The Advantage of Being Virtual—Target-Induced Adaptation and Selection in Dynamic Combinatorial Libraries

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Abstract: Numerical simulations are presented that describe the adaptive behavior of simple dynamic combinatorial libraries (DCLs) upon addition of a target. By studying the effect of various parameters such as the network topology, the initial concentrations, the association constants, and the binding affinities, general characteristics of such systems were derived. It is shown that the adaptation may lead to the amplification of molecules with a high affinity to the target, but only for specific boundary conditions. Furthermore, it is demonstrated that the selection process can be refined by using an evolutionary approach. These results are of importance for the design of selection experiments with DCLs.

Keywords: combinatorial chemistry · dynamic chemistry · host–guest chemistry · networks · numerical simulations

Introduction

Self-assembly processes are increasingly being employed for the construction of large and complex molecules with defined structures. But self-assembly can also be used to generate structural diversity in a very efficient way. The requirement for such a fuzzy assembly process is that several nearly isoenergetic structures can be built with the same reactive building blocks.[1] In recent years it became apparent that this diversity-orientated assembly offers unique possibilities for the detection of new receptors and catalysts as well as for the discovery of lead compounds in medicinal chemistry.[2,3]

A mixture of molecules, which is generated by reversible assembly of suitable building blocks under thermodynamic control, forms a chemical network that is able to adapt to the environment.[4] If a target molecule is added to such a system, it will re-equilibrate until the new thermodynamic minimum is established. It has been proposed that the re-equilibration will lead to the amplification of molecules with high affinity to the target molecule. Given that it is possible to detect the amplification by analytical techniques, this dynamic combinatorial library (DCL) could be screened for molecules that bind to the target in a single experiment.

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Starting with some seminal publications by Sanders and co-workers,[5] Lehn and co-workers,[6] and others[7] in the mid 1990s, selection experiments with DCLs have now been performed by using various target molecules such as alkali metal ions,[8] alkaline-earth metal ions,[9] alkylammonium ions,[10] anions,[11] *N*-heterocycles,[12] crown ethers,[13] uracil derivatives,[14] halocarbons,[15] biphenyl,[16] nucleic acids,[17] small peptides,[18] proteins,[19] protein crystals,[20] and transition state analogues.[21] Compared to this wealth of experimental data, there is a surprising lack of theoretical analyses about the adaptive behavior of DCLs.[22,23] An important contribution was published by Moore and Zimmerman, who have derived equations that describe the equilibrium concentrations of an infinitely large DCL with and without a target.[23] Under the assumptions that a) the members can be completely transformed from one into another and b) the equilibrium constants follow a normal distribution function with a standard deviation of one $\log K$ unit, the model predicts that the mean binding constant of the DCL can be shifted, but only to a very limited degree. Although based on a very simple model, this analysis is very useful in understanding some intrinsic limitations of selection protocols based on DCLs.

Herein comprehensive numerical simulations are presented of the adaptive behavior of selected DCLs. The underlying models were chosen to be minimal representations of typical classes of DCLs. By studying the effect of important parameters such as association constants and target concentration, some key characteristics of such chemical networks were derived. Importantly, it is demonstrated that the success of the selection process depends decisively on the design of the DCL and on the boundary conditions. Further-

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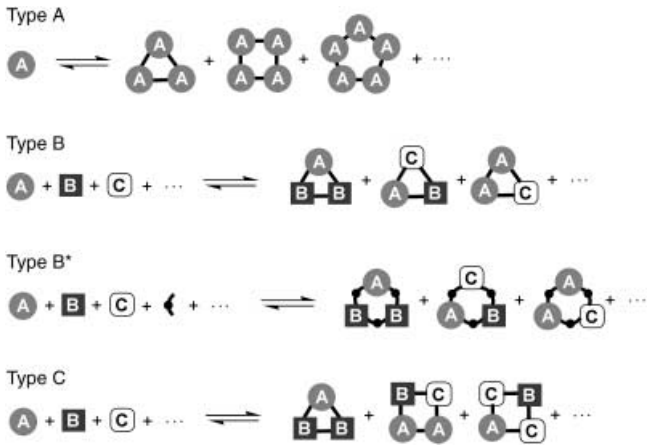
more, we will show for some specific models how the selection process can be refined using iterative procedures.

Results and Discussion

General considerations: For DCLs, which are obtained by assembly of reactive subunits, several different network topologies can be distinguished. For type A, only one building block is used and structural diversity is generated by disparity of the aggregation number (Scheme 1). For type B, two or more building blocks are used to assemble oligomeric species with uniform aggregation number but variable composition.[24] A variation of this class is the DCL of type B*, which is obtained by assembly of different building blocks but all members of the library have at least one common subunit (e.g. a spacer or a bridging ligand). Libraries of type C represent a hybrid of type A and type B because both the aggregation number as well as the building block is variable.[25]



Scheme 1. Different types of dynamic combinatorial libraries, obtained by self-assembly of reactive building blocks.

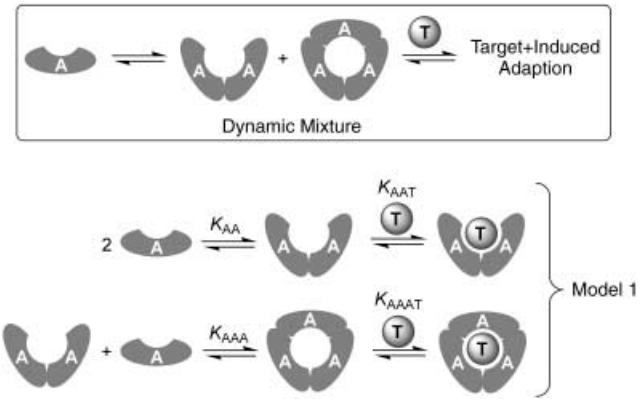
In the following the results of numerical simulations are described which were performed to determine the changes that occur upon addition of a target that binds to one or several members of the DCL. The underlying models were chosen to be minimal representations of the types A, B and B* discussed above. This work focuses on systems of low complexity because of the interest in deconvoluting the effects of key parameters such as target concentration and equilibrium constants. Of particular interest was the question of whether the adaptation that occurs upon addition of a target T is sufficient—in qualitative and quantitative terms—to be of use as an auto-selection procedure for the detection of molecules with high affinity to the target. For the discussion it is useful to introduce the amplification factor f_X, which is defined by the ratio of the steady-state concentration of the DCL member X after and before the addition of the target molecule. For values of $f_X > 1$, the species X is amplified as a result of the target T. The absolute value of f_X can be used as a quantitative measurement of the adaptation process. For competition situations in which several members of the library bind to the target with comparable affinity, it is furthermore important to discuss the selectivity factor $s_{X\text{-}Y}$ of two species X and Y. $s_{X\text{-}Y}$ is defined as the steady-state concentration of X divided by the steady-state concentration of Y.[26] In all cases, the steady-state concentrations of the corresponding species involved were simulated by using the program Gepasi.[27]

$$f_x = \frac{[X]_{ss} + [XT]_{ss} \text{ in the presence of T}}{[X]_{ss} \text{ in the absence of T}} \quad (1)$$

$$s_{X-Y} = \frac{[X]_{ss}}{[Y]_{ss}} \quad (2)$$

$[X]_{ss}$ = steady-state concentration of X
$[Y]_{ss}$ = steady-state concentration of Y
$[XT]_{ss}$ = steady-state concentration of X bound to T

Minimal models for DCLs of type A: The first model that was investigated is a minimal representation of a DCL of type A. A monomer A is in equilibrium with a dimer AA and a trimer AAA (Scheme 2). The corresponding association constants are K_{AA} and K_{AAA}. Both assemblies are able to bind to a target molecule T with the binding constants K_{AAT} and K_{AAAT}.[28] For all calculations, the initial monomer concentration was fixed to $[A]_i = 20$ mM.



Scheme 2. A minimal model of a DCL of type A ("Model 1"): a dimeric receptor AA and a trimeric receptor AAA are formed by assembly of the building block A. Both receptors are able to bind to a target T.

For a first series of calculations, the association constants were set to $K_{AA} = 1600$ mM^{-1} and $K_{AAA} = 20$ mM^{-1}. In the absence of a target T, these values lead to the formation of equal amounts of the two receptors ([AA] = [AAA] = 3.99 mM) together with a very small amount of the free building block A ([A] = 0.05 mM). If an excess of a target T ($[T]_i = 100$ mM) is introduced, the relative concentration of the two receptors changes depending on their affinity to the target. Under the assumption that only the dimer AA is able to bind the target with $K_{AAT} = 10$ mM^{-1}, the nearly quantitative transformation of AAA into AA is observed (Table 1, entry 2). Similar results are obtained if the affinity to the target is two orders of magnitude lower (Table 1, entry 3). If it is assumed that only the trimer AAA acts as a receptor with $K_{AAAT} = 10$ mM^{-1} or $K_{AAAT} = 0.1$ mM^{-1}, the pic-

Table 1. Calculated steady-state concentrations of the receptors AA, AAA, AAT and AAAT [mM] for different associations constants K_{AAT} and K_{AAAT} [mM^{-1}]. The calculations are based on Model 1 described in Scheme 2.

Entry	K_{AAT}	K_{AAAT}	$[T]_i$	[AA]	[AAT]	[AAA]	[AAAT]
1	–	–	0	3.99	0	3.99	0
2	10	0	100	0.01	9.99	0.00	0.00
3	0.1	0	100	0.92	8.41	0.44	0.00
4	0	10	100	0.06	0.00	0.01	6.62
5	0	0.1	100	1.09	0.00	0.57	5.37
6	0	0.1	1000	0.26	0.00	0.06	6.43
7	10	10	100	0.01	9.28	0.00	0.47
8	0.01	0.01	100	2.36	2.27	1.82	1.75

ture is reversed with nearly all AA being transformed into AAA during the adaptation process (Table 1, entries 4 and 5). A way to slightly improve the amplification is to augment the initial concentration of the target: if the amount of target is increased by a factor of 10 ($[T]_i = 1000$ mM), the amplification factor for AAA increases from $f_{AAA} = 1.49$ to $f_{AAA} = 1.63$ which corresponds to 98% of its maximum value (Table 1, entry 6).

The examples discussed so far represent extreme situations since the target binds exclusively to one of the two assemblies with high affinity. A more interesting and realistic situation is the case where both assemblies act as competing receptors. The steady-state concentrations for a system in which both receptors have the same affinity for the target T ($K_{AAT} = K_{AAAT} = 10\ mM^{-1}$) are given in Table 1, entry 7. Upon addition of the target, the system re-equilibrates considerably with substantial amounts of AA being formed from AAA ($f_{AA} = 2.33$). We thus observe the clear selection of the smaller receptor AA although the binding constants are the same. This result can be explained by the fact that the re-equilibration enhances the total number of receptor molecules (three AA can be formed from two AAA). For the system described in Table 1, entry 7 we have a total of 9.75 mM receptors bound to the target whereas without re-equilibration the maximum amount would be 2×3.99 mM = 7.98 mM. The preferential binding of the smaller assembly AA is less pronounced if we assume that the binding constants to the target are smaller. For $K_{AAT} = K_{AAAT} = 0.01\ mM^{-1}$, we find a total of 4.63 mM of AA and 3.57 mM of AAA (Table 1, entry 8).

The total concentrations of the assemblies AA and AAA[29] as a function of the binding constant K_{AAAT} with a fixed value for the affinity of the dimeric receptor AA ($K_{AAT} = 10\ mM^{-1}$) are shown in Figure 1. For a binding constant of $K_{AAAT} = 304\ mM^{-1}$, the relative concentration of $[AA]_t$ and $[AAA]_t$ are the same and correspond to the equilibrium concentrations in the absence of T. We therefore observe no adaptation of the dynamic mixture upon addition of 100 mM target T. For binding constants of $K_{AAAT} > 304\ mM^{-1}$, the trimeric assembly AAA is selected. At $K_{AAAT} = 3668\ mM^{-1}$ the amount of AAA ($[AAA]_t = 6$ mM) has reached 90% of its maximum value of 6.66 mM. For binding constants below $K_{AAAT} = 304\ mM^{-1}$, we observe the selection of the dimeric receptor AA. Interestingly, there is a region ($10\ mM^{-1} < K_{AAAT} < 304\ mM^{-1}$) in which AA is selected although the trimeric receptor AAA forms more stable complexes with the target.

As indicated above, the preferential selection of the dimer AA over the trimer AAA can be explained as a result of the increased total number of receptors, which are able to bind to the target when the system re-equilibrates to form AA from AAA. It was therefore of interest to see how the system behaves when we offer only a limited amount of target. Calculations, similar to those described in Figure 1, were performed by using an initial target concentration of only 4 mM (this corresponds to half of the total initial receptor concentration). The results are depicted in Figure 2. Clearly, the maximum adaptation observed for such a system is smaller. For very high values of K_{AAAT} the selectivity approaches a value of $s_{AAA\text{-}AA} = 2.75$, whereas for very small values of K_{AAAT} the selectivity is $s_{AA\text{-}AAA} = 2.92$. From an experimental point of view, this is a dis-

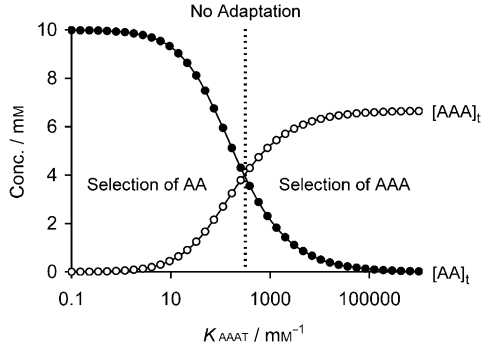


Figure 1. Total concentrations of the receptors AA (●) and AAA (○) as a function of the binding constant K_{AAAT} in the presence of 100 mM target T. The remaining variables were fixed to the following values: $[A]_i = 20$ mM; $K_{AA} = 1600\ mM^{-1}$; $K_{AAA} = 20\ mM^{-1}$; $K_{AAT} = 10\ mM^{-1}$. The calculations are based on Model 1 described in Scheme 2.

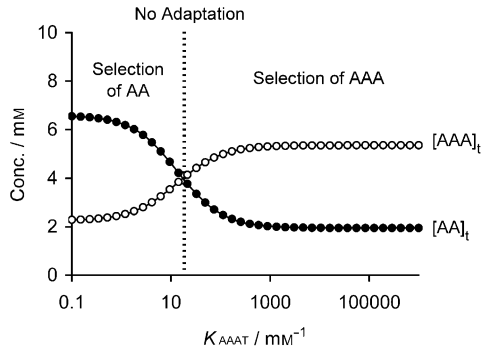


Figure 2. Total concentrations of the receptors AA (●) and AAA (○) as a function of the binding constant K_{AAAT} in the presence of 4 mM target T. The remaining variables were fixed to the following values: $[A]_i = 20$ mM; $K_{AA} = 1600\ mM^{-1}$; $K_{AAA} = 20\ mM^{-1}$; $K_{AAT} = 10\ mM^{-1}$. The calculations are based on Model 1 described in Scheme 2.

advantage because the overall changes that have to be detected by analytical means are smaller. But on the other hand, the region where AA is selected although the binding constant of AAA is higher, is much smaller: it goes from $K_{AAAT}=10\ mM^{-1}$–$18\ mM^{-1}$.

Experimentally, it may be possible to differentiate between free and bound receptors. In principle, this could be done by various spectroscopic techniques in homogeneous solution. Another possibility would be that the target is immobilized on a solid support and nonbound receptors are separated by filtration. For the situation described above with a substoichiometric amount of target this would be beneficial. Figure 3 shows the steady-state concentrations of

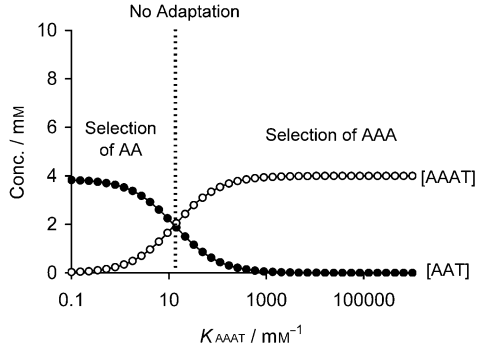


Figure 3. Steady-state concentrations of the adducts AAT (●) and AAAT (○) as a function of the binding constant K_{AAAT} in the presence of 4 mM target T. The remaining variables were fixed to the following values: $[A]_i=20$ mM; $K_{AA}=1600\ mM^{-1}$; $K_{AAA}=20\ mM^{-1}$; $K_{AAT}=10\ mM^{-1}$. The calculations are based on Model 1 described in Scheme 2.

the receptors, which are bound to the target T. Equal amounts of AAT and AAAT ("no adaptation") are observed for $K_{AAAT}=13\ mM^{-1}$ which is very close to the "ideal" value of $10\ mM^{-1}$. For binding constants of AAA which differ by a factor of 10 from that of AA ($K_{AAT}=10\ mM^{-1}$) we already observe a good selectivity for the respective adduct: the selectivity is $s_{AAT\text{-}AAAT}=12.2$ for $K_{AAAT}=1\ mM^{-1}$ and $s_{AAAT\text{-}AAT}=7.2$ for $K_{AAAT}=100\ mM^{-1}$. These results demonstrate that for a selection process with such a system, it is clearly advantageous to work with low target concentrations, especially if it is possible to differentiate between bound and free receptors. A requirement for such a procedure, however, is that the binding constants for target complexation are large enough to ensure significant adduct formation under the conditions employed.

So far it has been assumed that the association constants K_{AA} and K_{AAA} are sufficiently high that the equilibrium is nearly completely on the side of the receptors AA and AAA. By setting the association constants to the values of $K_{AA}=5.54\times10^{-4}\ mM^{-1}$ and $K_{AAA}=5.26\times10^{-2}$, a system is generated in which without target the monomer A is the dominating species ($[A]_i=19.00$ mM), and the two receptors are present in equally small amounts of $[AA]_i=[AAA]_i=0.20$ mM. The steady-state concentrations of the receptors as a function of the binding constant K_{AAAT} are shown in Figure 4. The graph resembles that of Figure 1: for $K_{AAAT}=K_{AAT}=10\ mM^{-1}$ we observe the clear domination of AA ($[AA]_t=6.43$ mM) over AAA ($[AAA]_t=1.20$ mM). The

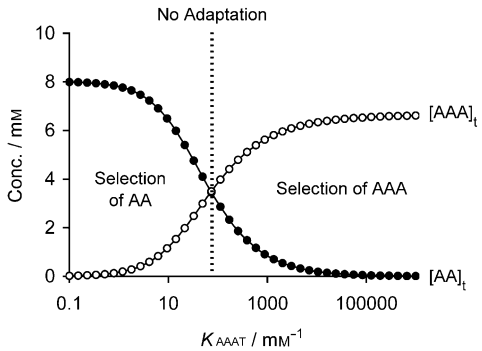
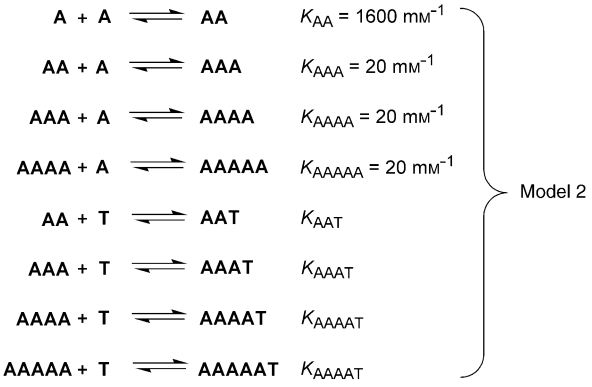


Figure 4. Total concentrations of the receptors AA (●) and AAA (○) as a function of the binding constant K_{AAAT} in the presence of 100 mM target T. The remaining variables were fixed to the following values: $[A]_i=20$ mM; $K_{AA}=5.54\times10^{-4}\ mM^{-1}$; $K_{AAA}=5.26\times10^{-2}\ mM^{-1}$; $K_{AAT}=10\ mM^{-1}$. The calculations are based on Model 1 described in Scheme 2.

region in which the "wrong" receptor (=lower affinity for T) is selected is smaller but it still covers almost one order of magnitude in K_{AAAT} ($10\ mM^{-1}<K_{AAAT}<73\ mM^{-1}$). The maximum values for $[AA]_t$ and $[AAA]_t$ are slightly lower than what was found for the calculations described in Figure 1. For such a system, it is not advantageous to differentiate between free and bound receptors since the total concentrations of AA and AAA are very close to the concentrations of the adducts AAT and AAAT.

To demonstrate that the characteristics described above are also found for more complicated DCLs of type A, the steady-state concentrations for a system were calculated, in which a monomer A is in equilibrium with a dimer AA, a trimer AAA, a tetramer AAAA, and a pentamer AAAAA (Scheme 3). The initial concentration of A (56 mM) and the



Scheme 3. A model of a DCL of type A ("Model 2"): receptors with aggregation number 2–5 are formed by assembly of the building block A. The association constants are fixed to $K_{AA}=1600\ mM^{-1}$ and $K_{AAA}=K_{AAAA}=K_{AAAAA}=20\ mM^{-1}$. All receptors are able to bind to a target T with a variable binding constant.

association constants were chosen in such a fashion that without target the four different aggregates are present in equal amounts (4.00 mM each). The steady-state concentrations for different binding constants and target concentrations are listed in Table 2.

It is assumed that all four assemblies compete for the target T. When the binding constants are all the same

Table 2. Steady-state concentrations of the receptors AA, AAA, AAAA and AAAAA [mM] for different associations constants [mM^{-1}] and initial target concentrations [mM]. The calculations are based on Model 2 described in Scheme 3.

Entry	$[T]_i$	K_{AAT}	K_{AAAT}	K_{AAAAT}	K_{AAAAAT}	[AA]	[AAA]	[AAAA]	[AAAA]
1[a]	0	10	10	10	10	4.00	4.00	4.00	4.00
2[a]	100	10	10	10	10	24.25	2.20	0.20	0.02
3[a]	100	0.01	0.01	0.01	0.01	5.43	4.56	3.83	3.22
4[a]	100	10	100	1000	10000	9.79	5.40	2.99	1.65
5[a]	8	10	100	1000	10000	2.12	1.66	2.06	7.70
6[b]	8	10	100	1000	10000	0.02	0.13	0.95	6.90

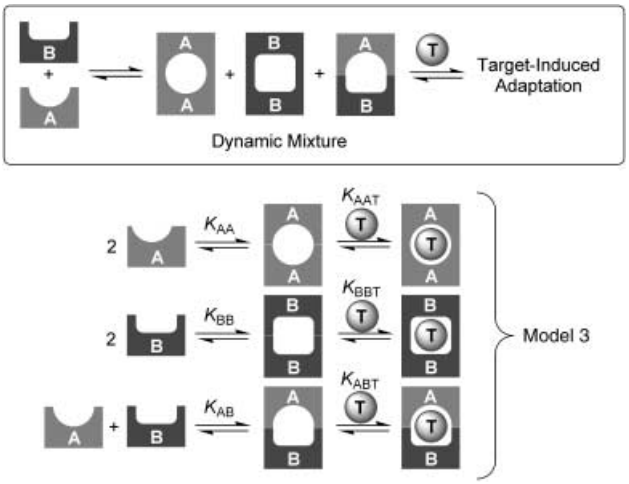
[a] The values listed correspond to the total concentrations of free + bound receptors. [b] The values listed correspond to the concentrations of the receptors bound to the target.

($10\ mM^{-1}$), we find pronounced differences in the steady concentrations of the total receptor concentrations. Whereas a strong amplification of the dimer AA (24.25 mM; $f_{AA}=6.06$) is observed, the concentrations of the higher aggregates are all diminished compared to the equilibrium concentration before addition of the target (Table 2, entry 2). Going from AA to AAAAA, the addition of each monomer A leads to a decrease of the total concentration by approximately one order of magnitude. Consequently, the pentamer AAAAA is present in only minute amounts (0.02 mM) although its affinity for the target equals that of the dimer AA. Again, the amount of re-equilibration is much less pronounced when it is assumed that all binding constants are smaller (Table 2, entry 3). Here the total concentration of AA is only 1.7 times higher than that of the pentamer AAAAA. If attempts are made to counterbalance the situation described in Table 2, entry 2 by increasing the affinity to the target in the order AA→AAAAA, the preferential formation of the dimer ($f_{AA}=2.45$) together with a slight amplification of the trimer ($f_{AAA}=1.35$) and a reduction of the tetramer ($f_{AAAA}=0.75$) and the pentamer ($f_{AAAAA}=0.41$) (Table 2, entry 4) are still observed. The concentration of the latter is only 17% of that of the dimer AA although its affinity for the target is 1000 times higher. It is clear that under those conditions, a selection process would give rise to totally misleading results.

If the initial concentration of the target is reduced from 100 to 8 mM (this corresponds to half of the total initial receptor concentration), the selection process shifts in favor of the pentamer AAAAA (7.70 mM; $f_{AAAAA}=1.93$). The smaller aggregates are present in roughly equal amounts with amplification factors of $f\sim0.5$ (Table 2, entry 5). If we focus only on the assemblies that are bound to the target (Table 2, entry 6), there is a clear differentiation between all receptors. Importantly, the relative concentrations correspond approximately to the relative binding constants. A selection process performed with a low target concentration and with focus on the adducts $(A)_xT$ could thus be used to determine the relative affinities.

The results obtained for these simple model systems point to some important characteristics of DCLs of type A: 1) It is not necessarily the assembly with the highest affinity for the target that displays the highest amplification factor. 2) In competition situations, there is an intrinsic bias for the selection of assemblies with a small aggregation number. 3) The preference for small assemblies is less pronounced for low binding constants. 4) It is possible that there is no re-equilibration upon addition of a target although the affinities of the DCL members to the target differ substantially. 5) Although it is advantageous to work with a large access of the target if only one member of the library is able to bind to the target, for competition situations it may be better to reduce the total amount of target. 6) If limited amounts of target are used, it is beneficial to perform the analysis based on the concentration of the DCL members that are bound to the target.

Minimal models for DCLs of type B: As a minimal representation of a DCL of type B, the model shown in Scheme 4



Scheme 4. A minimal model of a DCL of type B ("Model 3"): three dimeric receptors (AA, BB, and AB) are formed by assembly of the building blocks A and B. All receptors are able to bind to a target T.

was chosen. The reversible assembly of a binding site A and a second binding site B gives rise to a dynamic mixture of three receptors (AA, AB, and BB).[30] All receptors are able to bind to a target T. Equal amounts of the subunits A and B were used for all simulations ([A]=[B]=10 mM). Consequently, for high association constants the concentrations of the homo-dimeric assemblies AA and BB are always the same and adaptation changes only the relative concentration of the homo-dimeric assemblies AA and BB with respect to the hetero-dimeric assembly AB. It was furthermore assumed that the affinities between the building blocks are all the same. In the absence of a target molecule, the statistical distribution of the three receptors is thus expected to be [AA]:[AB]:[BB]=1:2:1. To account for the statistical preference of the heterodimer (AB=BA), we have set $=K_{AA}=K_{BB}=\frac{1}{2}K_{AB}$.

For the first model that was investigated, the association constants of the three receptors were fixed to $K_{AA}=K_{BB}=$

$^1/_2 K_{AB} = 1000\ mM^{-1}$. Under those conditions, the three receptors AA, AB, and BB are the dominant species in solution ([AA] = [BB] = 2.49 mM; [AB] = 4.99 mM) with a monomer concentration of only 0.05 mM. It should be pointed out that—contrary to what is found for DCLs of type A—the equilibrium concentrations of the receptors do not change upon addition of a target if we assume that the binding constants are all the same. This is generally true for DCLs of type B because re-equilibration will not affect the total concentration of the library members. Adaptation is therefore only expected if we introduce thermodynamic differences by variation of the binding affinities to the target. For the present system, it is assumed that receptor AA has a high affinity for the target with $K_{AAT} = 100\ mM^{-1}$ and that receptor BB has a low affinity with $K_{BBT} = 1\ mM^{-1}$. Using a large excess of the target T (100 mM), the total concentration of the assemblies AA and AB was determined as a function of the binding constant K_{ABT} of the hetero-dimeric receptor AB (Figure 5).

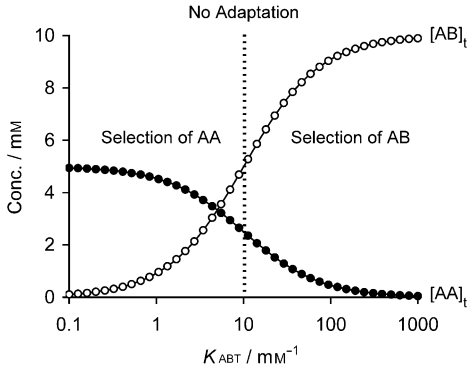


Figure 5. Total concentrations of the receptors AA (●) and AB (○) as a function of the binding constant K_{ABT}. The remaining variables were fixed to the following values: $[A]_i = [B]_i = 10$ mM; $[T]_i = 100$ mM; $K_{AA} = K_{BB} = ^1/_2 K_{AB} = 1000\ mM^{-1}$; $K_{AAT} = 100\ mM^{-1}$; $K_{BBT} = 1\ mM^{-1}$. The calculations are based on Model 3 described in Scheme 4.

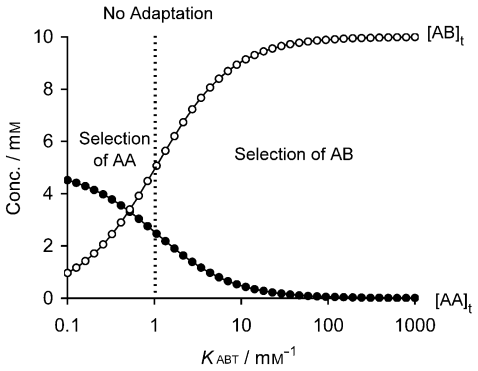


Figure 6. Total concentrations of the receptors AA (●) and AB (○) as a function of the binding constant K_{ABT}. The remaining variables were fixed to the following values: $[A]_i = [B]_i = 10$ mM; $[T]_i = 100$ mM; $K_{AA} = K_{BB} = ^1/_2 K_{AB} = 1000\ mM^{-1}$; $K_{AAT} = 100\ mM^{-1}$; $K_{BBT} = 0\ mM^{-1}$. The calculations are based on Model 3 described in Scheme 4.

Under the conditions specified above, virtually all receptors are bound to a target molecule. For a binding constant of $K_{ABT} = 10\ mM^{-1}$, the relative concentration of AA and AB ($[AA]_t = 2.49$ mM; $[AB]_t = 4.99$ mM) corresponds to the equilibrium concentration that is found without target. Clearly, there are other boundary conditions, which can give rise to steady-state concentrations that are indistinguishable from a system without target. From the observation that the addition of a target does not change the concentration of the DCL members, it is therefore not possible to conclude that there is no binding or equal binding to the target. On the contrary, it is possible that the binding constants vary substantially. In the special case of $K_{ABT} = 10\ mM^{-1}$, for example, an adaptation of the dynamic mixture is not observed although the other binding constants are quite distinct ($K_{AAT} = 100\ mM^{-1}$ and $K_{BBT} = 1\ mM^{-1}$).

For binding constants of $K_{ABT} > 10\ mM^{-1}$, the concentration of AB is higher than the statistically expected value of 5 mM. The dynamic mixture has thus adapted in a way that leads to selection of the hetero-dimeric receptor AB over the homo-dimeric receptor AA. Notably, there is again a region ($10\ mM^{-1} < K_{ABT} < 100\ mM^{-1}$) in which the selection does not give the right answer: AB is selected although the homo-dimeric receptor AA is more stable.

For binding constants of $K_{ABT} < 10\ mM^{-1}$, we amplify the homo-dimeric assembly AA. One should note, however, that we automatically co-amplify BB if we select AA. Unless we can differentiate the target-bound assemblies, we are not able to tell by simple measurements of the total concentrations whether AA or BB is the high affinity receptor that is responsible for the observed amplification. This is another general characteristic of a DCL of type B (given that the equilibrium is not on the side of the monomers; see below) because the depletion of hetero-assemblies to form a specific homo-assembly will automatically generate homo-assemblies comprising the other building blocks.

If we omit BB as a competitive receptor by setting the binding constant K_{BBT} to zero, we increase the region where the heterodimeric receptor AB is selected ($s_{AB\text{-}AA} > 2$ for $K_{ABT} > 1\ mM^{-1}$; Figure 6). The peculiar behavior of the latter system is further underlined by the following facts: if both receptors AA and AB have the same high affinity for the target T ($K_{AAT} = K_{ABT} = 100\ mM^{-1}$), the complete domination of the adduct ABT (9.89 mM; $f_{AB} = 1.98$) is observed over the adduct AAT (0.05 mM; $f_{AA} = 0.02$). If such a system were investigated experimentally, the less than 1% homo-dimeric receptor AA would be unlikely to be detected although it binds to the target with the same affinity as the selected receptor AB. On the other hand it is evident from Figure 6 that the selection of AA is only possible if the binding constants differ by more than two orders of magnitude ($K_{ABT} < 1\ mM^{-1}$). To understand these results in qualitative terms, it is important to realize that by converting one AA and one BB into two AB, the number of assemblies that are able to bind to the target, is increased from one to two.

Similar to what was found for DCLs of type A, the intrinsic preference of the hetero-dimer AB over the homo-dimer AA is less pronounced at lower binding constants. For $K_{AAT} = K_{ABT} = 0.01\ mM^{-1}$, for example, we find a total concentration of $[AB]_t = 5.81$ mM ($f_{AB} = 1.16$) versus $[AA]_t = 2.08$ mM ($f_{AA} = 0.84$). These values are much closer to a sit-

uation in which equal binding constants of AB and AA lead to equal amplification factors.

Next, the influence of the initial target concentration on the total concentration of AA and AB was investigated. The binding constants were fixed to $K_{AAT}=100\ mM^{-1}$, $K_{ABT}=10\ mM^{-1}$, and $K_{BBT}=1\ mM^{-1}$. As described above, for high target concentration these values lead to a situation where no adaptation is observed, that is the concentration of AA and AB correspond to the equilibrium concentration without target. The selectivity factor $s_{AA\text{-}AB}$ therefore equals 0.5. Similar values are found for $[T]_i=14$ mM (Figure 7). Going

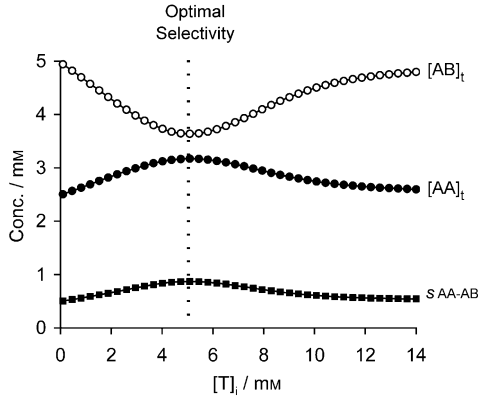


Figure 7. Total concentrations of the receptors AA (●) and AB (○) and the selectivity factor $s_{AA\text{-}AB}$ (■) as a function of the initial concentration of the target T. The remaining variables were fixed to the following values: $[A]_i=[B]_i=10$ mM; $K_{AA}=K_{BB}={}^1/_2\,K_{AB}=1000\ mM^{-1}$; $K_{AAT}=100\ mM^{-1}$; $K_{ABT}=10\ mM^{-1}$, $K_{BBT}=1\ mM^{-1}$. The calculations are based on Model 3 described in Scheme 4.

to smaller initial target concentrations, the total amount of AA slightly increases with a maximum value for $[T]_i=5$ mM and then decreases to the expected value of 2.5 mM for $[T]_i=0$ mM. Accordingly, the selectivity increases from $s_{AA\text{-}AB}=0.54$ to a maximum value of $s_{AA\text{-}AB}=0.87$ and finally decreases to $s_{AA\text{-}AB}=0.50$. From the data one can conclude that for these boundary conditions it is advantageous to work at low target concentrations around 5 mM but the overall benefit is modest.[31]

The picture changes substantially if we focus exclusively on the assemblies that are bound to the target T. The corresponding data are shown in Figure 8. For an initial target concentration of 14 mM, the assemblies AB and AA are almost exclusively bound to the target with concentrations close to what is found for $[T]_i=100$ mM. By reducing the available amount of target, we constantly reduce the concentration of adducts ABT and BBT. For the homo-dimeric assembly AA, on the other hand, we first observe an *increase* in concentration with a maximum at $[T]_i\sim6$ mM. The selectivity factor constantly increases to its maximum value of $s_{AAT\text{-}ABT}=5$. From an experimental point of view, these results are important. Given that we are able to differentiate between free and bound receptors, we can move from a situation at $[T]_i=14$ mM where the observed adducts approximately reflect the equilibrium concentrations without target to a situation at $[T]_i=4$ mM where we observe the preferen-

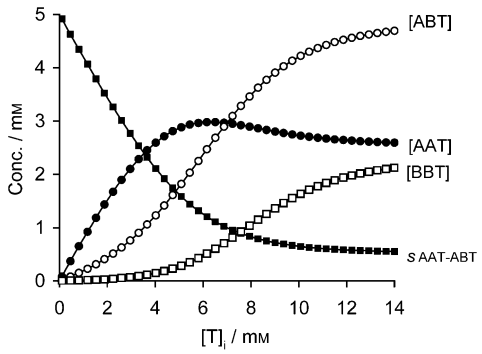


Figure 8. Steady-state concentrations of the adducts AAT (●), BBT (□) and ABT (○) and the selectivity factor $s_{AAT\text{-}ABT}$ (■) as a function of the initial concentration of the target T. The remaining variables were fixed to the following values: $[A]_i=[B]_i=10$ mM; $K_{AA}=K_{BB}={}^1/_2\,K_{AB}=1000\ mM^{-1}$; $K_{AAT}=100\ mM^{-1}$; $K_{ABT}=10\ mM^{-1}$; $K_{BBT}=1\ mM^{-1}$. The calculations are based on Model 3 described in Scheme 4.

tial formation of AAT (2.59 mM) over ABT (1.22 mM) and BBT (0.14 mM) in accordance with the relative binding constants. In terms of selectivity it would be best to work at very low concentrations of $[T]_i$ but in reality, the analytical technique employed will set a limit on the smallest amount of AAT that can be measured with sufficient accuracy.

The association constants of the receptors AA, AB, and BB were the last parameters, the influence of which we have investigated for Model 3 (Figure 9). The relative ratio

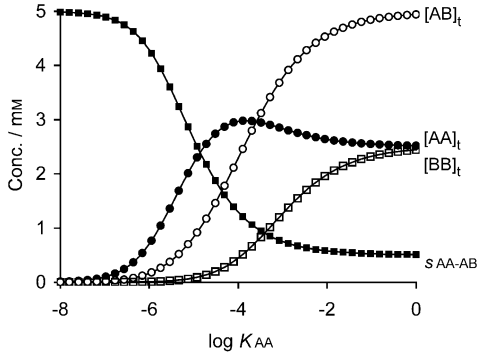


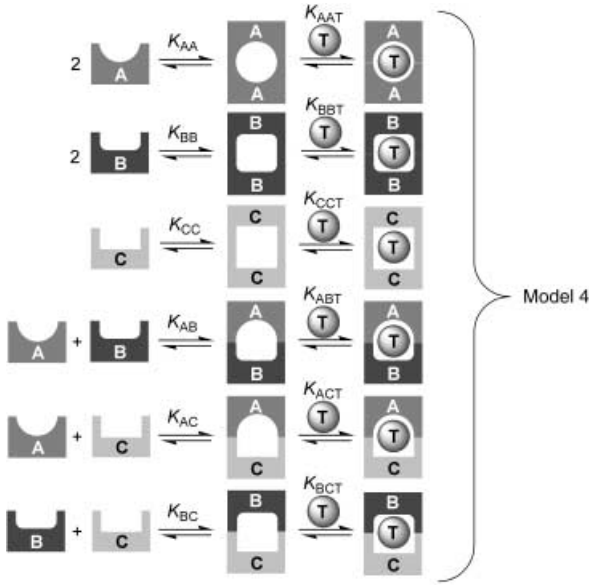
Figure 9. Steady-state concentrations of the receptors AA (●), BB (□) and AB (○) and the selectivity factor $s_{AA\text{–}AB}$ (■) as a function of the association constant K_{AA} (unit: mM^{-1}). The remaining variables were fixed to the following values: $[A]_i=[B]_i=10$ mM; $[T]_i=100$ mM; $K_{AA}=K_{BB}={}^1/_2\,K_{AB}$; $K_{AAT}=100\ mM^{-1}$; $K_{ABT}=10\ mM^{-1}$; $K_{BBT}=1\ mM^{-1}$. The calculations are based on Model 3 described in Scheme 4.

between the constants was fixed to $K_{AA}=K_{BB}={}^1/_2\,K_{AB}$ to simulate a statistical distribution, and the absolute values were varied from $1\times10^{-8}\ mM^{-1}\le K_{AA}\le1\ mM^{-1}$. The binding constants to the target were again set to $K_{AAT}=100\ mM^{-1}$, $K_{ABT}=10\ mM^{-1}$ and $K_{BBT}=1\ mM^{-1}$ with an initial target concentration of 100 mM. The shapes of the resulting curves resemble that of Figure 8. For an association constant of $K_{AA}=1\ mM^{-1}$, the total concentrations of the assemblies are close to the equilibrium concentration that is found without target (no adaptation). If we simultaneously reduce the stability of all assemblies, we initially increase the total amount

of AA but reduce the total amounts of AB and BB. The selectivity constantly increases to approach $s_{AA\text{-}AB}=5$ for very small values of K_{AA}. The maximum value of $[AA]_t$ (2.98 mM) is observed for $K_{AA}=1.5\times10^{-4}$ mM^{-1}. This association constant is so low that without a target, the building blocks A and B are the dominating species in solution ($[A]=[B]=9.94$ mM for $[T]_i=0$ mM) and only small amounts of the DCL members are formed ($[AA]=[BB]=15$ μM and $[AB]=30$ μM for $[T]_i=0$ mM). In a real experiment, the less than 1% aggregates that are present without a target are most likely not to be detected. We therefore have a situation in which the DCL is completely virtual.[32]

From a selection point of view, this virtual combinatorial library (VCL) is highly advantageous. The amplification factors are significantly higher than what is found for a "real" DCL with $K_{AA}>1$ mM^{-1}. For the high affinity receptor AA, for example, we find an amplification factor of $f_{AA}=201$ for the virtual DCL with $K_{AA}=1.5\times10^{-4}$ mM^{-1}. Likewise, the fact that the relative concentrations of the different aggregates AA, AB, and BB correspond to the relative affinities to the target is of importance. A selection process performed with this VCL would therefore provide the right answers: the assembly AA ($[AA]_t=2.98$ mM; $f_{AA}=201$) has the highest affinity for the target followed by AB ($[AB]_t=2.58$ mM; $f_{AA}=87$) and the low affinity receptor BB ($[BB]_t=0.56$ mM; $f_{AA}=38$). The VCL approach allows differentiation between the good receptor AA and the low affinity receptor BB without the need to separate bound and non-bound assemblies (in "real" DCLs, $[AA]_t$ always equals $[BB]_t$).

For the next model that was investigated, the complexity was increased slightly by introducing a third building block C (Scheme 5). Similar to A and B, C can form homo- and hetero-dimers. Therefore, the DCL comprises six competing receptors. Again, the association constants of the hetero-dimers were set to be two times higher than those of the homo-dimers to account for the statistical preference of the former ($K_{AA}=K_{BB}=K_{CC}=\frac{1}{2}K_{AB}=\frac{1}{2}K_{AC}=\frac{1}{2}K_{BC}$). For all calculations, the initial concentrations of the monomers were fixed to $[A]_i=[B]_i=[C]_i=12$ mM. The results of the simulations are summarized in Table 3.



Scheme 5. A model of a DCL of type B ("Model 4"): six dimeric receptors (AA, BB, CC, AB, AC, and BC) are formed by assembly of the building blocks A, B, and C. All receptors are able to bind to a target T.

Table 3. Steady-state concentrations of the receptors AA, BB, CC, AB, AC, and BC [mM] for different associations constants [mM^{-1}], binding constants [mM^{-1}] and initial target concentrations [mM]. The calculations are based on Model 4 described in Scheme 5.

Entry	$[T]_i$	K_{AA}	K_{AAT} [AAT] $[AA]_t$	K_{BBT} [BBT] $[BB]_t$	K_{CCT} [CCT] $[CC]_t$	K_{ABT} [ABT] $[AB]_t$	K_{ACT} [ACT] $[AC]_t$	K_{BCT} [BCT] $[BC]_t$
1	100	1000	100	100	100	100	100	100
			2.00	2.00	2.00	4.00	4.00	4.00
			2.00	2.00	2.00	4.00	4.00	4.00
2	100	1000	100	0.01	0.01	0.01	0.01	0.01
			5.88	1.39	1.39	0.06	0.06	2.78
			5.88	2.96	2.96	0.12	0.12	5.92
3	100	1000	100	0.01	0.01	100	0.01	0.01
			0.34	0.01	2.59	11.28	0.02	0.31
			0.34	0.02	5.61	11.28	0.04	0.67
4	100	1000	180	3.2	5.6×10^{-2}	24	3.2	4.2×10^{-1}
			2.04	2.03	1.76	4.07	3.82	3.75
			2.04	2.03	2.14	4.07	3.84	3.86
5	8	1000	180	3.2	5.6×10^{-2}	24	3.2	4.2×10^{-1}
			3.17	0.72	0.03	3.01	0.61	0.29
			3.27	2.01	3.01	3.74	1.71	4.21
6	100	1×10^{-4}	180	3.2	5.6×10^{-2}	24	3.2	4.2×10^{-1}
			3.11	1.02	0.06	3.56	0.84	0.48
			3.11	1.02	0.07	3.56	0.84	0.49
7	100	1000	4.2×10^{-1}	5.6×10^{-2}	7.5×10^{-3}	1.5×10^{-1}	5.6×10^{-2}	2.1×10^{-2}
			2.21	1.67	0.91	3.75	2.83	2.52
			2.27	2.01	2.32	4.04	3.41	3.92

The first three entries describe rather special cases to point out some important characteristics that appear to be general for DCLs of type B. For these simulations, the initial target concentration was set to 100 mM and the association constants fixed at $K_{AA}=1000$ mM^{-1}. Under these conditions, only very small amounts of the free building blocks A, B, and C are present in solution. If we assume that all receptors have the same high affinity to the target of $K_{XYT}=100$ mM^{-1}, the assemblies are all bound to the target but there is no adaptation and the total receptor concentrations $[XY]_t$ correspond to what is found without a target (Table 3, entry 1). Given that only the assembly AA displays a pronounced affinity for T and all other assemblies have the same low binding constant of 0.01 mM^{-1} (Table 3, entry 2), we observe a significant amplification of AA ($[AA]_t=$

5.88 mM, $f_{AA}=2.94$). But there is a concomitant amplification of species which comprise the other building blocks B and C, namely BB and CC ($[BB]_t=[CC]_t=2.96$ mM; $f_{BB}=f_{CC}=1.48$) and the hetero-assembly BC ($[BC]_t=5.92$ mM; $f_{BC}=1.48$). If we focus on those assemblies that are bound to the target, the concentration of the B/C receptors is much lower and AAT is the only species that is amplified relative to the equilibrium concentration. Entry 3 in Table 3 describes a system with two competing receptors AA and AB, both of which have a high affinity for the target ($K_{AAT}=K_{ABT}=100$ mM^{-1}). Now it is the hetero-dimer AB which is totally dominating the mixture ($[AB]_t=11.28$ mM; $f_{AB}=2.82$) with only small amounts of the homo-dimer AA being present ($[AA]_t=0.34$ mM; $f_{AA}=0.17$). The other species that is amplified is the low-affinity receptor CC with an amplification factor which equals that of AB ($[CC]_t=5.61$ mM; $f_{CC}=2.81$). If such a system would be investigated experimentally, we would obtain two hits, one of which is a false positive. The second good receptor AA, on the other hand, would stay unnoticed.

The remaining four entries in Table 3 describe a more complicated scenario. It was assumed that the target coordinates to the sites A, B and C with a binding energy of $\Delta G°=-15$, -10, and -5 kJ mol^{-1}, respectively, and that the overall binding energy for the dimeric receptor is strictly additive (e.g. -30 kJ mol^{-1} for AAT). The resulting binding constants range from $K_{AAT}=180$ mM^{-1} to $K_{CCT}=5.6\times10^{-2}$ mM^{-1}.[33] The steady-state concentrations of the assemblies using an excess of target T and a high association constant K_{AA} are given in Entry 4 in Table 3. Interestingly, there is basically no re-equilibration of the system when compared to the statistical distribution found without a target. Apparently, for this special combination of binding constants, the effects cancel out resulting in a nearly statistical distribution.

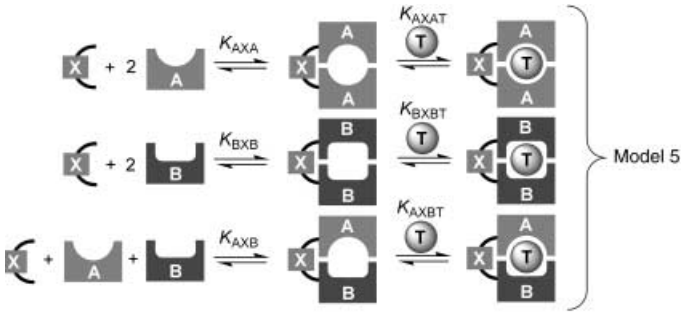
As already discussed for Model 3, there are several possibilities to improve the situation. First, one can reduce the amount of target added (Table 3, entry 5). With an initial target concentration of 8 mM, the highest amplification factor is found for the best receptor AA ($[AA]_t=3.27$ mM; $f_{AA}=1.64$). Furthermore, the total concentration of AA is now higher although the target concentration was reduced. But still, the low affinity receptor CC is likewise amplified to some extent ($[CC]_t=3.01$ mM; $f_{CC}=1.51$). If we focus on the target-bound receptors, however, the relative concentrations reflect nicely the relative binding constants.

A similar improvement is found if we work with a VCL (Table 3, entry 6). Again, it is the receptor AA that is amplified the most ($[AA]_t=3.11$ mM; $f_{AA}=219$) followed by the hetero-dimer AB ($[AB]_t=3.56$ mM; $f_{AB}=125$). It should be noted, however, that for a real experiment it might be difficult to determine the exact amplification factor due to the low steady-state concentration of the assemblies in the absence of the target (e.g. 14 μM for AA). Nevertheless, the good receptor AA can be identified simply by its concentration if we account for the statistical preference of AB over AA.

Finally, a system was investigated for which the binding affinities to the target were reduced to half of what was used for the simulations described in entries 4–6 in Table 3 (e.g. -15 kJ mol^{-1} for AA; Table 3, entry 7). Experimentally, this could be achieved by changing the solvent polarity or by changing the pH. Compared to entry 4 in Table 3, we observe a slight improvement: if we focus on the target-bound assemblies and account for the statistical preference of the hetero-dimers, we can identify AA as the best receptor but the differentiation is less good when compared to the systems described in entries 5 and 6 in Table 3.

From the simulations described above, the following characteristics of a DCL of type B can be deduced: 1) It is not necessarily the assembly with the highest affinity for the target that displays the highest amplification factor. 2) In competition situations, there is an intrinsic bias for the selection of hetero-assemblies. 3) It is possible that there is no re-equilibration upon addition of a target although the affinities of the DCL members to the target differ substantially. 4) Low target concentrations and/or low binding constants can give rise to selectivities which correspond more closely to the relative binding affinities, in particular if it is possible to differentiate between bound and non-bound members of the DCL. 5) For high association constants, the amplification of an assembly with a high content of one subunit will lead to the concomitant amplification of assemblies with a high content of the other subunits. 6) For selection processes with a DCL of type B, it is advantageous to work under conditions where the building blocks are the dominant species in solution and the aggregates are present in only small amounts (VCL approach).

A minimal model for a DCL of type B*: To simulate the adaptive behavior of a DCL of type B*, the minimal model described in Scheme 6 was used. The basic features are simi-



Scheme 6. A minimal model of a DCL of type B* ("Model 5"): three receptors (AXA, AXB, and BXB) are formed by assembly of the building blocks A and B and the spacer X. All receptors are able to bind to a target T.

lar to that of Model 3: two building blocks A and B contain binding sites for a target T. But this time, the self-assembly process requires a spacer X. The resulting receptors AXA, AXB, and BXB form a dynamic mixture, which is able to adapt upon addition of a target T.

To have a direct comparison with Model 3, boundary conditions were chosen which give rise to steady-state concentrations that are similar to what was calculated for a DCL without spacer. Thus, the association constant K_{AAA} was

fixed to 10000 mM^{-2} with $K_{AXA}=K_{BXB}=1/2\,K_{AXB}$ to ensure a statistical distribution of the receptors in the absence of a target. For $[A]_i=[B]_i=[X]_i=10$ mM, we find a steady-state concentration of 2.48 mM for AXA and BXB and of 4.98 mM for AXB. The affinities to the target T were chosen as before, that is the stability constants of the receptors were set to $K_{AXAT}=100$ mM^{-1}, $K_{AXBT}=10$ mM^{-1}, and $K_{BXBT}=1$ mM^{-1}.

If equal concentrations of A, B, and X are employed, this DCL behaves very similarly to what was found for Model 3. For $[A]_i=[B]_i=[X]_i=10$ mM and $[T]_i=100$ mM , for example, we basically observe no re-equilibration of the system. Interesting differences, however, arise if we vary the concentration of the building blocks A and B with respect to the spacer X. This is shown in Figure 10. As already described

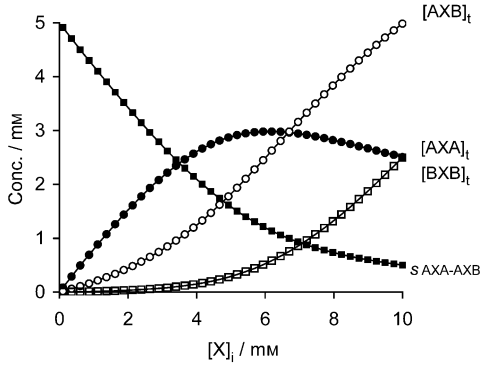


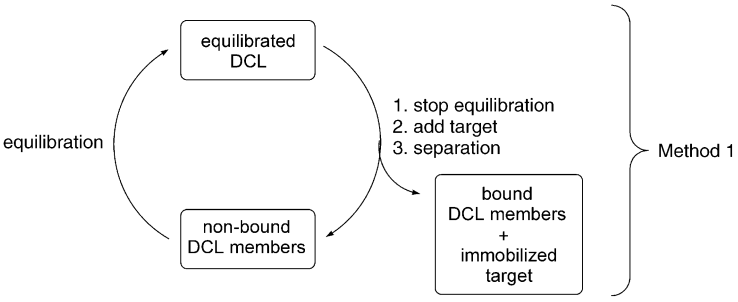
Figure 10. Steady-state concentrations of the receptors AXA (●), BXB (□) and AXB (○) and the selectivity factor $s_{AXA\text{-}AXB}$ (■) as a function of the initial concentration of the spacer X. The remaining variables were fixed to the following values: $[A]_i=[B]_i=10$ mM; $[T]_i=100$ mM; $K_{AXA}=10000$ mM^{-2}; $K_{AA}=K_{BB}=1/2\,K_{AB}$; $K_{AXAT}=100$ mM^{-1}; $K_{BXBT}=10$ mM^{-1}; $K_{BXBT}=1$ mM^{-1}. The calculations are based on Model 5 described in Scheme 6.

above, at an initial concentration of $[X]_i=10$ mM the system does not show any adaptation and the selectivity factor $s_{AXA\text{-}AXB}$ is 0.5. As soon as we deprive the mixture of the spacer X, the concentration of the receptors AXB and BXB decreases. The steady-state concentration of the high affinity receptor AXA, however, at first *increases* when the concentration of $[X]_i$ is lowered. This may seem surprising because we reduce the concentration of one of its building blocks but on the other hand we increase the amount of the free subunit A. Finally, the concentration of AXA also approaches zero because the availability of X becomes the limiting factor. Going from $[X]_i=10$ mM to $[X]_i=0.1$ mM, the selectivity factor constantly rises to its maximum value of $s_{AXA\text{-}AXB}=5$.

The data show that it is advantageous to perform a selection experiment at low concentration of the common building block X. If maximum selectivity were the only objective, it would be advantageous to work at *very low* concentrations of X. But from a practical point of view, it is most likely better to investigate the selection process at intermediate X concentrations where significant (and detectable) amounts of receptor are formed. Mechanistically, the reduction of X has a similar effect to working with a virtual DCL because both systems contain significant amounts of the free building blocks A and B. These free monomers act as a kind of buffer, which reduce the direct competition situation between the receptors (AB can only be formed on behalf of AA and BB).

For the optimal design of a selection process with a DCL of type B, these findings are of central importance. As demonstrated previously, DCLs of type B may face the problem that the assembly that is amplified most is not necessarily the assembly with the highest affinity to the target T. At least partially, this problem can be reduced if the experiment is performed with a virtual DCL, with a low target concentration, or by operating under conditions where the affinities to the target are not too high. But depending on the type of interaction that is used to assemble the subunits (covalent, noncovalent or metal–ligand bonds), it may be very difficult to adjust the association constants in such a way that a virtual DCL results. For systems that show a very low affinity for the target, the amplification may be difficult to detect. The introduction of a common building block X to generate a DCL of type B* represents another possibility for dealing with the above mentioned problem. In real systems, this building block may be a metal ion in a DCL based on metal complexes or a difunctional linker in a covalent DCL. By using sub-stoichiometric amounts of X with respect to the other building blocks, it is possible to enhance the selectivity factors and to reduce the possibility of false positives.

Evolutionary systems: A particularly interesting perspective of DCLs is the possibility of refining the selection using an iterative process. Here, two conceptually different approaches can be distinguished. The first method is schematically described in Scheme 7. An equilibrated DCL is subjected to conditions under which exchange reactions are slow. After addition of an immobilized target, the DCL members that are not bound to the target are separated and subsequently re-equilibrated under conditions that allow a fast exchange. This re-equilibration may lead to the reformation of DCL members with a high affinity to the target, which are then separated by using again the immobilized target.



Scheme 7. Iterative procedure for the selection of DCL members with a high affinity to an immobilized target ("Method 1").

Numerical simulations and experimental data (see below) on a system of this kind have been described by Eliseev.[22d]

Using a small DCL of three different compounds that can be completely inter-converted, they were able to demonstrate that it is possible to amplify the DCL member with the highest affinity to the immobilized target. The overall effect was shown to be the same as if the target was added directly to the equilibrating DCL. It was pointed out that the separation of the equilibration step from the selection step could be advantageous since the equilibration conditions are not necessarily compatible with the target.

The systems modeled by Eliseev are rather special since they are based on DCLs where each member can be converted into every other member. This is clearly not true for DCLs of type B and for DCLs of type A one has to account for the fact that the aggregation numbers are different for the various species. One of his main conclusions is valid for all types of DCLs: the final equilibrium for an evolutionary system with separated equilibration and selection steps (Scheme 7) will correspond to what is found for a system having identical boundary conditions except that the target is added directly to the dynamic mixture. The final concentrations for DCLs of types A and B will therefore be the same as described in the previous sections. It is important to note, however, that for high binding constants or for low association constants, the iterative process may require many cycles to reach the final equilibrium. This is illustrated by the following calculations.

To simulate an evolving DCL of type A, the model depicted in Scheme 2 was used with the exception that the equilibria between A, AA, and AAA were separated from the binding event to the (immobilized) target. The following fixed parameters were employed: $[A]_i = 20$ mM; $[T]_i = 100$ mM; $K_{AA} = 1600$ mM^{-1}; $K_{AAA} = 20$ mM^{-1}. As outlined above, these values give rise to an equimolar mixture of AA and AAA in the absence of T ($[AA] = [AAA] = 3.99$ mM). Using these concentrations, the amount of receptors that are bound to the immobilized target were calculated assuming that there is no interconversion between A, AA and AAA. With binding constants of $K_{AAT} = 1 \times 10^{-2}$ mM^{-1} and $K_{AAAT} = 1 \times 10^{-3}$ mM^{-1}, we find that 1.97 mM AA and 0.36 mM AAA are bound to the target. The solution concentrations of these species are 2.02 mM (AA) and 3.63 mM (AAA), respectively. Those values were employed as the input concentrations for the next equilibration step which gives rise to steady-state concentrations of $[AA] = 3.18$ mM and $[AAA] = 2.84$ mM. Addition of this solution to the immobilized target increases the concentration of AAT from 1.97 mM to 2.54 mM and decreases that of AAAT from 0.36 mM to 0.28 mM. The evolution of this system over the first six cycles is shown in Figure 11. Within six generations, the system has reached a stable equilibrium with final concentrations of $[AAT]_f = 2.85$ mM and $[AAAT]_f = 0.25$ mM. The iterative process therefore leads to an increase of the selection factor from $s_{AAT\text{-}AAAT} = 5.47$ to $s_{AAT\text{-}AAAT} = 11.40$. As mentioned above, these final values correspond exactly to what is calculated for a system where the target is added directly to the dynamic mixture of A, AA and AAA (Model 1, Scheme 2).

If we increase both binding constants by a factor of ten ($K_{AAT} = 1 \times 10^{-1}$ mM^{-1}; $K_{AAAT} = 1 \times 10^{-2}$ mM^{-1}), we also increase the maximum selectivity that is found for the final equilibrium to $s_{AAT\text{-}AAAT} = 21.43$. This effect is expected because the intrinsic preference for the smaller aggregate AA is higher for larger binding constants. But instead of six cycles, it now requires 16 cycles to establish the final equilibrium for this system. Experimentally, this trend may be a severe limitation because with binding constants above 1 mM^{-1}, the system evolves very slowly and many cycles are needed to reach the final equilibrium.

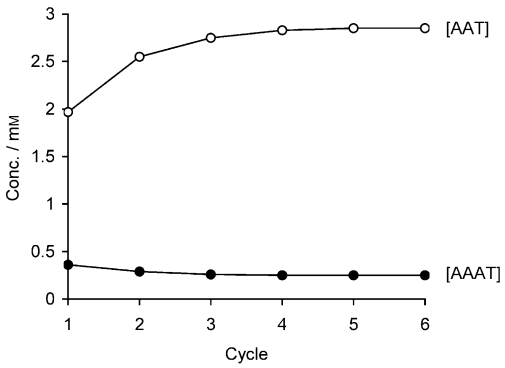
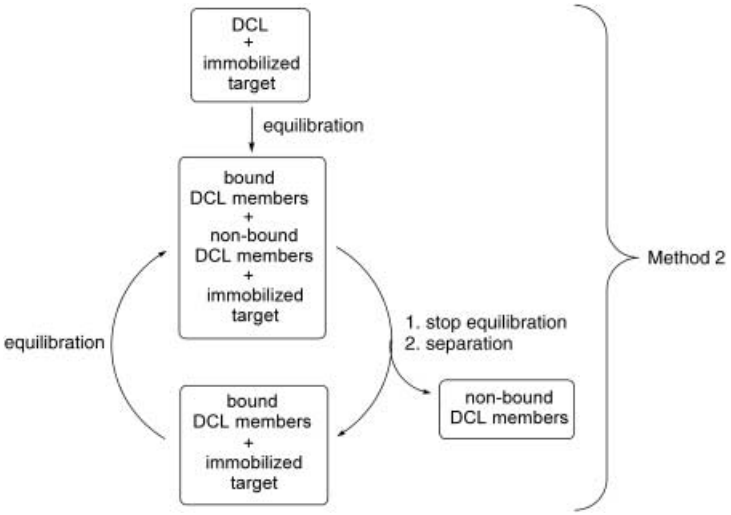


Figure 11. Steady-state concentrations of the target-bound receptors AAT and AAAT over the first five cycles of the evolutionary selection process for binding constants of $K_{AAT} = 1 \times 10^{-2}$ mM^{-1} and $K_{AAAT} = 1 \times 10^{-3}$ mM^{-1}. The remaining variables were fixed to the following values: $[A]_i = 20$ mM; $[T]_i = 100$ mM; $K_{AA} = 1600$ mM^{-1}; $K_{AAA} = 20$ mM^{-1}. The calculations are based on Method 1 described in Scheme 7.

The same problem applies to VCLs. Because VCLs display a low concentration of aggregates, the amount that can be bound to the target in each selection step is very small. As for systems with high binding constants, the number of cycles required to reach the final equilibrium is therefore large.

A conceptually very different evolutionary approach is depicted in Scheme 8. An immobilized target is added to a DCL. The mixture is then subjected to conditions under which adaptation occurs. When the new equilibration is es-



Scheme 8. Iterative procedure for the selection of DCL members with a high affinity to an immobilized target ("Method 2").

tablished, the system is "frozen" (e.g. by cooling or by a pH change) and the bound and the non-bound members of the library are separated by filtration. The fraction of the DCL that is bound to the target is then subjected again to conditions under which equilibration occurs. In each cycle of this process, a certain fraction of the DCL is lost in the separation step. However, given that the immobilized target is able to shift the composition of the DCL towards members with high binding constants, the *relative* concentration of these species will increase.

To simulate such an experiment, we have focused on the DCL illustrated in Scheme 5 (Model 4). For the boundary conditions, we have chosen the VCL situation described in Table 3, entry 6. Under those conditions we already observe a good selectivity for best receptor AA ($K_{AAT}=180\ mM^{-1}$). Together with the second best receptor AB ($K_{ABT}=24\ mM^{-1}$), these two DCL members are the dominant species that are bound to the target T ([AAT]=3.11 mM; [ABT]=3.56 mM). The low affinity receptors BB and AC ($K_{BBT}=K_{ACT}=3.2\ mM^{-1}$) are likewise bound to the target but the concentrations are approximately three times lower. The concentrations of the remaining receptors CC and BC are very low under these conditions (0.49 and 0.07 mM). Figure 12 shows the evolution of the system for the first six generations.

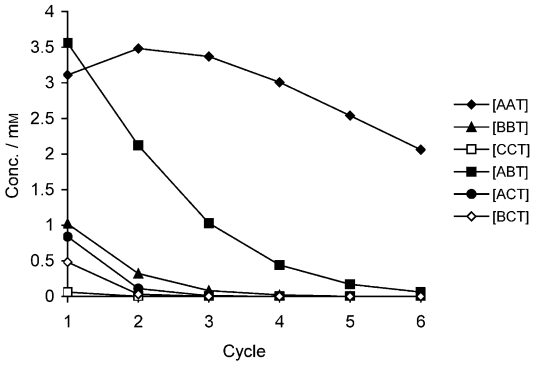


Figure 12. Steady-state concentrations of the target-bound receptors AAT, BBT, CCT, ABT, ACT, and BCT over the first six cycles of the evolutionary selection process. The remaining variables were fixed to the following values: $[A]_i=[B]_i=[C]_i=12\ mM$; $[T]_i=100\ mM$; $K_{AA}=1\times10^{-4}\ mM^{-1}$; $K_{AA}=K_{BB}=K_{CC}={}^1/_2\,K_{AB}={}^1/_2\,K_{AC}={}^1/_2\,K_{BC}$; $K_{AAT}=180\ mM^{-1}$; $K_{BBT}=24\ mM^{-1}$; $K_{CCT}=5.6\times10^{-2}\ mM^{-1}$; $K_{ABT}=24\ mM^{-1}$; $K_{ACT}=3.2\ mM^{-1}$; $K_{BCT}=4.2\times10^{-1}\ mM^{-1}$. The calculations are based on Method 2 described in Scheme 8.

In the theoretical paper by Moore and Zimmerman, an evolutionary protocol as described in Scheme 8 was disregarded as impractical because "the overall yield will plummet exponentially".[23] Although it is true that the *overall* yield decreases during such a process, the *relative* yields may change substantially. In our simulation, the concentration of the high affinity receptor AA even increases initially. After six cycles, we still observe 2.06 mM of AA, whereas the *total* concentration of all other DCL members is 0.06 mM. While this corresponds to a substantial loss of material (88%), such a situation can be highly advantageous under practical considerations. Without this iterative process, there are significant amounts of four DCL members, the separation of which might be analytically challenging. After six cycles, the concentration of the best receptor AA has dropped from 3.11 mM to 2.06 mM but there are essentially no other DCL members that would interfere with the measurement.

A related behavior is found if we calculate the evolution of a system with limiting amounts of target according to Table 3, entry 5. The concentrations of all assemblies drop with each cycle with the exception of the high affinity receptor AA. Even after 14 cycles, the concentration of the latter is higher than at the beginning (3.62 versus 3.17 mM; Figure 13), whereas the concentration of the competing receptor AB has dropped by 46%. The total concentration of all other receptors is less than 0.2 mM.

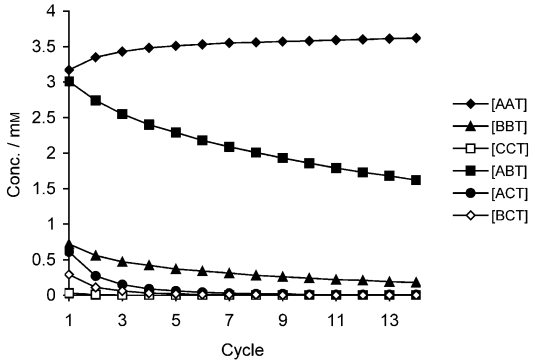


Figure 13. Steady-state concentrations of the target-bound receptors AAT, BBT, CCT, ABT, ACT, and BCT over the first fourteen cycles of the evolutionary selection process. The remaining variables were fixed to the following values: $[A]_i=[B]_i=[C]_i=12\ mM$; $[T]_i=8\ mM$; $K_{AA}=1000\ mM^{-1}$; $K_{AA}=K_{BB}=K_{CC}={}^1/_2\,K_{AB}={}^1/_2\,K_{AC}={}^1/_2\,K_{BC}$; $K_{AAT}=180\ mM^{-1}$; $K_{BBT}=24\ mM^{-1}$; $K_{CCT}=5.6\times10^{-2}\ mM^{-1}$; $K_{ABT}=24\ mM^{-1}$; $K_{ACT}=3.2\ mM^{-1}$; $K_{BCT}=4.2\times10^{-1}\ mM^{-1}$. The calculations are based on Method 2 described in Scheme 8.

Also of interest was the behavior of the system described in Table 3, entry 7. Compared to the previous system, the affinities for the target are reduced leading to a small amplification of the best receptor AA in the first equilibration step ($[T]_i=100\ mM$) but all competing receptors are likewise present in significant amounts. After 14 cycles, the concentration of AAT is as high as at the beginning (with an intermediate maximum after six cycles) but the concentrations of the remaining receptors have dropped between 66 and 99% (Figure 14). The final differentiation is remarkably good, given the fact that the binding constants are very similar (the binding constant of the best receptor AA and the worst receptor CC differ by a factor of 56).

These simulations show that an evolutionary protocol according to Scheme 8 is potentially very valuable, in particular for larger DCLs, where the separation of the different members becomes difficult. But what are the basic criteria that have to be fulfilled so that an iterative procedure will lead to the relative enrichment of the best binders? First of all, a repetitive selection protocol according to Scheme 8 is not useful for DCLs of type A. Since the assemblies are formed from a single building block, each equilibration step will produce all possible DCL members. Due to the fact that

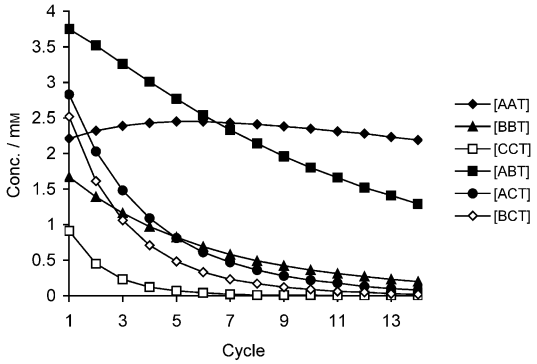


Figure 14. Steady-state concentrations of the target-bound receptors AAT, BBT, CCT, ABT, ACT, and BCT over the first fourteen cycles of the evolutionary selection process. The remaining variables were fixed to the following values: $[A]_i=[B]_i=[C]_i=12$ mM; $[T]_i=8$ mM; $K_{AA}=1000\ mM^{-1}$; $K_{AA}=K_{BB}=K_{CC}={}^1/_2\,K_{AB}={}^1/_2\,K_{AC}={}^1/_2\,K_{BC}$; $K_{AAT}=4.2\times10^{-1}\ mM^{-1}$; $K_{BBT}=5.6\times10^{-2}\ mM^{-1}$; $K_{CCT}=7.5\times10^{-3}\ mM^{-1}$; $K_{ABT}=1.5\times10^{-1}\ mM^{-1}$; $K_{ACT}=5.6\times10^{-2}\ mM^{-1}$; $K_{BCT}=2.1\times10^{-3}\ mM^{-2}$. The calculations are based on Method 2 described in Scheme 8.

the overall concentration decreases with each cycle, this re-equilibration will lead to a *relative* increase of assemblies with a low aggregation number.[34] The iterative process therefore enhances the bias for small assemblies. Unless the smallest assembly is also the best binder, the evolutionary process will be counterproductive.

For DCLs of type B, one should perform the experiment under conditions that already lead to an amplification of the best binders in the first cycle (e.g. with limiting target concentration, or ideally with a VCL). Furthermore, the binding constant of the assembly with the highest affinity to the target should be large enough that the majority of the respective assembly is bound to the target. Too much material is otherwise lost in the washing steps. The opposite is true for the binding constants of the low affinity receptors. Here, it is desirable that a significant fraction is removed by separation of the immobilized target.[35] One should note that the procedure described in Scheme 8 does *not* represent a simple affinity chromatography with several washing steps for which an increase in receptor concentration (as described in Figure 12 13, and 14) would be impossible.

Comparison with experimental data: An experiment, which corresponds to Model 1 (Scheme 2) was realized by using pseudo-peptide cyclic hydrazones.[10d] In thermodynamic equilibrium, a dimeric (88%) and a trimeric macrocycle (11%) were shown to be the dominant species in solution. Addition of acetylcholine as a target reversed the composition of the library (13% dimer, 86% trimer). We thus observe the strong amplification of the assembly with the higher aggregation number. Since the experiments were performed with a "real" DCL in the presence of an excess of target, our calculations predict that the observed selectivity is only possible if the binding constant of the trimer is significantly larger than that of the dimer. In the publication,[10d] the authors provide only data for the complexation of the trimer. It is therefore not clear whether we have a real competition situation between two receptors in this system. In a full account on this topic,[10c] the authors mention that for some targets such as $BnEt_3NCl$, it is possible to amplify higher oligomers than trimers. Unfortunately, there are again no detailed thermodynamic data on this—according to our calculations—unusual behavior.

A completely different response was observed for a structurally related pseudo-peptide hydrazone library using a crown ether as the target.[13] Here, the monomeric building block, which was present in trace amounts in the initial DCL, was amplified considerably so that it finally represented the dominant species in solution. Although we have not explicitly modeled a system where the monomeric building block can interact with the target, it is clear that such an interaction may lead to a very strong re-equilibration. This follows from the same argument that was outlined above: the formation of the monomer on behalf of the oligomers will increase the total amount of molecules that are able to interact with the target.

An examination of all the available experimental data on DCLs of type A[2] reveals that in most cases, it is an assembly with a small (but not necessarily the smallest) aggregation number that is amplified the most.[36] It is plausible that the receptors detected in these experiments are indeed the best binders but our simulations show that a receptor with a high aggregation number will have a hard time competing with the smaller ones and may thus escape detection.

For selection experiments with DCLs of type B, our calculations predict that there is an intrinsic advantage for hetero-assemblies that exceeds the simple statistical preference. This result was recently validated experimentally for a DCL of trinuclear metallamacrocycles.[22a] Steric interactions were used to introduce thermodynamic differences among the DCL members. In accordance with the predictions, it was not the thermodynamically most stable homo-trimer that was formed preferentially but the less stable hetero-trimers.

A DCL of type B which corresponds closely to Model 3 (Scheme 4) was described by Hioki and Still.[18b] Two different organic oligomers A and B, which can act as receptors for small peptides, were connected by means of a disulfide bridge. Under conditions that allow disulfide exchange, a nearly equal distribution of the species AA, AB, and BB was observed. Addition of an excess of a polymer-supported tripeptide as a target resulted in a pronounced re-equilibration with amplification of the homo-dimers AA and BB. According to calculations, this is only possible if the hetero-dimer AB has a significantly weaker binding affinity and this is exactly what the authors found. Since an immobilized target was employed, they were also able to separate the bound and non-bound receptors. This allowed the isolation the high affinity receptor AA in 97.5% purity.

The advantage of working with VCLs was demonstrated by Eliseev, Nicolau and co-workers.[19a,b] Using combinatorial mixtures of imines and the enzyme neuraminidase as a target, they were able to able identify potent inhibitors in single screening experiments. The key for the success of the experiment was that the conditions were chosen is such a fashion that the selected imines[37] were only formed in the presence of the target. In some cases, the observed amplifi-

cation factors were higher than 100, which allowed screening of mixtures of remarkable complexity. It should be noted that in addition to using a VCL approach, these experiments were also performed with assemblies that contain sub-stoichiometric amounts of a common building block (the amine). This might have contributed to the observed selectivity.

Remarkably high amplification factors were also reported in a recent publication by Nitschke and Lehn in which they studied the dynamic assembly of a metallosupramolecular grid.[38] Again a VCL approach was employed.

A clear demonstration that the introduction of a common building block can be beneficial for a selection process with DCLs was reported by the group of McLendon.[22b,39] Instead of screening the interactions with a target, they used a DCL to address the question of protein folding. For this purpose, they synthesized three peptide subunits containing 20 amino acids and a bipyridine ligand at the C-terminus. In the presence of iron(II), these peptides form a library of 11 exchange-labile three-helix bundles. Here, the metal ions act as a common building block. By determining the steady-state concentrations of the peptide-bundles in the presence of different concentrations of iron(II), they found that the most stable assembly was only selected when substoichiometric amounts of iron(II) were employed.

Although there are several reports about screening experiments with immobilized targets,[2] there are—to the best of our knowledge—only two publications about evolutionary systems.[7g,22d] Both are from the group of Eliseev who have used a photochemically induced isomerization process to generate a mixture of three different dicarbonic acids. Following a protocol that corresponds to Method 1, Scheme 7, the mixture was repetitively equilibrated and then allowed to interact with an immobilized arginin group. After 30 cycles, they observed a pronounced increase of the best receptor with respect to the other two. As predicted theoretically, the final concentration of the best receptor corresponds to what was calculated for a selection experiment where the equilibration is performed directly in the presence of the target.

A real refinement of the selection process should be possible with the method described in Scheme 8. Although conceptually simple, an experiment of this kind has not yet been performed (possibly discouraged by concerns as outlined by Moore and Zimmerman).[23] Our simulations show that such a process is potentially very useful, in particular for more complex DCLs with competing assemblies. It remains to be seen whether future experiments can confirm this prediction.

Conclusion

The theoretical analyses reported in this publication were carried out to gain further insight into the adaptive behavior of DCLs. Instead of presenting general mathematical descriptions, numerical simulations were performed on selected minimal models using a series of typical (or instructive) boundary conditions.[40] As expected for any complex network of interacting entities, some of the systems described in this study display a behavior that would be difficult to predict in detail without numerical simulations. For DCLs with an increased structural diversity, this is even more likely to be the case. The simulations have also revealed some fundamental trends and characteristics, which should be considered in the design of selection experiments with DCLs of types A and B.

A central finding is the fact that for both types of DCLs, it is not necessarily the assembly with the highest affinity to a given target that is amplified the most. Quite contrary, it is possible that the addition of a target molecule will lead to a decreased steady-state concentration of the best binder. This contradicts what has been claimed repeatedly in the literature.[41] Furthermore, it is possible that a target does not induce a re-equilibration of the dynamic mixture, even if the binding constants of the constituent members vary substantially.

For DCLs of type A, a bias is observed for the selection of assemblies with a small aggregation number, whereas for DCLs of type B there is an intrinsic preference for assemblies, the composition of which reflects the composition of the library. These preferences are most pronounced for systems with high association and binding constants containing an excess of target. To utilize DCLs in selection processes, it would be desirable if the highest amplification factors are found for the species with the highest binding constants. Our simulations show that such an "ideal" situation can be approached by an appropriate design of the selection experiment. An important parameter that is easy to control is the initial target concentration and for many systems it should be advantageous to work with relatively low amounts of the target. If the chemistry involved allows modulation of the association constants, it is recommended that the work is carried out with virtual libraries under conditions where the building blocks (and not the assemblies) are the dominant species in solution. Alternatively, one can design the DCL in such a fashion that the assemblies contain a common building block. Then it would be beneficial to use substoichiometric amounts of this building block with respect to the other subunits.

Evolutionary protocols represent an interesting alternative or extension to single DCL selection experiments. An experimental setup as described in Scheme 7 allows separation of the selection step from the equilibration step which may be advantageous for sensitive targets. According to the simulations reported herein, the repetitive equilibration in the presences of a target (Scheme 8) can be used to increase the signal-to-noise ratio of a selection experiment without a dramatic decrease of the signal intensity, at least for some systems. It will be interesting to compare these predictions with future experimental data.

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[24] For the present discussion it is assumed that every building block of a DCL of type B is able to associate with every other building block. Depending on the type of interaction that is used to link the subunits, this is not necessarily the case.

[25] It should be noted that in principle, all these types of DCL can be comprised of linear and/or cyclic assemblies—the graphical focus on cyclic assemblies in Scheme 1 was chosen arbitrarily.

[26] An alternative definition of the selectivity factor would be the ratio of amplification factors but the conclusions/trends are the same, regardless of the definition.

[27] All simulations were performed with Gepasi, version 3.30. The steady-state concentrations were calculated using the DCL models described in the text and the Newton algorithm that is implemented in the program. a) P. Mendes, *Comput. Appl. Biosci.* **1993**, *9*, 563–571; b) P. Mendes, *Trends Biochem. Sci.* **1997**, *22*, 361–363.

[28] In Schemes 2,4, and 5 the targets are shown as a substrate with the DCL members acting as receptors. The results of the simulations are equally valid for the reverse situation.

[29] Nomenclature: $[AA]_t = [AA] + [AAT]$.

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[31] The initial template concentration for which the maximum selectivity is observed depends on the absolute values of the binding constants. For low affinity receptors with $K_{AAT} = 1\ \mathrm{mM}^{-1}$; $K_{ABT} = 0.1\ \mathrm{mM}^{-1}$, $K_{BBT} = 0.01\ \mathrm{mM}^{-1}$, for example, the highest selectivity is observed for $[T]_i = 15$ mM.

[32] The term "virtual combinatorial library" (VCL) was introduced in 1997 by Lehn (see ref. [6c]).

[33] As described for Model 4, the simulation focuses on the dimeric receptors and does not include the possibility that the target binds to the monomeric building blocks.

[34] For a theoretical treatment of the concentration dependence of macrocyclizations see: G. Ercolani, L. Mandolini, P. Mencarelli, S. Roelens, *J. Am. Chem. Soc.* **1993**, *115*, 3901–3908.

[35] The washing step can be performed under conditions that are quite different from the equilibration conditions. To ensure that the weak binders are partially removed, it would be possible to decrease the binding constants by variation of the solvent polarity or the pH.

[36] A notable exception is described in reference [8]: the addition of sodium ions as a target was shown to increase the relative amount of a cyclic pentamer over a tetramer and a trimer.

[37] The imines were generated by condensation of a diamine and a set of ketones or aldehydes. Prior to analysis, the imines were reduced in situ to the corresponding amines.

[38] J. R. Nitschke, J.-M. Lehn, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 11970–11974.
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[40] It should be pointed out that there are several other variations of DCLs that were not discussed in this analysis. Apart from DCLs of type C (which should display the characteristics of both A and B), there is the possibility of using assembly reactions that are chemically orthogonal to each other (e.g. metal–ligand binding and imine bond formation; see: V. Goral, M. I. Nelen, A. V. Eliseev, J.-M. Lehn, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 1347–1352. Furthermore, one could couple a DCL with kinetically controlled reactions as discussed in reference [22c]. And these are just two recent developments in a rapidly evolving field.
[41] The statement that the addition of a target will shift the equilibrium toward the DCL member that binds the target most efficiently can be found in the references [11], [17a], [19a], [22c], among others. For a recent overview about DCLs entitled "Amplification of the Fittest" see: M. Freemantle, *Chem. Eng. News* **2002**, Sept. 2, 31–33.

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