Enantiomer Detection by MRI

Magnetic Relaxation Switch Immunosensors Detect Enantiomeric Impurities**

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Stereoisomers can differ profoundly from their corresponding enantiomers in pharmacological activity.[1–3] In fact, in some cases, such as with penicillamine, one enantiomer exhibits a therapeutic effect while the other is so toxic that even minute enantiomeric impurities result in severe noxious physiological consequences.[4] The legal and regulatory implications of these findings have led to a demand for methods to rapidly evaluate enantioselective syntheses and detect enantiomeric impurities.[5–8] As a result, a number of methods have been developed for the high-throughput screening of chiral compounds.[9–20] This has coincided with the rapid miniaturization of screening techniques.[10,21] However, there is still no universal method that combines high sensitivity ($< 0.1\,\%$, as required by some regulatory agencies), generality, speed, high-throughput capability, and a homogeneous format.

One promising approach for detecting minute traces of enantiomeric impurities utilizes stereoselective antibodies,[5,15,22,23] as was recently demonstrated with an immunoassay that allowed the determination of purity up to 99.998 % *ee*.[24] Moreover, since antibodies can be raised against virtually any compound, including low-molecular-weight drugs and hormones,[25] the use of immunoassays and -sensors represents a general and widely applicable approach for detecting enantiomeric impurities. Recent advances in molecular biology techniques means that the generation and engineering of large amounts of antibodies can also be carried out fairly inexpensively and quickly, that is, within a few weeks.[26] The chemistry for the synthesis of suitable immunogens as well as for the immobilization of antibodies or their targets is well established and can easily be adapted to a wide variety of different experimental setups.

Here, we present a novel homogeneous enantioselective immunosensor that utilizes magnetic relaxation switching as the detection element.[27–29] Magnetic relaxation switches (MRSs) consist of dextran-coated magnetic nanoparticles that alter the T2 relaxation time of water upon self-assembly.

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This system provides an attractive detection method because of its simplicity, homogeneous format, speed, and potential for high-throughput measurements. MRS assays have already been used to identify mRNA, proteases, restriction enzymes, and other biomolecules.[27,29,30] With this work we extend the usefulness of MRSs to the detection of enantiomeric impurities by combining it with the high specificity and generality of stereoselective antibodies. As a model system, α-amino acid enantiomers were used as analytes to evaluate the MRS immunosensor because of their biological importance and their inherent chiral structure.

The enantioselective MRS immunosensor was based on magnetic nanoparticles labeled with a derivative of D-phenylalanine (D-Phe). The magnetic nanoparticles consisted of a superparamagnetic iron oxide core with an aminated cross-linked dextran coating (CLIO). The CLIO–D-Phe nanoparticles were generated by first coupling tyramine to the primary amino groups of the CLIO nanoparticles by using a homobifunctional *N*-hydroxysuccinimide ester. Diazotization was then used to couple *p*-amino-D-phenylalanine through its side chain to the tyraminyl residues, thus preserving both the α-amino and carboxy groups attached to the stereogenic center. Scheme 1 shows the final azo compound that was produced on the CLIO nanoparticles.

When antibodies specific to D-amino acids (anti-D-AA) were added to the CLIO–D-Phe nanoparticles, the divalent nature of the antibodies resulted in the self-assembly of the nanoparticles, which led to a decrease of more than 100 ms in the T2 relaxation time. The presence of D-Phe impurities in samples of L-Phe was then determined by performing a one-step competitive immunoassay (Figure 1). Upon addition of mixtures of the enantiomers to the CLIO–D-Phe/anti-D-AA self-assembled structures, the presence of D-Phe impurities resulted in the dispersion of the nanoparticles by competing with the CLIO–D-Phe conjugates for antibody binding sites. This subsequently led to an increase in the T2 relaxation time. The presence of free D-Phe impurities could be detected within minutes, and the rate and magnitude of change in the T2 relaxation time was dependent on the concentration of impurities (Figure 2). The homogeneous format of the MRS immunoassay provides an important advantage over conventional solid-phase immunoassays, which require time-consuming incubation and washing steps. In addition, no derivatization of either the analyte or the antibody is necessary.

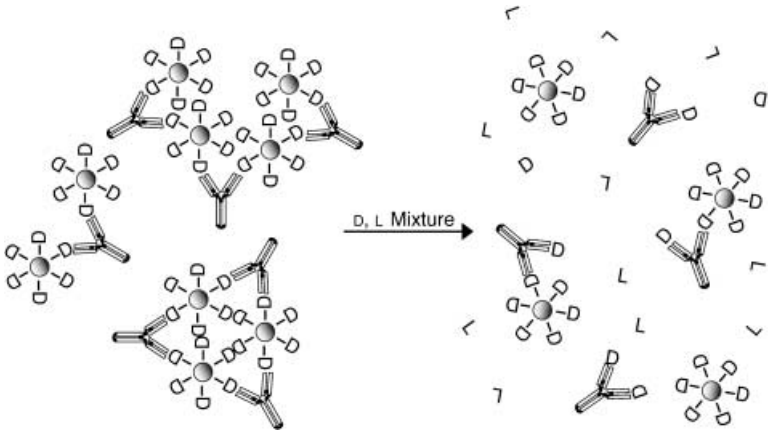


Figure 1. The addition of D-Phe impurities contained in L-Phe samples results in the dispersion of CLIO–D-Phe/anti-D-AA self-assemblies and a corresponding increase in the T2 relaxation time.

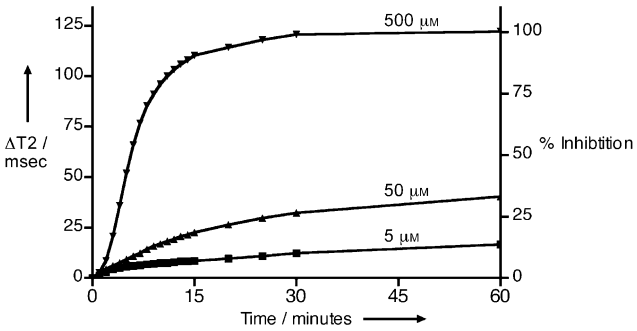
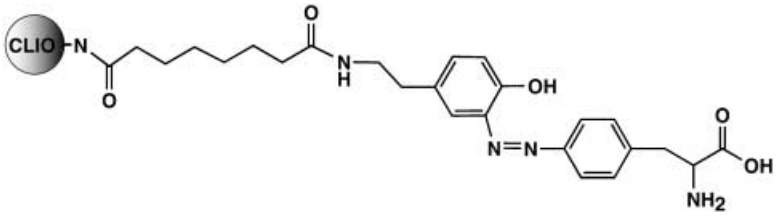


Figure 2. Kinetics of CLIO–D-Phe/anti-D-AA dispersion in the presence of D-Phe impurities. The increased rate and magnitude of change in the T2 relaxation time that accompanies the dispersion of CLIO–D-Phe/anti-D-AA self-assembled nanoparticles in the presence of increasing amounts of D-Phe is detected in real-time by using MRS immunosensors.



Scheme 1. Structure of the magnetic relaxation switch (CLIO–D-Phe) consisting of magnetic nanoparticles attached to a derivative of D-Phe.

The relative affinity and cross-reactivity of anti-D-AA for the phenylalanine enantiomers was evaluated in a series of competitive assays. Specifically, inhibition curves were obtained by measuring T2 relaxation times for CLIO–D-Phe/anti-D-AA samples in the presence of increasing concentrations of either D- or L-Phe. It was found that the concentration of D-Phe necessary to inhibit 50% of the anti-D-AA binding (the IC_{50} value) was 5.94 ± 0.14 μM, whereas the IC_{50} value for L-Phe was 7.91 ± 0.16 mM (Figure 3). This corresponds to a cross-reactivity of only 0.075%.

The stereoselectivity of anti-D-AA was subsequently utilized to analyze enantiomer mixtures of phenylalanine in competitive assays, in which the inhibitory effect of increasing concentrations of D-Phe on CLIO–D-Phe/anti-D-AA cross-linking was determined in the presence 0.01 mM, 0.1 mM, and 1 mM L-Phe (Figure 4a). It was found that the presence of L-Phe at these concentrations had no significant effect on the inhibition curve obtained with D-Phe. In fact, the IC_{50} values

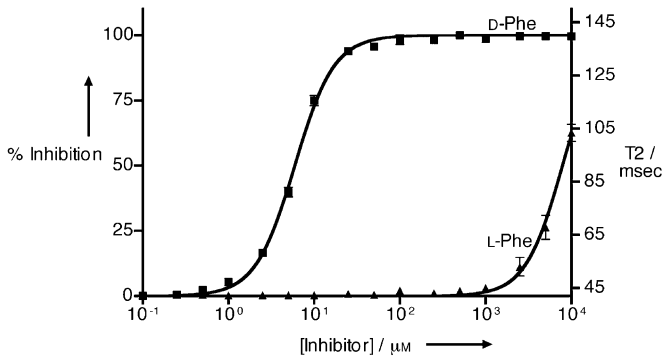


Figure 3. Inhibition of the CLIO–D-Phe/anti-D-AA self-assembly in the presence of increasing concentrations of L- or D-Phe as detected by changes in the T2 relaxation time.

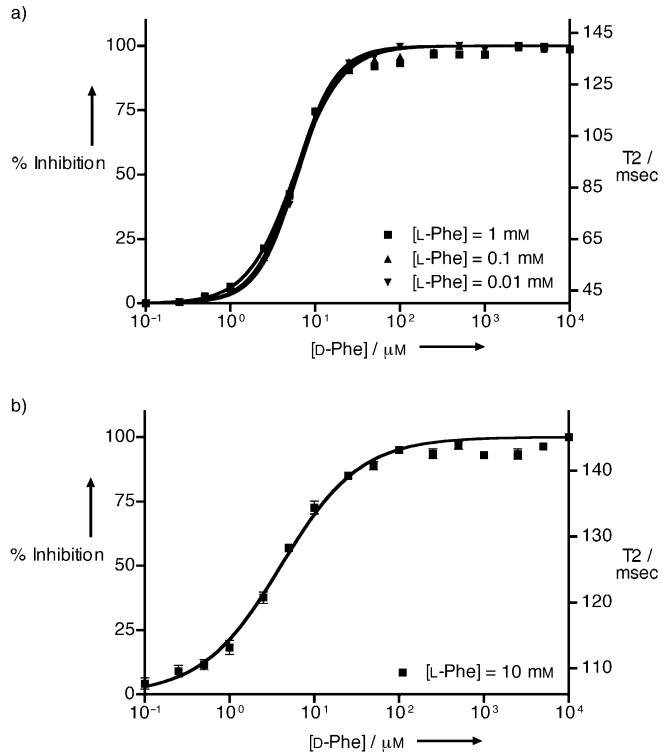


Figure 4. Inhibition of the CLIO–D-Phe/anti-D-AA self-assembly in enantiomer mixtures containing increasing concentrations of D-Phe in samples of L-Phe as detected by changes in the T2 relaxation time.

varied by less than 5%, and as little as 1 μM D-Phe could be detected in each case; the detection of D-Phe at a concentration of 1 μM in the presence of 1 mM L-Phe corresponds to 99.8% *ee*.

The *ee* detection limit of this MRS immunoassay was determined by adding increasing concentrations of D-Phe in the presence of 10 mM L-Phe to the CLIO–D-Phe/anti-D-AA self-assemblies (Figure 4b). Although L-Phe at this high concentration induces a T2 relaxation time that is nearly 60 ms higher than the background (Figure 3), minute traces of D-Phe impurities could still be detected. Since the T2 relaxation time is highly sensitive to any D-Phe impurities, quantification is possible if the "base level", caused by the major enantiomer alone, has been determined in a previous experiment. This is illustrated in Figure 4b, where as little as 0.1 μM D-Phe could be detected in the presence of 10 mM L-Phe; this is equivalent to 99.998% *ee*. Even at this level, the obtained data were highly reproducible with intra- and interassay standard deviations of less than 5%.

An important attribute of magnetic relaxation switch immunsensors is their potential for the rapid determination of enantiomeric excess in a high-throughput format. Several methods are already available that could be easily adapted to measure thousands of MRS samples per day, such as high-throughput NMR spectroscopy and magnetic resonance (MR) imaging.[31–34] Here, the feasibility of conducting high-throughput screening for enantiomeric impurities was demonstrated by using MR imaging. MR images were obtained for 60 MRS samples simultaneously in a 384-well plate (Figure 5). Data acquisition was complete in approximately two minutes. The T2 relaxation times were determined for CLIO–D-Phe/anti-D-AA samples in the presence of increasing concentrations of either D- (column 1) or L-Phe (column 2) alone, as well as for samples containing minute enantiomeric impurities of D-Phe in the presence of 10 mM L-Phe (columns 3 and 4). The relative affinity and stereoselectivity of anti-D-AA towards the phenylalanine enan-

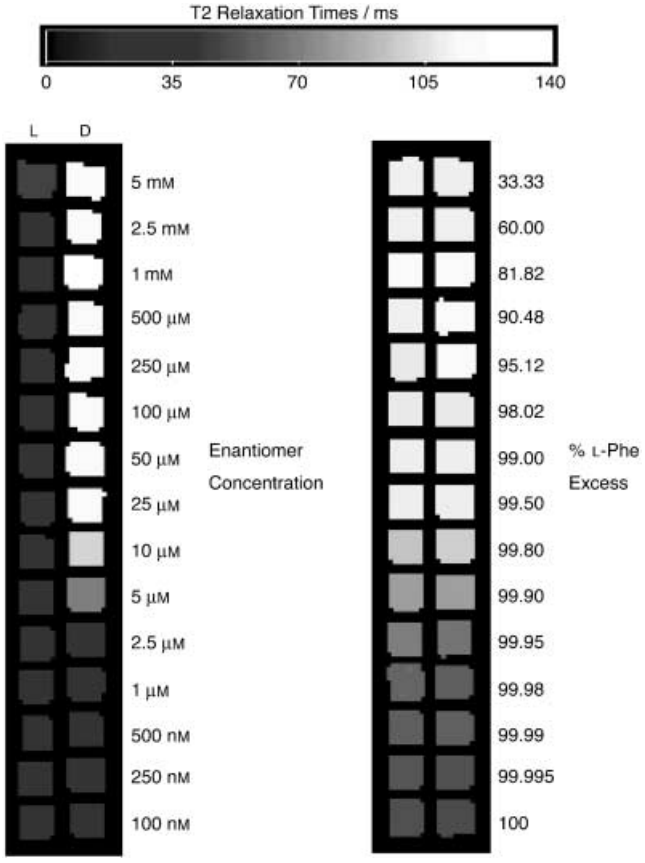


Figure 5. Magnetic resonance T2 maps of samples in a 384-well plate containing CLIO–D-Phe/anti-D-AA self-assemblies in the presence of increasing concentrations of D-Phe or L-Phe (columns 1 and 2, respectively) and increasing concentrations of D-Phe in 10 mM samples of L-Phe (columns 3 and 4).

tiomers as determined by the MR imager were found to correlate well with those values previously obtained with the NMR minispectrometer.

In conclusion, the one-step MRS immunoassay is a versatile tool for the ultrasensitive detection of enantiomeric impurities that could effectively be used to facilitate, for example, drug development. The ability to raise antibodies against virtually any compound makes this method a general approach for detection of enantiomers. The high degree of stereoselectivity possible with such antibodies allows for the detection of enantiomeric impurities at levels well-below the requirements of regulatory agencies. The MRS immunoassay may also be used to directly screen for the kinetic properties of enantioselective catalysts by taking advantage of the homogeneous format and the fact that T2 relaxation times are not altered by the optical properties of the reaction mixture.[27] Here the desired enantiomer, rather than the enantiomeric impurity, would be conjugated with the CLIO nanoparticle and used with the corresponding antibody in a competitive setup. A change in the T2 relaxation time would then reflect the catalytic production of the desired chiral compound. It is envisioned that the MRS immunoassay could serve as a universal approach for screening enantioselective catalysts that will expedite the production of enantiomerically pure compounds.

Experimental Section

Antibody production: Monoclonal anti-D-AA antibodies were produced as described elsewhere.[35] In brief, eight-week old BALB/c mice were immunized with *p*-azo-D-phenylalanine–keyhole-limpet-hemocyanin conjugates in complete Freund's adjuvant. Booster injections of immunogen in incomplete Freund's adjuvant and phosphate-buffered saline (PBS), pH 7.4, respectively, were administered twice at intervals of two weeks. Four and three days prior to fusion, final boosts were given intraperitoneally. Splenocytes of two mice showing strong immune responses were fused with NS0 myeloma cells by using polyethylene glycol. Hybridomas were selected in hypoxanthine/aminopterin/thymidine medium, and supernatants were screened by a noncompetitive enzyme-linked immunosorbent assay with three different solid-phase coatings: bovine serum albumin (BSA), *p*-azo-D-phenylalanine–BSA, and *p*-azo-L-phenylalanine–BSA. Hybridomas producing stereoselective antibodies were cloned at least twice by limiting dilution. Large quantities of the anti-D-AA antibody were obtained by the preparation of ascites fluid. The antibodies were purified by ammonium sulfate precipitation followed by ion-exchange chromatography on diethylaminoethyl-Sephacel with a gradient of 0–400 mM NaCl in 10 mM tris(hydroxymethyl)-aminomethane, pH 8.5, for elution.

Synthesis and characterization of CLIO–D-Phe: Superparamagnetic CLIO nanoparticles consist of an iron oxide core (5 nm diameter, 2064 Fe centers per particle, as determined by electron microscopy) and a thick shell of cross-linked dextran.[27–30,36] 10 μg of Fe per mL corresponds to a nanoparticle concentration of 89 nM. Nanoparticles with an aminated cross-linked dextran coating were synthesized and the number of amines per nanoparticle were quantified as previously described.[28,37] In brief, the number of amines were determined by a reaction with *N*-succinimidyl-3-(2-pyridyldithio) propionate[38] and treatment with tris(2-carboxyethyl)-phosphane (Pierce) to release pyridine-2-thione (P2T). Following filtration on a Microcon YM-50 filter (Amicon), released P2T was determined spectrophotometrically by using an extinction coefficient of $8080\,M^{-1}\,cm^{-1}$ at 343 nm. The CLIO–NH_2 nanoparticles were precipitated in four volumes of isopropanol and resuspended in dimethylsulfoxide to a final concentration of 10 mg of Fe per mL. They were then treated with an equal volume of 250 mM suberic acid bis(*N*-hydroxysuccinimide ester) (DSS) for 1 h at room temperature. Remaining DSS was removed by washing the nanoparticles three times by isopropanol precipitation. After the third wash, the nanoparticle pellet was resuspended with 250 mM tyramine in dimethylsulfoxide at a ratio of 62.5 μmoles per 1 mg of Fe, and the suspension was mixed overnight at room temperature. The remaining tyramine was removed by washing the nanoparticles three times by isopropanol precipitation, and the modified nanoparticles (CLIO–tyramine) were resuspended in 0.15 M sodium borate, 0.1 M NaCl, pH 9.0, to a concentration of 3.33 mg of Fe per mL. The suspension was then placed on ice.

Sodium nitrite ($35\,mg\,mL^{-1}$) was dissolved in ice-cold deionized water and incubated on ice. *p*-Amino-D-phenylalanine (9 volumes of $5.55\,mg\,mL^{-1}$) in chilled 0.2 M HCl was added, and the solution was mixed on ice for 1 h, to result in diazotized D-phenylalanine. The chilled CLIO–tyramine was mixed with diazotized D-phenylalanine (0.67 volumes), and the solution was mixed on ice for 4 h. The resulting conjugate, CLIO–D-Phe, was purified first on a Sephadex G-25 column and subsequently by dialysis against PBS. The amount of D-Phe conjugated per nanoparticle was determined as the difference in primary amines before and after conjugation of D-Phe to CLIO-NH_2. By using the molar equivalence between released P2T and reactive amines, and by assuming a value of 2064 Fe centers per nanoparticle, it could be calculated that each CLIO nanoparticle contained approximately 15 D-Phe groups.

MRS immunoassay kinetics: The kinetics of CLIO–D-Phe aggregation in the presence of anti-D-AA were determined by mixing CLIO–D-Phe (10 μg of Fe per mL) and anti-D-AA ($0.1\,mg\,mL^{-1}$) in PBS (200 μL), pH 7.4. Measurements of the T2 relaxation times were taken every 15 minutes for the first 150 minutes and then every 30 minutes until no change in the relaxation time could be detected. T2 relaxation times were recorded with a 0.47 T NMR Minispec apparatus (Bruker, Billerica, MA). The kinetics of the CLIO–D-Phe/anti-D-AA dispersion in response to the addition of free D-Phe were determined by first mixing CLIO–D-Phe (10 μg of Fe per mL) and anti-D-AA ($0.1\,mg\,mL^{-1}$) in PBS, pH 7.4, and allowing the samples to reach equilibrium. Then 5 μM, 50 μM, or 500 μM (final concentrations) D-Phe was added to the sample to give a total volume of 200 μL. Concentrations of CLIO–D-Phe, and anti-D-AA are based on the total volume after the addition of free D-Phe. T2 relaxation times were recorded every minute for the first 15 minutes, every 5 minutes until 30 minutes, and then at 60 minutes.

MRS competitive immunoassays: The inhibitory effect of D-Phe impurities on CLIO–D-Phe/anti-D-AA binding was determined by mixing CLIO–D-Phe (10 μg of Fe per mL), anti-D-AA ($0.1\,mg\,mL^{-1}$), and 0.1 μM–10 mM D- or L-Phe. The samples were incubated at room temperature until equilibrium was reached. Five T2 relaxation time measurements were then taken from each sample and averaged. This experiment was performed in triplicate and the average T2 relaxation time and standard deviation were calculated. The percentage inhibition was determined by using the equation: $\%\ \text{inhibition} = (T2-T2_{min})/(T2_{max}-T2_{min}) \times 100$, where T2 is the T2 relaxation time of the sample, $T2_{min}$ is the T2 relaxation time of the sample in the absence of free D-Phe, and $T2_{max}$ is the T2 relaxation time in the presence of 10 mM D-Phe. Similar experiments and analysis were also conducted with CLIO-D-Phe (10 μm of Fe per mL), anti-D-AA ($0.1\,mg\,mM^{-1}$), and 0.1 μM–10 mM D-Phe in the presence of 0.01, 0.1, 1, and 10 mM L-Phe.

MR imaging: MR images of 384-well plates were obtained by using a 4.7 T superconducting magnet (Bruker) by performing T2-weighted spin-echo sequences with echo times ranging from 20–

160 ms in increments of 20 ms and repetition times of 2000 ms. Maps of the T2 relaxation times were generated as described previously.[34]

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[1] D. R. Brocks, F. Jamali, *Pharmacotherapy* **1995**, *15*, 551.
[2] A. Brossi, *Med. Res. Rev.* **1994**, *14*, 665.
[3] J. Caldwell, *J. Chromatogr. A* **1995**, *694*, 39.
[4] E. Thall, *J. Chem. Educ.* **1996**, *73*, 481.
[5] R. L. Nation, *Clin. Exp. Pharmacol. Physiol.* **1989**, *16*, 471.
[6] K. M. Rentsch, *J. Biochem. Biophys. Methods* **2002**, *24*, 1.
[7] M. T. Reetz, *Angew. Chem.* **2001**, *113*, 292; *Angew. Chem. Int. Ed.* **2001**, *40*, 284.
[8] D. T. Witte, K. Ensing, J. P. Franke, R. A. De Zeeuw, *Pharm. World Sci.* **1993**, *15*, 10.
[9] M. G. Finn, *Chirality* **2002**, *14*, 534.
[10] G. A. Korbel, G. Lalic, M. D. Shair, *J. Am. Chem. Soc.* **2001**, *123*, 361.
[11] M. T. Reetz, K. M. Kuhling, H. Hinrichs, A. Deege, *Chirality* **2000**, *12*, 479.
[12] M. T. Reetz, K. M. Kuhling, A. Deege, H. Hinrichs, D. Belder, *Angew. Chem.* **2000**, *112*, 4049; *Angew. Chem. Int. Ed.* **2000**, *39*, 3891.
[13] M. T. Reetz, *Angew. Chem.* **2002**, *114*, 1391; *Angew. Chem. Int. Ed.* **2002**, *41*, 1335.
[14] M. P. So, T. S. Wan, T. W. Chan, *Rapid Commun. Mass Spectrom.* **2000**, *14*, 692.
[15] F. Taran, C. Gauchet, B. Mohar, S. Meunier, A. Valleix, P. Y. Renard, C. Creminon, J. Grassi, A. Wagner, C. Mioskowski, *Angew. Chem.* **2002**, *114*, 132; *Angew. Chem. Int. Ed.* **2002**, *41*, 124.
[16] C. Wolf, P. A. Hawes, *J. Org. Chem.* **2002**, *67*, 2727.
[17] M. T. Reetz, M. H. Becker, K. M. Kuhling, A. Holzwarth, *Angew. Chem.* **1998**, *110*, 2792; *Angew. Chem. Int. Ed.* **1998**, *37*, 2647.
[18] W. A. Tao, F. C. Gozzo, R. G. Cooks, *Anal. Chem.* **2001**, *73*, 1692.
[19] M. Tsukamoto, H. B. Kagan, *Adv. Synth. Catal.* **2002**, *344*, 453.
[20] P. Dutta, C. A. Tipple, N. V. Lavrik, P. G. Datskos, H. Hofstetter, O. Hofstetter, M. J. Sepaniak, *Anal. Chem.* **2003**, *75*, 2342.
[21] R. A. van Delden, B. L. Feringa, *Angew. Chem.* **2001**, *113*, 3298; *Angew. Chem. Int. Ed.* **2001**, *40*, 3198.
[22] P. A. Got, J. M. Scherrmann, *Pharm. Res.* **1997**, *14*, 1516.
[23] O. Hofstetter, H. Hofstetter, V. Schurig, M. Wilchek, B. S. Green, *J. Am. Chem. Soc.* **1998**, *120*, 3251.
[24] O. Hofstetter, H. Hofstetter, M. Wilchek, V. Schurig, B. S. Green, *Chem. Commun.* **2000**, 1581.
[25] O. N. Chappey, P. Sandouk, J. M. Scherrmann, *Pharm. Res.* **1992**, *9*, 1375.
[26] J. Hoogenboom, H. R. Chiswell, D. J. Eds, *Antibody engineering: a practical approach*, IRL Press, Oxford, **1996**.
[27] J. M. Perez, L. Josephson, T. O'Loughlin, D. Hogemann, R. Weissleder, *Nat. Biotechnol.* **2002**, *20*, 816.
[28] L. Josephson, J. M. Perez, R. Weissleder, *Angew. Chem.* **2001**, *113*, 3304; *Angew. Chem. Int. Ed.* **2001**, *40*, 3204.
[29] M. Zhao, L. Josephson, Y. Tan, R. Weissleder, *Angew. Chem.* **2003**, *115*, 1413; *Angew. Chem. Int. Ed.* **2003**, *42*, 1375.
[30] J. M. Perez, T. O'Loughlin, F. J. Simeone, R. Weissleder, L. Josephson, *J. Am. Chem. Soc.* **2002**, *124*, 2856.
[31] T. Hou, J. Smith, E. MacNamara, M. MacNaughton, D. Raftery, *Anal. Chem.* **2001**, *73*, 2541.
[32] P. J. Hajduk, T. Gerfin, J. M. Boehlen, M. Haberli, D. Marek, S. W. Fesik, *J. Med. Chem.* **1999**, *42*, 2315.
[33] P. A. Keifer, *Curr. Opin. Biotechnol.* **1999**, *10*, 34.
[34] D. Hogemann, V. Ntziachristos, L. Josephson, R. Weissleder, *Bioconjugate Chem.* **2002**, *13*, 116.
[35] O. Hofstetter, H. Hofstetter, M. Wilchek, V. Schurig, B. S. Green, *Int. J. Bio-Chromatogr.* **2000**, *5*, 165.
[36] T. Shen, R. Weissleder, M. Papisov, A. Bogdanov, Jr., T. J. Brady, *Magn. Reson. Med.* **1993**, *29*, 599.
[37] M. Zhao, M. F. Kircher, L. Josephson, R. Weissleder, *Bioconjugate Chem.* **2002**, *13*, 840.
[38] D. Hogemann, L. Josephson, R. Weissleder, J. P. Basilion, *Bioconjugate Chem.* **2000**, *11*, 941.

