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A novel protocol to accelerate dynamic combinatorial chemistry via isolation of ligand–target adducts from dynamic combinatorial libraries: A case study identifying competitive inhibitors of lysozyme

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ABSTRACT

A novel protocol based on size-exclusion chromatography (SEC) and MS was established to accelerate dynamic combinatorial chemistry (DCC) in this study. By isolating ligand–target adducts from the dynamic combinatorial library (DCL), ligands could be identified directly by MS after denaturation. Three new inhibitors for lysozyme were discovered by this SEC–MS protocol in a case study. K_m Data for these new inhibitors was also determined.

Although DCC has been used successfully to identify guest or host motifs for varied targets from small ions to large macromolecules, and even to drive self-assembly of supramolecular structures[2] or direct synthesis of tailored nanomaterials,[3] the application of the approach in lead discovery against pharmaceutically relevant protein targets such as enzymes and cellular receptors remains a major challenge. Chiefly, work to date has been hampered by the limited number of aqueous-compatible reactions amenable to DCL synthesis, and more importantly by the difficulty in characterizing binding 'hits', especially where larger library sizes are involved.[1]

Most likely due to their widespread availability, LC–MS or HPLC[4–7] are by far the most prevalent techniques used for deconvolution of dynamic libraries, possessing adequate sensitivity to detect subtle increases in concentration (amplification) of compounds of interest in the presence of a binding target. Although LC–MS identification of ephedrine binders from a library of several thousand members was reported recently,[8] large library sizes remain the exception rather than the rule with such an approach, since a mixture of many components cannot normally be adequately resolved by LC methods. This drawback limits library size to tens of compounds at most, rather than routinely enabling the screening of hundreds or thousands of prospective ligands in situ, as would be preferable. In as much as this is the case, the full potential of DCC in lead discovery against protein targets remains largely unfulfilled in practice.

In response to this technical challenge, a variety of alternative approaches to DCL hit characterization have been developed. Resin-bound dynamic combinatorial chemistry (RBDCC),[9] saturation transfer difference (STD) NMR assessment of direct binding,[10] and 'Tethering'[11,12]—also known as site-directed ligand discovery,[13] wherein a bifunctional linker is anchored to a suitable site on the protein of interest—all address the problems faced in screening DCLs from different points of view, and each has proven successful in delivering novel ligands for biological macromolecules. These techniques all involve considerable resource and specialized equipment, however, and as such we considered it would be of great benefit to develop complementary techniques more amenable to routine application in academic laboratories.

Direct isolation of ligand–target adducts from DCLs has largely been overlooked to date, perhaps due to assumption of immediate or fast dissociation of any such adducts on removal from the reaction mixture. We report here encouraging progress in developing the use of size-exclusion chromatography (SEC), coupled with MS analysis, as a rapid and generally applicable hit characterization method for DCLs. For the present model study, hen egg-white lysozyme (HEWL) was chosen as the protein target, since it is a readily available and well-studied enzyme. Lysozyme is a widely occurring glycosidase, and an important contributor to immune defense, which attacks and degrades bacterial cell walls (peptidoglycans)

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through hydrolysis of 1,4-β linkages to *N*-acetyl-D-glucosamine (NAG) in these polysaccharides.

To confirm the SEC method, NAG, which was a feedback inhibitor of HEWL with weak affinity (K_d) estimated in the range 20–60 mM,[14] was incubated with HEWL. D-Glucose was also added into the solution during incubation as a control small molecule. After SEC separation and denaturation (to release binder from HEWL), NAG was observed in MS. Whilst, there was no ligand detected when the solution without HEWL was passed through SEC in the control experiment (Fig. 1). This proved that SEC was an efficient tool to isolate protein and protein–ligand adduct from unbound small molecules.

According to the principle of fragment based drug discovery, D-glucosamine A1, which could be viewed a fragment of NAG, was employed to increase the possibility of generating new binders of HEWL. In DCL synthesis, a set of potential binders was designed based on analogous derivatives formed between amines A1–A4 (Scheme 1) and aldehydes B1–B10. Imine formation was selected since it is by far the most well attested reversible reaction employed in DCL synthesis.[15] Generally, reduction of the C=N bound (normally with $NaCNBH_3$) was performed to stabilize the structure in all most all the cases involved imine formation.[16] Same protocols were employed in this study.

After incubation of the DCL with its protein target, the library mixture was passed through a suitable SEC column to retain all the unbound small molecules in the library.[17] The eluent was then denatured by addition of acetonitrile to release potential binders prior to analysis. MS results suggested that three members of the DCL were discovered as binders of HEWL (Fig. 2). Meanwhile, a controlled library was synthesized with the same building blocks and protocol, but without HEWL as target, to make a comparison. After SEC, there was no compound detected in MS, which again, proved that the SEC column could retain all the unbound small library members and the three structures found in the DCL were binders of HEWL.

Since reduction of imine to amine can change the binding property of the structures dramatically, a control library without addition of reductive agent was also synthesized (Fig. 3). After SEC separation and denaturation, there was neither corresponding imine ligand nor corresponding aldehyde and amine (in case of imine hydrolysis) detected in MS. To exclude the possibility of detection limitation of MS, $NaCNBH_3$ was added into the above solution to reduce potential imine structures (if any structure was bound to HEWL and passed through SEC) prior to MS analysis. However, there was no ligand detected again. All these results suggested that the amine products were the ligands of HEWL, rather than imine intermediates. Meanwhile, the three amine ligands identified for HEWL showed a good degree of structural similarity (Scheme 1) suggesting a common binding mode, with the analogue containing the smallest sidechain **A1B2** seemingly present in significantly lower levels than related binders **A1B4** and **A1B6**.

To further confirm binding and quantify affinity of the protein target, each compound was resynthesized individually following a known procedure.[18] The inhibition mode and relative potencies of the new ligands were then investigated via an established protocol, measuring their effect on the lysis rate of Micrococcus lysodeikticus as described.[19]

A Lineweaver–Burk plot of the results (Fig. 4) shows approximately equal *y*-intercepts in each case, which strongly suggesting the newly identified ligands act as competitive inhibitors of HEWL. Values of the Michaelis constant, K_m (substrate concentration required for half-maximal velocity), calculated from the assay data indicate binding affinities at the enzyme active site follow the order A1B6 > A1B4 ≈ A1B2 (Table 1, entries 2–4). In contrast, the known, albeit weakly active, inhibitor NAG showed no discernible effect at 30 mM (entry 5). Observed IC_{50} values[20] were in the range of 2–3 mM for the new ligands.

Thus, a new dynamic combinatorial approach based on SEC–MS technique was established. In this case study, three novel binders to HEWL were successfully identified by this new protocol.[21] With this new procedure, ligands whichshowed significant binding affinity to the protein target of interest were isolated from the library mixture, greatly simplifying the detection of initial hits, as detailed analysis of the whole library was not required. The SEC–MS protocol thus offers the capacity to rapidly screen relatively large numbers of compounds, by simplify the analysis target to only library members show binding affinity. Its benefits can be made clear by comparison with a more typical DCL study targeting the same protein, which employed HPLC analysis to search for amplification of potential ligands.

Previously, a similar DCL against HEWL, based on reductive amination from 4-amino-4-deoxy-D-glucose analogues has been reported.[7] Even with a relatively small library size of 12 members (2 amines × 6 aldehydes), sub-libraries derived from each starting amine were required to fully deconvolute the HPLC chromatograms obtained both with and without the protein target, due to partial overlap of product peaks. The problems faced in this earlier work clearly illustrate the difficulties in DCL resolution often encountered using HPLC analysis, which are completely circumvented by our SEC–MS approach. Furthermore, the earlier study necessitated incorporation of a suitable chromophore into the amine starting materials to permit UV detection; another limitation not encountered in the present work.

However, there are also several limitations of this SEC–MS procedure. The major one is the capability of the SEC column (normally about 30 mM), which limits the concentration of total chemicals in the DCL. In this case, each single reaction in the library synthesis is limited at μM or even nM scale, which leads that the synthesis requires long time to get equilibrium. The other limitation is that the molecular weight cut of SEC column is always at KDa range, which means that this new protocol only works to DCC studies against biological macromolecules.

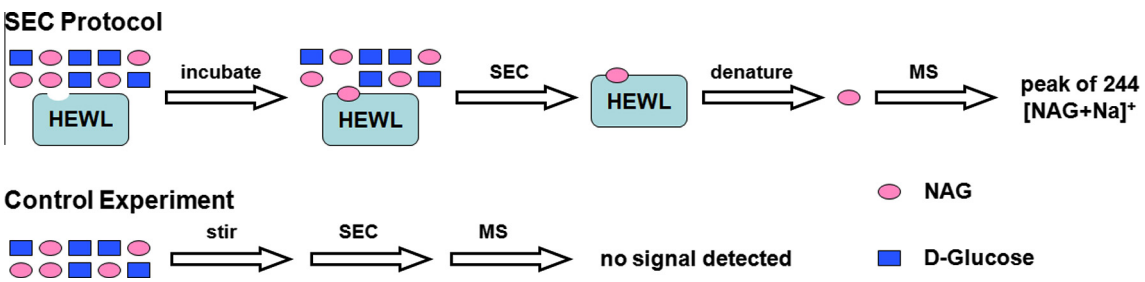

Figure 1. General protocol of SEC method.