Analysis of Protein−Small Molecule Interactions by Microscale Equilibrium Dialysis and Its Application As a Secondary Confirmation Method for on-Bead Screening†

Thomas Weidemann,‡ Jan-Marcus Seifert,§ Martin Hintersteiner,|| and Manfred Auer*,||

School of Biological Sciences, University of Edinburgh, The King's Buildings, CH Waddington Building 3.07, Mayfield Road, Edinburgh EH9 3JD, U.K., BIOTEC/Biophysics, Technische Universität Dresden, Tatzberg 47-51, 01307 Dresden, Germany, and Marinomed Biotechnology GmbH, Veterinärplatz 1, 1210 Vienna, Austria

Received March 31, 2010

On-bead screening of one-bead one compound (OBOC) libraries is an ultra fast surface based primary high-throughput screening (HTS) method. Typically the binding of a tagged target protein to bead immobilized compounds or its altered enzymatic activity are detected. For an efficient and reliable ligand discovery process secondary assays to confirm on-bead compound activity in homogeneous solution are key to exclude artifacts and weak binders. Ideally they should allow to flag hit compounds with undesirable biophysical properties such as aggregation, unspecific binding, or insufficient solubility and the like. Here we demonstrate that miniaturized and parallelized equilibrium dialysis is an excellent and generic secondary confirmation method for hit compounds identified by on-bead screening. We further show that microscale dialysis can be reliably performed prior to decoding and resynthesis even with hit-compounds cleaved from the single beads. Down-scaling of the method takes advantage of the fluorescent tag, AIDA, which is integrated as permanent tracer in our library design. Our results suggest that microscale equilibrium dialysis followed by high performance liquid chromatography (HPLC) analysis is a generic, cheap, and meaningful confirmation method for identifying the most promising candidates within a series hit compounds derived from fluorescently tagged one-bead one-compound libraries.

Introduction

Solid phase combinatorial synthesis provides a fast and cost-effective way to generate large combinatorial one-bead one-compound (OBOC) libraries.[1–5] In recent years, numerous new biologically active compounds have been identified by affinity-based on bead screening of OBOC libraries.[6–12] However, the high compound density on the bead, the presence of a bead matrix as screening compartment, and various other thermodynamic, kinetic, and electrostatic factors often lead to increased hit rates in on-bead screening as compared to solution screening.[13,14] Wang et al. addressed the high density problem by creating peptide libraries of topologically segregated beads.[15] In many cases, however, not all the actives in primary on-bead screening can be confirmed in homogeneous solution. Often, the target binding affinity of such a primary on bead hit can be weaker than the dynamic range of the solution assay. Consequently, to make on-bead screening an absolutely reliable high-throughput screening (HTS) method a series of secondary assays are needed to confirm the activity of primary hit-compounds in homogeneous solution. Artifacts and weak binders can therefore reliably be excluded at early stages within the screening process. An ideal secondary assay should also identify the best, that is, most potent, most specific, most soluble, least aggregating candidate compounds for chemical resynthesis and profiling. To link on bead screening with confirmation in solution, we have developed methods for generating (fluorescently) tagged bead based compound libraries: OBOC libraries with a built-in fluorophore[16,17] or fluorescent tagging site[8] offer a unique advantage for the development of such a generic secondary confirmation assay. A reporter group on every compound allows for a sensitive and specific detection of all bead-derived substances.

Equilibrium dialysis is a well-known biochemical method.[18,19] Recently it has been rediscovered as a robust method for the characterization of protein−ligand complexes in drug discovery.[20–24] Here we describe its application as a generic, highly miniaturized, and single bead-based solution confirmation method. The assay is run on a parallelized device with a high degree of miniaturization and a ratiometric data evaluation procedure in combination with high performance liquid chromatography (HPLC)-based compound analytics. Our microscale dialysis technique was applied to analyze fluorescently tagged hit compounds derived from on-bead screening of OBOC libraries. The AIDA-tag, the fluorescent UV label which was used in this study, facilitates

† This work was performed at Novartis Institutes for BioMedical Research, Brunnerstrasse 59, 1230, Vienna, Austria.

* To whom correspondence should be addressed. E-mail: manfred.auer@ed.ac.uk. Phone: +441316505346. Fax: +441316505088.

‡ Technische Universität Dresden.

§ Marinomed Biotechnology GmbH.

|| University of Edinburgh.

10.1021/cc100057e
Published on Web 08/03/2010

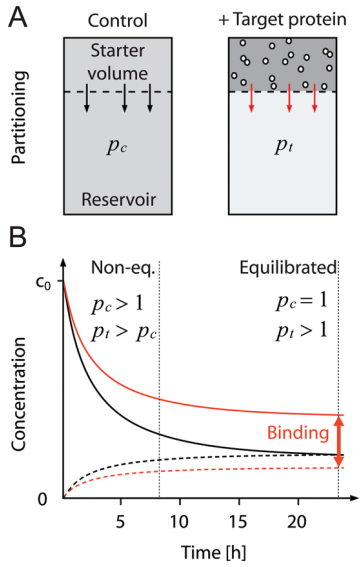

Figure 1. Dialysis principle. (A) Typical end point of a dialysis experiment in the presence and absence of target protein binding to a small molecule ligand (gray shading represents compound concentrations). (B) Expected time course of the compound concentrations equilibration in the starter volume (straight lines) and the reservoir (dashed lines). While in the control experiment without target (black) both concentrations level out, the presence of a target protein causes a ligand concentration difference between the two compartments (red).

compound detection and allows for analyzing cleaved fractions from individual hit beads.

Theoretical Basis

In a dialysis experiment, a small ligand (e.g., a compound) diffuses across a semipermeable membrane. The membrane separates two connected, liquid-filled compartments, V_1 and V_2, which may be of different size (Figure 1). The experiment is started by adding the compound to one of the compartments (starter volume V_1). Driven by Brownian motion, compounds cross the dialysis membrane, and a concentration gradient is formed by dilution into the reservoir V_2. Under ideal conditions the respective concentrations c_1 and c_2 will finally level out, meaning that equilibrium conditions are established.

If compartment V_1 is filled with both the small molecule and a target protein of higher molecular mass than the molecular weight cutoff (MWCO) of the dialysis membrane, a certain fraction of the compound will be retained in V_1 because of protein binding. Thus, the binding of the compound to the target protein results in a concentration difference between the two dialysis compartments (red curves in Figure 1) which reflects both the affinity and the concentration of the binding partners. The dialysis process can formally be described as (a) a reversible binding event of the compound to the target protein and (b) transport of the free compound across the membrane:

$$c_2 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} c_{1f} + c_{Pf} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} c_{1b} \qquad (1)$$

After addition of protein and test compound to the start volume the initial equilibrium is described as $c_1 = c_{1f} + c_{1b}$, with c_1, c_{1f}, and c_{1b} denoting the concentrations of total, free, and bound ligand. Equally, the mass balance for the protein is described as $c_P = c_{Pf} + c_{1b}$, with c_P, c_{Pf} denoting the concentrations of total and free protein. Using a large excess of protein ($c_P \approx c_{Pf}$) reduces the system to a series of (pseudo) first order reactions

$$c_2 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} c_{1f} \underset{k_{off}}{\overset{k'_{on}}{\rightleftharpoons}} c_{1b} \qquad (2)$$

with $k'_{on} = k_{on}c_P$. Accordingly, the concentration of free compound in the starter volume V_1 is governed by three independent rate constants: k_{on} and k_{off}, which describe target binding, and $k_{12} = -k_{21}$, the microscopic transfer rate of compound across the dialysis membrane.

We define the partitioning coefficient $p = c_1/c_2$, the ratio of total compound concentrations in the two compartments V_1 and V_2, as a useful observable to detect target protein binding in compartment V_1 (Figure 1). In control experiments (index c for *control,* no target protein), p_c values are high after spiking the start volume and decrease to ~1 at equilibrium. In target binding experiments p values are increased at all time points ($c_{1b} > 0$, $p_t > 1$, index t for *target* protein containing experiment). At low transport rates, for example, because of surface interactions of the compound, complete equilibration of the concentrations might not be reached, even in the absence of target protein. The p values with and without protein, however, can still be compared to detect binding ($p_t > p_c$) as the equilibration in the start compartment is fast as compared to the transport through the dialysis membrane. This holds true for most screening conditions which are characterized by diffusion controlled target association kinetics for small molecules, and slower compound redistribution between the two volumes.

If microscale dialysis is applied as a validation method for on-bead screening hits, the K_d detection range is a critical quality parameter. A simple estimation for the measurable affinities can be derived assuming true equilibrium conditions ($p_c^\infty = 1$). Under the aforementioned assumptions about the time scales, the concentration of free compound in the starter volume and the reservoir will be the same during the course of the experiment ($c_{1f} = c_2$). Using the definition for the dissociation constant K_d relates the partitioning end point to affinity as follows

$$p_t^\infty = \frac{c_{1f}^\infty + c_{1b}^\infty}{c_2^\infty} = \frac{c_{1b}^\infty}{c_{1f}^\infty} + 1 = \frac{k'_{on}}{k_{off}} + 1 = \frac{c_P}{K_d} + 1 \qquad (3)$$

Notably, this relation is independent of the size of the individual compartments. To save target protein, however, it is important to minimize the starting volume to the lowest experimentally possible size. The use of eq 3 for estimating the low affinity detection limit within a microscale dialysis experiment can be illustrated by a numeric example: When a concentration of 10 μM of a target protein with a single ligand binding site is used, the end point partitioning coefficient after dialysis of a binder with a K_d of 50 μM is $p_t^\infty = 1.2$. To detect such a 20% increase in p within a 2σ confidence interval demands an accuracy of about 3% in

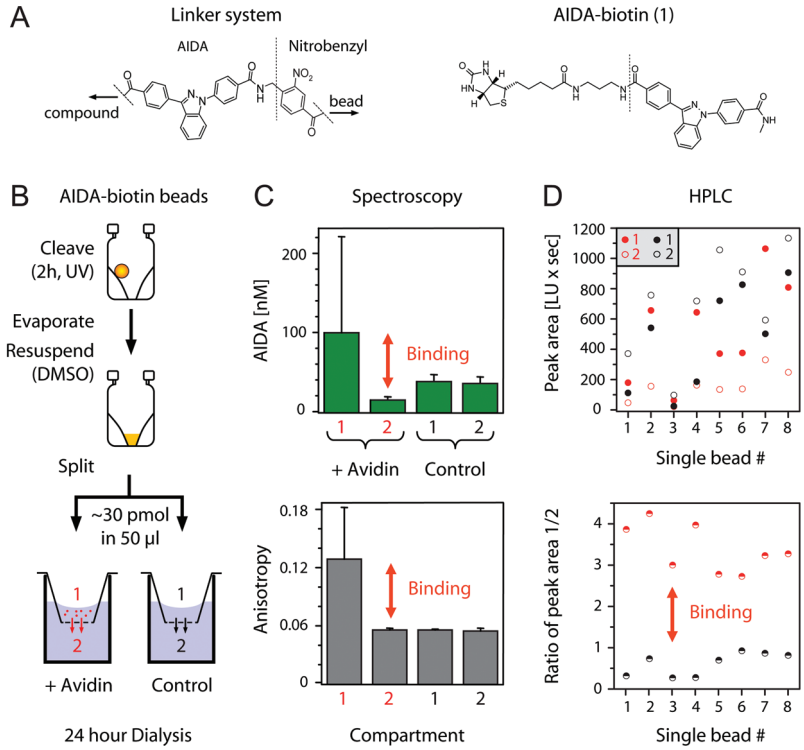

Figure 2. Single-bead dialysis experiments. (**A**) AIDA-linker as used for the combinatorial library design and the structure of AIDA-biotin. (**B**) Outline for sample preparation. (**C**) Fluorescence spectroscopy performed in each of the four compartments after dialysis (results averaged for the beads #5−8). Intensity and anisotropy changes indicate binding of AIDA-biotin to the target protein. (**D**) HPLC analysis of the recovered solutions. Peak area and partitioning value of the compounds are shown for all 8 individual beads in the presence (red) and absence (black) of target protein.

determining the individual compound concentrations c_1 and c_2 in the respective compartments. This requirement is well within the detection accuracy of standard HPLC systems.

Results and Discussion

Dialysis at the Single Bead Level. Ideally, primary on-bead screening hits are validated in solution without compound resynthesis. This requires downscaling of the experimental dialysis protocol to the single bead level. We used the Avidin−Biotin interaction system for method establishment. A typical on-bead screening situation was simulated with 8 TentaGel-beads containing biotin, labeled with a UV excitable dye (AIDA) which was used previously as fluorescence tracer in our one-bead one-compound libraries.[16,17,25] (Figure 2A). The beads were individually placed in a glass vial and processed separately as if they had been picked in an on-bead screen (Figure 2B). After compound cleavage from the bead and after removing the solvent under reduced pressure, the compounds were redissolved in 2 or 4 μL of dimethyl sulfoxide (DMSO), respectively. One of 2 and 2 of 4 μL were added to dialysis units containing either 300 nM Avidin (target chamber) in 50 μL of dialysis buffer or 50 μL of buffer without protein (control chamber). After dialysis, the solutions were recovered and fluorescence intensities were quantified on a fluorescence spectrometer.

Retention of AIDA-biotin because of Avidin binding during the dialysis experiment was clearly confirmed by detection of at least 5 fold increased AIDA fluorescence intensity in the protein compartment as compared to the reservoir (Figure 2C). In the buffer control chambers the AIDA-biotin concentrations had leveled out ($c_1 = c_2$). Moreover, only the compartment containing target protein showed an increased anisotropy suggesting that compound retention was due to protein binding.

The AIDA-biotin fluorescence intensities were related to compound concentrations with a calibration curve derived from a linear dilution series starting from a 1 mM AIDA-biotin stock solution (see Supporting Information, Figure 1A). Averaged over 4 beads, the amount of AIDA biotin found in the individual compartments were as follows: 5 pmol in 50 μL of the target protein containing compartment, 9 pmol in 450 μL of the respective reservoir, and 20 pmol of AIDA-biotin in 500 μL of the buffer control (Figure 2C). This resulted in a total of 34 pico-moles of AIDA-biotin cleaved from individual 90 μm TentaGel beads, respectively. This amount of compound is within the expected loading range of the resin and the typical photolytic compound cleavage efficiency. From the error bars in Figure 2C it is evident that intensity-based calibration showed considerable variations.

We therefore reasoned that a reversed phase HPLC analysis performed under the usual denaturing conditions would provide a standardized protocol for the determination of AIDA-conjugated substances recovered from the individual microscale dialysis compartments. The bound compound is separated from the protein by elution with an acetonitrile gradient on a reversed phase column. Signal variations because of an altered quantum yield of the dye in the bound state are thereby prevented. Compound concentrations after dialysis were therefore quantified via peak area integration of HPLC traces. The ratio of peak area measured

in two connected dialysis compartments directly reflects the partitioning coefficient p (data are summarized in Figure 2D). Although, by following the HPLC quantification protocol, surface exposure to tips and wells is reduced to a single transfer step, the absolute amount of cleaved compound still considerably varied between the individual beads (upper panel, Figure 2D). However, replacing the individual peak area measures by the ratio of the peak areas in the target and reservoir compartments resulted in much more robust parameter (p_t/p_c) for detecting ligand-target binding (Figure 2D, lower panel). For this initial experiment the dialysis membranes have not been pre-boiled, which led to significant volume shifts explaining the variations in Figure 2D. Nevertheless, comparing the partitioning values for each individual pair of dialysis wells with and without Avidin revealed the binding event in all 8 samples, even in sample #3, which contained a very low compound concentration.

The data in Figure 2D show that the partitioning coefficient of AIDA-biotin was independent of the individual starting concentrations in each well. This implies that equal splitting of the redissolved compound is not critical for a successful binding experiment. The optimized procedure for microscale dialysis followed by ratiometric HPLC analysis comprised the dilution of the single bead cleaved compound in 4.2 μL followed by spiking 50 μL of the two starter volumes with 2 μL of the stock solution in DMSO. This keeps the DMSO concentration below 5%, which might be crucial for protein integrity and folding.

Solution Properties of AIDA-Tagged Compounds. To test the applicability of microscale dialysis as a secondary assay in a high throughput screening process, we selected a diverse set of 45 resynthesized AIDA-tagged hit-compounds, resulting from a series of on-bead screens with diverse target proteins. From DMSO stock solutions, a typical amount for single bead analysis (25 picomols) was dialyzed against buffer in the absence of protein. Automated HPLC peak detection was used to quantify the compound concentration in each dialysis compartment and to calculate the corresponding partitioning coefficient (Supporting Information, Figure 2A). The area under the main HPLC peak was converted into absolute substance amounts based on a previously recorded calibration curve (Supporting Information, Figure 1B). The initial compound concentration was determined by HPLC analyzing an aliquot of the stock-solution without prior dialysis under identical conditions. By comparison of the amount of AIDA-fluorescence in the dialysis compartments with that of the reference run from the stock solution, the compound loss during the experiment was calculated (recovery, Supporting Information, Figure 2B). After dialysis, the liquid-filled volume of the starter compartment V_1 was measured using a pipet (Supporting Information, Table 1) to account for any possible volume changes due to evaporation and osmotic pressure.

During the first dialysis experiments with AIDA-biotin, volume shift in the dialysis compartments were recognized to be an issue. This problem was solved by including a pre-boiling step for the dialysis membranes. It seems that complete hydration and swelling of the membrane before loading the compound is critical to avoid liquid redistribution.

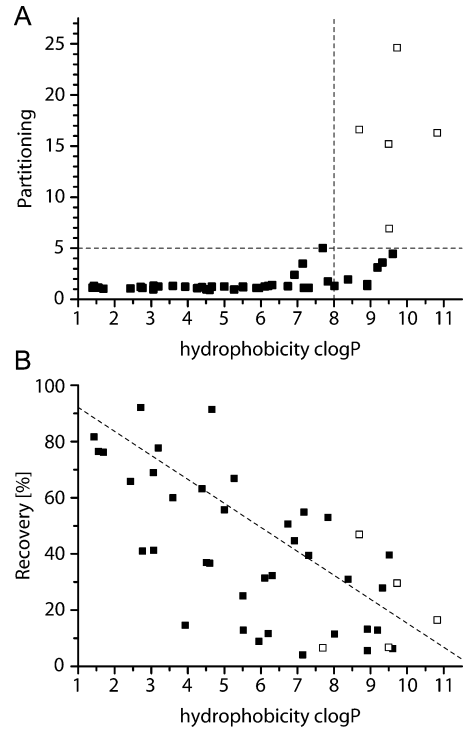

Figure 3. Dialysis and hydrophobicity. (A) Partitioning coefficients of individual compounds plotted against their predicted cLogP. We defined a cutoff criterion for further hit compound investigation of $p_c < 5$ (broken line) (B) Recovered amount of sample after dialysis in comparison to the initially spiked compound correlated with cLogP (broken line). Compounds with $p_c > 5$ are highlighted (open symbols).

When using 450 μL of dialysis buffer in the reservoir, the starter volume V_1 was on average slightly increased from 50 to 62.7 $\pm$ 6.7 μL after 24 h. The increase may be due to a small osmotic or hydrostatic pressure difference between the two compartments. As noted above the end-point partitioning coefficients p_t^{∞} are not affected by volume shifts. However, too small volumes containing the target protein may not be compatible with automated HPLC injection.

The average starting concentration for a spiking experiment was 300 nM in a volume of 50 μL and therefore each dialysis tube contained on average 15 pmol of compound. After dialysis the concentration decreased to less than 30 nM (1/10th) because of compound dilution into the reservoir and some adsorption. On average 50% of the spiked compound could be recovered after 24 h. Despite these losses, AIDA fluorescence intensity exceeded the sensitivity limit of the HPLC detector (10 Lu $\times$ sec $\approx$ 0.1 pmol/50 μL = 2 nM AIDA). After 24 h dialysis at room temperature, the samples reached an average partitioning coefficient of $p_c = 3$ ($n = 45$, $stdev = 4.7$); however, this value was biased by a small subset of extreme outliers. Applying an arbitrary cutoff criterion ($p_c > 5$) results in a mean partitioning value of $p_c = 1.5$ ($n = 40$, $stdev = 0.79$) for the remaining samples. Thus, equilibrium was almost fully reached in this subgroup (Supporting Information, Table 1).

We then asked whether there is a systematic correlation between the hydrophobicity of the compound and the partitioning and recovery after a dialysis experiment (Figure 3). For each structure, the cLogP values, that is, the partitioning coefficient between water and octanol phases,

was calculated using a chemo-informatics tool (*Biobyte*, Novartis Institutes for BioMedical Research). A comparison of these cLogP values with the experimentally determined partitioning coefficients revealed a remarkable independence of these two properties. The measured p_c values were almost constant up to clogP-values of about 6. Some compounds with log p values between 6.5 and 8 show stronger retention. However, their pc values are generally still below 5. Therefore even compounds with less ideal properties can be analyzed for target binding. A limited number of outliers populate the quadrant with clogP > 8 and p_c > 5. Overall, these data demonstrate that small molecule dialysis provides a suitable method for characterizing hit-compounds over a wide range of cLogP values. In fact, the entire cLogPs range commonly considered appropriate for drug like molecules (clogP < 5) and even beyond is covered.

In contrast to the partitioning coefficient, the compound recovery from dialysis chambers correlates negatively with the calculated cLogPs. Compounds with a smaller cLogP exhibit a preferentially better recovery. Although this correlation is significant, Figure 3B illustrates that each compound shows unique adsorption properties to the material of the dialysis chamber which cannot be predicted from hydrophobicity alone. We emphasize that even with compound recoveries as low as 10% a reliable partitioning coefficient can still be determined.

Dialysis at the Single Bead Level. For integration into a bead-based screening process, equilibrium dialysis must be applicable to compounds cleaved from individual hit beads. 90 μm TentaGel beads, which are commonly used for on-bead screening, contain ~50 pmol of substance. Three key questions related to the miniaturization of the dialysis experiment needed to be addressed: (i) What is the minimal amount of AIDA-conjugated compound that can reliably be detected via HPLC-analysis after dialysis? (ii) How many dialysis runs can be carried out with single bead derived substance? and (iii) What is the well-to-well variability of partitioning coefficients for individual hit compounds?

To address these questions 11 exemplary compounds including AIDA-biotin were selected. For each compound, two beads were manually removed from the resin batch and placed in two sample tubes. The compounds were then cleaved from the resin and processed as outlined in Figure 4A: 20% of substance was kept for monitoring the cleavage yield; 50% or 30% were dialyzed against buffer. Measuring the volumes of the start compartment V1 after dialysis using a pipet showed that the presence of protein does not introduce additional osmotic pressure, since the volume shift of in average 20% was unchanged as compared to the buffer controls (Supporting Information, Figure 3). A corresponding shift towards dissociation because of protein dilution can therefore be neglected. The obtained dialysis results from the bead cleaved material were in good agreement with what was observed for resynthesized and purified hit compounds. The absolute amounts of compound obtained from single bead cleavage as well as the compound recoveries after dialysis varied considerably, whereas the partitioning values showed significantly less variability (Figure 4B). On average the 10 compounds dialyzed with $p = 1.3 \pm 10\%$. The

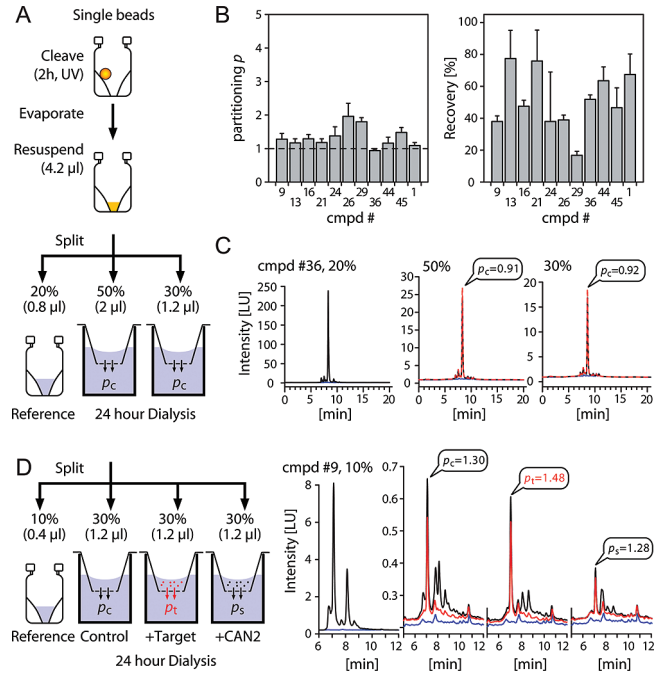

Figure 4. Dialysis of compounds cleaved from single beads AAA. (A) Dialysis process scheme for single beads to address variability and sensitivity of the dialysis experiment. Eleven beads were processed in duplicates. (B) Partitioning and recovery for each compound (# identifier as in Supporting Information, Figure 2). Errors are derived from the 4 independent dialysis experiments. (C) HPLC chromatograms for compound (**36**): 20% reference directly from the redissolved stock (black) in comparison to the solutions after dialysis in compartments V_1 (starter volume, black) and V_2 (reservoir, red) spiked with 50% and 30% of bead material, respectively. A blank HPLC run (PBS, blue) is shown. (D) Alternative strategy for splitting the single bead probe to include a protein specificity control. The 4-fold split strategy is exemplified using compound (**9**). HPLC traces from left to right: Reference, buffer control (p_c), target protein (p_t), specificity control (p_s).

accuracy of HPLC detection is remarkable: A comparison of the data achieved with the 50% split fractions and the 30% split fractions shown in Figure 4C demonstrates that the partitioning coefficient was reproduced within 1%. For compounds with reduced solubility this accuracy is reduced because of incomplete dialysis. Because of the high detection sensitivity revealed in initial experiments described above, we decided to include a further compound split in the dialysis process scheme to test for potential unspecific binding events with primary hit compounds. As outlined in Figure 4D, we dialyzed one aliquot of compound against buffer, a second aliquot in the presence 5 μM of the corresponding target protein, and a third in the presence of 5 μM of the ubiquitous cellular house keeping protein Carboxyanhydrase 2 (CAN2). The 30% subdivision allows addressing hydrophobicity (p_c), binding potential (p_t), and specificity (p_s) with the substance obtained from one individual bead. The ratio of peak areas of the two control dialyses without protein and with CAN2 was almost identical ($p_c = p_s \approx 1.3$). The dialysis in presence of target protein, however, yielded a higher partitioning factor, $p_t = 1.43$ therefore indicating a weak, specific target protein binding of compound (**9**).

Compound Profiling by Microscale Dialysis. To demonstrate the usefulness of microscale dialysis as a method

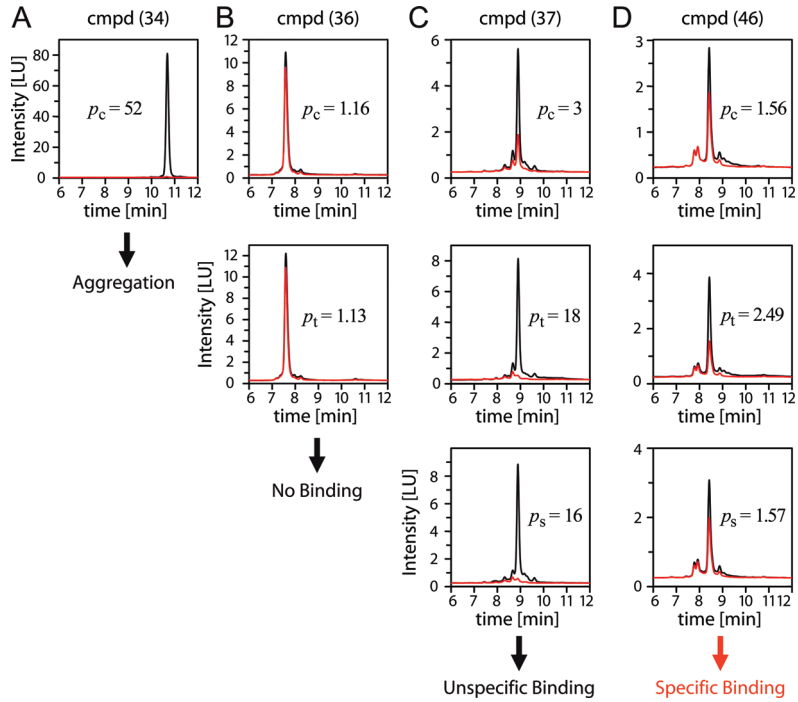

Figure 5. Microdialysis for compound profiling HPLC analysis (start volume trace in black, reservoir trace in red) of resynthesized compounds dialyzed in the absence (p_c) and presence of 5 μM target protein (p_t) or a house keeping protein (CAN-2) as a specificity control (p_s). Within the group of four chemically related compounds (1−4) (A) is sticky or aggregates in solution, (B) equilibrates well but does not bind to the target, (C) binds unspecifically to both tested proteins, and (D) binds specifically to the target protein.

to validate compounds from bead-based screening and profile their biophysical properties, we investigated a subset of 4 chemically related, resynthesized AIDA-compounds. The original hits were picked based on a target protein binding to the bead immobilized compound in the course of an on-bead screening experiment. All of these compounds were active in spectroscopic and cellular assays (not shown). For microscale dialysis we used 100 pmol of compound for each experiment as outlined in Figure 4D.

The experiment showed that the solution properties of the four compounds differed substantially (Figure 5). While compound (**34**) exhibited aggregation or surface adsorption, compound (**36**) equilibrated almost completely, however, without any target protein binding as indicated by a less than 3% difference in the partitioning coefficients p_t, p_c. Although the partitioning values for compounds (**37**) and (**46**) indicated target protein binding ($p_t > p_c$), only compound (**46**) dialyzed equally well in presence of CAN2 and in the buffer only control ($p_t \sim p_c$). Compound (**37**) turned out to be unspecific (i.e., $p_t \sim p_s > p_c$). Therefore compound (**46**) was the only validated hit of the subset with the favorable biophysical properties.

Conclusion

To expand the repertoire of generic secondary assays which can be carried out with the ∼50−100 pmol of substance, cleaved from one hit bead, we looked into methods which require less sophisticated equipment and are universally applicable. Equilibrium dialysis is a well established biochemical method. It is, however, not widely used in drug screening and profiling. In this study we successfully explored the possibility to adapt equilibrium dialysis combined with HPLC analytics to confirm, validate, and profile on-bead screening identified hit compounds cleaved from single 90 μm TentaGel beads. Because the compounds contained AIDA as a generic fluorescent tag, we were able to probe up to three different experimental conditions: Solution properties, target protein binding, and specificity. Thus, in addition to detection of binding, our approach delivered important information about the molecular properties of the compounds such as purity, solubility, binding selectivity, and tendency to aggregate in a very early phase of the selection process.

It cannot be ruled out that the AIDA-moiety influences the measured properties to a certain extent. However, on the basis of substantial experience in on-bead screening of AIDA tagged libraries we can conclude that there is generally a good correlation between physicochemical profiles of tagged and not-tagged compounds. AIDA was designed as a relatively inert chemical entity to allow subsequent combinatorial expansion of the library rather than to generate an optimized fluorescent tracer. The placing of the AIDA moiety as the first building block in a combinatorial synthesis might partly be responsible for the relatively unchanged binding properties of an AIDA tagged compound. The number of experiments which may be performed with single-bead cleaved material increases with detection sensitivity. Therefore, fluorescent tags with enhanced brightness would be beneficial. Our recently introduced post-screening library tagging system, which is suited for a "single bead-single molecule" fluorescence spectroscopic analysis and subsequent K_d determination allows for a far greater flexibility in choosing a dye tracer.[8]

In contrast to standard dialysis, the compound concentrations were detected in both the protein and the buffer containing compartment. Because our method combines the

resolving power of HPLC analytics with an upstream equilibrium binding assay, it allows for identification of the active chemical entity above a background of side products. Side products derived from a single bead may be assigned by retention time and ranked according to their partitioning values. Subsequent MS analytics may then be used to decode the active species. This may be particularly important for OBS, because on bead library production inherently lacks the possibility for efficient post-synthesis purification steps on the solid support. Therefore side products present in screening situations cannot always be avoided to the same degree as in homogeneous solution HTS. Multiplexed detection, however, may even be exploited purposefully by designing libraries displaying more than one compound class per bead as long as the respective HPLC peaks are separable.

To deal with the possible issues arising from handling and analysis of picomole amounts we introduced the concentration ratio of the compound in both dialysis compartments, the partitioning coefficient, as a remarkably robust and reproducible readout. It is well-known that multiple effects like electrostatics, adsorption to walls, or the formation of higher order aggregates can complicate the quantitative analysis of equilibrium binding data. Using a ratiometric approach, most of these negatively influencing factors cancel out. As a next development step a multi well format and progressive automation could be easily implemented.[21,24] In addition, combined HPLC and mass spectrometric (LC-MS) decoding strategies may significantly enhance the scope of this approach for triaging of on-bead screening hit lists.[23] Currently, the partitioning values provide a fast and easy method for relative ranking of the hit-compounds according to their affinity for a target protein. Equation 3 describes a simple relation between the K_d of a compound and the partitioning value for an idealized sample at full equilibration. However, the long dialysis times which are required to reach a fully equilibrated state of the sample make the use of this equation impractical. In addition, non-specific interactions of the compounds with components of the dialysis compartment would lead to a significant overestimation of absolute affinities for many compounds. We are therefore in the process of developing a theoretical framework based on differential equations for the determination of absolute affinities from non-equilibrium dialysis data.

Experimental Section

Library and OBS. Details about the library design and the screening procedures leading to OBS hit-compounds is described elsewhere.[16,17] In brief, AIDA is an indazole-based UV-dye which was attached via a photocleavable nitrobenzyl linker to TentaGel beads as the first element of our on-bead library design. The dye is followed by a three-carbon atom diaminopropane spacer unit connecting different heterocyclic scaffolds to the AIDA tag. The scaffolds generally contain 4 combinatorial positions used for diversification during a split-mix-and-divide combinatorial synthesis scheme. For OBS, the beads were distributed in a monolayer in 96-well glass-bottom plates, incubated with fluorescently labeled target protein and subjected to confocal scanning by the CONA method on the PickoScreen platform.[7] After data analysis, the picking device integrated in our screening instruments allowed picking of individual hit beads and their deposition in HPLC auto sampler vials for further processing. For compound cleavage from resin, the beads were placed in a solution of methanol, containing 1% TFA. and exposed to UV illumination for 120 min using a Stratalinker 1800 UV illumination cabinet (Stratagene, La Jolla, CA, U.S.A.), fitted with five 365 nm UV bulbs (Type NEC black light T5FL8BL, 26 cm length, 8 W each). Irradiation power was 1070 μJ/min. The beads were removed from the solution afterward, and the samples dried and redissolved in DMSO.

Dialysis. For dialysis, Slide-A-Lyzer MINI units (Pierce #69570, MWCO 10 kDa) were placed into 48-well microplates (Falcon #351178). Prior to the experiment, the Slide-A-Lyzer MINI units were shortly boiled in distilled water to remove trace contaminants of metals and glycerol and to accelerate hydration of the membrane. The units were equilibrated in dialysis buffer in 48-well plates overnight, emptied by upside down centrifugation with 15 g for 1 min (Heraeus Megafuge 1.0R) and filled with either 50 μL of 5 μM target protein solution (300nM Avidin for the AIDA-biotin experiment) or 50 μL of dialysis buffer (PBS, 0.005% Tween20, Sigma) as a control. The filled units were placed into the reservoir plate, containing 450 μL of dialysis buffer per well. Dialysis was started by adding equal volumes of the single bead compound stock solution in DMSO (typically 1.8 μL, i.e. < 2.5 μL $= 5\%$ DMSO) to 50 μL of the starter volume. The units were then covered with adhesive sealing film (Exel Scientific, #100-SEAL-PLT), the lid was closed and sealed with parafilm to avoid evaporation. Dialysis was allowed for 24 h at room temperature without mechanical agitation.

HPLC. For analysis, 50 μL of each, the starter volume and the reservoir, were supplemented with 10 μL of acetonitrile in HPLC autosampler vials. When less than 50 μL volume could be recovered, a 1:1 ratio was maintained, and the reduced amount adjusted to 60 μL final volume. In all cases 55 μL were injected into an Agilent 1100 HPLC instrument equipped with DAD (G-1315B) and fluorescence detectors (G1321A). Either: Separation was done on a Merck EcoCart cartridge system with Lichrospher C18 SelectB column (5 μm, 60 Å, 3 × 125 mm +4 × 4 mm precolumn) with a gradient from A: 95/5 water/acetonitrile, 0.1% TFA to B: 95/5 acetonitrile/water, 0.1% TFA (1 min 0% B, 9 min 5−95% B, 4 min 95% B, 3 min 95−5% B, 3 min 5% B) and a flow of 0.7 mL/min. Or: Separation was done on a Waters Symmetry C8 column (3.5 μm, 60 Å, 4.6 × 50 mm +4 × 4 mm Merck Lichrospher RP-8 precolumn) at 40 °C with a gradient from A: 95/5 water/acetonitrile, 0.1% TFA to B: 95/5 acetonitrile/water, 0.1% TFA (1 min 0% B, 5 min 5−95% B, 1 min 95% B, 3 min 95−5% B, 2 min 5% B) and a flow of 1.2 mL/min. The compounds were monitored by UV absorption at 215, 254, and 280 nm and fluorescence detection at ex 333 nm/em 410 nm, gain 10. The fluorescence from the AIDA-tag of the compound was used to quantify the amount of substance by automatic peak integration. Individual peaks of the chromatograms at 410 nm emission were batch-reintegrated with the Agilent

Chemstation software. Data for retention time and peak area were exported as text with a custom-programmed macro program.

Fluorescence Spectroscopy. Spectra were measured with a Fluorolog $\tau-3$ spectrofluorometer (Jobin Yvon/Horiba, Germany) equipped with double monochromators in the excitation and emission path. Exciting at 333 nm, we integrated emission spectra from 350−450 nm, using slits at 5 nm bandwidth, 5 s integration time with a wavelength increment of 1 nm. A WG320 UV filter in the emission path was used for stray light attenuation. Raman scattering was subtracted by a buffer spectrum. AIDA samples were quantified using an AIDA-calibration curve, which was reproduced from a 1 mM stock-solution of AIDA-biotin. Anisotropy was measured using 333 nm excitation and 410 nm emission in repetitions to less than 0.1% error. All measurements were done with 60 μL fluorescence quartz cuvettes (26-50-F Q, Starna, U.K.) at room temperature (∼22 °C).

Acknowledgment. We thank Adolf Adam for providing a useful MS Access-based evaluation tool (*myD*) for automatic peak analysis and Franz Amberger for "beta-testing" the experimental procedure. Marion Kala is kindly acknowledged for editing parts of the manuscript.

Supporting Information Available. Additional information is provided in Supplementary Figures 1−3 and Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Blackwell, H. E.; Perez, L.; Stavenger, R. A.; Tallarico, J. A.; Cope Eatough, E.; Foley, M. A.; Schreiber, S. L. *Chem. Biol.* **2001**, *8* (12), 1167–1182.
(2) Clemons, P. A.; Koehler, A. N.; Wagner, B. K.; Sprigings, T. G.; Spring, D. R.; King, R. W.; Schreiber, S. L.; Foley, M. A. *Chem. Biol.* **2001**, *8* (12), 1183–1195.
(3) Kodadek, T.; Bachhawat-Sikder, K. *Mol. BioSyst.* **2006**, *2* (1), 25–35.
(4) Lebl, M.; Krchnak, V.; Sepetov, N. F.; Seligmann, B.; Strop, P.; Felder, S.; Lam, K. S. *Biopolymers* **1995**, *37* (3), 177–198.
(5) Reddy, M. M.; Bachhawat-Sikder, K.; Kodadek, T. *Chem. Biol.* **2004**, *11* (8), 1127–1137.
(6) Lehman, A.; Gholami, S.; Hahn, M.; Lam, K. S. *J. Comb. Chem.* **2006**, *8* (4), 562–570.
(7) Hintersteiner, M.; Buehler, C.; Uhl, V.; Schmied, M.; Muller, J.; Kottig, K.; Auer, M. *J. Comb. Chem.* **2009**, *11* (5), 886894.
(8) Hintersteiner, M.; Kimmerlin, T.; Kalthoff, F.; Stoeckli, M.; Garavel, G.; Seifert, J. M.; Meisner, N. C.; Uhl, V.; Buehler, C.; Weidemann, T.; Auer, M. *Chem. Biol.* **2009**, *16* (7), 724–735.
(9) Pei, D.; Wavreille, A. S. *Mol. BioSyst.* **2007**, *3* (8), 536–541.
(10) Lathrop, J. T.; Fijalkowska, I.; Hammond, D. *Anal. Biochem.* **2007**, *361* (1), 65–76.
(11) Luo, J.; Zhang, H.; Xiao, W.; Kumaresan, P. R.; Shi, C.; Pan, C. X.; Aina, O. H.; Lam, K. S. *J. Comb. Chem.* **2008**, *10* (4), 599–604.
(12) Maillard, N.; Clouet, A.; Darbre, T.; Reymond, J. L. *Nat. Protoc.* **2009**, *4* (2), 132–142.
(13) Chen, X. W.; Tan, P. H.; Zhang, Y. Y.; Pei, D. H. *J. Comb. Chem.* **2009**, *11* (4), 604–611.
(14) Dixon, S.; Ziebart, K. T.; He, Z.; Jeddeloh, M.; Yoo, C. L.; Wang, X.; Lehman, A.; Lam, K. S.; Toney, M. D.; Kurth, M. J. *J. Med. Chem.* **2006**, *49* (25), 7413–7426.
(15) Wang, X.; Peng, L.; Liu, R.; Xu, B.; Lam, K. S. *J. Pept. Res.* **2005**, *65* (1), 130–138.
(16) Meisner, N. C.; Hintersteiner, M.; Seifert, J. M.; Bauer, R.; Benoit, R. M.; Widmer, A.; Schindler, T.; Uhl, V.; Lang, M.; Gstach, H.; Auer, M. *J. Mol. Biol.* **2009**, *386* (2), 435–450.
(17) Meisner, N. C.; Hintersteiner, M.; Uhl, V.; Weidemann, T.; Schmied, M.; Gstach, H.; Auer, M. *Curr. Opin. Chem. Biol.* **2004**, *8* (4), 424–431.
(18) Klevens, H. B.; Carr, C. W. *J. Phys. Chem.* **1956**, *60* (8), 1245–1249.
(19) Westphal, U.; Ashley, B. D.; Selden, G. L. *J. Am. Chem. Soc.* **1958**, *80* (19), 5135–5138.
(20) Ji, Q. C.; Rodila, R.; Morgan, S. J.; Humerickhouse, R. A.; El Shourbagy, T. A. *Anal. Chem.* **2005**, *77* (17), 5529–5533.
(21) Banker, M. J.; Clark, T. H.; Williams, J. A. *J. Pharm. Sci.* **2003**, *92* (5), 967–974.
(22) Kariv, I.; Cao, H.; Oldenburg, K. R. *J. Pharm. Sci.* **2001**, *90* (5), 580–587.
(23) Wan, H.; Rehngren, M. *J. Chromatogr., A* **2006**, *1102* (1−2), 125–134.
(24) Wong, S. Y.; Chan, T.; Putnam, D. *J. Comb. Chem.* **2009**, *11* (2), 202–205.
(25) Gstach, H.; Pflugseder, K.; Prechelmacher, S.; Monteux, D.; Lewis, S. E.; Storz, T.; Früchtel, J. S.; Graf, C.; Pruckner, A.; Auer, M. A Powerful Tool for Generic Fluorescence Labelling of Combinatorial Compound Libraries, 5. International Electronic Conference on Synthetic Organic Chemistry (ECSOC-5), Basel, Switzerland, 2001; http://www.mdpi.org/ecsoc/ecsoc-5/Papers/b0008/b0008.htm.

CC100057E