A photoimmobilisation strategy that maximises exploration of chemical space in small molecule affinity selection and target discovery†

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We show that the use of multiple photochemistries is necessary to ensure diverse immobilisation of small molecules for binding of polypeptides using phage display and antibody libraries.

The search for small molecule–protein partnerships is significant and challenging, cutting across many areas of chemical biology.[1] An accepted approach for facilitating such discovery is the immobilisation of a bioactive molecule such as a natural product or drug onto an appropriate surface (*e.g.* bead,[2] 96-well plate, glass slide microarray[3,4]) which then can be used to probe interactions with a library of proteins.[5,6] Immobilisation of bioactive molecules requires their presentation at an interface in a form that does not compromise biological activity; this is frequently labour intensive and requires prior knowledge of detailed structure–activity relationships. Schreiber has noted[7] that it is necessary to develop methods where small molecules can be immobilised on surfaces in multiple orientations[5] which enhance their potential bioactivity in screening protocols. His proposed solution utilises the reaction between functional groups present in the ligand with isocyanate decorated surfaces giving a mixture of products, but one that may be biased to a particular mode of chemical reactivity. The result when used in an affinity selection is that only a subset of available chemical space around the ligand will be explored. An alternative approach is the use of photoimmobilisation[8] (using for example carbenes, nitrenes *etc.*) allowing a more stochastic exploration of, for example regioisomers, although such an approach has been reported to generate too many false positives in subsequent screens.[7] We note that it is possible even in a supposedly 'non-selective' reaction applied to small molecules for the photoactive group to react preferentially with the ligand in only a few positions. Thus it is unlikely that any single chemistry will universally capture all small molecules in a form that is active in the chosen bioassay. In this communication we report the use of a parallel photoimmobilisation strategy, using five different reactive intermediates in a 96-well plate format, to maximise the bioactivity of immobilised small molecules when screening libraries of expressed proteins displayed on bacteriophage (Fig. 1).[9]

In a ligand/receptor screen at an interface it is important to: (i) minimise non-specific binding between the immobilisation surface and non-target proteins; (ii) ensure that the ligand is displayed in a bioactive orientation, and (iii) use a rapid method for identification of the target proteins. Our approach addresses all three of these prerequisites. We have recently evaluated chemistries that confer resistance to protein adsorption on silica, 'Lantern' surfaces[10] and TentaGel™ allowing us to select from a variety of surfaces with confidence.[11] To screen against the immobilised ligand we chose to use a bacteriophage complementary DNA (cDNA) library encoding proteins displayed on the bacteriophage head, selecting the tightest binding proteins by elution with free ligand.[6] The key advantages of this technique over conventional affinity chromatography are: the rapid screening of highly complex libraries; effective signal amplification and the physical link between gene and encoded protein, allowing rapid identification of selected protein targets by PCR and sequencing of the bacteriophage cDNA.

In order to explore the effect of photoimmobilisation upon adduct regiochemistry, we screened five different photoactive groups[12] (or 'Tags') **1–5** (Fig. 2; see Supporting Information for preparation) which would access three different reactive species namely carbene, nitrene and di-radical intermediates. For the precursors **2–5** we also changed the electronic nature of the *p*-aromatic substituent linking the photoactive site of the molecule with the surface. In this study we derivatised amine functionalised Corning Stripwell™ (96-well) plates, initially giving the amine **6**, acid **7** and chloride **8** groups (Fig. 2). Coupling both **6** and **7** using EDC and the appropriate organic partner then furnished the

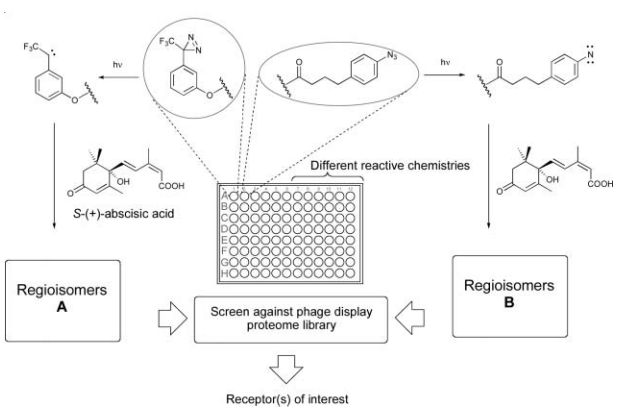

Fig. 1 Illustration of the Magic Tag® display and screening process; different sets of regioisomers (A, B…) result from capture of *S*-(+)-abscisic acid with each different chemistry under particular reaction conditions.

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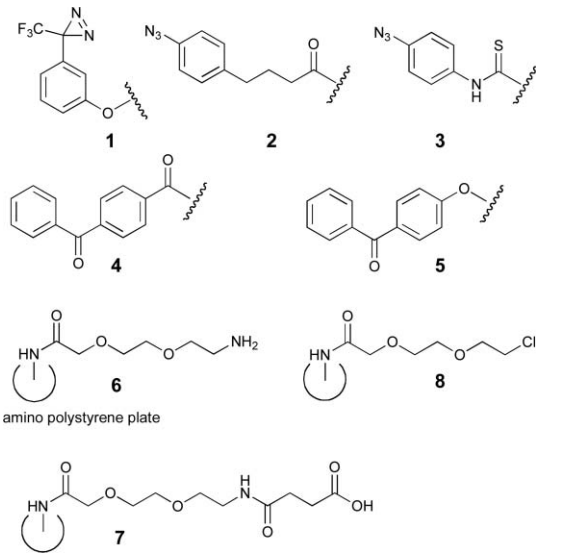

Fig. 2 Photoactive precursors **1–5** and linkers to 96-well plate **6–8**.

supported Tags **1–4** (each in a different Stripwell™). In order to access supported Tag **5** we reacted chloride **8** with 4-hydroxybenzophenone using excess sodium ethoxide.

We tested our parallel immobilisation strategy using the endogenous plant hormone abscisic acid (ABA),[13] Fig. 1. Thus a solution of *S*-(+)-ABA (1 mg ml^{-1} solution in phosphate buffered saline, PBS) was added to each photoactive Tag and irradiated at 254 nm for 5 minutes followed by washing with PBS. In order to assess the bioactivity profile of the immobilised *S*-(+)-ABA for each Tag type we incubated the 96-well plate-bound *S*-(+)-ABA with either an antibody raised to a C-1 carboxyl conjugated ABA hapten (Olchemim 0052707, raised in rabbit) or a C-4′ enone conjugated equivalent (MAC252, raised in rat) visualised using an anti-rat or anti-rabbit FITC conjugate. We also compared the results of each photoaffinity Tag with those obtained from a commercial equivalent, Corning's Universal-BIND plate (Fig. 3). The differences in response across the five chemistries is striking: for example *S*-(+)-ABA captured by **1** shows a significant response to C-4′ raised antibody, but none to C-1 raised antibody. The reverse is true for the adduct with the Corning reagent. *S*-(+)-ABA bound to benzophenone **5** elicits no response from either antibody, notable in the context of the results below. Whilst one might have concluded that Tag 5 was unsuccessful in capturing ABA from

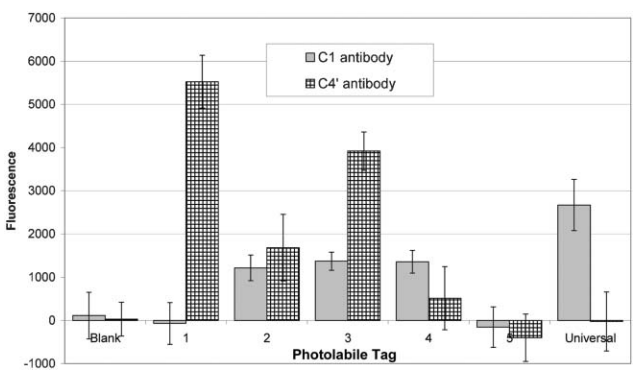

Fig. 3 Recognition of immobilised *S*-(+)-ABA with FITC-antibodies.

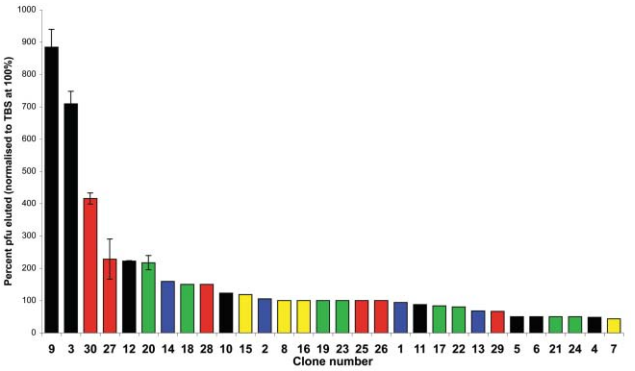

Fig. 4 Elution specificity showing percent pfu removed with *S*-(+)-ABA relative to percent removed with tris-buffered saline (TBS). *Key*: blue = **1**; yellow = **2**; red = **3**; green = **4**; black = **5**. Error bars represent standard deviations of duplicate experiments.

solution, our subsequent studies (Fig. 4 and controls; Fig. 5) show that immobilisation is indeed successful. The predominant regioisomers for the Tag 5 – *S*-(+)-ABA product are however not recognised by either antibody.

This range of immobilised *S*-(+)-ABA was then probed using phage display to identify possible target proteins. Thus *S*-(+)-ABA was subjected to three rounds of biopanning against a bacteriophage λ display library containing cDNA representing the expressed genes of the *Arabidopsis thaliana* genome.[11] Wells containing Tags that had been treated only with buffer, that is they had not been exposed to *S*-(+)-ABA, were subjected to identical biopanning as control experiments. After three rounds of biopanning, 20 individual phage plaques from each Tag type/control were selected for PCR and sequencing giving a total of 200 sequences. After subtracting sequences common to both control and test surfaces, 30 gene products remained as putative *S*-(+)-ABA interactors. The different 'hits' were distributed across all Tag types (**1**, $n = 4$; **2**, $n = 4$; **3**, $n = 6$; **4**, $n = 8$; **5**, $n = 8$: Fig. 4 for details) highlighting the necessity for multiple chemistries in order to properly explore chemical space. These 30 bacteriophage λ clones were then subjected to secondary screening to ensure their specificity to *S*-(+)-ABA, by determining the number of plaque

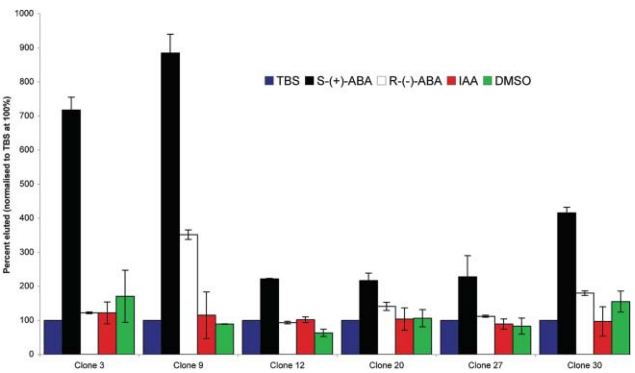

Fig. 5 Selective elution with *R*-(−)-ABA. *Key* (cluster, l to r) blue = TBS, black = *S*-(+)-ABA, white = *R*-(−)-ABA, red = indole acetic acid, green = dimethylsulfoxide. Error bars represent standard deviations of duplicate experiments.

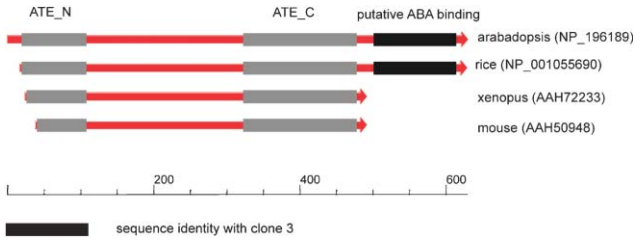

Fig. 6 Alignment of ATE1 proteins showing known transferase domains and the putative ABA binding domain from clone 3.

forming units (pfu) in the eluate from each well (rank ordered in Fig. 4).

The top six candidates (clones 9, 3, 30, 27, 12, and 20) were then chosen to undergo a third screen in which each was selectively eluted from the relevant well with: the antipode of the plant hormone, *R*-(−)-ABA;[14] a structurally unrelated plant hormone, indole acetic acid (IAA); the solvent dimethylsulfoxide (DMSO) each at 1 mg ml^{-1}. As depicted in Fig. 5, each clone shows a stereoselective response to *S*-(+)-ABA with clones 3 and 9 eluting both in largest quantity and with greatest selectivity. Bioinformatic searches using the NCBI BLAST database (see Supporting Information) revealed the insert in clone 3 to match the gene expressing ATE1 (AT5g05700, corresponding to amino acids 500 to 618), an arginyl-tRNA:protein arginyltransferase, which transfers arginine to the *N*-terminus of proteins, shown to be involved in leaf senescence.[15]

The bacteriophage lambda clone that bound specifically to *S*-(+)-ABA (Fig. 5) displayed the domain that is distal to the transferase domains ATE_N and ATE_C (Fig. 6). This region is also found in the ATE1 rice protein (NP001055690) but is lacking in ATE1 proteins from outside the plant kingdom, which tend to be about 100 amino acids shorter in length. It is therefore possible that in plants this region modulates transferase activity *via* ABA binding. The insert in clone 9 corresponded to amino acids 203 to 269 in a putative protein kinase (NP_177210: see Supporting Information for detail). Neither of these putative ABA binding regions showed any significant homologies ($e < 0.1$) to FCA proteins,[16] Mg-chelatase H subunits[17] or the G-protein coupled receptor[18] that have recently been shown to bind ABA, nor solved structures in the Protein Databank.

Clone 3 was selected from the well bearing Tag **5** (Fig. 2), suggesting that the binding motif discovered through this surface exhibits low chemical space homology with either antibody used above (*cf.* Figs. 3 and 4). The functional significance of this and other selective ABA-binding sequences reported here continues to be investigated by expression of soluble recombinant protein from *A. thaliana* cDNA clones together with binding studies using radiolabeled ABA and isothermal titration calorimetry. These important experiments will offer further insight into the nature of the recognition processes discovered during ligand immobilisation and phage biopanning. We observe that affinity based screens have not hitherto explored the diversity inherent in complex natural products, a problem addressed through the use of this more chemo- and regiodiverse ligand display strategy.

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