PNA-Based Dynamic Combinatorial libraries (PDCL) and screening of lectins

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ABSTRACT

Selections from dynamic combinatorial libraries (DCL) benefit from the dynamic nature of the library that can change constitution upon addition of a selection pressure, such as ligands binding to a protein. This technology has been predominantly used with small molecules interacting with each other through reversible covalent interaction. However, application of this technology in biomedical research and drug discovery has been limited by the reversibility of covalent exchange and the analytical deconvolution of small molecule fragments. Here we report a supramolecular approach based on the use of a constant short PNA tag to direct the combinatorial pairing of fragment. This PNA tag yields fast exchange kinetics, while still delivering the benefits of cooperativity, and provides favourable properties for analytical deconvolution by MALDI. A selection of > 6 000 assemblies of glycans (mono-, di-, tri-saccharides) targeting AFL, a lectin from pathogenic fungus, yielded a 95 nM assembly, nearly three orders of magnitude better in affinity than the corresponding glycan alone (41 μ M).

Keywords:

Dynamic Combinatorial libraries

Peptide Nucleic Acid (PNA)

Screening

Aspergillus fumigatus lectin (AFL)

Ralstonia solanacearum lectin (RSL)

1. Introduction

Drug discovery is empowered by screening technologies,¹ however, synthesizing and screening diverse libraries containing meaningful molecular complexity is not an easy task. This challenge continues to inspire the development of enabling technologies, some of which have been embraced in the drug discovery process such as phage display,²⁻⁴ fragment-based approach,⁵ and DNA-encoded libraries.⁶⁻¹⁰ Dynamic combinatorial library (DCL) offers an elegant solution to the generation of libraries by linking fragments transiently through reversible covalent chemistry, thus generating higher molecular diversity that can adapt to a selection pressure from a target of interest (Figure 1A).¹¹⁻¹⁸ Following the seminal report of Huc and Lehn demonstrating the selection of a carbonic anhydrase inhibitor from a library a dynamic imine,¹¹ progress has been hampered by two main challenges; identifying robust and general chemistries that allows dynamic exchange on the time-scale of the screening conditions and deconvolution of the selected product. While a number of reactions have been reported in DCL,^{11-12, 19-21} difference in reactivity amongst the fragments can lead to biases in the product distribution and hence, identification of the fittest combination.²²⁻²³ Ideally, for screens against a protein, fragment recombination is highly dynamic at conditions close to physiological environment of the protein to allow for the constitution of the library to respond to the selection pressure. This challenge is exacerbated by the limitations imposed by the

Figure 1. A) Dynamic Combinatorial library (DCL), B) PNA-based Dynamic Combinatorial library (PDCL).

Figure 2. A) General scheme selection protocol, B) Schematic representation of the correlation between stringency of washes and retained compound on the protein, C) Mass spectrum of the starting library (Panel 1), flow through (Panel 2), first wash (Panel 3), 6th wash (Panel 4) and elution (Panel 5) of a selection of fucose vs glucose-PNA conjugates against RSL, D) Percentage based on intensities of the peaks of PNA-fucose and PNA-glucose obtained by MALDI of the starting library and elution step of the selections done with RSL or no protein by using the 1:1 or 1:99 fucose/glucose libraries.

selection conditions and, is proportional to library size. For instance, the combination of two sets of 100 fragments can generate a combinatorial output of 10 000 combinations. Under ideal thermodynamic equilibrium, each permutation will be present at 0.01% of the library concentration; however, in the presence of the selection pressure, the fittest combination could rise to 1% of the library concentration. The larger the fragment set, the more amplification for a given combination can be achieved following a selection pressure. Recently, the groups of Yixin Zhang and Xiaoyu Li reported important advances in the field by including DNA-tags that direct the association of the fragments and encode their structure, thus enabling sequencing technologies to analyze the selection outcome.²⁴⁻²⁷ We have recently demonstrated that the oligomeric interaction of a lectin with epithelial cells can be inhibited with a fucose-PNA conjugate that does not form stable dimer in solution but benefits from cooperativity between ligand-protein interaction and PNA hybridization.²⁸ Herein, we explore the utility of such highly dynamic assemblies for DCC screening (Figure 1B) and demonstrate a simple MALDI analysis of the selection outcome (libraries contain > 6 000 possible assemblies).

2. Results and discussion

The use of hybridization to pair ligand and gain in affinity through cooperative interactions with a target is well established.²⁹⁻³⁴ However, the majority of studies reported so far used relatively long duplexes to achieve stable pairing between ligands. We showed that in one case, this could be reduced to an extremely short length PNA oligomer (4-mer) while preserving the cooperativity.²⁸ Peptide nucleic acids (PNAs) is a convenient platform to tag ligands since its chemistry is comparatively more permissive than that of DNA and is compatible with standard solid phase synthesis of the library.³⁵⁻³⁸ The use of such short

sequences ensures a highly dynamic exchange (duplex $K_d > 1 \mu\text{M}$; $k_{on} \approx 10^6 \text{ s}^{-1} \text{ M}^{-1}$; $k_{off} > 1 \text{ s}^{-1}$).³⁹ Short PNA ionize very well by MALDI and a 4-mer PNA is sufficient to push the molecular weight of any adduct outside of the noise arising from the matrix. We reasoned that a constant tag in a library could harmonize the detection of selected library member in a MALDI-based MS-analysis. This deconvolution would be desirable in accelerating the analysis of selection compared to DNA sequencing approaches.

To investigate the utility of the PDCL in order to identify new bidentate ligands, we focused our efforts on the discovery of new binders for medically relevant lectin. AFL (or FleA) is a carbohydrate-binding protein from *Aspergillus fumigatus* lectin which plays a key role in the *A. fumigatus* colonization of human host and the development of the infection.⁴⁰⁻⁴² There are current fungicides against *A. fumigatus*,⁴³⁻⁴⁴ however, the increasing resistance of *A. fumigatus* strains to these treatments urges alternative approaches targeting different pathways.⁴⁵⁻⁴⁸ The best ligand reported to date is a hexavalent cluster with K_d of 18 nM.⁴⁸ The bacterial lectin from *Ralstonia solanacearum* (RSL) has strong similarity with AFL and almost identical fucose binding sites.⁴⁹ Binding of this lectin to epithelial glycans is sufficient to induce membrane invagination and uptake.⁵⁰⁻⁵¹

2.1. Selection and detection of PNA-Dynamic Combinatorial library screens

We envisioned the selection to be performed by affinity purification of the fittest assembly as shown in Figure 2A. The library, which is in dynamic equilibrium, is challenged with the target, immobilized on magnetic beads. During the incubation, a self-sorting should drive the equilibrium towards enrichment of the fittest assembly which remains associated to the protein. Washing the beads then removes the excess library members.

Figure 3. A) General scheme for the split and mix library synthesis. B) Structure of library building blocks.

However, there is a fine balance between the stringency of the equilibration and washes (the more stringent, the higher the probability to drive the equilibrium to the optimal solution and remove suboptimal binders) and detection (the more stringent the washes, the lower the quantity of recovered material), as illustrated in Figure 2B. The parameters are likely to be unique for each library/target combination and a rapid assessment of a selection would greatly facilitate optimization. This work flow was tested for RSL with two fragments: fucose that binds with low μM affinity and glucose which does not have measurable affinity. The ratio of the two fragments was measured by integrating the intensity of the MS peaks obtained by MALDI. As shown in Figure 2C, only the glucose fragment is detected in the selection flow through, and the desired fucose fragment can be recovered using an elution at 95 °C. It was observed that the addition of detergent (0.05% Tween-20) into the buffer was crucial in order to remove of all the non-binders. As a negative control, the same library was subjected to a selection against no protein (only the streptavidin beads), and none of the members of the library members were detected in the elution (Fig. 2D). Next, we envision mimicking a larger PDCL by including only 1% of the fucose-PNA fragment (i.e. mimicking a 100 x100 fragment = 10 000 assemblies). This library was screened under the same conditions (0.05 μg of protein immobilized on 10 μL of resin, 10 pmol of Fuc-PNA, 2 nmol of Glc-PNA, incubated in 50 μL of buffer; 6 x 50 μL washes), and only the fucose fragment was detected upon elution (Fig. 2D). This shows the potential of dynamic combinatorial libraries in which starting from a 0.01% of Fuc-Fuc dimer in the library, addition of the protein and equilibration for 1h (i.e. raising the concentration of Fuc-Fuc up to 1% of library) is sufficient to yield detectable amount of

product. However, under the same conditions, selection against AFL did not afford any product. The weaker affinity of AFL for fucose or the lack of structural diversity in this pilot study were thought to contribute to this unproductive selection. We thus set out to prepare a library which included 2'-fucosyllactose, as a more representative member of epithelial glycans involved in lectin adhesion. Furthermore, this validation was performed with a palindromic PNA tag (GGCC) which can self-hybridized but does not allow to discriminate between two members self-pairing individually or forming a heterodimer (AA + BB vs AB). To avoid this ambiguity, the library was designed with sequences that are not palindromic and can only hybridize to the designed complementary strand.

2.2. Synthesis of the 5,000-member library

A library (150 different members, resulting in 5 000 assemblies) of different glycans conjugated to the PNA via diverse linkers (varying length, flexibility and electrostatic nature) and position (glycan on the same end or opposite ends of the hybridization complex) was prepared using split and mix synthesis⁵²⁻⁵³ (Fig. 3A). The resins bearing the different PNA sequence necessary for the assembly, CGGC with an amine for functionalization at the N-terminus and C-terminus that are complementary to GCCG with an amine for functionalization at the N-terminus were mixed and split into five pools. To avoid mass redundancy, a serine at the C-terminus of the third sequence was included. Standard peptide couplings were used to introduce the linkers (a flexible PEG, a rigid pentaproline, a positively charged pentalysine and a negatively charged pentaglutamic acid) and the pools were mixed and split again for the conjugation to 10

Figure 4. A) PNA-glycan conjugates nomenclature. B) T_m measurement of AFL and RSL. C) Mass spectrum of the 5,000 library selection. Starting library (Panel 1), elution of the AFL selection (Panel 2), elution of the RSL selection washing at r.t (Panel 3) and elution of the RSL selection washing at 60°C (Panel 4). Black dots denote the [M+Na]⁺.

different glycans (mono-, di- and trisaccharide). The glycans were functionalized with an azide except the Glc-NAc cluster which contained a carboxylic acid moiety. In the latter case, it was conjugation via amide bond formation, in all other cases, the linker was extended with propargyl glycine in order to perform the CuAAC conjugation (see scheme S1 for full synthetic details). Each pool was analyzed by MALDI to confirm the completion of the synthetic path (>10% truncated sequences present in the library). One side reaction was noted during this analysis, partial acylation of a lysine in compounds containing the pentalysine linker. Since this side reaction was not as detrimental to the library, no efforts were invested in circumventing this side reaction. Considering this permutation, more than 6 000 assemblies are present in the dynamic library.

2.3. Screening of AFL and RSL

With this library in hand, we set out to perform the screen against AFL and RSL (Figure 4). As discussed in the screening optimization, a key factor in the selection is the stringency of the selection conditions. The simplest factor to change is the temperature at which the washing steps are done. For this purpose, the T_m of both AFL and RSL was measured using Differential Scanning Fluorimetry (DSF), yielding a T_m of 52.1°C for AFL and 87.5°C for RSL (Fig. 4B) which is in agreement with previously reported values.^{40,54} Keeping a margin of 20°C to avoid protein denaturation, the washing steps for AFL

and RSL could be performed at up to 30°C and 60°C respectively. With this information in hand, the screening the PDCL (>6 000 assemblies) was performed according to the same conditions used in the optimization (0.05 µg of protein immobilized on 10 µL of resin, 10 pmol of each member of the library, incubated in 1 mL of buffer; 6 x 50 µL washes). While MALDI analysis of the starting library is so densely packed with signal (MW range of the library: 1.5 KDa - 3.3 KDa), it appears as a noisy baseline, the analysis of product recovered after selection against AFL shows distinct peaks (Fig. 4C, Panel 1). The four most intense peaks contain 2'fucosyl lactose, exclusively pairing ligand at opposite ends of the PNA duplex (M+N, no significant selection of M+C). Selection against of RSL at room temperature afforded a larger set of compounds. While this selection yielded exclusively assemblies bearing the 2'fucosyl lactose, there is no clear selection for a particular linker type (Fig. 4C, Panel 3). Performing the selection under more stringent conditions at 60 °C yielded a single prominent assembly: 2'fucosyl lactose linked at opposite ends with a pentalysine linker (MKA+NKA, Fig. 4C, Panel 4).

2.4. Validation of the hits

In order to validate the results obtained during the selection of the PDCL, the sequences selected for both AFL and RSL were individually synthesized. For comparison, the sequence bearing the PEG linker (X) was also included since it was used in the

selection performed in Figure 2 and had been previously reported.²⁸ The affinity of AFL and RSL for the glycan itself, 2'-fucosyl lactose, is vastly different (41 μ M and 0.38 μ M for AFL and RSL respectively). While the linker-PNA adduct has a slight detrimental impact on the affinity towards RSL, it yielded very significant gain in affinity for AFL (41 μ M $\rightarrow$ 0.34 μ M) with the pentalysine linker (MKA-N). Indeed, the three best assemblies for AFL contain a pentalysine linker (entry 7, 12, 13) with an affinity ranging from 0.094 μ M to 0.16 μ M. While the difference between assemblies with a single pentalysine linker and two lysine linker is moderate (entry 7, 10, 11, 12 vs 13), the difference between the best assembly (two pentalysine linkers and the assembly bearing two neutral linkers of comparable atomic length (two PEGs) is notable (0.094 vs > 1.0 μ M, entry 13 vs 9). Overall, the affinities measured closely parallel the results obtained in the selection and the best assembly showed a 430-fold enhancement of affinity relative to the trisaccharide glycan alone. Similar trends are evident in the RSL selection with the three highest affinity assemblies having a lysine linker (entry 7, 11, 12). However, the comparison with the selection performed at 60 °C should be treated with caution since the entropic contribution to binding may be significantly affected between the SPR experiment performed at room temperature and the selection performed at 60 °C. Nonetheless, the highest affinity assembly (0.0051 μ M, entry 11) represent a significant affinity gain with respect to the glycan alone (75-fold gain) or an assembly with comparable linker length but lacking charges (4 fold, entry 8).

Entry	Linkers	AFL (μ M)	RSL (μ M)
1	2'-Fucosyllactose	41	0.38
2	MOA-N	>2.0	0.91
3	MXA-N	>2.0	0.61
4	MKA-N	0.34	0.62
5	MOA-NOA	0.29	0.023
6	MOA-NXA	0.42	0.023
7	MOA-NKA	0.16	0.0055
8	MXA-NOA	0.55	0.021
9	MXA-NXA	>1.0	0.034
10	MXA-NKA	0.17	0.0087
11	MKA-NOA	0.18	0.0051
12	MKA-NXA	0.16	0.0063
13	MKA-NKA	0.095	0.012

Figure 5. A) Values of the K_D measured by SPR of the individually re-synthesized hits of the 5,000-library selection for AFL and RSL.

3. Conclusion

The present work demonstrates the selection of highly dynamic supramolecular assemblies, PDCL, with a simple MALDI readout. The PNA tag facilitates MALDI analysis by providing a more homogeneous ionization and favorable mass range. The fact that a 4-mer PNA is sufficient to achieve cooperativity between ligands makes the preparation of libraries, fast, simple and compatible with solid phase split and mix synthesis. The

constant PNA tag ensures homogenous distribution of assemblies while the fast kinetics of hybridization enables fast equilibration following a selection pressure. We anticipate that further finetuning can be achieved with different PNA length and oligomerization order which may be important for specific targets. The selection and MALDI analysis can be performed in 3 h and multiple experiments can be performed in parallel. The identification of an assembly with a dramatic gain of affinity for AFL (95 nM) compared to the glycan alone (41 μ M) illustrates the potential of PDCL. The affinity of this dimeric assembly is within five fold of a hexameric cluster.⁴⁸

4. Experimental

4.1 Synthesis of PNA sequences

NovaPEG® Rink amide resin (5.0 mg, 0.44 mmol/g, NovaBiochem) was swollen in CH_2Cl_2 for 10 min and washed two times with DMF. Iterative cycles of amide coupling, capping of the resin, deprotection of the main chain protecting group and a final CuAAC coupling were done in order to synthesize the PNA-sugar conjugate. Once all the sequence was synthesized, the desired compound was cleaved from the resin (TFA) and acetates protecting groups removed (NaOH). For individual sequence syntheses, the compounds were purified using HPLC. For mix and split combinatorial synthesis, the library was used without further purification. Full synthetic details and physical characterization can be found in the SI.

4.2. Synthesis of the glycans

All the glycans used in the library have been synthesized as previously described. 2'-fucosyllactose,⁵⁵ xylose,⁵⁶ glucose,²⁸ glucosamine,⁵⁵ maltose,⁵⁵ cellobiose,⁵⁵ Glc-NAc,⁵⁵ Gal-SPhe,⁵⁵ Mannose⁵⁵ and Glc-NAc cluster.

4.3 Synthesis of the library with split and mix

NovaPEG® Rink amide resin (150 mg, 0.44 mmol/g, NovaBiochem) were divided into 3 columns and the synthesis of three different PNA sequences was done following the protocol previously reported for peptide synthesis:⁵⁷

M (Main): Resin-cGgC-Mtt

N' strand (N): Resin-Ser-gCcG-Mtt

C' strand (C): Resin-Lys(Alloc)-gCcG-Ac

(Capital letters used Ser modified PNA, lower case letter use for unmodified PNA) (C' to N')

The Mtt or Alloc groups were removed and the 3 pools were mixed. Next, the resin was divided in 5 pools in which the 5 different linkers were introduced via reiterative coupling of Fmoc protected amino acids. The pools were mixed and split in 10 different pools. For the conjugation of all glycans (except the Glu-NAc cluster), Fmoc-Pra-OH was coupled, deprotected and acetylated. The azide-functionalized glycans were coupled by CuAAC.⁵⁸⁻⁵⁹ For the coupling of the Glu-NAc cluster, the activated ester was directly coupled to the free amine of the linker. Each pool was cleaved individually (TFA) and acetates on the glycans removed (NaOH). Each pool was analyzed by MALDI. See SI for detailed scheme (Scheme SI-1) and full synthetic details as well as MALDI analysis of each pool.

4.4 Library Screening

Recombinant lectins were produced in *Escherichia coli* and purified on affinity column as previously reported.^{40, 49} Biotinylated protein (RSL or AFL, 0.05 μ g) was incubated with the library (10 pmol of each member of the library) PBS-t (0.01

M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, with 0.05% tween 20). The solution was mixed gently on an orbital shaker for 1h at room temperature. After this incubation period, 10µL of Streptavidin Dynabeads™ M-280 (prewashed with PBS-t) were added and protein loading on the bead was allowed to proceed for 45 min at room temperature. Then the magnetic beads were washed with PBS-t (specified wash number for selection performed in Figure 2; 4 times for selections shown in Figure 4, 3 min incubation for each wash). Two additional wash under the same conditions were performed with PBS in order to remove Tween. Finally, 10µL of water were added to the beads and brought to 95°C for 5 min in order to recover the members bound to the protein (elution). The solutions were spotted on a MALDI plate using DHB matrix and spectra were measure in positive linear mode (Bruker Daltonics Autoflex)

4.5 Tm measurement

AFL or RSL lectin was diluted to a final concentration 1 µM in 20 mM Tris pH7.4, 0.1 M NaCl containing the Orange SYPRO dye 5X. The protein – dye mixture (20µL) was distributed on 384 White LightCycler® 480 MultiWell plates (cat- 04 729 749 001- Roche). The plate was placed on a LightCycler® 480 II Roche and the experiment was performed according to the manufacturer's protocol. Starting from 20°C the temperature was increased at a rate of 0.06°C/s to reach 85°C target temperature.

4.6 SPR measurements

SPR experiments were performed on a Biacore T200 instrument (GE Healthcare) at 25 °C in HBS-EP+ buffer (10 mM HEPES, 150mM NaCl, 3mM EDTA, pH 7.5, 0.05% v/v surfactant P20) at a flow rate of 30 µL min⁻¹. The protein was loaded on a research grade CM5 chip at 350 RU were immobilized (channel 4 for AFL and channel 2 for RSL) using an amine coupling procedure according the manufacturer's protocol. Experiments consisted of injections (association 180 s, dissociation 1000 s) of decreasing concentration of PNA-sugar compound (2-fold cascade dilutions from indicated started conformation top line). The bottom line corresponds to control injection with only buffer. The chip was fully regenerated by two injections of 1 M fucose in running buffer for 60 s. Binding was measured as resonance units over time after blank subtraction, and the data interpreted using the Biacore T200 software, version 3.2. The K_d were calculated based on steady-state affinity analysis.

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