A Mega-high-throughput Screening Platform for the Discovery of Biologically Relevant Sequence-defined Non-natural Polymers

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Abstract

Combinatorial methods enable the synthesis of chemical libraries on scales of millions to billions of compounds, but the ability to efficiently screen and sequence such large libraries has remained a major bottleneck for molecular discovery. We developed a novel technology for screening and sequencing libraries of synthetic molecules of up to a billion compounds in size. This method utilizes Fiber-optic Array Scanning Technology (FAST) to screen bead-based libraries of synthetic compounds at a rate of 5 million compounds per minute (~83,000 Hz). This ultra-high-throughput screening platform has been used to screen libraries of synthetic "self-readable" non-natural polymers that can be sequenced at femtomole scale by chemical fragmentation and high-resolution mass spectrometry. The versatility and throughput of the platform was demonstrated by screening two libraries of non-natural polyamide polymers with sizes of 1.77M and 1B

compounds against the protein targets K-Ras, asialoglycoprotein receptor (ASGPR), IL-6, IL-6 receptor and TNFα. Hits with nanomolar binding affinities were found against all targets, including competitive inhibitors of K-Ras binding to Raf and functionally active uptake ligands for ASGPR facilitating intracellular delivery.

Main Text

Natural biological polymers such as peptides, proteins, and nucleic acids have evolved molecular recognition functionalities to produce highly specific binding interactions and catalytic enzymatic functions. In an empirical approach to discover novel affinity agents, catalysts, and materials, biotechnologists have exploited these rich functional properties to create therapeutics, diagnostics, sensors, industrial reagents, and biomedical research tools by using powerful biological tools to screen large libraries of natural polymers of 10^{7-12} unique molecular sequences.[1-4] As data regarding the structures of biopolymers have amassed, so has the ability to rationally design *de novo* proteins and other biopolymers.[5-7]

There is a growing interest in expanding polymer discovery into the field of sequence-defined non-natural polymers using chemical rather than biological synthesis to further access to molecular diversity and functionality and improve biostability and physicochemical properties.[8-14] At present, there is insufficient sequence and related structural data to design non-natural polymers *de novo;* as such, screening libraries to find polymers with desired properties is currently the most practical solution.

A chemical synthetic methodology for building libraries of non-natural polymers is established on solid support beads via the "one-bead one-compound" (OBOC) method.[15, 16] This combinatorial "mix and split" method has the potential to make libraries of similar size to

biological methods; however, in practice, it has traditionally produced small libraries on the scale of only thousands to hundreds of thousands of compounds because of the high cost associated with screening and hit sequencing.[17] The current commercially available technology with the highest throughput for bead screening is fluorescence-activated cell sorting (FACS)[18, 19], which has a theoretical throughput of ~10^8 in a 10-hour period. In practice, however, hit enrichment from large libraries (>10^6) using pull-down methods is the only way to achieve time- and cost-effective FACS screening that reduces the number of beads to 10^{4-5}.[19] Sequencing of α-amino acid library hits on beads to identify peptides is routinely done by liquid chromatography with tandem mass spectrometry (LC-MS/MS)[20, 21], which works well for short oligomer peptides and peptides but does not translate well to novel non-α-amino acid backbone polymers. Many methods have been developed to improve the sequencing process such as introducing fluorescent dyes,[22] isotopic tags,[23] DNA encoding,[24] ladder-sequencing[25-28] and chemical encoding methods.[29] However, none of these sequencing methods are sensitive enough for libraries synthesized on beads of less than 30 μm in diameter[19] because these libraries contain ~4 picomoles of polymer / bead and more typically use bead sizes of >90 μm containing >100 picomoles of polymer per bead to ensure there is sufficient material for hit identification (**Table S1**). Large libraries of beads with these diameters are prohibitively expensive (in materials costs) to synthesize and screen, which is another reason why such chemical libraries sizes have been limited to date.

Here, we present a novel platform that enables the production of large libraries of synthetic, sequence-defined non-natural polymers on the scale of $10^7 – 10^9$ members for mega-throughput screening using a platform based on a Fiber-optic Array Scanning Technology (FAST™) that screens up to ~5 million polymers a minute. Furthermore, we describe a method for sequencing single bead hits down to 10 μm in diameter with femtomole sensitivity. We demonstrate the

platform's broad use in screening against five targets of biomedical interest to identify biologically relevant non-natural polymers with affinities in the nanomolar to sub-nanomolar range that can inhibit protein – protein interactions (PPIs) and have exceptional biological activity and stability.

Results

FAST Screening Approach

FAST was originally developed to identify rare circulating cancer cells in blood with high sensitivity and specificity (**Figure 1**).[30, 31] In this application, cells preincubated with fluorescently labeled cell surface markers are plated as a monolayer on 108- x 76-mm glass slides, which are then scanned by excitation with a 488-nm laser. Emitted fluorescence is collected through a fiber-optic bundle, and the collected light is passed through a bandpass filter and analyzed by a photomultiplier tube to measure emission at 520 nm (green) and 580 nm (red/orange). Cartesian coordinates of fluorescently labeled objects are located on a pixel map, along with fluorescent intensity measurements at the two emission wavelengths. In this well-free assay format FAST can routinely identify the location of single rare cells in a milieu of 25 million white cells in a 1-minute scan with an ~8-µm resolution. In optimizing the FAST system for bead screening the only major modification to the scanning process was the need to plate beads at a lower density than cells due to their propensity to aggregate and need to extract them post-analysis for sequencing. Empirical optimization of bead plating density revealed that 10-µm diameter TentaGel™ beads plated with a density of 5 million beads per plate gave a relatively well-dispersed monolayer enabling automated analysis and bead picking for down-stream processing. Similarly, 20-µm beads were optimally plated at a density of 2.5 million beads per plate. Detection sensitivity was assessed by spiking biotin-labeled beads into a pool of underivatized beads and incubating with

Alexa Fluor 555-labeled streptavidin for 1 hour before plating. The FAST screening process gave a detection sensitivity of over 99.99% (**Table S2** and **Fig. S1**).

Self-Readable Polymers and the "Ptych" Approach

We created a novel self-readable sequencing approach to polymer library design called the "ptych" (pronounced "tick") design. **Figure 2a** depicts a "tetraptych" (from Greek meaning "four-fold" from *tetra*, i.e., "four" and *ptysso*, i.e., "to fold"), which is a term used in the art world to describe a panel painting divided into three sections that can be folded to display a composite scene. In our application, a tetraptych is defined as a set of four monomers that make up one diversity element in a longer sequence. The full sequence can then be formed by linking multiple ptychs together. Each tetraptych, a four-monomer element in this example, is selectively composed of monomers that enable diversity of physiochemical and structural properties at the individual ptych level. With this approach, the scale and diversity can be built by choosing the number of ptychs in a linear sequence and the number of ptych variants at each position (see example library design – Figure 3). The size of the ptych is also flexible. For example, two monomers define a diptych; four monomers define tetraptychs (**Figure 3b**); and six monomers define hexaptychs (**Figure 3a)** and so forth.

By connecting ptychs via chemically cleavable linkers that can be cleaved under orthogonal conditions to those used in library synthesis and screening, the sequence of a ptych polymer can be directly read by mass spectrometry (MS). **Figure 2a** depicts a general polymer design with three tetraptychs, each consisting of four diversity-building block monomers and a cleavable linker. Cleavage of the linker monomers yields a mixture of the three tetraptychs. As a preliminary proof-of-concept, we used the phenyl-acetamido-methylene (PAM)[32] linker as a cleavable

monomer building block. This monomer is normally used as a cleavable linker between a peptide and resin in Boc solid-phase peptide synthesis (SPPS).[32] In our application, it provides an ester bond between ptychs that is stable to the Fmoc/tBu/Alloc protection strategy[33, 34] to build the polymers, but it can be readily cleaved using aqueous base such as ammonium hydroxide or sodium hydroxide. **Figure 2b** depicts a general polymer design with multiple tetraptychs, each consisting of a PAM linker and three diversity building-block monomers. Hydrolysis of the esters yields a mixture of the different tetraptychs.

The most important aspect of the ptych design is that building blocks can be selected so each ptych diversity element in a sequence has a unique molecular weight. As a result, each of the ptychs present in the mixture after cleavage can be identified by its mass using high-resolution LC-MS and an electrospray source on an LTQ-Orbitrap XL mass spectrometer that can detect molecular ions at the femtomole level; this provides three orders of magnitude more sensitive than the MS fragmentation methods used in peptide and protein sequencing.[35, 36] The approach can also sequence virtually any chemical building block, whereas MS fragmentation is extremely dependent on the nature of the fragmentation patterns of the backbone chemical bonds of the building blocks and requires extensive characterization in each case. The 10-μm diameter beads at ~0.2mmol/g loading typically carry ~100 fmols of compound that is readily detectable by high-resolution LC-MS (**Table S1**). Using the ptych design, polymer libraries can be synthesized on much smaller diameter beads to create much larger libraries using far fewer synthetic building blocks. Combined with the FAST platform, this enables screening and hit identification of much larger synthetic bead-based polymer libraries than has previously been possible.

In a validation study to determine the reliability of sequences from individual beads, a set of 90 individual 10-μm beads, each containing one of four possible unique sequences, were mixed and then picked from a plate and cleaved and sequenced. We were able to obtain the full correct

sequence for 81 of the picked beads (90%) (**Tables S3a-d)**. There were no incorrect sequence assignments in any of the validation samples in which ptychs were detected. The samples that did not yield an identifiable sequence also did not yield any ptych assignments, indicating the beads were likely not deposited correctly in the vial or were otherwise lost during sample processing, which is a consideration in microscale handling.

Figure 4 summarizes the screening process of a tetraptych polymer library. The library is incubated with a fluorescently labeled target, washed and then FAST screened to identify positive hits defined as fluorescently labeled beads that indicates binding to the target (**Figure 3a**). The plate and the hit location data from FAST are transferred to an automated fluorescence microscope and picking robot (ALS CellCelector) (**Video S1**) for preliminary hit quality control. Confirmed hit beads are individually transferred into vials and treated with cleavage solution to hydrolyze the backbone esters yielding a mixture of, in this case, tetraptychs which are sequenced by LCMS (**Figure 3b**). The hit sequences are re-synthesized for validation and further testing (**Figure 3c-d**).

Library Design and Screening Against Multiple Targets

To demonstrate the full process, we synthesized two large libraries (the non-natural polymer library 1 and 2 - NNP1 and NNP2). The NNP1 (**Figure 3a**) consists of six hexaptychs as the diversity elements in which each ptych was composed of four D - amino acids or glycine and an L-amino acid or glycine ester linked to a PAM linker. This produced polymers 36 monomers in length with an average molecular weight of ~5 kDa. Each ptych was designed to have one of 11 possible hexaptychs per diversity position (listed under Hexaptych 1, Hexaptych 2 etc. in **Figure 3a**), making a 11^6 or an ~1.77 million compound library. This corresponds to 66 hexaptychs, each of which was designed through a selection of monomers to give a range of physicochemical

properties in each sequence position and a unique molecular weight for each hexaptych. Before synthesizing the library, we confirmed synthetic feasibility of each individual hexaptych as a reference to determine the retention time by LC-MS and facilitate the sequencing of hits (**Table S4**). We made 75 copies of the library on 20-µm diameter monosized amino TentaGel microsphere resin beads with a loading of 0.27 mmol/g, which required only ~550 mg of bead resin.

Figure 3b shows the design for the NNP2 that consists of nine tetraptychs constituting a total polymer length of 36 monomers. For each ptych in the sequence, there were 10 possible tetraptychs, constituting a total of 90 tetraptychs and creating a library of 10^9 or one billion compounds. We believe this is the largest bead-based synthetic sequence-defined NNP library reported to date.[19] This library was constructed on 10-µm beads and required only 1.5 g of resin for production of three copies. The individual ptychs in this library were also synthesized as controls and validated for sequencing (**Table S5**). After constructing the libraries, all side-chain protecting groups were removed and the libraries were screened against multiple biological targets.

The NNP1 was screened against K-Ras and the asialoglycoprotein receptor 1 (ASGPR1). K-Ras is an oncology drug target that is mutated in ~30% of cancers and is associated with uncontrolled cell proliferation—particularly in deadly pancreatic and lung cancers.[37] ASGPR1 is a glycoprotein receptor predominantly found on the surface of liver hepatocytes and has been utilized as a mediator for liver-specific intracellular drug delivery of nucleic acid-based therapeutics.[38] Disruption of K-Ras binding to its singling partner requires a PPI inhibitor, and ASGPR1 is a C-type lectin glycan receptor; both are challenging targets for traditional small-molecule approaches and therefore represent interesting test cases for NNP ligands. Prior to library screening, we

performed a preliminary titration screen using varying concentrations of targets against the library and naked control beads (**Tables S7-S11**). Based on these results, we selected relative protein concentrations and set a background threshold for hit fluorescent signals. We then incubated the libraries with a fluorophore-labeled version of the target protein in blocking buffer (50% Odyssey buffer and 0.5% CHAPS) followed by a sequence of washes (**Table S6**).

In the case of the K-Ras screen, we also included fluorescently labeled recombinant Ras-binding domain (Raf) as a counter target to specifically screen for inhibitors that would bind to K-Ras and block its interaction with its downstream signaling partner Raf (**Figure 5a-b**). We labeled primary screening targets with dyes maximally excited at ~555 nm (AF555 or CF555) and counter targets with dyes maximally excited at ~647 nm wavelength (AF647 or CF647). After FAST screening, we identified a preliminary hit list of 381 K-Ras binding beads. We measured the mean fluorescence intensity (MFI) of each bead was measured and quantified results by fluorescence microscopy so we could prioritize the hits using both the overall brightness of the bead as a qualitative measure of binding affinity and the MFI ratio of the target to the counter target (**Figure** 5**b**). We identified a total of about 200 K-Ras selective binding beads, and a total of 120 beads were successfully isolated during bead picking. Most bead-picking losses were a result of bead aggregates that were difficult to separate using the capillary extractor.

The library size of NNP1 was 1.77M members on 20-μm beads, and it was screened at a 2.8-fold redundancy with 5M beads on two plates (2.5M beads per plate). We cross-validated hits via hit redundancy in the screen and found several hits with the same sequence (or 4-5 out of 6 hexaptychs in common, i.e., 67%-83% similarity within sequences). Hit sequences in the K-Ras screen were pooled into 14 clusters, and the most prevalent sequences in each case were

selected from each cluster. Similarly, 190 hits sequences from the ASGPR1 screen were grouped into 19 clusters and individual hits from each cluster were selected for hit confirmation.

After sequencing, hits were synthesized and purified by preparative high-performance LC (HPLC). A preliminary study of IL6R hits showed switching the backbone ester bonds to amides had only minor effects on measured binding affinities (**Table S12**), and as this greatly simplified hit resynthesis because all hits were prepared as the full backbone amide versions of the original hits. We measured resynthesized hit binding affinities for their respective targets using microscale thermophoresis (MST) (**Figure 5c**). The analysis confirmed binding in the majority of hits and revealed a number of hits in all cases; binding affinities were in the low nanomolar range and some were at the sub-nanomolar levels (see Table S13 for a complete list of hits and affinities). Equilibrium binding affinities (K_D) for K-Ras hits ranged from 18 – 180 nM, and 34 – 230 nM for ASGPR. Additional hits showed binding interactions indicated by changes in initial fluorescence (IF) on the MST instrument, but these were deprioritized because additional analyses are required to establish the K_D.

With a library size of 1B members on 10-μm beads, the NNP2 would require 200 plates to screen the entire library at 5M beads per plate. With a custom industrial robotic high-throughput screening (HTS) suite, this would be fairly straightforward—the entire library could be FAST screened in ~10 hr. In this proof of concept study, we manually screened a 10 – 15 million portion of the library, corresponding to 2 – 3 screening plates against three targets: IL-6, TNFα, and soluble IL-6 receptor (IL-6R). The targets are cytokines and a cytokine receptor involved in immunological signaling processes and are FDA-approved antibody-based therapeutic targets. As with the Ras – Raf screen, we performed the IL-6 screen using IL-6R labeled with a distinct fluorophore. This allows identification of polymers with compounds capable of blocking its

binding interaction with IL-6R. Similarly, we performed the screen against IL-6R using counter labeled IL-6. For the screens with both a target and anti-target, we observed MFI distribution ratios of the targets and anti-targets on beads in each screen. We selected and isolated the hits with the highest target to anti-target MFI ratios. For TNFα, we were primarily interested in finding selective affinity agents and did not conduct a competition screen. Binding affinities ranged from 25 – 500 nM for IL6 , 0.6 – 330 nM for IL6R and 23 – 270 nM for TNFα. The most potent hit in the NNP2 screen was a K_D of 620 pM against IL-6R (Table S13).

Some target hits required additional MST optimization to derive a reliable K_D value. These were deprioritized for further evaluation, and we focused only on the hits that gave a clear and immediate value of K_D (see Table S13 and Table S14 for a hit summary of all the five targets). We expect that MST optimization would increase the number of strong binders.

Discovery of PPIs and Receptor Mediated Intercellular Delivery Agents

To demonstrate the biological significance of NNPs, we focused on two targets (K-Ras and ASGPR) and tested the hits for functional biological activities. Our goal was first to confirm the hits identified in the screen inhibited the K-Ras-Raf interaction, (as screened for in our library screen design). We tested this in an MST competition binding assay (**Figure 6a**) and measured the K-Ras/Raf interaction alone, without any disruption. for which K_D values of 50 nm and 107 nm were obtained in two technical runs. Then, we measured the same interaction with three different K-Ras lead hits (16796-1-4, 16796-1-8 and 16796-1-13) separately pre-incubated with K-Ras at room temperature for 15 minutes. The K-Ras-Raf interaction was measured, and the three NNPs showed a range from complete inhibition (16796-1-8), partial inhibition (16796-1-13), and no inhibition (16796-1-4). We inspected the three hit-beads in the original library and the MFI ratio of the target (K-Ras-CF555) to the counter target (Raf-AF647) for these screen and found the ratio

for 16796-1-8 was significantly higher than that of the other two hit-beads (MFI ratios: 16796-1-9 = 2.55; 16796-1-4 = 1.89 and 16796-1-13 = 1.53). This suggests the FAST competition screening assay can enrich the NNP hits for functional inhibitors of PPIs, which is important for the biological activity. Second, the MFI ratio provides feedback on how to choose the hit beads for increased specificity and activity, which is important and useful data.

We focused on ASGPR as our second target for biological activity analysis. ASGPR is a glycoprotein receptor, and all the published ligands are glycans that so far mimic the native substrates.[39-41] We wanted to determine if NNP hits could specifically internalize liver cells with high expression of ASGPR but not cells lacking ASGPR expression; also, we wanted to discern if cell uptake was receptor-mediated. Therefore, we compared ASGPR-mediated uptake of the two lead NNP hits (16768-9-4 and 16768-9-6) in the HepG2 human hepatocarcinoma cell line and HEK293 cell line; these represent high-level and non-expressing cells, respectively (**Figure 6b**). As a positive control, we utilized the known ASGPR ligand N- acetylgalactosamine (GalNAc)[39] and a non-hit NNP from the same library (16796-1-14) as a negative control. All compounds were labeled with fluorescein and analyzed by flow cytometry (see Online Methods). Internalization of the two NNP hits was significantly higher in HepG2 cells compared to in HEK293 cells, even higher than of the positive control GalNAc, and certainly than the non-hit negative control. Furthermore, competitive cell uptake assays were performed utilizing the two NNP hits and the positive control trivalent GalNAc ligand for competition in receptor-mediated uptake in HepG2 cells with asialofetuin, a naturally occurring serum protein ASGPR[42] as the competing ligand (**Figure 6b**). In this assay, the cells were pre-incubated with two concentrations of asialofetuin, 20 μM and 60 μM, which represent 67 and 200-fold excess compared with the test compound (0.3 μM). An uptake assay was performed in HepG2 cells; NNP hits decreased with increasing concentrations

of asialofetuin, and mostly abolished with 60 μM of asialofetuin. These results indicate these ligands compete on the same receptor, and that uptake is ASGPR-mediated.

Biological Stability of NNP Hits

To demonstrate the superiority in stability of NNPs over peptides, we investigated the stability of an IL-6R hit from NNP2 to proteinase K and in human plasma. For the proteinase K stability assay, we compared the original hit with its fully L- amino acid variant. The L-amino acid variant was completely degraded within less than 2 hours in the presence of proteinase K (**Figure 6c**), whereas minimal degradation was observed for the NNP hit after an overnight incubation. For the stability assay in human plasma, the stability of the hit was compared to the natural peptide Angiotensin I. Angiotensin I was completely degraded within 4 hours in human plasma (**Figure 6c**), whereas NNP2 hits stayed largely intact even after overnight incubation.

Discussion

Using the FAST and ptych design, we demonstrated a mega-throughput screening and sequencing strategy for the discovery of potent and functional non-natural polymers. The novel ability to screen at the femtomole scale on 10-μm beads will enable the time and cost-effective screening with much larger chemical diversity than previously been reported. In this proof-of-concept study, we used commercially available building blocks and well-established solid phase chemistry to construct these first NNP libraries. Using the same approach, it is relatively straightforward to move into increasingly novel synthetic building blocks and coupling chemistry[43] as well as different cleavable linkers enabling an unlimited access to polymer diversity through library synthesis and empirical screening. With the library size and throughput, we have shown here that we can find low nanomolar to picomolar hits from primary screening

and have used this to validate biological selectivity and activity in a range of molecular targets. Besides strong binding affinity, we have also shown biological functionality of the hits of two targets as representative use cases; the ability to disrupt PPI by inhibition of the K-Ras/Raf interaction, and ASGPR-mediated cell uptake. As observed by others,[19] The NNPs show unique stability against biological degradation as described for the hits herein.

Transition melt temperatures across all hits ranged from 39 – 65^{O}C (data not shown), which is within the range of folded proteins of similar lengths (for example see [44]) and indicates that tertiary structure is probably important for binding of these hits. As the diversity of synthetic polymers expands, 3D structures of hits by crystallography or nuclear magnetic resonance (NMR) will most likely identify templates for novel secondary and tertiary structural motifs that can be rapidly refined by building focused libraries for secondary screening.

The overarching message is that the ptych design is extremely relevant. As structural motifs become better understood, individual ptychs can be engineered to promote intra- and inter-molecular recognition to stabilize structure and maximize affinity. We used a regular repeating ptych design for libraries, but more elaborate designs that use different numbers and types of monomers in ptych positions are possible; these will provide further chemical diversity for primary screening and strategies that allow optimization of hits.

In summary, we have demonstrated a method for stepping outside of the bounds of natural polymers and moving into a new field of designer polymers with completely new structure and function through empirical screening. The application area of this platform is vastly broad and includes therapeutics for drug discovery, affinity reagents for sensors and diagnostics, and reagents for catalysis.

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Author Contributions:

NC conceived of the concepts presented here with support from PBM and MAS. MAS, TS, XL, PB, JS, CR, AL, ATO, KY and LS ran the experiments. MAS, PBM and NC wrote the manuscript.

Competing Interests:

The authors state no competing interests.

Additional Information

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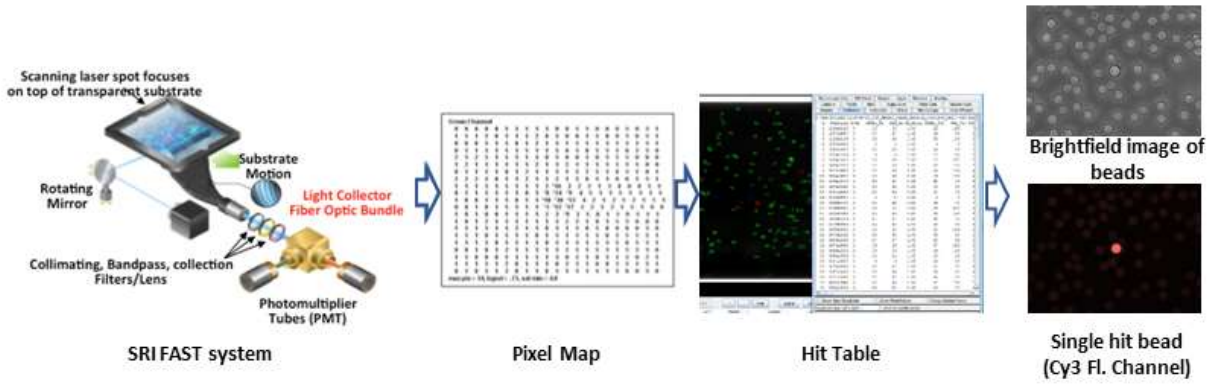
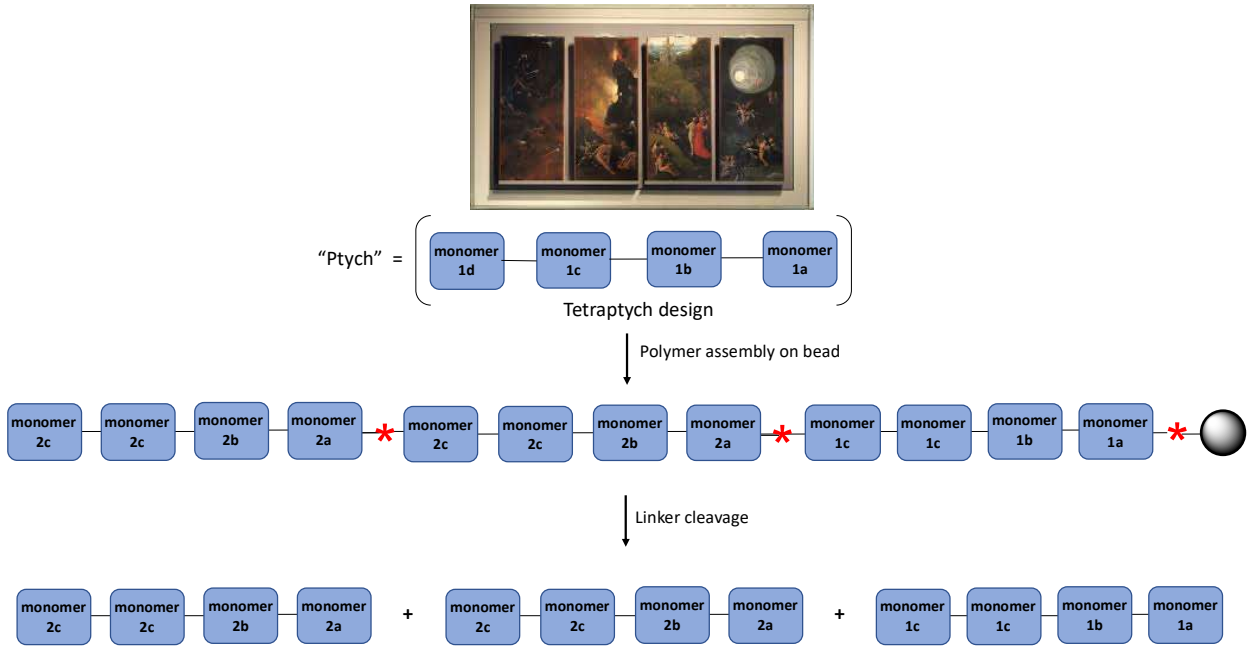


Figure 1. Diagram of the SRI FAST (Fiber-optic Array Scanning Technology) system. The FAST system uses rapid laser scanning with sensitive photomultiplier tube (PMT) fluorescence emission detection to rapidly generate a pixel map indicating the position of fluorescently-labeled beads. Analysis of the pixel map generates a hit table with cartesian coordinates and multiple calculated fluorescence metrics to detect hit beads high sensitivity and specificity. The coordinates of the hits can then be transferred to other microscopy systems for additional multi-wavelength imaging analysis or bead extraction**.**

a.



b.

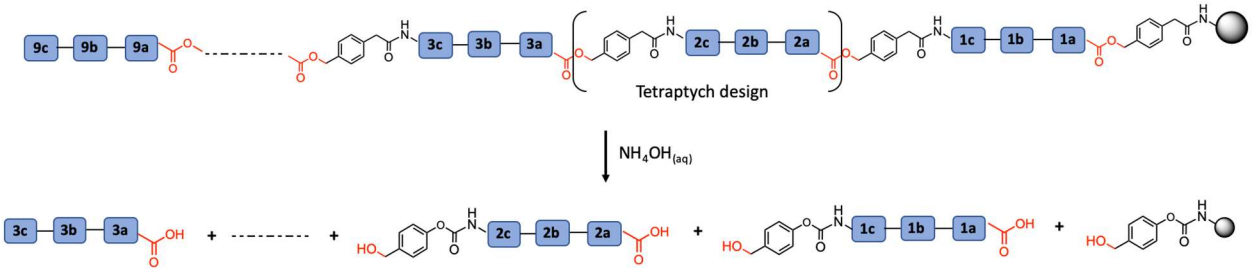
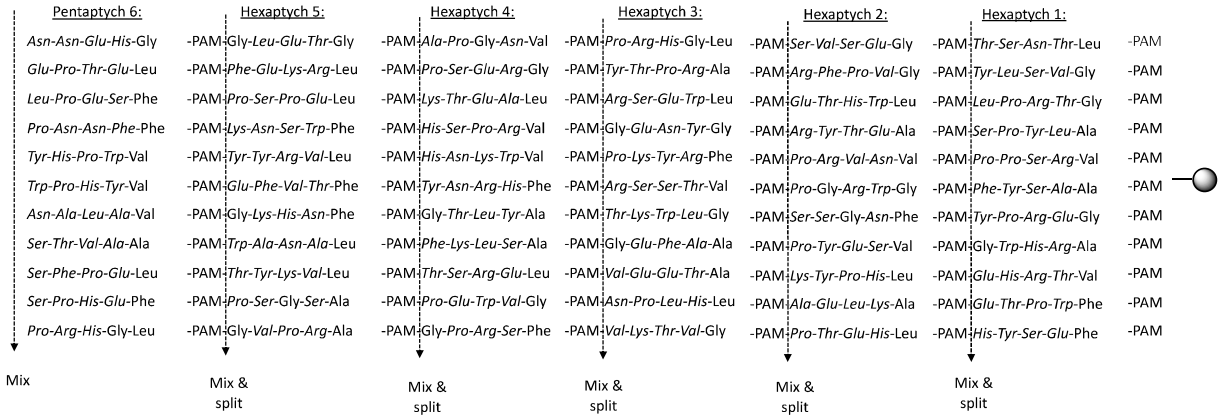


Figure 2. Description of ptych design. a. Example of a tetraptych painting (visioni dell'aldilà [Visions of the Hereafter] by Hieronymus Bosch) and the analogous ptych design of the polymers. Each polymer is constructed of a sequence of multiple triptychs, connected to each other by a cleavable linker. When sequenced, each polymer separately is subjected to the suitable cleavage reaction in which the linkers are

cleaved, to generate a mixture of the ptych fragments. b. Tetraptych-based polymer with PAM (phenyl-acetamido-methylene) with a chemical structure shown in black, as one of the monomers in each tetraptych and an ester bond (in red) as the cleavable linker. Once subjected to a solution of ammonium hydroxide, the ester linkers are cleaved to generate a mixture of all the tetraptychs constituting the polymer, allowing the sequence analysis of the polymer.

a.



b.

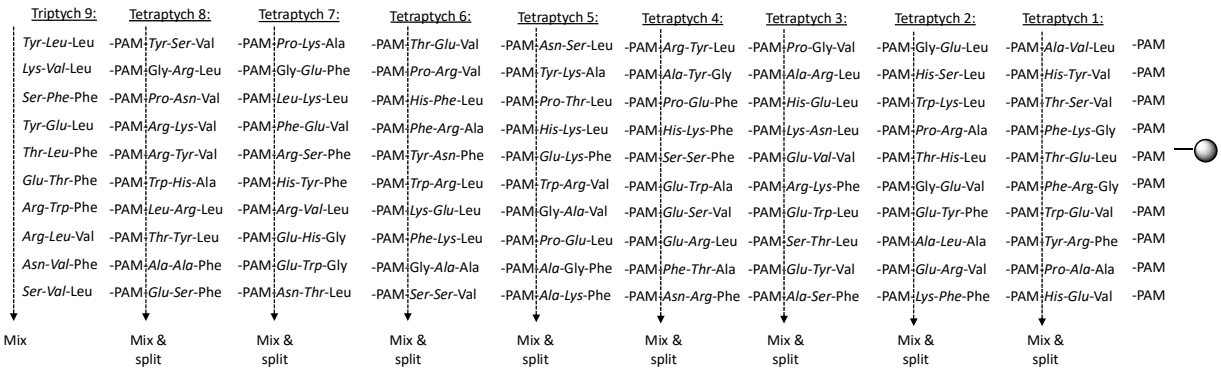
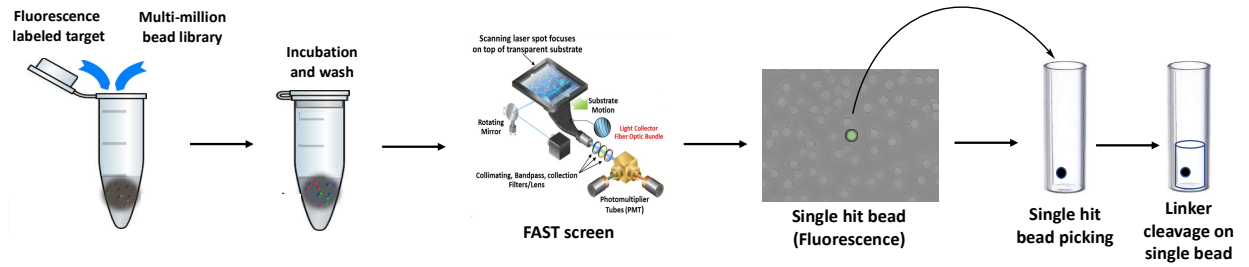
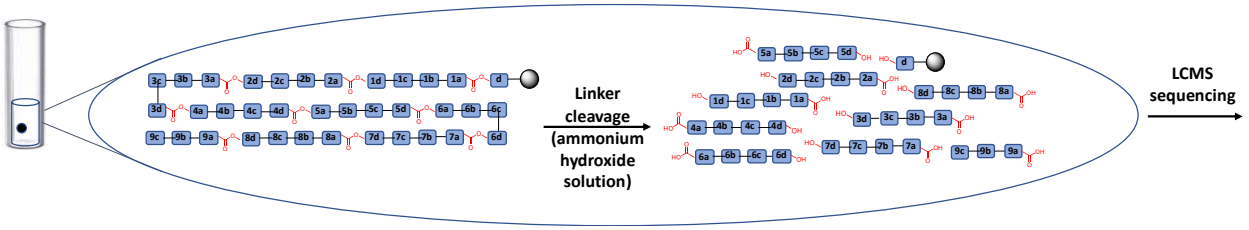


Figure 3. Libraries design and validation. a. Hexaptych design for the ~1.77 million compound library NNP1. b. Tetraptych design for the 1B compound library NNP2. ● indicates the resin beads. *Italics* indicates D-amino acid. In our design there are D-amino acids and PAM linker, which are both non-natural building blocks. We chose to use L-amino acids at the position next to PAM because some fmoc-L-amino acid-PAMs were commercially available, whereas the D form was not. We synthesized in solution the rest of the fmoc-L-amino acid-PAMs that were not commercially available.

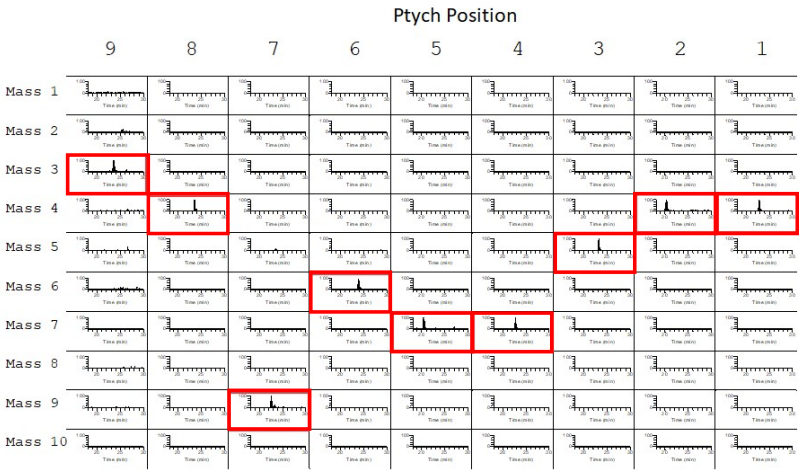
a.



b.



c.



d.

Fluorescein-(D)Asn-(D)Val-(L)Phe-PAM-(D)Tyr-(D)Ser-(L)Val-PAM-(D)Arg-(D)Ser-(L)Phe-PAM-(D)Phe-(D)Arg-(L)Ala-PAM-(D)Tyr-(D)Lys-(L)Ala-PAM-(D)Glu-(D)Arg-(L)Leu-PAM-(D)Ala-(D)Arg-(L)Leu-PAM-(D)Pro-(D)Arg-(L)Ala-PAM-(D)Phe-(D)Lys-Gly-PAM

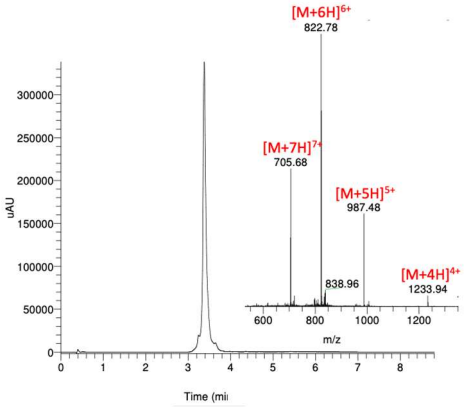
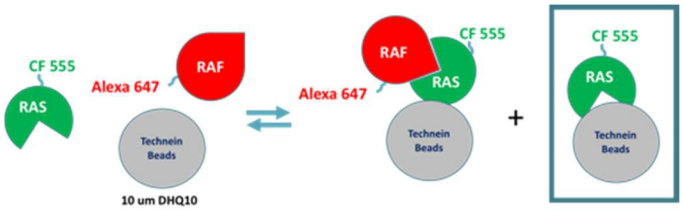


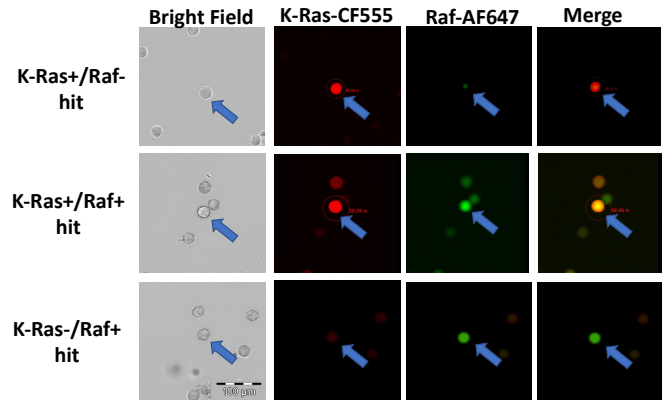
Figure 4. Summary of the screening process of a bead-based library. a. The polymer-bead library is incubated with a fluorescently labeled target of interest, washed and then screened in FAST to identify positive hits. After evaluating the single bead hits, each bead hit is picked into a separate LC-MS vial and is subjected to the cleavage solution. b. Upon treatment with ammonium hydroxide, all the esters are

hydrolyzed to yield a mixture of the different tetraptychs of a single sequence in each vial. c. Analysis of ptych fragment masses allows reconstruction of the ptychs into a specific sequence based on the library design. d. LC-MS chromatogram of a purified full-length 36-mer (9 tetraptych) polymer of which the sequence analysis is shown in Figure 3c. The compound hit was characterized by LC-MS (ESI); observed: 4931.9 ± 0.9 Da; calculated: 4932.5 Da.

a.



b.



c.

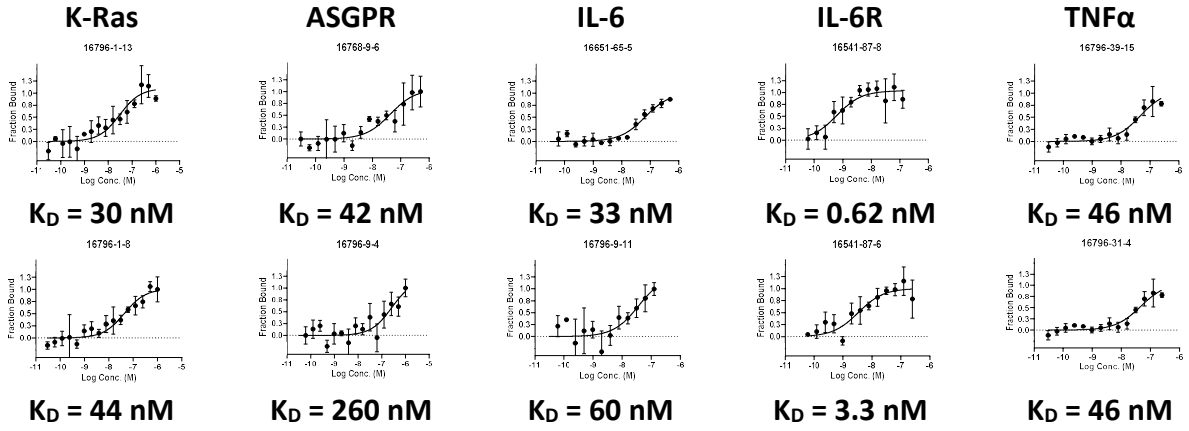
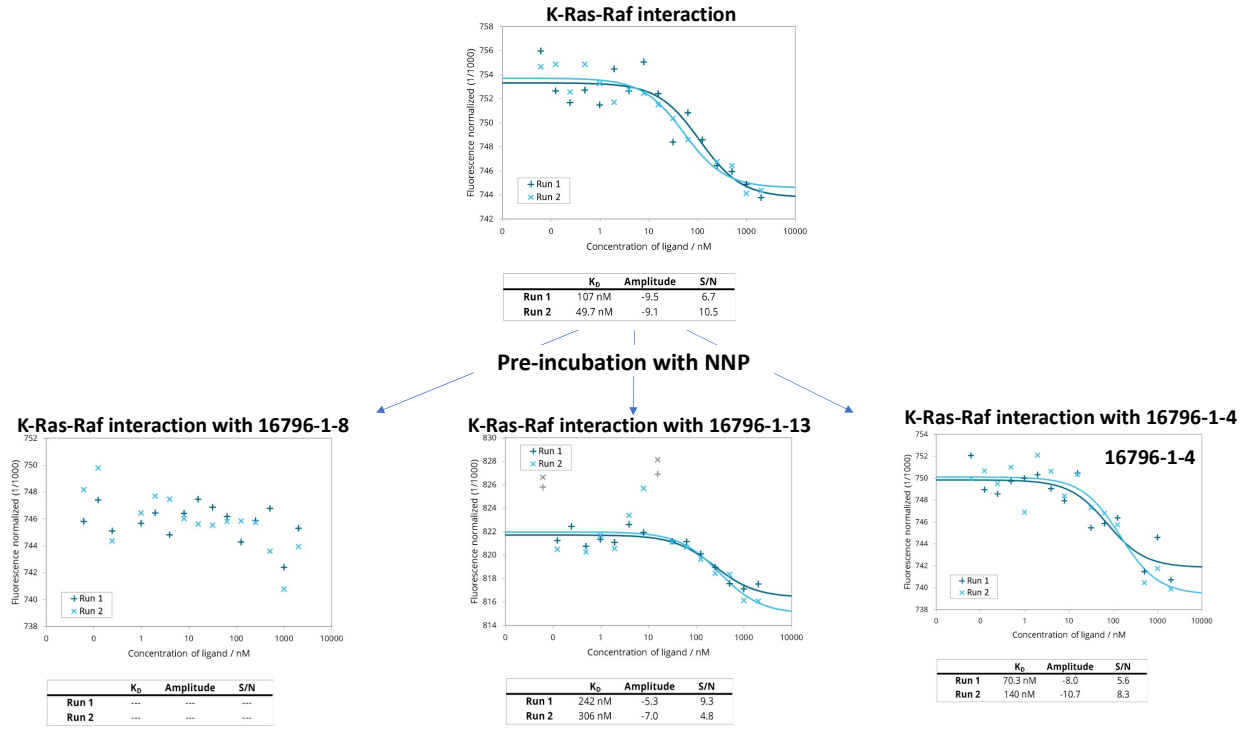


Figure 5. Screening data and Representative MST data. a. Competition screen of library beads against the primary target K-Ras labeled with CF555 in the presence of Raf-RBD as a counter target labeled with Alexa 647. Only the single positive beads with fluorescence at 555 nm were picked and sequenced. This strategy was performed to enrich for inhibitors that would bind to Ras while blocking its interaction with its downstream signaling partner Raf. b. Automated digital microscopic images demonstrating three types of hits. Top: single positive bead binds the primary target K-Ras but not the counter target Raf. Middle: double single positive bead binds both the primary target K-Ras and the counter target Raf. Bottom: single positive bead binds the counter target Raf but not the primary target K-Ras. c. Representative of MST data showing the binding of resynthesized purified polymers to their targets and the calculated KD values.

a.



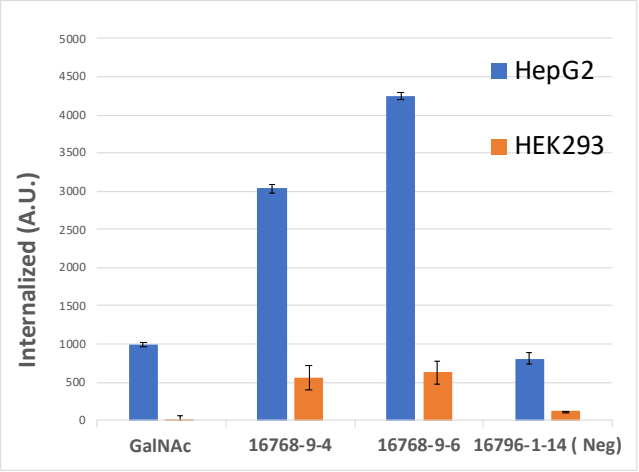
	K_D	Amplitude	S/N
Run 1	107 nM	-9.5	6.7
Run 2	49.7 nM	-9.1	10.5

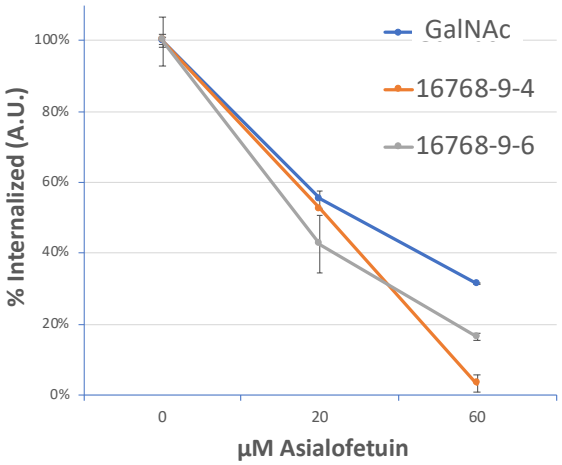
	K_D	Amplitude	S/N
Run 1	---	---	---
Run 2	---	---	---

	K_D	Amplitude	S/N
Run 1	242 nM	-5.3	9.3
Run 2	306 nM	-7.0	4.8

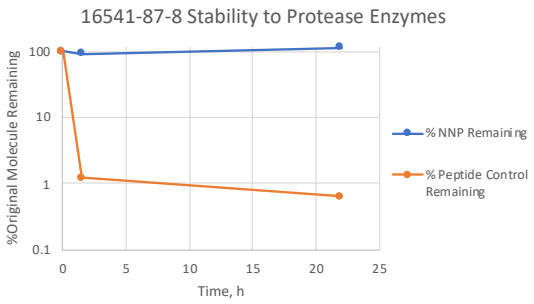
	K_D	Amplitude	S/N
Run 1	70.3 nM	-8.0	5.6
Run 2	140 nM	-10.7	8.3

b.





c.



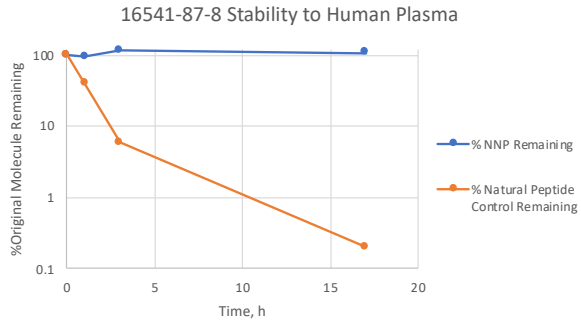


Figure 6. Biological relevance and stability of NNPs in biological matrices. a. K-Ras-Raf inhibition assay in MST. The K-Ras-Raf interaction was measured by MST in the absence (top) and in the presence (bottom) of 3 NNP hits. NNP 16796-1-8 showed complete inhibition, NNP 16796-1-13 showed partial inhibition, and NNP 16796-1-4 showed no inhibition. b. Left: cell uptake of GalNAc (positive control), two NNP hits (16768-9-4 and 16768-9-6) and a non-hit NNP (16796-1-14, negative control) in HepG2 (high on ASGPR) vs. HEK293 (low on ASGPR) cells lines. Right: competition uptake assay of GalNAc and two NNP hits (16768-9-4 and 16768-9-6) in HepG2 cells after preincubation with different concentrations of asialofetuin

(a naturally occurring serum protein ASGPR ligand). Decrease in cell uptake with increasing concentrations of asialofetuin indicates ASGPR-mediated uptake. c. Stability data for NNP 16541-87-8 in the presence of proteinase K (left) and in human plasma (right) compared with a natural peptide.

Online Methods

Library Synthesis. All libraries were synthesized using "one-bead-one-compound" and "mix-and-split" methods of solid-phase synthesis3 on TentaGel® amine 10 µm or 20 µm resin. The polymer library was synthesized using standard Fmoc-based amide coupling conditions, with 5.5 eq. acid monomer, 5 eq. HATU and 10 eq. DIPEA for 30 minutes each coupling. The Fmoc protecting group was removed using two treatments of 20% piperidine/DMF for 5 + 10 minutes. At the end of the polymer's construction, side chain protecting groups were removed with a cleavage cocktail of TFA:water:Triisopropylsilane 95:2.5:2.5 (v/v/v).

Screening of OBOC Libraries on FAST. FAST screening assay were specifically optimized for each target in terms of probe concentration, blocking and washing stringency etc. The probe binding to the D-AA library beads was performed in tubes. In brief, typically the libra bray or control beads were hydronated in the buffer (1% PEG, 50mM Tris, pH 7.5, 25% Odyssey PBS (LI-COR Biosciences)) for 30 min at RT with vortex followed by 1 min of sonication to break apart the large bead clumps. Beads were then centrifuged down, and the bead pallets were washed 2X with Odyssey/PBS buffer, the bead suspension was further filtered through a 30-um size cell strainer to remove bead aggregates. The concentration of the hydrated beads was determined based on bead counting using a hemocytometer. Aliquots of the bead suspension with the required number of beads then were centrifuged down and resuspended in blocking buffer (100% Odyssey, 0.5% Chaps, 200mM NaCl in PBS) and incubated overnight at RT with gentle rotating. After blocking, the beads were palleted and resuspended in 100% Odyssey buffer and then mixed at 1: 1 volume ratio) with the CF555 or AF555 conjugated probe that was diluted in the pre-binding buffer (1% Chaps, 400mM NaCl, 2mM TCEP, in PBS) to 2X final working concentration. The probe/library bead mixture were incubated for 1 hour at RT with gentle rotation to allow

probe bind to the library beads. After incubation, the beads were palleted and the unbound probes were aspirated, followed by 3 X of washes with 10 ml of wash buffer ((0.5% Chaps, 200mM NaCl, 1mM TCEP in PBS)), 5 mins/time and additional 2 X washes with 10ml of 0.5% Chaps /PBS. After the last centrifugation, the buffer was aspirated but left final ~ 500ul to resuspend the beads in this residual buffer and sonicate them for 30 second to dissociated newly formed bead clumps. Then 1.5 ml prepared 0.3% low melting agarose (LMT) that were kept in 37C water-bath before use were added to into the re-sonicated beads to make the bead/soft agar suspension.

Beads in the LMT suspension were then transferred and evenly plated onto the FAST slide (the screening plates) and then the slides were placed on cold tray to accelerate the curing and immobilization of the beads. Following the gel formation, a layer of mounting medium (e.g. 500 ul Live-Cell medium) was gently placed on top of the gel to keep the beads from rapid drying or photo-quenching of the fluorescence. The sample slides (plates) were scanned and analyzed using the FAST system.

Bead Analysis and Picking using ALS CellCelector. which the hits were identified and then coordinate list of the hit were generated and transferred to the CellCelector for Automated Digital Microscopy (ADM)'s further imaging the hits with multiple channels at higher resolution. Hit beads were then QC/QA reviewed based on the morphology and fluorescence staining, and fluorescence of selected channels were quantified to rank the top hits for isolation, then each selected single hit-beads was isolated with CellCelector individually into the HPLC vials in ddH_2O for MS based sequencing.

Processing and sequence analysis of picked beads

Beads were deposited directly into glass autosampler vials containing deionized water. The vials were inserted into deep-well 96-well plates and dried in a vacuum centrifugal concentrator (GeneVac II Plus) at 40 °C. To hydrolyze the inter-ptych ester linkages 50 uL of 7% aqueous ammonium hydroxide or 150 mM NaOH was added and the samples incubated at 37 °C for 6 h and then evaporated under vacuum in the centrifugal concentrator. The samples were then prepared for analysis by adding 50 uL of 5% acetonitrile in water with 0.1% formic acid. and analyzed by capillary reversed-phase gradient LC-MS/MS using an Agilent capillary HPLC pump and CTC Analytics autosampler coupled to an LTQ-Orbitrap mass spectrometry system. Expected masses of hydrolysis products were loaded into an inclusion list for targeted MS/MS when detected above threshold in a high resolution Orbitrap scan. Data analysis used both MS and MS/MS data to assign high confidence hits for assembling sequences for the hits.

Hit Re-Synthesis. Hits were synthesized on ChemMatrix RAM resin by an automated peptide synthesizer using standard Fmoc-based amide coupling conditions with DIC/Oxyma as the coupling reagents.

Hit Characterization. Hits were tested on Microscale Thermophoresis (MST) to determine their binding affinity to various targets. MST experiments were performed on a Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany). Measurements were performed in triplicate with 16 point, two fold dilutions series. The buffer for the Asialoglycoprotein receptor contained 20mM HEPES pH 7.4, 150mM NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% Pluronic F-127, and 1mM DTT; for IL-6 contained 20mM HEPES pH 7.4, 150mM KCl, 10mM $MgCl_2$, and 0.1% Pluronic F-127; for the soluble IL-6 receptor 20mM HEPES pH 7.4, 150mM NaCl, 10mM $MgCl_2$, and 0.05% Tween-20; for K-Ras 20mM HEPES pH 7.4, 150mM NaCl, 10mM $MgCl_2$, 0.05% Tween-

20 and 1mM DTT; for TNFα 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.05% Polysorbate-20. Triplicate data was analyzed using MO.AffinityAnalysis software (NanoTemper Technologies GmbH).

K-Ras- Raf inhibition assay. Interaction between the target protein K-Ras4B G12V and the ligand protein Raf-RBD was investigated using a MicroScale Thermophoresis (HTS-MST) assay in the absence and presence of three NNP hits: 16796-1-4, 16796-1-8 and 16796-1-13. Loading of KRas4B G12V with the GppNHp (non-hydrolyzable GTP analog) was performed according the following protocol: The 200-µM stock solution of the target protein was diluted to 10 µM in 20 mM HEPES pH 8.0, 150 mM NaCl, 10 mM MgCl2, 1 mM TCEP, 0.05% Tween-20 (total volume 110 µL). 10 µL were set aside for later labeling quality control. EDTA pH 8.0 (stock concentration 10 mM) was added to the protein solution to a final concentration of 80 µM. GppNHp (stock concentration 50 mM) was added to the protein solution to a final concentration of 750 µM. The solution was incubated at 30°C for 2 hours (PCR tube) and then placed on ice for 2 minutes. $MgCl_2$ was added to the protein solution to a final concentration of 100 mM. The resulting protein solution was buffer exchanged into 100 mM HEPES pH 6.5, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM TCEP. The resulting concentration of the target protein was 8.9 µM, which was used for Maleimide-647-dye labeling. <u>For the interaction between KRas4B G12V and Raf with no NNP present</u> two technical runs with the same samples were performed between GTP-loaded K-Ras4B G12V and Raf-RBD in the same buffer conditions that were used to test the interaction between K-Ras and the NNP hits: 20 mM HEPES pH 7.4, 150 mM NaCl, 10 mM MgCl2, 1 mM DTT, 0.05% Tween-20. <u>For the interaction between KRas4B G12V and Raf in the presence of NNPs</u> the labeled target protein KRas4B G12V was diluted to 10 nM in assay buffer containing 2 µM NNP and incubated at room temperature for 15 min. This solution was then mixed with the ligand protein Raf serial dilution 1:1 to yield the final assay samples with 5 nM target protein and 1 µM NNP.

Cell culture for ASGPR uptake assay. The HEK293T (human embryonic kidney cells) and human hepatoma HepG2 cells were grown according to the protocols provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). When cells reached cell were seeded at ~1.5 X 10^5cells/well in 24-well culture plate for the uptake assay. After at least 16 hours of culture to allow cell attach and equilibrate, the compound treatment was set up for the uptake assay.

ASGRPR uptake assay. The fluorescein-labeled Techneins or fluorescein-labeled N-acetylgalactosamine (GalNAc) recognizing ASGRPR were added to wells at indicated concentrations and incubated for 2 hours. Two plates were prepared for each treatment condition, one serving as 4◦C no internalization control that was kept on ice during incubation, while the second plate was incubated at 37◦C to allow for energy dependent internalization. Following the incubation period, all plates were placed on ice and washed three times with ice-cold PBS/3% BSA/2 mM EDTA and then lifted with trypsin. Cells were transferred to a 96-well round bottom plates in FACS buffer (BD Biosciences, San Jose, CA, USA). Cells were then analyzed by flowcytometry using LSR-II with HTS sampler (BD Biosciences, San Jose, CA, USA). Data (mean fluorescence intensities) and further analyzed using Flowjo software (BD Biosciences, San Jose, CA, USA). The internalized fraction was expressed as the difference between the corresponding 4◦C and 37 ◦C MFIs as previously described.[38]

For the competitive uptake assay with the natural ligand of ASGPR (asialofetuin), cells were pre-incubated with 20 μM and 60 μM asialofetuin (Sigma-Aldrich, USA) on ice or at 37◦C for 1.5 hours, followed by the treatment with compounds for additional 2 hours before flow cytometric analysis as described above. The reduction of the uptake under asialofetuin competition was expressed by the percentage against the same treatment condition without asialofetuin.[45]

Stability Assays. For proteinase K stability, solutions of each tested compound (200 µM) in 10% DMSO and 20 mM Tris HCl at pH 8 were prepared. Proteinase K was added to a final concentration of 100 µg/mL and 100 µM of the tested compound in 5% DMSO and 10 mM Tris HCl at pH 8. The solutions were incubated at 37 °C and aliquots after 0 h, 1.5 h and 16 h were analyzed by LC-MS.

For human plasma stability, lyophilized human plasma (Sigma) was reconstituted in sterile water for injection, aliquoted into 200 uL aliquots and stored frozen at -80 °C prior to the stability studies. For the stability studies three aliquots per NNP were thawed at room temperature. An additional set of three aliquots for a positive control peptide (Angiotensin I, Sigma) were also thawed. The incubations for the plasma stability were initiated by mixing 2 uL of a 2 mM stock solution of NNP 16541-87-8 or positive control in DMSO with the 200 uL thawed plasma aliquot. After briefly vortex-mixing, 50 uL zero-time-point samples were removed, mixed with 50 uL of water and 400 uL of acetonitrile and frozen on dry ice until all time point samples had been collected. The samples were incubated at 37 °C with samples removed and water/acetonitrile added at 1 h, 3 h and 17 h. Proteins were precipitated by centrifuging the samples at 17,000 G, 4 °C for 1 h. The supernatants were removed and concentrated in a centrifugal vacuum concentrator (GeneVac Genie II) at 45 °C until the volume had been reduced to ~60 uL. The samples were then diluted with 95% water 5% Acetonitrile and 0.1% Formic Acid to volume of 200 uL, 2 uL of an internal standard peptide (Val5-Angiotensin I, Sigma) were added prior to samples analysis by LC-MS on the LTQ-Orbitrap XL system described above.