| Boronate ester exchange | \( \text{R}_1^1\text{OH} + \text{R}_2^3\text{BH}_2 \xrightarrow{\text{pH}} \text{R}_1^1\text{BO}^{-}\text{R}_2^3\) | Requires pH-pKa boronic acids. |
| Imine exchange | \( \text{R}_1^1\text{NH} + \text{R}_2^3\text{NH} \xrightarrow{\text{pH}} \text{R}_1^1\text{N}==\text{R}_2^3\) | pH range (4-7) and post reduction with NaBH4CN prior analysis. |
| Hydrazone exchange | \( \text{R}_1^1\text{N}==\text{R}_2^3\) | pH range (4-8). A catalyst is needed to work at moderately basic pH. |
| Acylyldrazine exchange | \( \text{R}_1^1\text{NH} + \text{R}_2^3\text{NH} \xrightarrow{\text{pH}} \text{R}_1^1\text{N}==\text{R}_2^3\) | pH range (4-8). A catalyst is needed to work at moderately basic pH. Acyl group stabilizes the exchange. |
| Oxime exchange | \( \text{R}_1^1\text{NH} + \text{R}_2^3\text{OH} \xrightarrow{\text{pH}} \text{R}_1^1\text{N}==\text{R}_2^3\) | pH range (4-8). A catalyst is needed to work at moderately basic pH. |
| Disulfide exchange | \( \text{R}_1^1\text{S}==\text{R}_2^3\) | pH 7. Recommended to use GSH/GSSG buffer as catalyst. |
| Thio-Michael exchange | \( \text{R}_1^1\text{S}==\text{R}_2^3\) | pH 7. Mild conditions in aqueous media. |
| Hemithioacetal exchange | \( \text{R}_1^1\text{S}==\text{R}_2^3\) | pH 7. |
| Thioxeter exchange | \( \text{R}_1^1\text{S}==\text{R}_2^3\) | pH 7. Mild conditions in aqueous media. |

### CARBON–NITROGEN BOND FORMATION

The common method for preparing C═N related compounds is the acid-catalyzed reaction of aldehydes or ketones with amine derivatives. Schiff discovered this reaction, and since then, imines are usually referred to as Schiff’s bases. Imine exchange (\(\text{R}_1^1\text{C}==\text{N}==\text{R}_2^3\)) is a reversible reaction that needs the water removed to force the C═N formation.8 If the reaction between the aldehyde/ketone is carried out with an amine that has one lone pair of electrons adjacent to the attacking nitrogen such as hydrazine, hydroxylamine, or semicarbazide, the equilibrium shifts to the adduct formation. Thus, alternative libraries based on C═N exchange have been developed such as hydrazone/acylhydrazone and oximes.

**Exchange of Imines.** Imine formation usually occurs from moderately acidic pH (5.0) to basic pH (8.5) at room temperature. Aromatic aldehydes are preferred since they give the most stable adducts, and a reducing agent such as NaBH₄CN is added at the end of the DCL to “freeze” the equilibrium and to produce stable DCL members to high performance liquid chromatography (HPLC) analysis.

An imine exchange system in the presence of hen egg-white lysozyme (HEWL) was presented by Fang et al. (Figure 3).9

The group developed a new methodology by size-exclusion-chromatography coupled to mass spectrometry (SEC-MS) for the DCL analysis. N-Acetylglucosamine (NAG), a well-reported inhibitor (\(K_I = 20–60 \text{mM}\)) of HEWL was added as a control to the library of several aldehydes and amines (Figure 3b), as well as the reducing agent (NaBH₄CN) and β-N-glucose. The pre-equilibrated DCL was incubated with the protein before passing through the SEC column to retain the nonbinders. After denaturing the protein in order to release the best binders, three compounds were detected in MS. The Michaelis–Menten constant (\(K_m\)) showed similar values to the control inhibitor (Figure 3c).

**Exchange of Hydrazones.** Hydrazones (\(\text{R}_1^1\text{C}==\text{N}==\text{N}==\text{R}_2^3\)) are generated by the reaction between a hydrazine and an aldehyde or ketone. Hydrazones are thermodynamically

The reaction needs to (i) be reversible on a reasonable time scale; (ii) be compatible with the experimental conditions of the selection process (e.g., solvent, pH), including the functional groups on the building blocks and template; (iii) be mild, not to interfere with the delicate noncovalent interactions involved in molecular recognition; (iv) ensure the solubility of all the library members at equilibrium because insoluble material could act as a kinetic trap; (v) be easily turned off to kinetically “freeze” the DCL, enabling the isolation of their members.

In some reversible reactions a small biocompatible catalyst is added to promote the exchange (e.g., hydrazine formation). The P-D DCC should be “frozen” prior to analysis to ensure a stable composition. For instance, imine exchange can be “turned off” by the irreversible functionalization of the imines to amines using a reducing agent such as NaBH₄CN, and in
disulfide exchange the chemistry is frozen by changing to acidic pH.

### BORON–OXYGEN BOND FORMATION

Boronic ester exchange: Boronate ester (\(\text{R}_1^1\text{C}==\text{O}==(\text{O}==\text{R}_2^3)\)) results from the attack of a diol to a boronic acid. This exchange requires from slightly acidic to slightly basic pH depending on the pKₐ of the building blocks of the library.1 Therefore, it is suitable for a wide range of proteins.

Leung et al. originally reported a serine protease alpha-chymotrypsin (α-CT)-DCL using this reversible chemistry.6 Then, Demetriades et al. used the prolyl hydroxylase domain isofrom 2 (PHD2), a Fe²⁺ and 2-oxoglutarate (2OG)-dependent oxygenase that regulates the human hypoxic response.7 The experiment was performed using several diols at pH 7.5 and two boronic acid fragments as support ligands (Figure 2). The boronic acid derivatives were designed as “war-head” building blocks to participate in Fe²⁺-II chelation in the active site and through the incorporation of a boronic acid moiety, participate in boronate ester exchange. The DCLs were analyzed by mass spectrometry (MS) and the binding constants measured by nuclear magnetic resonance (NMR) (Figure 2c). An analogue of these compounds (compound 4) showed a \(K_D = 0.8 \mu\text{M}\) and \(IC_{50} = 1.3 \text{nM}\) values (Figure 2d).

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ACS Omega 2020, 5, 26307−26315

https://dx.doi.org/10.1021/acsomega.0c03800

26309
more stable than imines and kinetically inert under neutral conditions. The hydrolysis and exchange become slower because of the mesomeric effect. This reaction is chemoselective, compatible with a broad range of solvents and templates, and orthogonal to most of the functional groups.

Soubhye et al. applied a DCC approach to develop inhibitors of myeloperoxidase (MPO), an enzyme involved in oxidative stress and inflammation. Two DCLs that included hydralazine and isoniazid (irreversible inhibitors) were set up. Four compounds showed the best inhibitory capacity with a lowest IC50 value of 79 nM.

Recently, the use of a MS-based screening applied to the discovery GABA uptake inhibitors was reported by Hauke et al. The gamma-aminobutyric acid transporter 1 (GAT1) removes GABA, which is an important inhibitory neurotransmitter in the central nervous system. This approach enabled the screening of a vast diversity of lipophilic residues in a nicotinic acid derivative hydrazine library. A hit was...
protein-directed DCC is the system set up by Garcia et al. to detect protein from others benchmark GAT1 inhibitors such as tiagabine. Other example of multiprotein directed DCC combining the zwitterionic acylhydrazone moiety structurally resembles a peptide bond stable to be used in dynamic libraries. Moreover, the withdrawing group (EWG), the hydrazone tends to be too favoring the exchange. In the absence of the acyl electron withdrawing group (EWG), the hydrazone tends to be too stable to be used in dynamic libraries. However, the acylhydrazone moiety structurally resembles a peptide bond offering donor and acceptor hydrogen bonds. However, it is still relatively slow taking days to equilibrate at physiological pH (7−8). Bhat et al. pioneered the use of high concentrations of aniline as a nucleophilic catalyst in a protein-directed DCL to speed up this exchange at physiological pH from days to hours. The aniline catalyzed the acylhydrazone exchange through the formation of a Schiff’s base intermediate, which is more reactive than the parent aldehyde. Glutathione-S-transferase (GST) was the protein to direct the DCL catalyzed by aniline, which reached equilibrium in 6 h instead of days.

The bacterial adhesin FimH, a protein involved in urinary tract infections, was the target of the P-D DCC work of Frei et al. They chose small DCLs of aldehydes and acylhydrazides in the presence of aniline as catalyst. The compounds identified showed affinity in the micromolar range. $K_D$ values obtained by surface plasmon resonance (SPR) were in agreement with the amplification factors found in the DCL.

Fu et al. combined DCC with the in situ fluorescence polarization (FP) screening to identify inhibitors of UDP galactopyranose mutase (UGM), an essential enzyme in the biosynthesis of mycobacterial cell wall. The inhibitor found displayed activities against an attenuated strain of $M. tuberculosis$ and provides a promising novel class of inhibitors.

A slightly different approach using $19\text{F}$-NMR to monitor the DCL targeting $\beta$-ketoacyl-ACP synthase III (FabH) was presented by Ekström et al. The DCL contained fluoroticromatic aldehydes and several acylhydrazides. Unfortunately, the catalyst aniline was interfering with the DCL binding FabH so it was replaced by 4-amino-L-phenylalanine (4-APA). One of the compounds found decreased the enzyme’s activity to 50% at 3 mM concentration. Das et al. designed a multiprotein directed DCC combining the zwitterionic “thermal-tag” and the differential scanning fluorimetry (DSF) to detect protein–ligands interactions. They reported the first subfamily selective of ALKBH3 inhibitors. Other example of protein-directed DCC is the system set up by Garcia et al. They tested Trypanosoma cruzi bromodomain-containing protein TbBDF3, involved in Chagas disease, in a small library of acylhydrazones using aniline as catalyst. The compound found showed interesting antiparasitic activity and selectivity index.

Since the discovery of aniline, several catalysts have been reported. Among them, $p$-anisidine is able to perform the exchange at low temperatures. Recently, we reported the first stabilizer of a protein–protein interaction between the neuronal calcium sensor I (NCS-1) and the guanine exchange factor Ric8a (Figure 4).

The equilibration time was reached using of $p$-anisidine in less than 5 h under physiological conditions (pH 7.4) and low temperature (4 °C). The library was formed by one aldehyde and five acylhydrazides in the presence and absence of the protein (Figures 4a,b). Compound 10b (Figure 4c) exhibited affinity to the protein in the micromolar range. The mode of action of the compound was studied using co-immunoprecipitation (Co-IP), NMR, and X-ray crystallography. The in vivo results in an animal model of Alzheimer’s disease showed that 10b was able to recover the number of synapses to normal levels.

Few examples have been reported with bis-acylhydrazones. Clipson et al. published bivalent acylhydrazones in the presence of GST enzyme, and Mondal et al. reported a DCC plus fragment-linking approach using the aspartic protease endothiapepsin to optimize previously reported inhibitors. The most potent endothiapepsin inhibitor showed an IC$_{50}$ value of 54 nM, around a 240-fold improvement in contrast to the parent hits.

Exchange of Oximes. Oximes (R$_1$−C=O−R$_2$) are generated by the acid catalyzed reaction of an aldehyde and a hydroxylamine derivative. At physiological conditions, the exchange rate is negligible; thus, aniline derivatives can be used to accelerate the exchange. Oximes from aromatic aldehydes are preferred because of their stability in aqueous solution. Kern et al. described the use of MS binding assays to optimize inhibitors of mGAT1. In this work, the DCL conditions could not be tolerated by mGAT1. Therefore, the pre-equilibrated libraries were set up at pH 6.0 in the absence of mGAT1 and then diluted with phosphate buffer pH 7.1 for the biological testing. Three of the inhibitors found presented nanomolar binding affinities and significant subtype selectivity to mGAT1.
SULFUR–SULFUR BOND FORMATION

Thiol–Disulfide or Disulfide Exchange. Disulfide exchange (R₁−S−S−R₂) is one of the most biocompatible DCC. The reaction occurs by the nucleophilic displacement of a thiolate anion from a disulfide through the nucleophilic attack by another thiolate anion. Neutral-basic conditions (pH 7–9) favors the exchange and the reaction stops at acidic pH (pH < 5).23a The addition of small amount of DMSO as cosolvent has been reported to speed the DCL equilibration time.23b Under dilute DCL conditions, the exchange requires long equilibration times what could be an issue when the template is a protein.

A DCL directed by the E. granulosus thioredoxin glutathione reductase (TGR), a NADPH-dependent selenoenzyme which reduces oxidized thioredoxin (Trx) and glutathione (GSSG) was explored by Saiz et al. (Figure 5).24

They set up a pre-equilibrated DCL using 5-thio-2-nitrobenzoic acid (TNB, 11a) as fragment anchor, GSH (11b) and thiols 11c–f. The redox buffer GSH/GSSG (4:1) was used to favor the thiol–disulfide exchange. The interconversion was stopped by addition of trichloroacetic acid (50% aq.). Figure 5c shows the resulting hit compound 12af and the synthesized analogue 12aj with IC₅₀ values in the micromolar range.

CARBON–SULFUR BOND FORMATION

Hemithioacetal Formation/Exchange. Hemithioacetal (R₁−C(OH)−S−R₂) is the reaction of a thiol to a given aldehyde or ketone. Caraballo et al. published one of the first examples of this chemistry in protein templated DCLs (Figure 6).

They prepared a DCL to target β-galactosidase, an enzyme that catalyzes the hydrolysis of O-glycosidic linkages of β-galactosides. Figure 6b shows the library of two aldehydes based on a known inhibitor, an enzyme’s substrate and five thiols to react under thermodynamic control. The binders were
identified by proton saturation transfer difference NMR spectroscopy (1H-STD-NMR). At neutral pH, the HTA formation and dissociation are much faster than the NMR relaxation time leading to a virtual DCL formation. Therefore, the HTA products bound to the target were detected only in the presence of enzyme. To confirm the inhibitory capacity of the compounds identified (Figure 6c), an inhibition assay was performed using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The formation of ONP (o-nitrophenol) in the presence of the inhibitor was followed by NMR. The combined results from the inhibition studies and the 1H-STD NMR experiments confirmed the formation of virtual HTA systems of β-D-galactosidase inhibitors. The HTA identified inhibitors decreased the rates of substrate hydrolysis by 12- and 4-fold, respectively.

### TECHNIQUES APPLIED TO P-D DCC ANALYSIS

Comparative and noncomparative analysis can be applied to P-D DCLs (Figure 7). A comparative analysis needs a blank reaction without the target, to run concurrent with a templated one such as HPLC-MS\(^5\),\(^{12,19}\) and DSF.\(^6\) In the non-comparative approach, the hits can be analyzed in complex with the target, and no amplification rate is calculated like in ligand-observed nuclear magnetic resonance techniques,\(^6\),\(^{25}\) SEC-MS,\(^9\) and non-denaturing MS.\(^{26}\) In this approach, the absence of a blank DCL makes it difficult to check that the thermodynamic equilibrium has been reached.

HPLC is one of the most applied comparative method to analyze P-D DCLs. The dissociation of the target-ligand complexes prior the analysis is essential since the detection of ligands bound to the protein may not be consistent. This dissociation could come after denaturation of the protein target (e.g., heat, addition of a solvent or (ultrafast) centrifugation). The need of a chromatogram with separate signals to get the right species concentration could be considered time-consuming and therefore a disadvantage of this method. Small differences between the blank and templated chromatograms are detected by using the relative peak area (RPA) of each compound, that is, the sum of all peak areas was set to 100% and each peak was assigned its percentage of the blank and templated DCL (Figure 8). The amplification factor can be obtained from dividing RPA\(_{\text{templated}}/\text{RPA}_{\text{blank}}\).\(^{13}\) By comparing between the control and templated chromatogram it can be checked that the system has reached the equilibrium (blank DCL) and therefore measured the amplification rate of the compounds in the presence of the target.

NMR has been applied in numerous publications.\(^6\),\(^{15,25}\) From \(^{11}\)B-NMR\(^6\) and \(^{19}\)F-NMR spectroscopy\(^{15}\) to ligand-observed NMR techniques such as 1H-STD NMR spectroscopy,\(^25\) which is able to measure weak bindings and requires small amounts of nonisotopically-labeled protein (ca. 100 nmol) or water ligand observed through gradient NMR spectroscopy (waterLOGSY).\(^6\) Unambiguous signal assignment in medium-size DCLs and the distinction between specific and nonspecific binding could be somehow challenging.

MS coupled to HPLC has been widely used in DCC (Table 1). In some cases, ligand target adducts were previously isolated using SEC, and after denaturation of the protein, the released ligands were identified by MS.\(^9\) Furthermore, this technique has been successfully applied in non-denaturing MS experiments\(^26\) where native ligand–target complexes can be detected.
CONCLUSIONS AND OUTLOOK

Protein directed dynamic combinatorial chemistry has materialized as an efficient hit-identification tool. This chemical strategy combines the synthesis of ligands with the screening of potential binders against the protein targets in situ, speeding up the drug design and discovery development.

Several proteins have been targeted using different reversible covalent chemistries, and strategies such as site-directed P-D DCC have been successfully applied to explore additional protein binding pockets. However, the present P-D DCC faces some limitations such as the use of large and complex libraries, where the current analytical methods are unable to ensure the unambiguous identification of the best binders, and the long equilibration times that could risk the stability of the protein target.

A promising combinatorial approach that could address the size limitation issues in P-D DCC is DNA-encoded dynamic libraries (DEL). In DEL, each compound is linked with a unique DNA tag, all having a common sequence that can form dynamically exchanging duplexes through noncovalent interactions. DELs can contain a huge amount of different compounds and the library selection can be feasibly decoded using DNA sequencing. Nevertheless, it still needs to improve the current limited chemical diversity that makes it unsuitable for de novo ligand discovery. In addition, resin-bound DCC (RBDCC) successfully applied in DNA/RNA-templated libraries could be considered as well as an alternative to simplify the analytical challenges of complex libraries in P-D DCC.

Furthermore, other druggable proteins like membrane-bound proteins or the protein–protein interactions could be explored broadening the scope of the P-D DCC approach.

Efforts should be made toward the development of high throughput screening P-D DCC methodologies leading to the combination of the powerful DCC in situ ligand synthesis with an efficient biological screening platform that enables the hit identification of unexplored druggable targets.

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Notes

The authors declare no competing financial interest.

Acknowledgments

This work was supported by the grant PID2019-108587RB-I00 from the Spanish Ministry of Science and Innovation (MCI).

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Biographies

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Ruth Pérez-Fernández. Ruth received her Ph.D. in chemistry from Universidad Autónoma de Madrid working on supramolecular chemistry under the supervision of Prof. J. de Mendoza. She studied as a postdoctoral researcher at the University of Cambridge in 2005–2007 with Prof. J.K.M. Sanders, investigating molecular recognition through dynamic combinatorial chemistry and biphasic reversible exchange/transport in solution. In 2008, she joined Consejo Superior de Investigaciones Científicas (CSIC) and in 2009 she was appointed as a tenured scientist. Since 2015 she is working at Centro de Investigaciones Biológicas ”Margarita Salas” (CIB-CSIC) and her research explores dynamic chemical processes in biological environments gathering insights on diseases etiology.
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