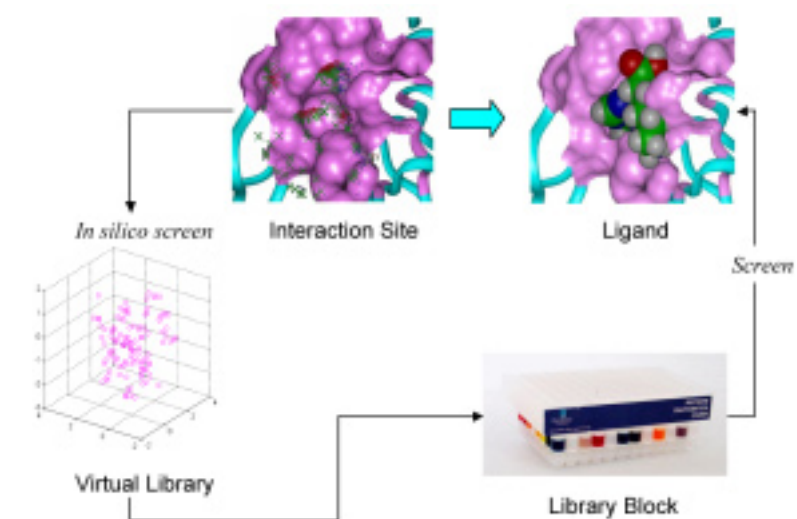


ABSTRACT

The use of affinity chromatography at an early stage in downstream processing is widely acknowledged as a desirable objective owing to the increased yields and reduced cost of goods provided. However, as therapeutic protein targets become increasingly diverse, there is a general lack of affinity adsorbents which possess the required selectivity and robustness to be used in primary capture applications. We report the development of a new generation of stable synthetic affinity ligands derived from combinatorial ligand libraries. This approach was used to identify affinity ligands for the capture of a variety of protein targets from differing sources including a tPA-Urokinase fusion protein from mammalian cell culture and a variety of human plasma proteins including fibrinogen and vWF-Factor VIII complex. Recoveries of bound protein were typically in the region of 90% with purities in the range 90 - 99% following direct capture and elution. The high level of purity obtained significantly simplified subsequent purification steps which provided appreciable reductions in total manufacturing costs.

RATIONAL LIGAND DESIGN

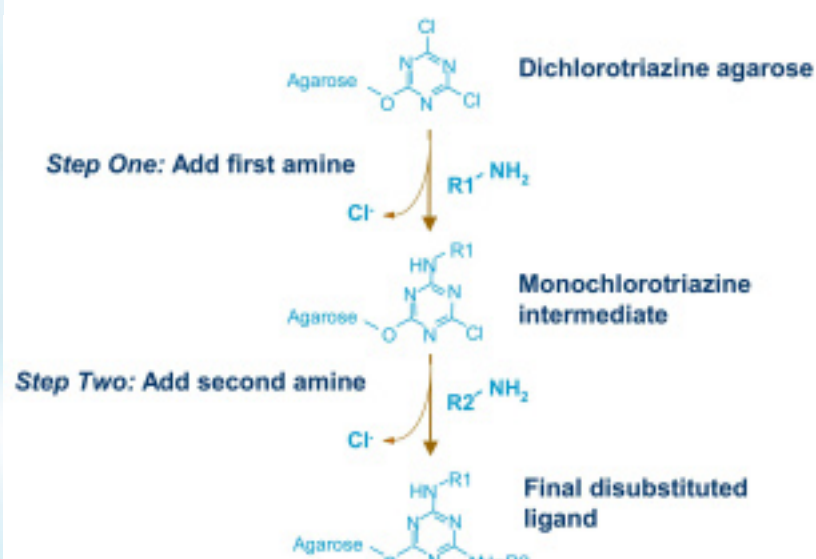


DESIGN SPECIFICATIONS

- Direct capture from feedstock
- Column Capacity: 5 - 20g/l (target protein dependent)
- Flow rate (loading): 100 - 300cm/hr (15cm bed length)
- Recovery: 80%
- Purity: 80%
- Alkali stable (0.5M NaOH sanitization)

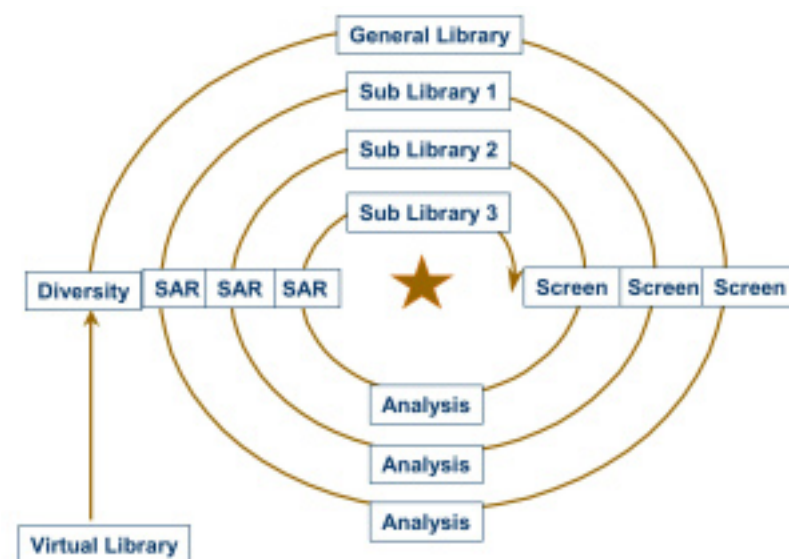
TRIAZINE LIGAND LIBRARIES

Diverse libraries of affinity ligands were obtained by stepwise reaction of primary amine-containing compounds with immobilised dichlorotriazine groups [1]. Such Mimetic™ ligands have proven utility for downstream process applications and are inherently inert due to the lack of fissile bonds.



SCREENING STRATEGY

Combinatorial libraries were constructed which contain 2-dimensional arrays of triazine ligands derived from primary amine compounds with chemically diverse "R" groups. Ligand libraries were prepared with each containing 64 ligands/library using combinations of 24 different primary amines. These "General Libraries" contained a wide spectrum of ligand functionalities including combinations of anionic, neutral, cationic, hydrophilic and hydrophobic groups. Included within these libraries were analogues of chemical structures known to interact with the target protein. The libraries were screened in 96 column blocks (each column containing 250 ml of adsorbent) using a Tecan Genesis robotic liquid handling system in a chromatography mode. Equilibration and elution buffers were selected to maintain the target protein integrity during binding and elution.



SCREENING CONDITIONS

- Equilibration: 3mL, Neutral pH buffer
- Load: 1 - 2mL feedstock containing target protein, followed by 0.25mL equilibration buffer
- Wash: 1.5mL of equilibration buffer
- Elution: Either by increasing salt or pH (sometimes in combination) or by inclusion of polarity modifying agents (1 - 2 elution systems per screen)
- Sanitisation: 1mL 30% isopropanol/ 0.2M NaOH
- Target protein determined by activity or specific ELISA. Protein concentration is determined by Bradford assay.

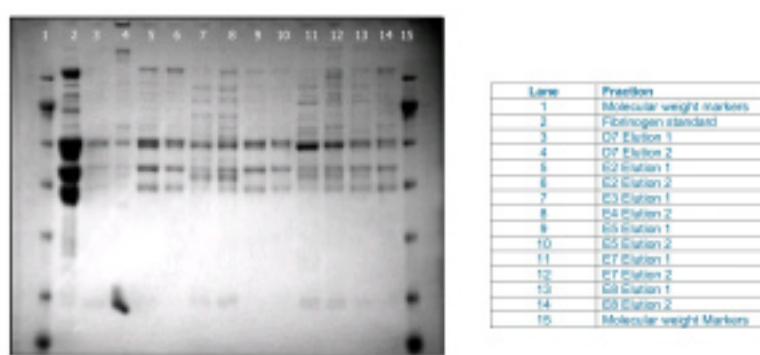
PRIMARY SCREENING

General libraries were screened for target protein binding and specific elution. Typically several ligands in the general libraries screened were found to be effective for capture of the target protein directly from applied feedstock. A proportion of these ligands also allowed the specific release of bound protein under the elution conditions employed. On the basis of these findings, 64 ligand sub-libraries were constructed using analogues of the primary amine compounds which demonstrated the best target binding properties during the initial screening round. The most promising ligands from sub-library screening were selected for secondary screening at the 1 to 5 ml column scale.

	1	2	3	4	5	6	7	8	Scale
A	41	19	99	21	>500	15	98	142	Lowest
B	250	38	134	26	>500	45	76	57	
C	39	21	157	27	>500	27	63	166	
D	>500	33	333	15	>500	34	138	52	
E	60	>500	137	>500	83	43	223	>500	
F	>500	9	57	11	>500	66	67	44	
G	386	26	51	10	417	73	92	29	
H	>500	14	35	14	398	65	120	34	Highest

(µg/fraction)
Sub-Library Screening for Fibrinogen recovery from human plasma

Reducing SDS-PAGE (10% Bis-Tris) for selected fractions from Fibrinogen Sub-library #172 screening



SECONDARY SCREENING

Example: tPA-Urokinase fusion protein from cell culture

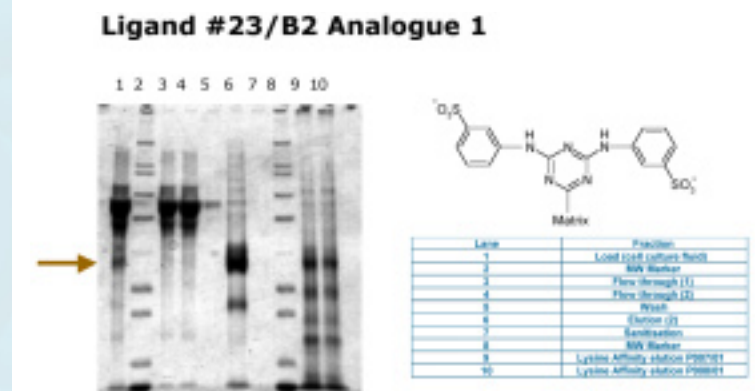
- Scale: 1mL of adsorbent (1 cm bed height)
- Flow Rate: 1.2 mL/min
- Equilibration: 5 column volumes of 25mM sodium phosphate, pH 7.5, 100 mM NaCl
- Load: Cell culture containing approximately 5mg tPA-Urokinase
- Wash: To baseline (5-10 column volumes of equilibration buffer)
- Elution 1 (E1): 10mM sodium citrate, 20% ethylene glycol, 250mM NaCl, pH 3.5
- Elution 2 (E2): 25mM sodium phosphate, 40% ethylene glycol, 500mM NaCl, pH 7.5
- Sanitisation: 30% isopropanol/0.2M NaOH

Ligand	E1 Purity (%)	E2 Purity (%)	µg Rec E1	Eg Rec E2	Total Eluted	Bind Cap	% Recovery
#23/B2	n/a	72	0	4247	4247	>4247	>90
#23/B8	n/a	n/a	0	0	0	1833	0
#85/H8	77	52	64	193	257	421	61
#86/F1	100	n/a	261	n/a	261	609	43
#85/H7	68	n/a	274	n/a	274	336	82
#86/F2	100	27	284	29	313	885	35
#85/G4	100	85	255	208	463	969	48
#85/H5	91	13	295	19	314	501	63
#85/A3	100	2	217	17	234	364	64
#23/H8	25	1	313	22	335	335	>90
#85/B2	52	5	248	27	275	275	>90
#85/A7	45	1	167	16	183	413	44
#86/C1	100	9	1281	62	1343	1961	68

Ligand B2 from library #23 exhibited the highest capacity with reasonable purity. A further 2 additional ligands (near-neighbour compounds of ligand #23/B2) were made and tested:

Ligand	E1 Purity (%)	E2 Purity (%)	E1 Recovery (mg)	E2 Recovery (mg)	Total eluted (mg)	Recovery (%)
Analogue 1	n/a	91	0	4370	4370	92
Analogue 2	n/a	91	0	3720	3720	75

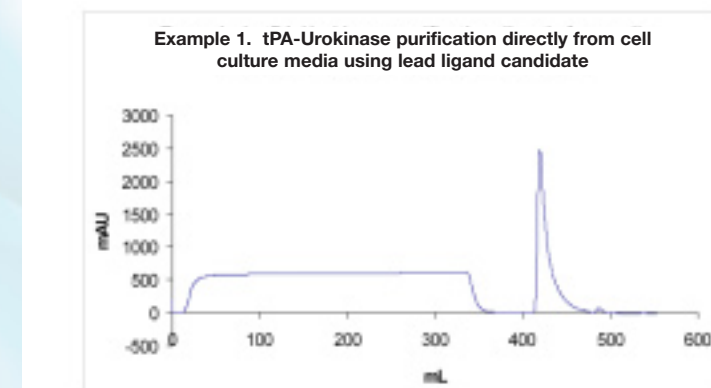
LEAD LIGAND SELECTION



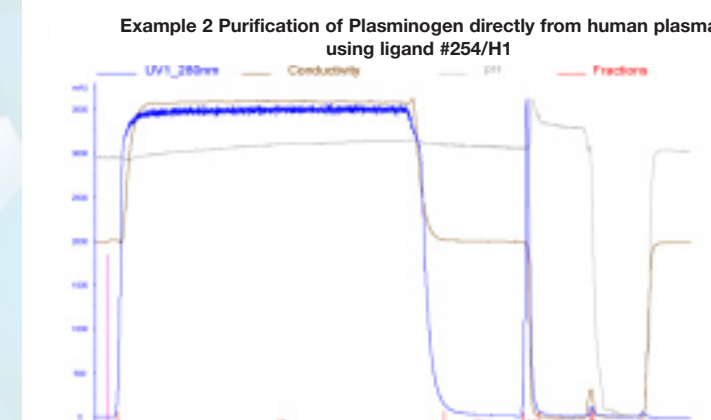
STABILITY

Ligand loss (24h; 20 - 25°C)	Ligand Loss in 0.5M NaOH at 60°C
1M NaOH <0.002%	Graph showing Ligand Loss (%) vs Time (Hours) for 0.5M NaOH at 60°C. The loss increases over time, reaching approximately 100% loss after 100 hours.
30% propan-2-ol/ 0.2M NaOH <0.002%	
20% Ethanol <0.002%	

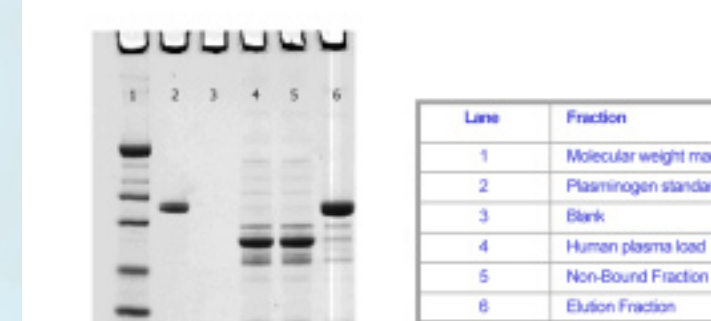
PERFORMANCE VERIFICATION



- Capacity: 11.4mg/mL
- Purity: ≥ 99%
- Recovery: 92%
- Loading flow rate: 300cm/hr
- Alkali Stable (0.5M NaOH)



- Capacity: 5mg/mL
- Purity: ≥ 95%
- Recovery: 90%
- Loading flow rate: 100cm/hr
- Alkali Stable (0.5M NaOH)



SUMMARY

By screening combinatorial libraries of triazine based affinity ligands, compounds can be identified which provide improved capture and purification of diverse proteins from relatively clean cell culture media or highly complex feedstock such as plasma. This work demonstrates the benefits of a combinatorial approach to ligand discovery and the utility of triazine ligands in downstream process applications for purification of therapeutic proteins.

REFERENCE

- 1) International patent application PCT/DK96/00399