Combinatorial Chemistry

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Microparticle Matrix Encoding of Beads**

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The combinatorial approach remains one of the most powerful avenues for the discovery of new active compounds.[1] Solid-phase screening of combinatorial libraries is associated with: 1) millimolar rather than nanomolar concentrations, b) nontrivial structural analysis, and c) a non-biocompatible resin environment. In addition to chemical[2–4] and radio-frequency[5] encoding, split/mix libraries[6,7] have been encoded by using several optical methods, which include spherical encoding by dye diffusion,[8] laser-etched barcodes,[9,10] in situ composite particle labeling,[11a] and infrared tags in resins[12] (Figure 1). In another version of microparticle composite labeling, Battersby et al. performed pre-synthesis-coating of beads with microparticles for fluorescence and light-scattering-based decoding.[11b] Despite these advances, the number of codes used in libraries has remained quite low.

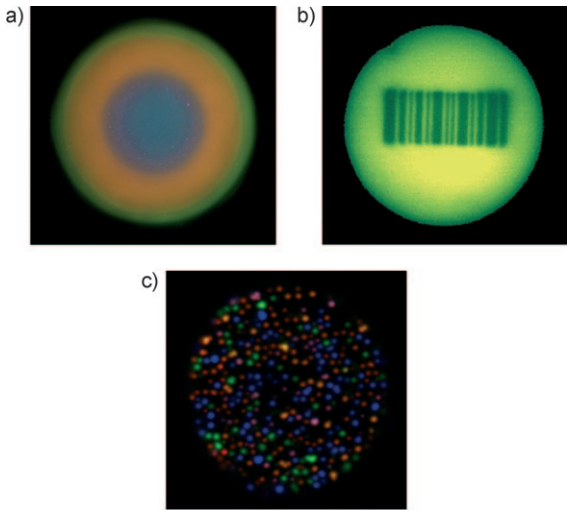

Figure 1. Examples of optical encoding comprise a) diffusion of reactive fluorophores,[8] b) laser bleaching,[9] and c) in situ encoding with particles.[11a]

Efficient encoding requires simultaneous reliable production of beads with a random code distribution.[13] In principle, the method allows encoding of an infinite number of beads,[14] although in practice the encoding is limited to the number of beads(e.g., 30000), which have been handled in one experiment. An encoding process that is independent of the chemistry employed and allows a variety of assays is therefore advantageous. We present herein a versatile, simple encoding principle that may solve many problems in solid-phase screening and decoding: optical microparticle matrix encoding (MPM encoding) with fluorescent microparticles. The utility of this technique has been demonstrated in the identification of avidin ligands from a focused library, in which compounds could not be distinguished with mass spectrometric (MS) methods. The library design was based upon previously reported L and D amino acid libraries for avidin and streptavidin, thus indicating selectivity (R_0) for aromatic residues.[15–18] It is not clear whether histidine and proline (in the histidine–proline–-glutamine (HPQ) sequence binding to streptavidin) are required for avidin recognition.

MPM encoding (Figure 2) is efficient and satisfies the prerequisites for functional encoding (see theoretical determination of encoding potential in the Supporting Information). Uniform 10 μm Tentagel microparticles labeled with a chemically stable fluorophore ATOTA (tris(dialkylamino)-trioxatriangulenium ion)[19] were randomly distributed in a bis(acrylamido) polyethylene glycol (PEG) macromonomer[20] (64000 cm^{-3}) by ultrasound and homogenization. Inverse suspension polymerization of the microparticle-containing macromonomer in the presence of bis(diphenylmethylsilyl) PEG 1500 stabilizer provided 500000 uniform,

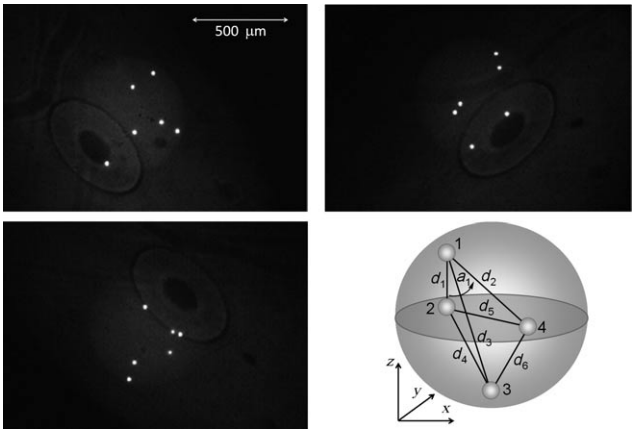

Figure 2. Images of one encoded bead, recorded using telecentric optics with the instrument shown in Figure 4. The image coordinates are converted into 3D-interparticle vector lengths (d) and angles (a) between all vector pairs.

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amino-functionalized, uniquely encoded beads (72 mL; ca 6.5 g dry weight), which contained an average of 8 microparticles per bead, with a bead size of (550 ± 100) μm.

The 3D matrix presented by the random positions of microparticles can be viewed as a set of unique vectors that connect each microparticle with all other microparticles in the bead. The vector lengths and angles between pairs of vectors represent a large rotation- and translation-independent parameter set. This dataset constitutes a unique fingerprint that is easily compared by computational methods.

In order to record the codes, beads were gently sucked onto 150 μm capillaries in a carousel controlled by a stepper motor, and transported to three orthogonally positioned cameras. The beads were illuminated using a laser and the images recorded. Image analysis accurately provided the 2D coordinates of centers of the microparticles in each image and 3D coordinates were determined by correlation as outlined in Figure 3. A second station on the carousel was dedicated to

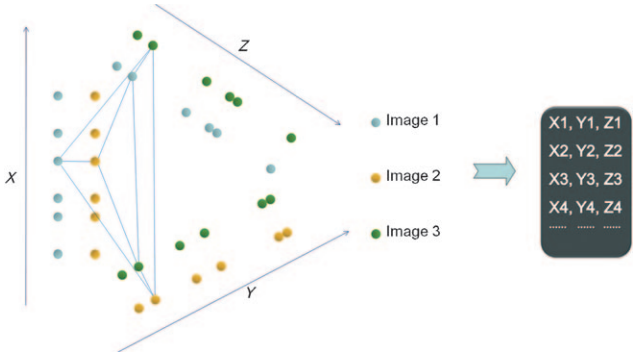


Figure 3. Fitting procedures that compare axis projections from all three images and optimize the indicated connectivity are used to reliably transform the three sets of 2D coordinates into one set of 3D coordinates.

recording the intensity of a biochemically derived fluorescent signal, which is different from that of the microparticles.[21] The carousel (Figure 4) was further connected to a gear pump that provided a controlled pressure drop over the capillaries. The equipment rapidly provided high-quality images and bioassay readout in a short period of time (0.3 s per bead). The short exposure eliminated the photobleaching often observed under a fluorescence microscope.

The images were analyzed with two computer programs:[22] ImToCoord provided 3D coordinates and H2S-Compare transformed the coordinates and compared splits with hits. Microparticle coordinates were converted into interparticle distances and angles between particle-to-particle vectors, and datasets were matched and scored. Selected hit/split pairs were visually compared by simulated annealing alignment of the microparticles.

For identification of avidin ligands, a focused library was screened by using MPM encoding. Ligand concentration on the solid support was reduced 10-fold to differentiate tight from low-affinity binders.[23] A library of 343 compounds, $R^2R^1R^0$-G-PEGA (PEGA = polyethylene glycol-polyacrylamide copolymer), was assembled according to Scheme 1

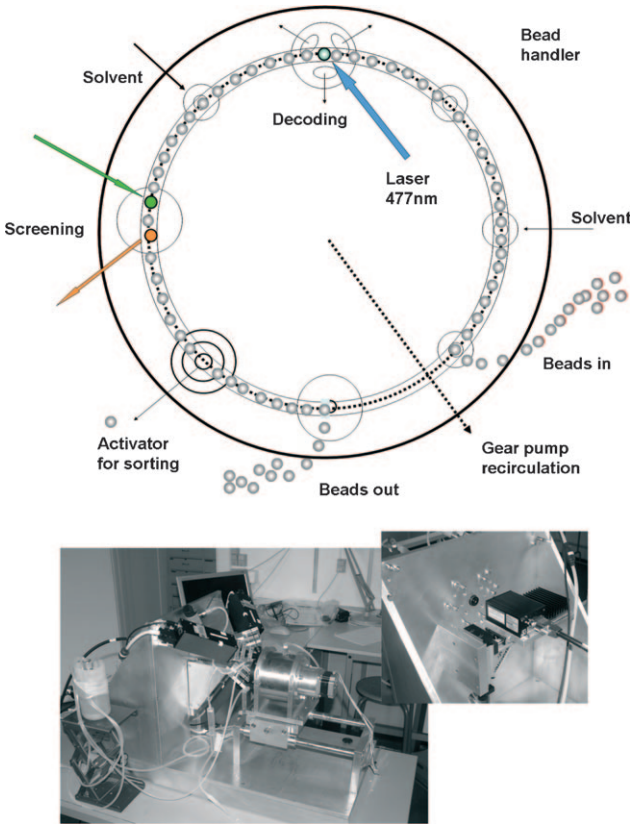


Figure 4. The core of the decoder consists of a rotating Kalrez disk with pressure-drop capillaries. Output from bioassays may be read in-line and directly correlated with the structure of compound.

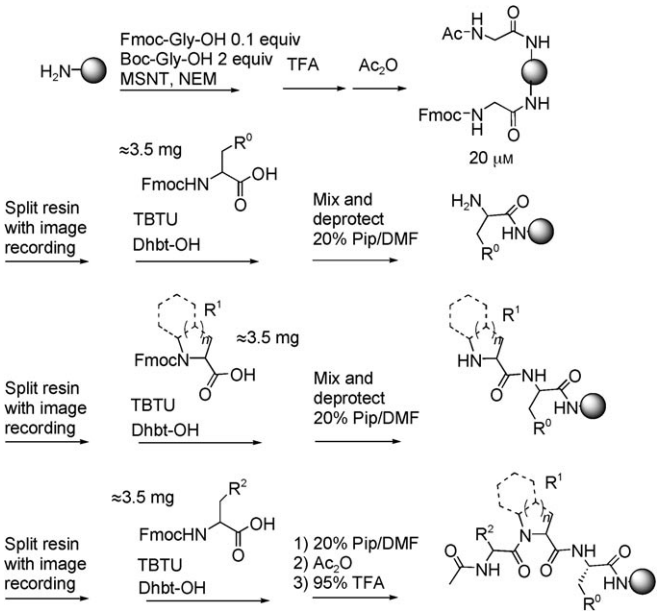
and Table 1 at a loading of approximately 1 μmol ligand per milliliter of resin. The split/mix synthesis was performed on 3000 encoded beads by splitting the resin with image recording prior to each coupling. The progress of the peptide couplings was monitored using the yellow coloration the Dhbt-OH coupling catalyst developed with residual free amino groups.

The library was incubated in bovine serum albumen (BSA) buffer with ROX-labeled avidin, and 30 beads with high fluorescence intensity were isolated and decoded. Of these beads, 20 structures were determined and 9 were

Table 1: Amino acids used for synthesis of the library in Scheme 1.[a]

R^2	R^1	R^0
val	Aze	Gln
His	Pro	gln
his	pro	Phe(4-NO_2)
Tha	*N*-Me-Ala	phe(4-NO_2)
Ala(3-Pyr)	Nca	Cit
Ala(4-Pyr)	Pca	Phe(3-CN)
Gly(4-Pip)	Oic	Phe(4-CN)

[a] Ala = alanine, Aze = azetidine-2-carboxylic acid, Cit = citrulline, Gln = glutamine, His = histidine, Nca = nipecotic acid, Pca = pipecolic acid, Phe = phenylalanine, Pro = proline, Pyr = pyridyl, Oic = (2*S*,3a*S*,7a*S*)-octahydroindole-2-carboxylic acid, Val = valine. Codes with an uppercase letter represent the L-amino acid, while codes in lower case represent the D-amino acid.



Scheme 1. MPM-encoded synthesis of a split/mix library containing several isobaric pairs of building blocks. The initial loading was reduced by acylation with a mixture of Fmoc-Gly-OH and Boc-Gly-OH. Boc = *tert*-butoxycarbonyl, Dhbt-OH = 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine, Fmoc = 9-fluorenylmethyloxycarbonyl, MSNT = 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole, NEM = *N*-ethyl morpholine; Pip = piperidine, TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, TFA = trifluoroacetic acid.

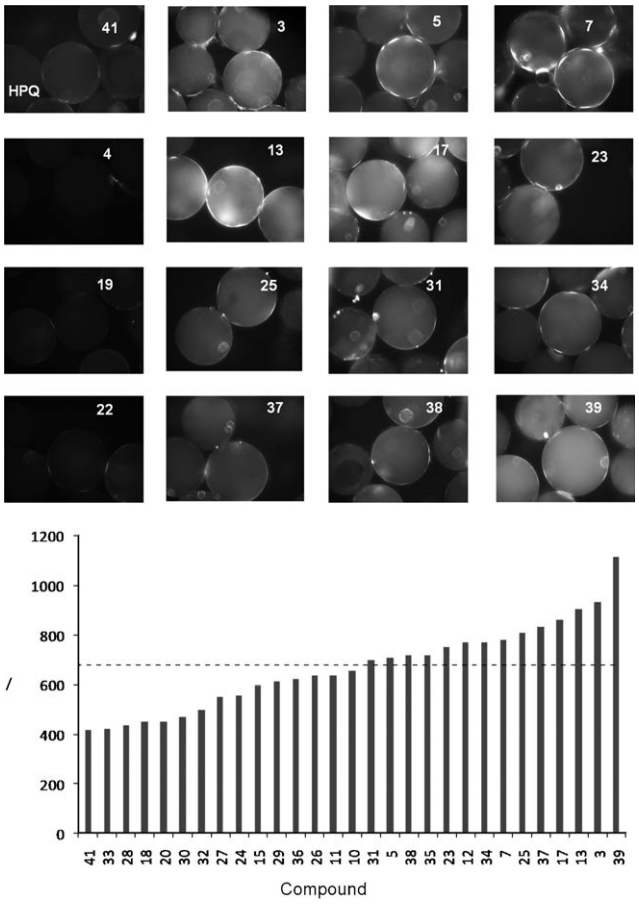


Figure 5. Binding of ROX-avidin (ROX = 6-carboxy-X-rhodamine) to resynthesized hits. Compound 41 is HPQ and compounds 4, 19, and 22 are inactive controls. The numbers 1–41 represent compounds tabulated in the Supporting Information.

Table 2: Data for the binding of peptides to immobilized avidin as determined by SPR.

Compound	*m*/*z* (calcd)	K_d [M]
His-Pro-Gln-Gly-OH (41)	480.1641 (480.2206)	1.21×10^{-3}
his-Aze-Phe(4-CN)-Gly-OH (**39**)	510.2091 (510.2101)	2.33×10^{-6}
his-Oic-phe(4-NO_2)-Gly-OH (**23**)	598.2611 (598.2625)	1.42×10^{-5}
Tha-Nca-phe(4-NO_2)-Gly-OH (**7**)	574.1982 (574.1971)	5.00×10^{-5}
his-Pro-phe(4-NO_2)-Gly (**5**)	544.2144 (544.2156)	4.60×10^{-5}
Tha-*N*-MeAla-Phe(4-CN)-Gly-OH (**34**)	512.2263 (512.2258)	6.99×10^{-6}
his-Aze-phe(4-NO_2)-Gly-OH (**13**)	530.2002 (530.2000)	3.72×10^{-6}
Tha-Aze-Gln-Gly-OH (**25**)	482.1696 (482.1710)	2.13×10^{-5}
4-PyA-Aze-phe(4-NO_2)-Gly-OH (**37**)	541.2048 (541.2047)	7.05×10^{-5}
His-pro-Phe(4-CN)-Gly-OH (**31**)	524.2251 (524.2258)	2.08×10^{-5}
his-Oic-Phe(4-CN)-Gly-OH (**17**)	578.2719 (578.2727)	6.60×10^{-6}
4PipG-Oic-phe(4-NO_2)-Gly-OH (**38**)	601.2972 (601.2986)	8.90×10^{-6}
His-*N*-MeAla-gln-Gly-OH (**3**)	468.2199 (468.2207)	2.70×10^{-5}
Tha-Pro-phe(4-NO_2)-Gly-OH	560.1815 (560.1815)	8.73×10^{-5}
Val-Aze-phe(4-NO_2)-Gly-OH	492.2084 (492.2094)	7.80×10^{-5}
Cyclic peptide ligands[a]		
***c*-A-His-Pro-Gln-FPAEK-OH**	503.7885 (503.7591)	6.70×10^{-2}
c-A-his-Oic-Phe(NO_2)-FPAEK-OH	562.7830 (562.7803)	2.44×10^{-6}
c-A-his-Oic-phe(NO_2)-FPAEK-OH	562.7805 (562.7803)	1.78×10^{-6}
c-A-His-pro-Phe(4-CN)-FPAEK-OH	525.7618 (525.7617)	5.34×10^{-6}
c-A-his-Oic-Phe(4-CN)-FPAEK-OH	562.7830 (562.7803)	5.18×10^{-7}
c-A-His-Pca-Phe(4-NO_2)-FPAEK-OH	542.79 (542.7644)	2.67×10^{-7}

[a] *c*- represents cyclization between Glu side chain and *N*-terminal Ala. Cyclopeptides were detected as *m*/2*z*. FPEAK = Phe-Pro-Glu-Ala-Lys. These cyclopeptides are reported to significantly increase the binding of their tripeptide inserts.

partially determined (2–3 possible structures; see Table 1 in the Supporting Information).

The identified hits were synthesized for solid-phase binding assays and the binding affinity was investigated by using surface plasmon resonance (SPR). The binding assay is presented in Figure 5 and correlates well with the SPR results (Table 2). Immobilization of avidin and binding of twofold dilutions of ligands generally provided good binding curves that show some nonstoichiometric binding. Accordingly, fitting was performed on the rise of the association curves and the latter section (15–300 s) of the dissociation curves. The K_D values ranged from 10^{-2} to 10^{-7} M (Table 2). Incorporation into cyclic peptides[24] (Figure 6) increased the affinity by approximately one order of magnitude (Table 2).

The ligand preference was surprisingly variable for R^1 and R^2, whereas a 4-substituted phenyl ring was clearly preferred for R^0. The relative configuration of the three amino acids varied, with a D amino acid preferred for R^0 and an L amino acid for R^1. Similarly, the structural R^1 residue did not have a preference for one particular ring size although Pro, Oic, and Aze were predominant.

In conclusion, a concept and a practical method of encoding beads with a matrix of fluorescent microparticles have been presented. The encoding potential is large and

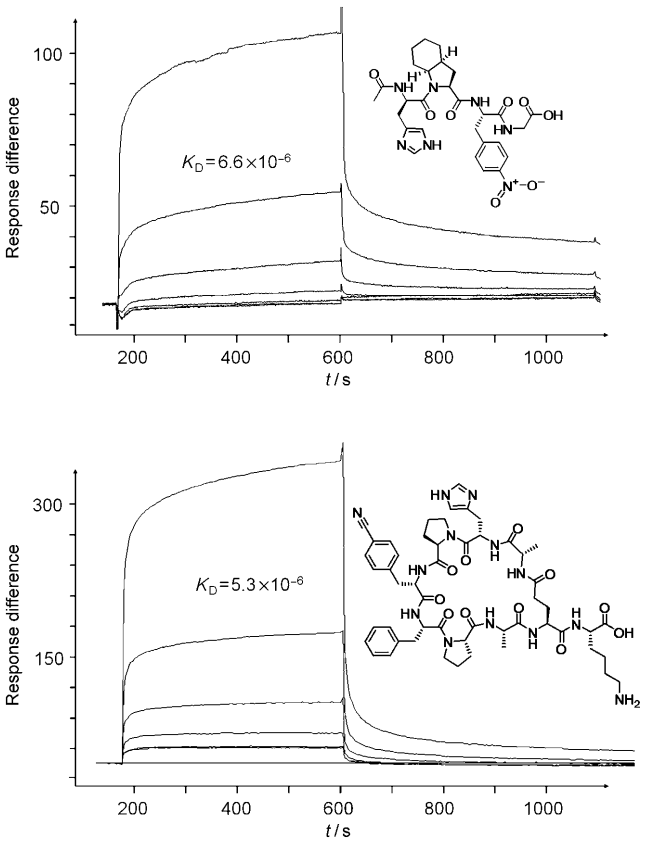


Figure 6. Examples of sensograms showing binding of a linear and a cyclic ligand to avidin immobilized on a CM5 chip using a Biacore 3000 instrument.

depends on the ability to record and analyze high-quality images of the beads as well as the number of beads that can be handled in practice, which is currently 30 000 beads (ca. 4 mL of resin with 550 μm beads). The recording method is rapid and has been automated; fast algorithms that permit real-time conversion of images to a bead signature have been developed. The structure identification can be combined with direct in-line screening in the instrument; correlation of structure with screening results is described in a separate communication.[21] The method was validated by screening a highly focused library of avidin ligands, and ligand structures, which can not be by distinguished by using conventional MS-MS techniques, were determined. The technology is promising for the screening of libraries of complex molecules and of solid-phase-bound ligands at biologically relevant concentrations. Practical microparticle detection and bead handling limits the current scaling of the system, but the synthesis of 150 μm beads with 4 μm microparticles is feasible.

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[1] J. P. Kennedy, L. Williams, T. M. Bridges, R. N. Daniels, D. Weaver, C. W. Lindsley, *J. Comb. Chem.* **2008**, *10*, 345 – 354.
[2] Z.-J. Ni, D. Maclean, C. P. Holmes, M. M. Murphy, B. Ruhland, J. W. Jacobs, E. M. Gordon, M. A. Gallop, *J. Med. Chem.* **1996**, *39*, 1601 – 1608.
[3] M. H. J. Ohlmeyer, R. N. Swanson, L. W. Dillard, J. C. Reader, G. Asouline, R. Kobayashi, M. Wigler, W. C. Still, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 10922 – 10926.
[4] S. Brenner, R. A. Lerner, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5381 – 5383.
[5] K. C. Nicolaou, X.-Y. Xiao, Z. Parandoosh, A. Senyei, M. P. Nova, *Angew. Chem.* **1995**, *107*, 2476 – 2479; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2289 – 2291.
[6] K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, R. J. Knapp, *Nature* **1991**, *354*, 82 – 84.
[7] A. Furka, F. Sebestyen, M. Asgedom, G. Dibo, *Int. J. Peptide Protein Res.* **1991**, *37*, 487 – 493.
[8] R. A. Farrer, G. T. Copeland, M. J. R. Previte, M. M. Okamoto, S. J. Miller, J. T. Fourkas, *J. Am. Chem. Soc.* **2002**, *124*, 1994 – 2003.
[9] K. Braeckmans, S. C. d. Smedt, C. Roelant, M. Leblans, R. Pauwels, J. Demeester, *Nat. Mater.* **2003**, *2*, 169 – 173.
[10] D. C. Pregibon, M. Toner, P. S. Doyle, *Science* **2007**, *315*, 1393 – 1396.
[11] a) B. J. Battersby, D. Bryant, W. Meutermans, D. Matthews, M. L. Smythe, M. Trau, *J. Am. Chem. Soc.* **2000**, *122*, 2138 – 2139; b) B. J. Battersby, G. A. Lawrie, A. P. R. Johnston, M. Trau, *Chem.Commun.* **2002**, 1435 – 1441.
[12] H. Fenniri, L. Ding, A. E. Ribbe, Y. Zyrianov, *J. Am. Chem. Soc.* **2001**, *123*, 8151 – 8152.
[13] S. F. Christensen, J. H. Truelsen, M. Meldal, R. Michael, I. Johannsen, US 127369, **2006**.
[14] S. F. Christensen, I. Johannsen, J. M. Carstensen, L. Kuhlmann, M. Meldal, WO 061094, **2005**.
[15] K. S. Lam, M. Lebl, *ImmunoMethods* **1992**, *1*, 11 – 15.
[16] B. Gissel, M. Roland Jensen, K. Gregorius, H. Elsner, I. Svendsen, S. Mouritsen, *Pept. Sci.* **1995**, *1*, 217 – 226.
[17] S. Østergaard, P. H. Hansen, M. Olsen, A. Holm, *FEBS Lett.* **1995**, *362*, 306 – 308.
[18] T. Gaja, S. C. Meyera, I. Ghosh, *Protein Expression Purif.* **2007**, *56*, 54 – 61.
[19] B. W. Laursen, F. C. Krebs, *Angew. Chem.* **2000**, *112*, 3574 – 3576; *Angew. Chem. Int. Ed.* **2000**, *39*, 3432 – 3434.
[20] F.-I. Auzanneau, M. Meldal, K. Bock, *J. Pept. Sci. J. Peptide Sci.* **1995**, *1*, 31 – 44.
[21] J. E. Rasmussen, C. B. Schiødt, S. F. Christensen, L. Nørskov-Lauritsen, M. Meldal, P. M. St. Hilaire, K. J. Jensen, *Angew. Chem.* **2010**, DOI: 10.1002/ange.200906602; *Angew. Chem. Int. Ed.* **2010**, DOI: 10.1002/anie.200906602.
[22] Available from the author upon request.
[23] M. Conza, H. Wennemers, *Chem. Commun.* **2003**, 866 – 867.
[24] Y.-P. Chang, Y.-H. Chu, *Anal. Biochem.* **2005**, *340* 74 – 79.

