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Affinity chromatography in dynamic combinatorial libraries: one-pot amplification and isolation of a strongly binding receptor

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We report the one-pot amplification and isolation of a nanomolar receptor in a multibuilding block aqueous dynamic combinatorial library using a polymer-bound template. By appropriate choice of a poly(*N,N*-dimethylacrylamide)-based support, unselective ion-exchange type behaviour between the oppositely charged cationic guest and polyanionic hosts was overcome, such that the selective molecular recognition arising in aqueous solution reactions is manifest also in the analogous templated solid phase DCL syntheses. The ability of a polymer bound template to identify and isolate a synthetic receptor via dynamic combinatorial chemistry was not compromised by the large size of the library, consisting of well over 140 theoretical members, demonstrating the practical advantages of a polymer-supported DCL methodology.

Introduction

Dynamic combinatorial chemistry has been developed as a new technique aimed at the discovery of synthetic receptors[1] or ligands for biomacromolecules[2] with unprecedented binding affinity and without any need for the time-consuming and tedious steps of designing, screening and testing. Equilibrium mixtures or libraries of oligomeric receptors or hosts, that are themselves formed via reversible covalent bonds, are responsive towards the addition of potential guests; the host with the highest affinity is usually amplified at the expense of the weaker binding members of the library.

This approach has led regularly to unpredictable molecular recognition events and the identification of new synthetic receptors.[3] Some recent examples for the selection approach of dynamic combinatorial chemistry include the discovery of donor-acceptor [2]-catenanes,[4] applications in self-synthesising molecules[5] and supramolecular materials.[6] However, with only a few exceptions the size of the libraries has most often been small.[2b, 2g, 7, 12h] This is slightly surprising since, at least in theory, the probability of identifying a potential hit increases with the size of the library.[8] The analytical challenges and limitations associated with larger and more diverse libraries has been one of our main motivations in developing a polymer-supported methodology for the simultaneous selection, amplification and isolation of a synthetic receptor in DCLs. Since the first report from Sanders' laboratory[9] we have accumulated a considerable body of knowledge and experimental know-how vis-à-vis how the nature of the polymer matrix, its morphology and the loading of solid-phase bound guest affect molecular recognition selectivity and efficiency, both in organic solvents[10] and under aqueous conditions.[11] Using a polymer-supported methodolgy [2b, 2g, 9, 12] we first of all explored the thermodynamically controlled, simultaneous synthesis and isolation of macrocyclic receptors.[10, 11] In addition we could demonstrate enantio- and diastereoselective separations of static mixtures of pseudo-dipeptide based macrocylces via an affinity chromatography protocol in polar organic environments.[10]

During our development of an adamantyl amine derivative as a cationic template immobilised on a lightly crosslinked water-compatible polymer support, it became obvious to us that selective molecular recognition of anionic hosts in water, free from unwanted simple ion-exchange effects, generally represents an exceptional challenge. Interestingly, whereas a poly(*N,N*-dimethylacrylamide)-based resin was able to achieve this, a corresponding polyacrylamide-based resin was not.[11] In this context we now report on the application of the same successful support and immobilised adamantyl amine derivative as the template, **DMAM GT 1** (Figure 1), in synthesising a much larger library, involving an increased number of building blocks, including library members with binding affinities in the micromolar range and below. We will demonstrate clearly the practical advantages a polymer-supported methodology offers for the identification and separation of a favoured synthetic receptor in a dynamic combinatorial library.

Fig. 1 Synthesis of gel-type (**GT**) polymer-supported template **DMAM GT 1**, using *N,N*-dimethylacrylamide (**DMAM**) structural comonomer, methylene bisacrylamide (**MBA**) as crosslinker and **1** as functional comonomer (0.5 mmol·g^{-1}); insert shows a transmission optical microscope photograph of H_2O swollen gel beads **DMAM GT** at a magnification of x10. [The detailed synthesis of monomer **1**, blank polymer **DMAM GT** and functional polymer **DMAM GT 1** has been reported previously.[11]]

Fig. 2 Anionic building blocks **2**, **3**, **4** and **5** and cationic guest **6** for DCLs based on disulfide exchange.

Results and discussion

We set out to use a target library of dithiol building blocks **2**, **3**, **4** and **5** (Figure 2), that reversibly form disulfides under slightly basic aqueous conditions.[3b, 3f, 3g, 3i, 3j, 4, 7b, 7c, 11, 12c, 12f-h, 13] The size of the targeted library with four building blocks can be estimated using DCLCount; [7c, 14] when limiting the potential size of oligomers to tetramers, the largest library would in theory be composed of a maximum of 141 distinct members. While we can usually detect macrocycles up to tetramers using mass spectrometric analysis, the theoretical library size increases rapidly from a first conservative estimate to more than 1400 theoretical library members taking all species up to hexamers into account.

Fig. 3 HPLC analyses of a DCL prepared from building blocks **2**, **3**, **4** and **5** (2 mM in total): (A) in absence of template; and (B) after 72 h exposure to **6** with a template/building block mole ratio of 1/1.

If an equimolar mixture of the four building blocks **2**, **3**, **4** and **5** at a total concentration of 2 mM is equilibrated at pH 8 (50 mM borate buffer) for three days, analysis of the reaction mixture by LC-MS demonstrates that many products coelute and/or disappear below the detection limit. In fact, we have only managed to assign very few macrocycles with high confidence (Figure 3). If the same library is exposed to the template **6**, the product distribution changes dramatically. Most pronounced of all is the amplification of different isomers of $(\mathbf{5})_4$, as well as compounds **(2)(3)(4)**, $(\mathbf{2})(\mathbf{4})_5$, $(\mathbf{2})_2(\mathbf{4})(\mathbf{5})$ and **(2)(3)**. The formation of the $(\mathbf{5})_4$ receptor (as a mixture of regioisomers) was observed previously and its templated amplification from an octameric [2]-catenane $(\mathbf{5})_8$ was disclosed in a separate study.[3g] However, the four latter species have not been described thus far and it remains unclear from the two initial solution-based experiments whether they are indeed amplified via selective binding to the template, or remain in the equilibrated mixtures as leftovers of a library starved of building block **5**. One labour intensive approach to shed light on this system would be to isolate all

Fig. 4 HPLC analyses of a DCLs prepared from building blocks **2**, **3**, **4** and **5** (2 mM in total): (A) in absence of template; (B) filtrate and (C) aqueous wash after 72 h exposure to **DMAM GT 1** (4 mg·ml^{-1}) with a template/building block mole ratio of 1/1; (D) EtOH elution of **DMAM GT 1**.

Fig. 5. Compositions of DCLs[15] using adamantyl *N*,*N*-dimethylamine template **6** and polymer-supported adamantyl amine template **DMAM GT 1** (4 mg·ml^{-1}) employing building blocks **2**, **3**, **4** and **5** (2 mM in total), with a template/building block mole ratio of 1/1: (A) in absence of template; (B) after 72 h exposure to **6**; (C) sum of filtrate and aqueous wash after 72 h exposure to **DMAM GT 1**; and (D) EtOH elution of **DMAM GT 1**.

candidates and determine their individual binding affinity for the target. However, use of polymer-supported template **DMAM GT 1** constitutes a much more straightforward approach. We anticipated that different elution behaviour of receptors from the solid-phase might occur depending on their binding affinity for the target guest. Using polystyrene-supported guests we have already shown the possibility of separating small mixtures of hosts in static libraries via an affinity chromatography type application in organic solvents.[10] The experimental conditions for the DCLs in the presence of resin **DMAM GT 1** were the same as for the two previous aqueous solution-based libraries,[11] using an overall equimolar building block concentration of 2 mM, keeping the template to building block ratio fixed at 1/1 (see experimental section). After equilibration, the beads were filtered off (Figure 4B), washed several times with borate buffer (pH 8) to remove any weakly bound species (Figure 4C), and then eluted with ethanol to liberate the more strongly retained host molecules (Figure 4D). Figure 5 shows the results of this procedure more clearly, comparing the library composition in the absence of template (A) , with that in the presence of **6** (B) with the material obtained in the filtrate + wash of the resin (C) with the material that finally eluted from the resin (D). Comparing Figure 5D with Figure 5B confirms that the compounds which are selectively amplified by solution phase template **6** are also amplified and retained by resin **DMAM GT 1**. Furthermore, the difference in composition of the fraction eluted with aqueous buffer alone, Figure 5C, compared to that of the fraction from the ethanol elution step, Figure 5D, suggests strongly that $(\mathbf{5})_4$ is the strongest binding library member, a conclusion that could not be made based solely on consideration of the amplifications observed in solution-based DCLs. The amplification of **(2)(3)** does not lead to any retention on the polymer-bound template. Inspection of its CPK model indicates that it does not contain a cavity that may act as a binding pocket so it is therefore very unlikely to act as a receptor. Its abundance in the library may be explained from the fact that it is a dimer and small macrocycles tend to dominate over larger ones.[18e]

Even in the presence of an extremely large number of polyanionic potential hosts the results presented in Figure 5 demonstrate the feasibility of applying polymer-supported cationic templates in aqueous phase DCLs, avoiding simple ion-exchange effects, and relying fully on efficient molecular recognition events in determining the selectivity of host-guest interaction.

Having demonstrated the one-pot identification and isolation of a high affinity synthetic receptor $(\mathbf{5})_4$, we decided in a second step to amplify and isolate that particular receptor from a DCL composed only of one building block **5** (Figure 6). **DMAM GT 1** exhibited excellent selectivity compared to an unfunctionalised control polymer, **DMAM GT**, and allowed receptor $(\mathbf{5})_4$ to be obtained in good purity and recovery.

Fig. 6 Use of unfunctionalised control polymer **DMAM GT** (4 mg·ml^{-1}) and polymer-supported adamantyl amine **DMAM GT 1** (4 mg·ml^{-1}) in DCLs using building block **5** (2 mM), and a template/building block mole ratio of 1/1.

Conclusions

We have demonstrated the ability of a polymer-supported template to identify and isolate a synthetic receptor via dynamic combinatorial chemistry in a one-pot procedure. In multibuilding block libraries, consisting of well over 140 theoretical members, unselective ion-exchange type behaviour between the solid-phase bound cationic template and polyanionic macrocyclic receptors in solution was avoided by appropriate choice of a poly(*N,N*-dimethylacrylamide)-based support: the selectivity in solution-based molecular recognition events was fully translocated to the solid-phase. In addition we have achieved the near quantitative one-pot amplification and isolation of the identified synthetic receptor, previsouly shown to bind to its target with an affinity of at least 1 x 10^7 M^{-1}.

The practical feasibility and selectivity were influenced negligibly by the large size of the library. These results pave the way for the use of dynamic combinatorial libraries that are much larger than currently common. Overall the present work has confirmed the original suggestion by Sanders and Brady[16] over 10 years ago that the use of polymer supports and appropriate solid phase chemistry holds out the prospect of making a significant contribution in the successful development and exploitation of dynamic combinatorial chemistry.

Experimental

Materials

Reagents and solvents were purchased from commercial sources and used without further purification. LC-MS solvents and formic acid were purchased from Romil or Rathburn.

Borate buffer (50 mM, pH 8.0) was prepared by dissolving 174 mg (2.5 mmol) of B_2O_3 in 100 mL of MilliQ water. The solution was adjusted to pH 8.0 by careful addition of a 1.0 M KOH solution.

The synthesis of the soluble templates, polymer-supported templates and the various building blocks for DCL experiments has been reported previously:

Blank polymer **DMAM GT**, polymer-supported template **DMAM GT 1** and soluble template **6**, see reference 11;
building block **2**, see reference 3f;
building block **3**, see reference 17;
building block **4**, see reference 3b;
building block **5**, see reference 3g.

Experimental procedure

Dynamic combinatorial libraries preparation:

Experimental conditions using polymer-supported conditions were similar to the conditions described previously:[10, 11] DCL syntheses took place in 50 mM borate buffer pH 8 in the presence of polymer-supported templates, the reaction vials were put on a horizontal shaker. After 3 days the resins were filtered off through syringe filters (0.45 µm cellulose membrane filters), the vials rinsed with a small amount of borate buffer which was then filtered also through the syringe filter, and both filtrates combined. The beads were then washed repeatedly using 2 x 2.0 ml of each of the appropriate solvent (each washing step consisted of shaking the beads for 10 min): borate buffer for the non-disruptive wash which removes unselectively bound oligomers; ethanol for the disruptive wash or elution, which releases selectively amplified and bound receptors by disrupting non-covalent interactions between host and guest or receptor and template bound on the polymer support. An internal standard was added to each solution: 50 µl of a 3,5-dihydroxybenzoic acid solution (20 mM) in borate buffer (50 mM, pH 8).

Throughout all of the solution- and solid-phase based experiments the template to building block ratio of 1 was kept constant. In this particular system the supression of a high affinity binder in favour of a minor binder (e.g., a hetero- or small oligomer)[18] could not be observed.

Analytical instrumentation

General LC-MS analysis:

Samples were analysed using an Agilent 1100 series HPLC coupled to a diode array detector (signal set at 260 nm, reference at 550nm) and an Agilent XCT Ion-Trap. Analyses were performed using a reversed phase HPLC column (Agilent C8 Zorbax Eclipse XBD, 2.1 x 150 mm, 3.5 µm), using an injection volume of 5.0 µL, a flow rate of 0.20 mL/min and a gradient (5% to 95% in 15 min, held at 95% for a further 10 minutes) of acetonitrile in water (both containing 0.1% formic acid) at 318K. Negative ion mass spectra were acquired in standard enhanced mode using electrospray ionisation (drying temperature: 350 ºC; nebuliser pressure: 35 psi; drying gas flow: 8 L/min; HV capillary: 4000 V; ICC target 20,000).

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Notes and references

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Figure 1 Exposing a DCL composed of anionic building blocks **1** and *rac*-**2** to cationic guest **3** leads to the amplification of receptors (**1**)(**2**)$_2$ and (**1**)$_3$.

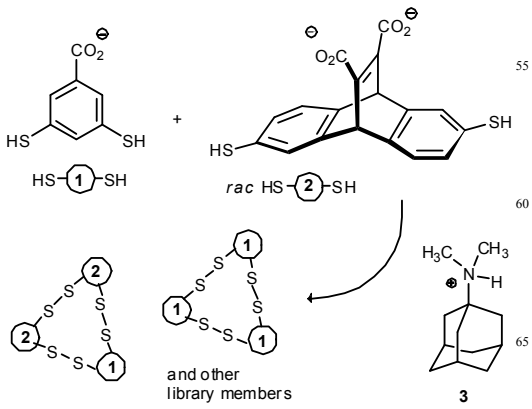

Figure 2 HPLC analyses of a DCL made from building blocks **1** and *rac*-**2** (2 mM in total) (a) in absence of template, and (b) after 72 h exposure to template **3** (2 mM).

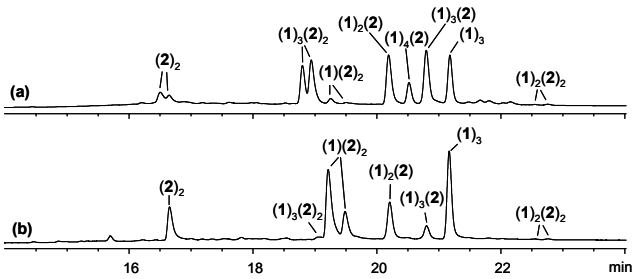

Figure 3 Synthesis of polymerisable template **4b**: (a)[35] MsCl, Ag_2O, CH_2Cl_2, rt, 48h, 48%; (b)[35] NaN_3, dry DMF, N_2, 120 °C, 2h, 95%; (c)[35] (i) Ph_3P, dry THF, N_2, rt, 12h (ii) H_2O, rt, 10h, 88%; (d)[36] ethyl trifluoroacetamide, Et_3N, MeOH, rt, 12h, 90%; (e)[37] MsCl, Et_3N, CH_2Cl_2, rt, 12h, 96%; (f)[38] N-(1-adamantyl)-N-methylamine, NaI, K_2CO_3, CH_3CN, reflux, 36h, 82%; (g)[36] 6M NaOH, rt, 12h, 97%; (h)[36] acryloyl chloride, DMAP, K_2CO_3, CH_2Cl_2, rt, 12h, 99%.

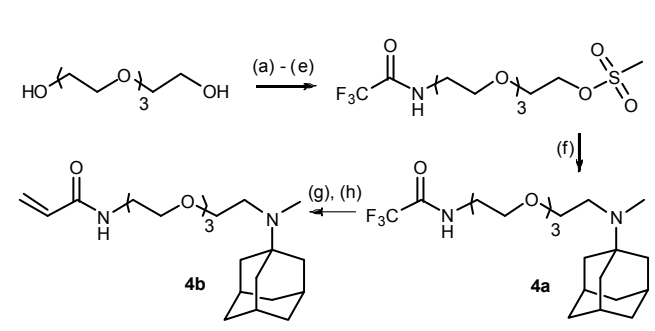

Figure 4 Synthesis of polymer-supported adamantylamine derivative **AM / DMAM GT 4b** in an inverse-suspension polymerisation using 4b (20 wt%), **MBA** (4 wt%), **AM or DMAM** (76 wt%) to yield a template loading of 0.5 mmol **4b** /g.

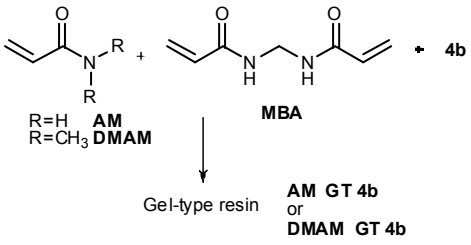

Figure 5 Transmission optical microscope photographs at a magnification of x10 showing gel-type beads swollen in H_2O. Left: resin **AM GT**; Right: resin **DMAM GT**.

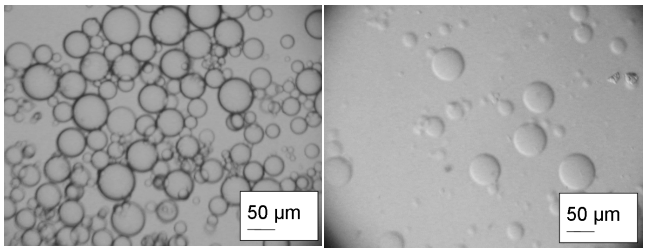

Figure 6 HPLC analyses of a DCL made from building blocks **1** and *rac*-**2** (2 mM in total) (a) in absence of template, and (b) after 72h exposure to **DMAM GT 4b** (4 mg/ml), (c) borate buffer wash of **DMAM GT 4b**, and (d) elution with ethanol.