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Determination of binding affinities on solid supports: influence of the loading and the nature of the solid support

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The scope of a solid phase binding assay for the determination of binding affinities between a solid supported substrate and a coloured host has been studied by investigating the influence of the nature and the loading of the solid support.

Solid-phase supported chemistry has become an important tool for applications ranging from traditional solid phase synthesis, the use of solid supported reagents and scavenger resins to the generation of split-and-mix libraries and their testing in on-bead binding assays.[1] We and others have used a solid phase assay for the determination of binding affinities between a solid supported substrate and a dye-marked receptor in solution, or *vice versa*.[2,3] The assay provides a convenient and fast estimate of the intermolecular binding strength simply by measuring the UV/VIS absorbance of the coloured receptor before and after the incubation with the solid supported substrate (Fig. 1). For the calculation of the binding constant it is assumed that all of the immobilised guest molecules can participate in the intermolecular association with the host molecule. Neither the solid support is taken into consideration nor are possible effects arising from the aggregation of the substrate within the resin bead, the polarity of the resin, its swelling and diffusion properties.

To gain insight into the macroscopic influence of the solid support we determined the binding energies between a peptidic guest immobilised on different kinds of resins towards its dye-marked receptor in chloroform solution. The present work describes our findings on the influence of the loading and the nature of the solid support on the solid-phase binding affinity measurement.

As a test system we used the diketopiperazine receptors **1** and **2** as dye-marked hosts and the tripeptide Ac-D-Val-D-Val-D-His-linker-resin as a solid supported guest (Fig. 2). This highly specific intermolecular interaction had previously been found in combinatorial on-bead assays between the dye-marked receptors and a 24389-membered tripeptide library on polystyrene resin.[3a]

In order to analyze the impact of the capacity of the solid support on the measured binding affinity we functionalized the same type and batch of polystyrene (PS) beads with different amounts of the peptide, Ac-D-Val-D-Val-D-His-NH$(CH_2)_5$CO–PS. To allow a wide range of different loadings we used a 1% crosslinked aminomethyl-PS (200–400 mesh) with an initial loading of 1.13 mmolg^{-1}.[4] Different degrees of peptide functionalization were accomplished by coupling different ratios of N-Fmoc-aminohexanoic acid (Fmoc-Ahx) and acetic acid onto the PS-support. Diisopropylcarbodiimide (DIC) and

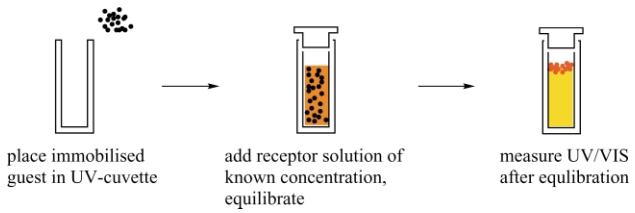
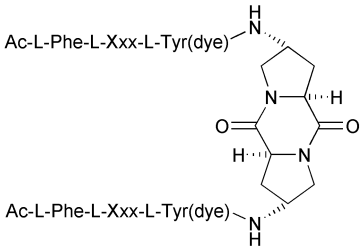


Fig. 1 Solid phase binding assay.



receptor **1**: Xxx = Asn(Trt)

receptor **2**: Xxx = Gln(Trt)

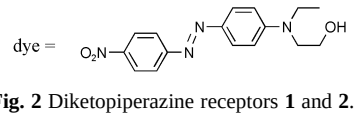


Fig. 2 Diketopiperazine receptors **1** and **2**.

1-hydroxybenzotriazole (HOBt) served as coupling reagents for this initial coupling as well as for the following amino acid couplings with N-α-Fmoc-D-His and N-α-Fmoc-D-Val. † The loading of each resin was determined by the quantitative Fmoc-test.[5] By using mixtures of Fmoc-Ahx and acetic acid ranging from 100:0 to 10:90 for the initial resin functionalisation, seven different resins with peptide loadings between 1.13 mmolg^{-1} and 0.11 mmolg^{-1} were prepared. These macroscopic loadings correspond to average peptide concentrations on the beads swollen in chloroform ranging from $\approx$300 mM^{-1} to $\approx$30 mM^{-1}.

For the determination of the binding affinity a precisely measured amount of the solid supported peptide was placed in a UV-cuvette and 1 ml of the receptor in chloroform solution ($\approx$14 μM) was added.[6] The mixture was tightly sealed and allowed to equilibrate by slight agitation for at least 48 h. After this time period, the absorbance of the remaining receptor concentration did no longer change when measured after allowing the beads to float to the top of the chloroform solution (Fig. 1).

The binding constants and affinities were calculated under the assumption of a simple bimolecular receptor–peptide complex and participation of all peptides in the intermolecular binding by eqn. (1).

$$K_a = [RP]/\{([R_0] - [RP])\,([P_0] - [RP])\} \quad (1)$$

[RP] = concentration of the receptor–peptide complex at equilibrium, $[R_0]$ = initial receptor concentration, $[P_0]$ = initial peptide concentration.

In control experiments with PS-resin that was acetylated in place of the peptide no binding was observed. Thus, unspecific background absorption of the receptors to the resin matrix can be neglected. The data listed in Table 1. demonstrate that receptor **1** binds the peptide Ac-D-Val-D-Val-D-His-NH$(CH_2)_5$CO–PS generally by $\approx$1 kcal mol^{-1} tighter than receptor **2** independent of the loading (except at a loading of $\geq$1 mmol g^{-1}). Thus, the relative binding strengths are retained regardless of the resin capacity. In contrast, the absolute binding affinity depends on the capacity of the resin. The binding

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Table 1 Binding affinities ($\Delta G = -RT\ln K_a$) measured between receptors **1** and **2** and the peptide Ac-D-Val-D-Val-D-His immobilised on PS-resin with different peptide loadings[a]

Entry	Resin loading (mmol g^{-1})	Receptor **1** ΔG (kcal mol^{-1})	Receptor **2** ΔG (kcal mol^{-1})
1	1.13	−2.9	−2.8
2	0.90	−4.3	−3.4
3	0.79	−4.6	−3.8
4	0.57	−5.4	−4.4
5	0.40	−5.6	−4.6
6	0.28	−5.7	−4.7
7	0.11	−5.7	−4.8

[a] All measurements were repeated multiple times to ascertain the accuracy of the binding affinities within an error of ±0.2 kcal mol^{-1}.

affinity increases for both receptors with reduced resin loading and reaches a constant value at resin loadings of $\leq$0.3 mmol g^{-1} which corresponds to peptide concentrations on each bead of $\leq$90 mM^{-1}.

The observed lower binding affinities with higher loaded resins indicate that not all of the peptides are able to participate in the intermolecular interaction with the receptor. This could either be due to hindered accessibility of the receptor molecules to the inner spheres of the beads or to aggregation of the peptides with each other. These results suggest that resins with low loadings represent a solution-like situation where the guest molecules are free to interact with the receptor independently from each other. Thus, the solid phase binding assay might serve as a means to extrapolate to the binding affinities of a host-guest system in solution.[7]

To analyze the impact of the nature of the solid support on the binding affinity the peptide Ac-D-Val-D-Val-D-His was immobilized on supports with different polarity and hydrophilicity, namely Polyethyleneglycol polystyrene (Tentagel), Polyethyleneglycol acrylamide (PEGA), and Polyacrylamide (SPAR).[4] The supports were chosen with the same bead size (200–400 mesh)[8] as the PS-resin and comparable macroscopic loadings. Binding affinities were determined as described above and are listed in Table 2.[9]

As observed for the comparison of the effect of different loadings, the relative binding affinity differences between receptor **1** and **2** towards the immobilised peptide remain $\approx$1 kcal mol^{-1}. Thus, the relative binding strengths neither depend on the loading of the resin nor on the resin type. The binding affinities of both receptors determined on Tentagel, PEGA and PS (entry 5, Table 1) with loadings of $\approx$0.4 mmol g^{-1} are identical within the error (±0.2 kcal mol^{-1}) of the assay.[10] The same is observed for the binding affinities using SPAR or PS (entry 3, Table 1) with loadings of 0.8 mmol g^{-1}. The measured binding affinity is therefore independent of the nature of the resin. Among the tested solid supports the equilibrium is reached within a few hours by using PEGA while all other resins require an equilibration time of $\geq$ 48 h until the UV/VIS absorbance remains constant. Solid supports like PEGA with good diffusion properties[11] are therefore optimal choices for binding affinity measurements on a solid support.

Table 2 Binding affinities ($\Delta G = -RT\ln K_a$) measured between receptors **1** and **2** and the peptide Ac-D-Val-D-Val-D-His immobilised on different resins[a]

Entry	Resin loading (mmol g^{-1})	Receptor **1** ΔG (kcal mol^{-1})	Receptor**2** ΔG (kcal mol^{-1})
1	Tentagel (0.44)	−5.2	−4.6
2	PEGA (0.40)	−5.6	−4.4
3	SPAR (0.80)	−4.5	−3.3

[a] All measurements were repeated multiple times to ascertain the accuracy of the binding affinities within an error of ±0.2 kcal mol^{-1}.

In conclusion, while the nature of the solid support has no significant impact on the determination of binding constants on a solid support, care has to be taken by comparing binding constants that have been determined on resins with different loadings. Most importantly, relative binding affinities remain identical regardless of the capacity or the type of resin used. Thus, the determination of binding constants on a solid support is not only a convenient but also reliable means for obtaining relative measures of intermolecular binding affinities.

Notes and references

† To ascertain the identity of the immobilised peptide, a sample was prepared in parallel on Wang resin. The peptide was cleaved off the solid support with octylamine in CH_2Cl_2 (1:3) and was deprotected with TFA:CH_2Cl_2 (1:5) to isolate Ac-D-Val-D-Val-D-His-NH-$(CH_2)_7CH_3$: ^{1}H NMR (500 MHz, CD_3OD, 25 °C): δ = 7.81 (s, 1H; His_{imi}), 6.94 (s, 1H; His_{imi}), 4.57 (dd, J = 8.3, 6.2 Hz, 1H; His-Hα), 4.15 (d, J = 7.6 Hz, 1H; Val-Hα), 4.11 (d, J = 7.6 Hz, 1H; Val-Hα), 3.13 (t, J = 7.1 Hz, 2H; $NCH_2(CH_2)_6CH_3$), 3.06 (dd, J = 14.9, 6.2 Hz, 1H; His-Hβ), 2.96 (dd, J = 14.8, 8.3 Hz, 1H; His-Hβ′), 2.02 (m, 2H; 2Val-Hβ), 2.00 (s, 3H; $COCH_3$), 1.44 (m, 2H; $NCH_2CH_2(CH_2)_5CH_3$), 1.28 (m, 10H; $NCH_2CH_2(CH_2)_5CH_3$), 0.94–0.88 (m, 15H; Val-CH_3, $NCH_2CH_2CH_3$); ^{13}C NMR (500 MHz, CD_3OD, 25 °C): δ = 174.2, 173.6, 173.3, 172.6, 136.0, 133.8, 118.6, 60.7, 60.5, 54.6, 40.5, 33.1, 31.8, 31.6, 30.5, 30.4, 30.3, 30.1, 28.0, 23.8, 22.4, 19.8, 19.7, 19.0, 18.9, 14.5; MS (ESI): m/z (%): 507.5 (100) $[M + H]^+$.

1 K. C. Nicolaou, R. Hanko and W. Hartwig, *Handbook of Combinatorial Chemistry*, Wiley VCH, 2002.

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3 (*a*) H. Wennemers, M. Conza, M. Nold and P. Krattiger, *Chem. Eur. J.*, 2001, **7**, 3342–3347; (*b*) M. Conza and H. Wennemers, *J. Org. Chem.*, 2002, **67**, 2696–2698; (*c*) H. Wennemers, M. C. Nold, M. M. Conza, K. J. Kulicke and M. Neuburger, *Chem. Eur. J.*, 2003, **9**, 442–448.

4 Polystyrene and PEGA were purchased from Novabiochem, Tentagel from Rapp Polymers and SPAR was obtained from Advanced ChemTech.

5 J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*; Pierce Chemical Company: Rockford, IL, 1984.

6 A receptor concentration of ≈14 μM was chosen as an initial concentration since it corresponds to a UV/VIS absorbance of $A \approx 0.9$ and therefore allows to conduct the binding assay in the most sensitive absorbance range. The use of different initial concentrations also led to the same binding affinities.

7 Binding constants could not be determined accurately in solution since no variable (^{1}H NMR, UV/VIS) could be found that allowed a monitoring over a sufficiently large concentration range. The NMR-binding titration of receptor **2** with Ac-D-Val-D-Val-D-His-PEG-OCH_3 (PEG = polyethyleneglycol, M = 750) allows for an estimate of the binding affinity in the order of 4–6 kcal mol^{-1}.

8 PEGA could only be obtained with bead sizes of 50–100 mesh.

9 As for PS, no binding was observed when the receptors were mixed with Tentagel, PEGA or SPAR resins that were acetylated in place of the peptide, indicating that unspecific absorption of the receptors to the resin matrix can be neglected.

10 The comparison with PEGA is restricted by its different size (50–100 mesh) and swelling property (13 ml g^{-1}) in chloroform compared to PS and Tentagel (≈6 ml g^{-1}). The peptide concentration on a single bead of PEGA is approximately half the concentration of PS.

11 (*a*) T. Groth, M. Grøtli and M. Meldal, *J. Comb. Chem.*, 2001, **3**, 461–468; (*b*) M. Meldal, *Tetrahedron Lett.*, 1992, **33**, 3077–3080.