**Compound Interaction Screen on a Photoactivatable Cellulose Membrane (CISCM) Identifies Drug Targets**


Identifying the protein targets of drugs is an important but tedious process. Existing proteomic approaches enable unbiased target identification but lack the throughput needed to screen larger compound libraries. Here, we present a compound interaction screen on a photoactivatable cellulose membrane (CISCM) that enables target identification of several drugs in parallel. To this end, we use diazirine-based undirected photo-affinity labeling (PAL) to immobilize compounds on cellulose membranes. Functionalized membranes are then incubated with protein extract and specific targets are identified via quantitative affinity purification and mass spectrometry. CISCM reliably identifies known targets of natural products in less than three hours of analysis time per compound. In summary, we show that combining undirected photoimmobilization of compounds on cellulose with quantitative interaction proteomics provides an efficient means to identify the targets of natural products.

Phenotypic screening has emerged as a key driver for biomedical innovation allowing the discovery of unknown therapeutic mechanisms of small molecules. A major challenge in such forward chemical genetic approaches is the chemoproteomics-based deconvolution and characterization of protein targets and mode of action of the identified small molecule.[1–3] Classical affinity-based target identification (ID) involves one distinct derivatization of the compound by one linker trajectory. This requires extensive efforts by structure activity relationship (SAR) studies to obtain suitable small molecular probes for affinity-based pulldown assays – a tedious and time consuming-process which may even unintentionally exclude additional target proteins.[4,5] This applies even more for large natural product derived molecules, where a synthetic access is not available or tractable. For kinase targets, an alternative method builds on affinity beads that are broadly specific for a wide range of cellular kinases. Using these beads in competition with free kinase inhibitors of interest in different concentrations enables the target ID.[6,7] One drawback of this approach is its limitation to kinase inhibitors. More recent proteomic approaches like thermal proteome profiling (TPP) and limited proteolysis-small-molecule mapping (LiP-SMap) do not require compound tagging or immobilization.[8,9] However, these methods require deep characterization of proteomic samples and therefore long mass spectrometric measurement times. Therefore, target ID studies based on TPP and LiP-SMap are typically limited to a single compound.

Undirected photocrosslinking is an attractive alternative to immobilize small molecules on an affinity matrix.[10–14] The chemo- and site-nonspecific nature of the photocrosslinking reaction leads to a distribution of differently tagged products for each small molecule with no prior derivatization required. This enables simultaneous and parallel immobilization of multiple small molecules in an array format. Such arrays can be probed with a single tagged protein, isolated or in a whole cell protein extract, to assess its interaction with multiple small molecules (multiple compounds, one candidate target protein).[15] Photoimmobilized small molecules can also be used to fish for interaction partners in whole cell protein extracts followed by unbiased target ID.[16–18] However, since distinguishing specific target proteins and non-specific contaminants is challenging, such target ID experiments were so far limited to single compounds (one compound, multiple target proteins).

To the best of our knowledge, undirected photocrosslinking was not yet described for high-throughput target ID of multiple compounds in parallel (multiple compounds, multiple candidate target proteins).

Quantitative affinity purification combined with mass spectrometry (q-AP-MS) uses quantification to distinguish specific

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[a] F. T. I. Melder, Prof. Dr. M. Selbach
Proteome Dynamics Lab
Max Delbrück Center for Molecular Medicine in the Helmholtz Association
Robert-Roessler-Str. 10, 13125 Berlin (Germany)
E-mail: matthias.selbach@mdc-berlin.de

[b] Dr. P. Lindemann, Dr. M. Nazaré
Medicinal Chemistry
Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP)
13125 Berlin (Germany)
E-mail: nazare@fmp-berlin.de

[c] Dr. A. Welle, S. Heißler
Institute of Functional Interfaces and Karlsruhe Nano Micro Facility (KNMFi)
Hermann-von-Helmholtz-Platz 1
76344 Eggenstein-Leopoldshafen (Germany)

[d] V. Trouillet
Institute for Applied Materials (IAM-ESS) and Karlsruhe Nano Micro Facility (KNMFi)
Karlsruhe Institute of Technology (KIT)
Hermann-von-Helmholtz-Platz 1
76344 Eggenstein-Leopoldshafen (Germany)

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interaction partners and non-specific contaminants.\(^{[19,20]}\) This efficient and automated identification of specific interactors enables large-scale interaction screens. In this context, cellulose provides a cheap, easy to handle, lightweight and printable solid support platform for large scale interaction screens. For example, synthetic peptides immobilized on cellulose arrays via SPOT synthesis\(^{[21]}\) can be used to screen for interacting proteins in whole cell extracts.\(^{[22-24]}\) On the one hand, the high local concentration of peptide ligands on the cellulose matrix and the mild washing conditions preserve even weak interactions, enabling interaction screens with high sensitivity. On the other hand, comparing protein abundance across different pull-downs can distinguish specific interactors from non-specific contaminants, which leads to high specificity.

We reasoned that photomobilizing small molecules on cellulose membranes should allow us to generate affinity matrices to probe the interaction of many proteins with multiple small molecules in parallel (multiple compounds, multiple target proteins). To this end, we devised a prototypic compound interaction screen on a cellulose membrane (CICSM) for complex natural products consisting of six steps easy to carry out (Figure 1). First, a cellulose membrane (CM) is functionalized with a photocrosslinker. Second, small molecules are spotted onto this membrane. Third, the spotted small molecules are immobilized via undirected photocrosslinking. Fourth, the membranes are incubated with protein extracts (e.g. whole cell lysates), followed by mild washing. Fifth, individual spots are excised, proteins are digested and analyzed by mass spectrometry using LC-MS gradients of 45 minutes per replicate. Finally, specific interaction partners of individual compounds are identified via label free quantification (LFQ).\(^{[26]}\)

For undirected photocrosslinking we selected trifluoromethylphenyldiazirine (TPD) as a photoreactive precursor because of its superior crosslinking efficiency compared to other photocrosslinkers.\(^{[22,28]}\) and its reactivity with a broad range of functional groups.\(^{[29]}\) We functionalized the cellulose membrane using a N-hydroxysuccinimide (NHS)-based approach\(^{[30]}\) and an amine-functionalized diazirine with a PEG-spacer (Scheme 1a). Direct coupling of oxidized cellulose (2) with amine-containing trifluoromethylphenyl diazirine (TPD-3) would be an alternative strategy. This approach employs cellulose immobilized photo-generated carbene species that form covalent bonds with proximal small molecules by non-selectively inserting into carbon–heteroatom (C–Cl), heteroatom–hydrogen (O–H, N–H) and even carbon–hydrogen (Csp\(^3\)=H, Csp\(^2\)=H) bonds (Scheme 1b).

To confirm the efficient functionalization of the cellulose we followed each of the functionalization steps shown in Scheme 1a (bottom) using X-ray photoelectron spectroscopy (XPS, Figure 2a) and attenuated total reflection fourier-transformed infrared (ATR-FTIR) spectroscopy (Figure 2b, Figure S1). Comparison of high resolution C 1s spectra of unmodified (1) versus oxidized cellulose (2) shows the appearance of a peak of weak intensity at 289.2 eV binding energy, which corresponds to the newly formed carboxylic group in 2. The formation of a carbonyl group could also be observed by an ATR-FTIR signal for carbonyl vibrations at 1599 cm\(^{-1}\) for the oxidized cellulose (2). Subsequent functionalization of the oxidized cellulose with EDC and NHS led to the detection of N 1s at 402.0 eV binding.
energy corresponding to the succinimide nitrogen bound to the oxygen of the ester group (1.0 atomic percent).[31] A further species at 400.0 eV can be explained by the remaining of EDC which contains N–C–N groups and amine (5.6 atomic percent). The functionalization of cellulose with NHS could also be detected in ATR-FTIR showing vibrations for the amide group (1705 cm⁻¹) over a mass range of m/z 1160–1220. (d) ToF-SIMS spectrum in the mass range of the amino acid fingerprinting signal of N-methylated Leu (C₅H₁₁N⁺, m/z 100) across different samples: CsA-spotted NHS-activated cellulose (purple), CsA-spotted UV-treated NAC (pink), CSA-spotted TFC (green) and CSA-spotted TFC and treated with UV (brown). (e) Spectra were acquired at three different lateral positions across the corresponding sample.

To evaluate if the TFC matrix allows photocrosslinking of small molecules we first performed experiments using cyclosporine A (CsA) as a large polyfunctional natural product, (Figure 2c–e) using Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) since it allows detection of both intact and small molecules onto a cellulose matrix, we next tested if this platform provides a suitable affinity matrix to assess protein–small molecule interactions. To this end, we selected cyclosporine A (CsA), tacrolimus (FK506) and sirolimus (rapamycin) as model compounds because of their well characterized protein binding partners and their high structural complexity.[32–34] We also included lenalidomide as a member of the group of immunomodulatory drugs (IMiDs) that can pull-down their target ligand cereblon.[35] All compounds were spotted onto the TFC membrane (4) in triplicates and immobilized via photocrosslinking. Unmodified, oxidized and TPD-functionalized cellulose without spotted compounds were used as controls. To obtain protein extracts we lysed Jurkat cells in lysis buffer. For the interaction screen, cellulose membranes were incubated with the lysate for two hours at 4°C. After three washes with a detergent-free lysis buffer, membranes were air-dried. Cellulose spots corresponding to individual photocrosslinked compounds were excised and transferred into 96-well plates containing digestion buffer and processed for shotgun proteomic analyses using standard methods. All 21 samples (four compounds and three controls, in triplicates) were analyzed by high resolution LC-MS/MS on a Q Exactive HFX mass spectrometer.

Data analysis with MaxQuant[36] identified 3,383 protein groups (protein and peptide FDR of 1%) in all samples combined. The corresponding proteomics data is openly accessible on ProteomeXchange. To identify the proteins interacting specifically with a given immobilized compound we used label-free quantification (LFQ).[37] Hierarchical clustering of differentially abundant proteins (ANOVA, FDR 5%) revealed clustering of replicate samples (Figure 3 a). To identify specific targets we compared protein abundances in the three replicates for a given compound to all other samples using the

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**Figure 2.** XPS, ATR-FTIR and ToF-SIMS spectra of stepwise cellulose functionalization. (a) C 1s XPS spectra for unmodified (CM) and oxidized (ox) cellulose (left), N 1s photoelectrons for oxidized and NHS-activated cellulose (NAC, center) and F 1s photoelectrons corresponding to covalently bound fluorine atoms of the trifluoromethyl group. Both XPS and ATR-FTIR data confirmed a successful attachment of the oxygen of the ester group (1.0 atomic percent). The decreased ATR-FTIR signal and showed a signal at 1650 cm⁻¹.

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proteins did not show preferential binding and can thus be
previous results.\(^{(35)}\) As expected, the vast majority of identified
changes of at least four. For cyclosporine, this identified PPIF,
sirolimus (sir) and (e) lenalidomide (len), respectively, against all other
grouped triplicates of (b) cyclosporine (CsA), (c) tacrolimus (FK506), (d)
sirolimus (sir) and (e) lenalidomide (len), respectively, against all other
samples. Proteins with t-test p-values < 0.01 and fold changes of at least
four are labeled and known protein targets marked in blue.

Student's t-test and presented the data as volcano plots
(Figure 3 b–e). As expected, the vast majority of identified
three controls. Each column is an individual replicate of the total 21 samples.
(b–e) Volcano plots displaying the log\(_2\) fold change (x-axis) against the
Student's t-test derived -log\(_{10}\) p-value (y-axis) for pairwise comparison of
grouped triplicates of (b) cyclosporine (CsA), (c) tacrolimus (FK506), (d)
sirolimus (sir) and (e) lenalidomide (len), respectively, against all other
samples. Proteins with t-test p-values < 0.01 and fold changes of at least
four are labeled and known protein targets marked in blue.

Figure 3. Differential protein identifications derived from AP-MS data. (a) Heatmap of Z-scores computed for ANOVA significant (FDR 5%), 250 randomizations) protein identifications of four immobilized compounds and three controls. Each column is an individual replicate of the total 21 samples.
(b–e) Volcano plots displaying the log\(_2\) fold change (x-axis) against the
Student's t-test derived -log\(_{10}\) p-value (y-axis) for pairwise comparison of
An important finding of the work was that undirected photocrosslinking
not be efficient enough for fragment-like small molecules like
lenalidomide only exhibiting a limited number of reactive
attachment sites. In addition, functionalization itself and/or the
linker we used might prohibit the interaction. In fact, the
interaction between lenalidomide and cereblon has been
reported to be sensitive to changes in functionalization sites
and linker chemistry.\(^{(40)}\) Also, while quantitative affinity purification
and mass spectrometry can in principle detect relatively
weak interactions,\(^{(41)}\) the fact that the interaction between
lenalidomide and cereblon is weaker than that of CsA, FK506 and
sirolimus and their respective targets provides an addi-
tional explanation. Whatever the specific reasons are, our data
for lenalidomide shows that CISCM is not a one-size-fits-all
approach for all drug target combinations.

In summary, we present a compound interaction screen on
a photoactivatable cellulose membrane (CISCM) that is able to
rapidly screen for drug targets in a parallel fashion. A key
advantage of the approach is that it does not require tedious
functionalization and previous SAR studies for linker
implementation. While undirected photocrosslinking has been used
before to assess interaction of individual candidate proteins
with drug libraries, we show that q-AP-MS enables unbiased
proteome-wide screening. To our knowledge, CISCM is the first
method that allows high-throughput target ID of multiple
compounds in parallel (multiple compounds, multiple candidate
target proteins). Diazirine-based photocrosslinking enables
immobilization of a broad range of functional groups.\(^{(29)}\) Thus,
while crosslinking via isocyanate or NHS potentially provides
higher conversion for specific nucleophiles,\(^{(42)}\) the diazirine-

Compared to immobilization-free methods like TPP and LIP-
SMap that require long LC-MS measurement times, the high
throughput of CISCM enables multiplexed analysis of several
compounds in parallel. Current limitations of our method are
due to the known limitations of diazirine-based photocrosslink-
ing strategies. For example, the approach does not seem to
work efficiently for small fragment-like compounds. However,
itself to reliably detect targets of larger and complex
natural products makes it particularly suitable for this important
class of drug compounds. Given its simplicity and throughput,
CISCM is an attractive method, complementing existing
approaches in particular in the context of drug leads derived
from natural products.\(^{(43,44)}\)

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in proteomeXchange at https://www.proteomexchange.org, reference number PXD033050, and in DOI: 10.35097/583.

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