2d: m.p.: 101 – 102 °C (decomp); ^{1}H NMR (400 MHz, CD_3CN): $\delta = 9.12$ (s, 1H; H-2), 8.80 (d, $J = 6.2$ Hz, 1H; H-6), 8.75 (d, $J = 8.1$ Hz, 1H; H-4), 8.09 (dd, $J = 8.1$, 6.2 Hz, 1H; H-5), 7.35 (d, $J = 8.0$ Hz, 2H; Ph), 7.29 (d, $J =$ 8.0 Hz, 2H; Ph), 7.13 (br. s, 1H; $CONH_2$), 6.57 (br. s, 1H; $CONH_2$), 5.70 (s, 2H; CH_2Ph), 2.35 (s, 3H; CH_3); ^{13}C NMR (100 MHz, CD_3CN): $\delta = 163.7$, 146.9, 145.4, 145.0, 141.4, 135.5, 131.1, 130.5, 130.4, 129.5, 65.7, 21.3; elemental analysis: calcd for $C_{14}H_{15}F_6N_2OP$ (372.25): C 45.17, H 4.06, N 7.53; found: C 45.33, H 3.93, N 7.52.

Cyclic voltammograms of the NAD^+ model compounds were measured in acetonitrile solution that contained tetra-*n*-butylammonium tetrafluoroborate (0.1M) as the supporting electrolyte. An ALS/CHI CHI-620 electrochemical analyzer, with a Pt disk working electrode, an $Ag/AgNO_3$ (0.01M) reference electrode, and a Pt counter electrode was used for the analysis.

Received: May 18, 2000
Revised: July 13, 2000 [Z15140]

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A New Self-Assembling System for Targeted Gene Delivery**

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The goal of targeted nonviral gene therapy has aroused considerable interest amongst scientists in recent years.[1] The ability to deliver recombinant DNA both selectively and efficiently to a given cell-type requires several features working effectively in harmony. Preferably the DNA needs to be compacted to protect it from enzymatic attack,[2] and tightly bound to a suitable targeting ligand.[1b] The resultant vector needs to be efficiently delivered to the target cell population with sufficient selectivity to minimize unwanted interaction with other cell types. Finally, once internalized into the desired cell the DNA must escape from the endosome into the cytoplasm[3] and enter the nucleus while being protected from the action of nucleases en route. Each of the above stages presents a major scientific challenge and different strategies have been formulated. Cationic liposomes, for example, have been studied in detail,[4] but lack specificity in targeting and generally yield heterogenous complexes with DNA. Cationic polymers, notwithstanding their toxicity in vivo, strongly bind DNA and may afford a pH-dependent release mechanism through a conformational change in the polymer in the more acidic medium of an endosome.[5] We now report our preliminary work to develop a new modular supramolecular approach to overcome the challenges associated with targeted gene delivery.

Compaction of DNA in nature is achieved through charge neutralization of the polyanion by interaction with the protonated polyamine spermine.[6] Accordingly, we have prepared a series of amphiphilic tetra- and hexaamines **1** – **12**, which as a consequence of the C_3 or C_4 spacing between each nitrogen site possess 3.6 or 5.2 positive charges at

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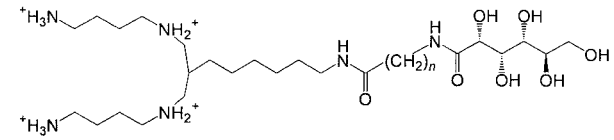
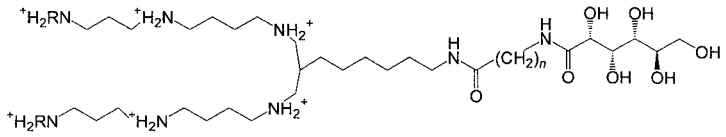
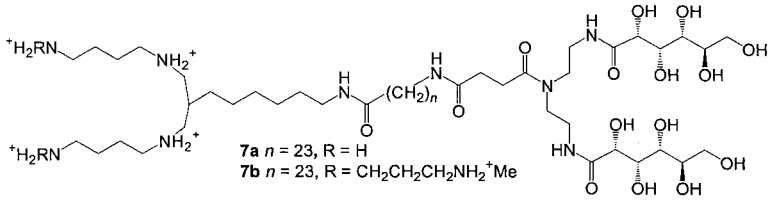
[**] We thank the BBSRC Chiroptical Service for CD studies, Brian McManus (Optokem Instruments, Nercyws, Flintshire) for assistance with the light-scattering studies, Dr. Clive Roberts (Molecular Profiles Ltd.) for AFM studies, Professor J.-H. Fuhrhop for many discussions, Dr. I. S. Blagbrough for related synthetic work, the EPSRC for support, and the Royal Society for a Leverhulme Trust Senior Research Fellowship (D.P.).



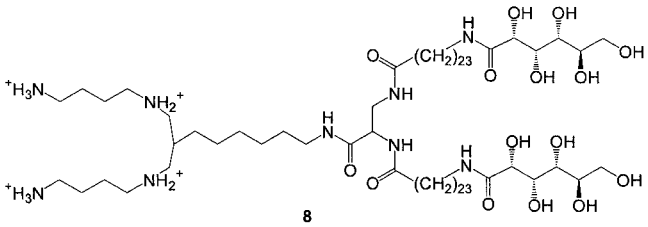
1 n = 17
2 n = 11
3 n = 23



4a n = 23, R = H
4b n = 17, R = H
5 n = 23, R = Me
6 n = 35, R = Me



7a n = 23, R = H
7b n = 23, R = $CH_2CH_2CH_2NH_2^+Me$



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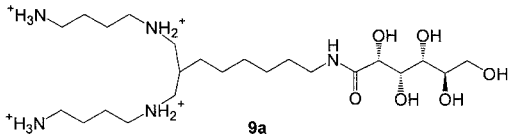
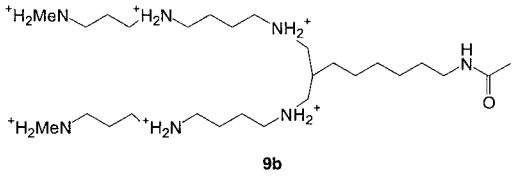
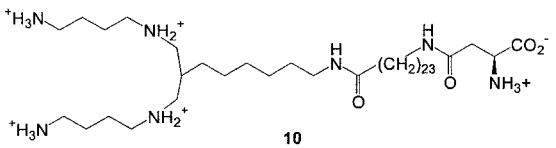


9a



9b



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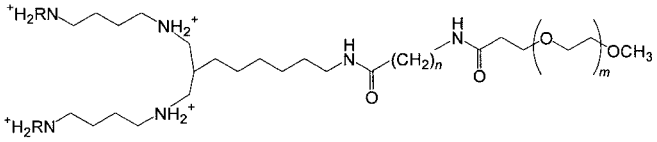


11 n = 23, m = average 45, R = H
12 n = 23, m = average 45, R = $CH_2CH_2CH_2NH_2^+Me$

ambient pH (7.4).[7a] The DNA-binding polyamine "head" group is separated from a hydrophilic "tail" group by a long-chain hydrocarbon varying in length from C_{12} to C_{36}. The hydrophilic tail groups are either sugars, such as those shown in structures **1**–**9** (prepared by the reaction of δ-gluconolactone with the appropriate primary amine), zwitterionic amino-acids, for example, **10**, or polyoxyethylene glycol chains (PEG, MW 2000) for example, **11** or **12**. Such non-ionic surfactant tail groups improve water solubilty, are biocompatible, and may reduce nonspecific interactions such as protein binding and subsequent uptake by the reticuloendothelial system. The amphiphilic structures **1**–**12** resemble bolaamphiphiles[8] and may be expected to self-assemble in solution. Similar aggregates may also be expected to form readily in mixtures where the long-chain hydrocarbon and polyamine groups are held constant but the hydrophilic tail group is varied. Control over DNA compaction and surface-coating characteristics in a supramolecular assembly comprised of a bolaamphiphile and condensed DNA may be achieved by varying the relative proportion of each component.[7c]

Preliminary DNA-binding experiments were carried out with super-coiled plasmid DNA (pH 7.4, 150 mM NaCl) to rank the relative DNA-binding affinities of bolaamphiphiles ("bolas") **1**–**12**. Binding affinity was monitored by a dye-exclusion assay where the titration of the bola (increasing equivalents of the bolaamine versus a constant amount of DNA phosphate, "N:P ratio") caused a reduction in the intensity of the fluorescence emission from an ethidium bromide/DNA complex.[9] The following observations were made: a) bolas **8** (with two C_{24} chains) and **10** (with an amino acid tail group) bound comparatively weakly, and b) DNA-binding affinity varied with the length of the hydrocarbon chain for a given polyamine and tail group and followed the order $C_{12} < C_{18} < C_{24} > C_{36}$. Differences in the binding strength between tetraamine- and hexaamine-based bolas were apparent for the C_{18} chain ($N_6 > N_4$), but were not evident for C_{24}-chain bolas, for example, **7a** versus **7b**.

The particles that formed at N:P ratios of 0.5:1 and above were stable over the pH range 3.5 to 7.5, as judged by the dye-exclusion assay and agarose gel electrophoresis. However, the particles formed at N:P ratios of 2:1 and above resisted the attack of added Dnase I or of foetal calf serum when tested for plasmid integrity.[10] Photon correlation spectroscopy (PCS) suggested that the particles which formed at N:P ratios of about 0.7:1 or at greater than 2:1, without the inclusion of any PEG-bola (**11** or **12**), had a mean diameter of the order of 300 nm. Considerably larger aggregates formed at N:P ratios between 1:1 and 2:1, presumably as a

  0044-8249/00/11222-4230 $ 17.50+.50/0 *Angew. Chem.* **2000**, *112*, Nr. 22

result of charge neutralization allowing the formation of lattices. More compact particles however were observed to form across the same range of N:P ratios with the inclusion of as little as 2% of a PEG derivative (**11** or **12**). Field-flow fractionation (a separation technique based on size) coupled with multi-angle laser-light scattering allowed time-dependent DNA-compaction studies to be carried out.[11] Examination of the DNA particles formed from 13 mol% of **12** and 87 mol% of **5** at a N:P ratio of about 1:1 (Figure 1) revealed behavior consistent with the formation of well-defined overall

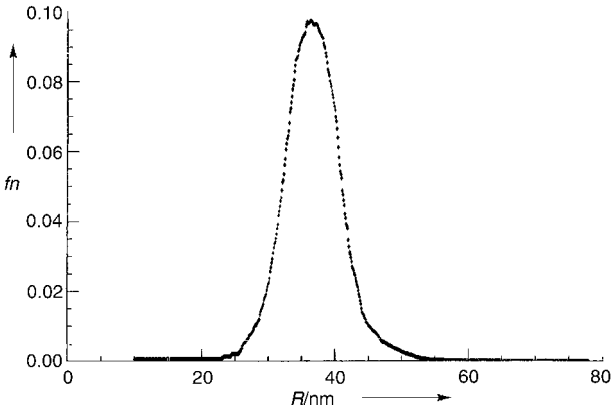


Figure 1. Field-flow fractionation data using multi-angle laser light scattering highlighting the dispersity in particle size for the complex of **5**:**12** (87:13 mol%) and 50 μM plasmid DNA (N:P ratio 0.86:1, 293 K, 150 mM NaCl, pH 7.4, 20 mM HEPES), with the differential number fraction *fn* expressed as a function of the root-mean-square radius (nm).

spherical particles. A time-independent (over two days) root-mean-square radius (R_{rms}) of 41 nm was observed, a value that compares well with the hydrodynamic radius (R_w) of 48 nm as measured by PCS (theoretical value of $R_w = 1.29 \times R_{rms}$ assuming a spherical structure[11, 12]). Measurements of the zeta potential on the same preparations over a N:P ratio of 0.5:1 to 2:1, revealed a surface charge on the particles ranging between -5 and $+10$ mV. In contrast, particles formed from **5** in the absence of PEG-bola exhibited a surface charge ranging from -40 mV to $+20$ mV, which strongly suggests that the PEG tail groups shield the charge on the particle surface.

The nature of the DNA in the particles was examined by circular dichroism (150 mM NaCl, 20 mM *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), pH 7.4). As the bolas were added to the B-form DNA the intensity of the longest wavelength (positive) band shifted (from 268 to 290 nm) and reduced in overall intensity to reach a limiting value at an N:P ratio of 1:1 or more. The position of zero-CD (cross-over from negative to positive dichroism) shifted simultaneously from 257 to 268 nm (Figure 2), which is consistent with the retention of the B-form DNA in the bound state.[13, 14] Taken together, these physicochemical studies are consistent with the formation of spherical or toroidal particles in which the polyamine-bound DNA is compacted inside the supramolecular assembly, with the hydrocarbon chains separating it from the hydrophilic tail groups and bulk water.

The plasmid DNA pEGlacZ,[12] which codes for the enzyme β-galactosidase, was condensed with the sugar-bola **5** or PEG-

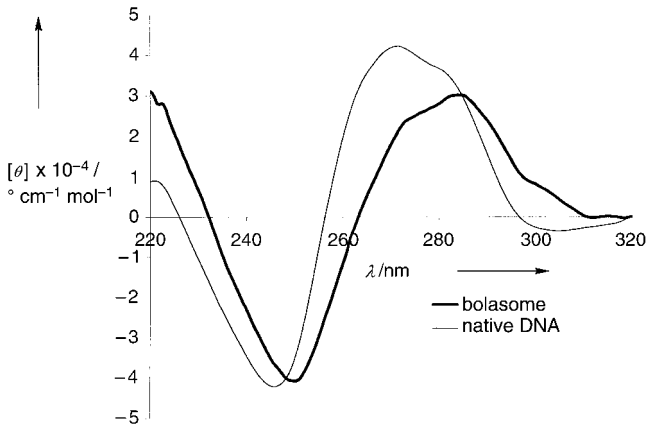


Figure 2. CD spectra of 50 μM plasmid DNA (293 K, 150 mM NaCl, 20 mM HEPES, pH 7.4) in the absence (thin line) and presence (bold) of the bolaamphiphile **2** (0.6:1 N.P ratio).

bola **12** at N:P ratios of 0.5:1, 1.5:1, and 4:1 and tested for transfection competence in Chinese hamster ovary cells.[15] The PEG-bola derivative resulted in reporter-gene activity at around the detection limit of the β-galactosidase assay (Figure 3). In contrast the sugar-bola derivative gave a clearly

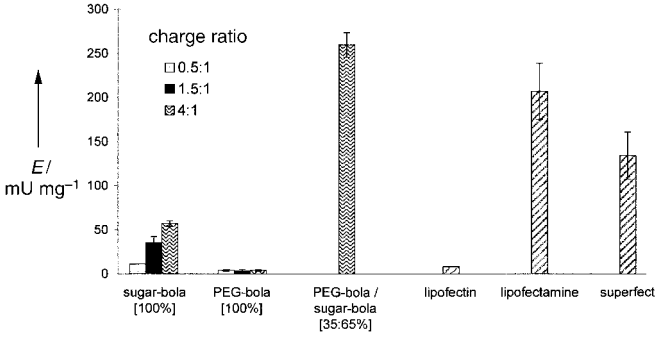
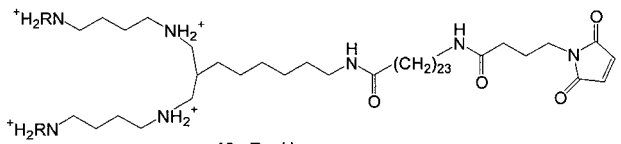


Figure 3. Transfection of Chinese hamster ovary cells with bola/DNA complexes and comparison with commercial transfection reagents.

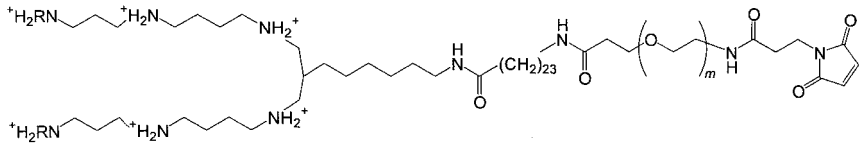
detectable level of β-galactosidase for particles prepared at the 0.5:1 N:P ratio, and increased by threefold at 1.5:1 and by fivefold at 4:1 N:P ratio. For comparison results are shown for the commercial transfection reagents lipofectin, lipofectamine, and superfect tested with the same plasmid under optimal conditions according to the manufacturer's instructions. The rank order of reporter-gene expression obtained was lipofectamine > superfect > lipofectin, with the sugar-bola at an N:P ratio of 4:1 giving a transfection level intermediate between that of superfect and lipofectin.

When a mixture of sugar-bola and PEG-bola (65:35 mol%) was used to condense plasmid DNA at a 4.1 N:P ratio the resulting reporter-gene expression increased by over fourfold to that obtained with the sugar-bola complex and was comparable to that obtained with lipofectamine. No acute toxicity was found at up to 70 μg bola/DNA doses in mice.

To demonstrate that antibody-mediated targeting of the bola-condensed DNA particles or "bolasomes" occurred, an antibody Fab′ fragment with an exposed hinge cysteine thiol residue was selectively conjugated to the maleimide group of **13** or the PEG-bola derivative **14**. An anti-CD3 antibody was



13a R = H
13b R = $CH_2CH_2CH_2NH_2^+Me$



14 *m* = average 45, R = Me

chosen to direct the targeting to the T cell receptor complex present on human T lymphocytes and on Jurkat cells, a human T-cell line which has been reported to be relatively refractive to transfection.[16] Accordingly, a mixture of the anti-CD3-Fab′-bola conjugate (0.004 mol %), sugar-bola **5** (67 mol %), and PEG-bola **12** (33 mol %) was used to form anti-CD3 bolasomes at a 3:1 N:P ratio. Control bolasomes containing a Fab′ fragment of irrelevant specificity were also prepared.

An AFM image[17] (Figure 4) of such particles revealed similar overall dimensions to those characterized by PCS, the majority with a diameter of about 80 nm. The transfection competence of both types of particle was tested on Jurkat cells[18] and resulted in a significant level of reporter-gene

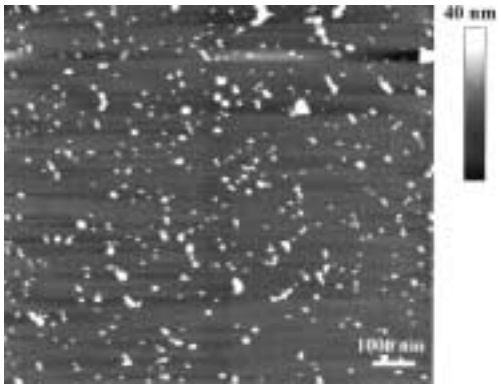


Figure 4. AFM image of particles comprising **5** (67 mol %) and **12** (33 mol %) with 0.004 mol % of the anti-CD3-Fab conjugate of **14**, 3:1 N:P ratio (295 K, 10 mM HEPES).

expression (733 ± 300 pg of β-galactosidase per mg of cytosolic protein of cell extract) for the anti-CD3 bolasomes compared to undetectable expression for the control bolasomes. Futhermore, the specificity of the transfection was confirmed by competitive displacement of the anti-CD3 bolasome in the presence of a large excess ($20\ \mu mL^{-1}$) of free anti-CD3 IgG, which resulted in complete abolition of transfection. Antibody targeting of the bolasome to the T cell receptor complex has thus allowed uptake and intracellular release of the plasmid DNA, presumably by a receptor-mediated endocytosis pathway.

These studies augur well for the future development of a robust and selectively targeted DNA-delivery system. A new class of water-soluble DNA-compacting agents has been defined which not only induce self-assembly of plasmid DNA into discrete particles of around 100 nm diameter, but also provide the means of regulating surface properties through their hydrophilic tail groups. Importantly, the surface charge of the particles may be controlled by the introduction of PEG groups which serve to minimize certain nonspecific interactions in vivo. Furthermore, the incorporation of a mixture of PEG-bola and sugar-bola, in a defined ratio, improves the transfection competence of the bolasome. Finally, targeted transfection of a chosen cell type can be achieved using a bolasome that includes a bola-conjugated antibody.

Received: June 6, 2000 [Z15233]

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[7] a) A. E. Martell, R. M. Smith, *Critical Stability Constants*, *Vol. 1–6*, Academic Press, New York, **1974–1989**; *pK*a values for **9a** and **9b** were measured using potentiometric methods (0.1M NMe_4Cl, 298 K) followed by data analysis using SUPERQUAD: **9a**: 10.76(3), 10.03(2), 9.29(3), 7.59(4); **9b**: 10.49(4), 10.16(3), 9.31(3), 8.61(3), 8.00(2), 7.10(4). Individual data sets typically gave statistical σ and ψ^2 values of 0.27 and 6; b) details of these and additional syntheses are given in: M. A. W. Eaton, T. J. Norman, D. Parker, T. S. Baker, A. N. Weir, C. F. Catterall, WO-A 99/52858, **1999** [*Chem. Abstr.* **1999**, *131*(22), 2993156]; c) syntheses[7b] were carried out using standard polyamine and peptide coupling procedures and each compound (**1–12**) gave satisfactory ^1H NMR, ES-MS, and microanalytical data in accord with the proposed structures. A representative synthesis for **1a** involved the condensation of diethyl (2-benzyloxyhexyl)malonate with excess diaminobutane, followed by borane reduction ($BH_3 \cdot THF$), Boc protection (Boc_2O/MeOH), hydrogenolysis ($Pd(OH)_2$/C, H_2-*t*BuOH), mesylation ($MeSO_2Cl/Et_3N/CH_2Cl_2$), and amination via the primary

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azide (NaN_3-DMF then H_2/Pd-C/MeOH) to give a Boc-protected primary amine. This was coupled with 24-(penta-*O*-acetylglucuronyl)amino-tetracosanoic acid (prepared by the opening of δ-gluconolactone with 24-amino-tetracosanoic acid (MeOH, 60 °C, DBU, 10 mins), followed by peracetylation (Ac_2O, pyridine)) using EDC/NHS/NMM-Et_3N/CH_2Cl_2. Deacetylation (aqueous ammonia/MeOH) and removal of the Boc groups (CF_3CO_2H 96%, H_2O 4%, 20 °C, 30 mins) yielded the tetra-trifluoroacetic acid salt of **1** as a colorless lyophilisate. Boc = *tert*-butoxycarbonyl; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide; NHS = *N*-hydroxysuccinimide; NMM = *N*-methylmorpholine.

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[10] Samples of 7.67 kb plasmid DNA[12] or bola-condensed DNA were analyzed by gel electrophoresis following incubations (30 min, 37 °C, pH 7.4, 150 mM NaCl) in the absence and presence of either nuclease or of 50% foetal calf serum. For nuclease treatment, DNase I (Pharmacia, FPLC pure) was added at a concentration of 1 unit μg^{-1} of DNA. Condensed DNA was not visible by ethidium bromide staining, but was revealed in parallel tracks where the sample had been incubated with 0.5% sodium dodecyl sulphate.

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[15] Transfection complexes were prepared in 10 mM HEPES pH 8.0 buffer by adding a plasmid solution dropwise to an equal volume of bola solution to give a final concentration of 30 $\mu g\,mL^{-1}$ DNA. N:P ratios were calculated assuming the nucleotide residue had a formula weight of 310. The transfection reagents lipofectin and lipofectamine were obtained from Life Technologies Ltd. and superfect from Qiagen Ltd. Cell culture media and additives were obtained from Life Technologies Ltd. Chinese hamster ovarian cells were plated out in 24-well cell culture plates at 10^5 cells per well in 1 mL culture medium (Iscoves complete medium containing 2 mM glutamine and 10% foetal calf serum). After 24 h the cells were washed with 1 mL of Optimem medium per well. Transfection was carried out over 4 h at 37 °C in the presence of 500 µL of Optimem and 167 µL of condensed DNA (5 µg), then the medium was replaced with 1 mL of culture medium and the incubation continued for a total of 48 h. The levels of the activity of the β-galactosidase reporter gene in the cells were assessed using an enzyme activity kit (Promega Ltd) according to the manufacturer's instructions, where the hydrolysis of the substrate *o*-nitrophenyl-β-D-galactopyranoside was measured spectrophotometrically in washed, lysed-cell supernatants. The assay was calibrated against a β-galactosidase standard (Boehringer Mannheim Ltd.; 0.775 mU enzyme activity per ng of protein). The results were normalized for supernatant protein concentration following measurement of the latter using a BCA protein assay kit (Pierce and Warriner Ltd.)

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[17] Samples were imaged under ambient conditions (10 mM HEPES, pH 8.0) using either a DI3000 or a DI Multimode atomic forcr microscope, in the tapping mode with a scan frequency of 1 – 3 Hz.

[18] Jurkat E6.1 cells (ECACC) were cultured in Dulbecco's modified eagles medium (DMEM) (Life Technologies Ltd.) that contained 10% foetal calf serum, 2 nM of glutamine, 50 units mL^{-1} of penicillin, and 50 $\mu g\,mL^{-1}$ of streptomycin. For transfection, cells were washed and re-suspended in serum-free DMEM at 2×10^6 cells mL^{-1} and 0.5 mL per well added to 24-well culture plates. Bola-condensed DNA (200 µL) was added at 4 $\mu g\,well^{-1}$ and the plates incubated for 1 h at 37 °C in 5% CO_2. Incubation was continued for a total of 24 h following addition of 300 µL of 33% foetal calf serum in DMEM. Cells were harvested by centrifugation and washing with phosphate buffered saline. Cells were lysed and supernatants assayed using a β-galactosidase enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Ltd). Reporter gene levels were normalized for cytosolic protein.[15]

A Versatile Periodate-Coupled Fluorogenic Assay for Hydrolytic Enzymes**

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The development of new catalysts is being increasingly followed by using combinatorial and evolutionary methods.[1–3] These approaches require the ability to assay large numbers of samples in parallel. This can be achieved using solid-phase bound assays related to immunoassays[4] and a variety of spectroscopic methods, such as IR thermography[5] and mass spectrometry.[6] One of the most popular methods is the use of chromogenic and fluorogenic substrates as product formation sensors.[1, 7, 8] Herein, we report a new versatile fluorogenic assay for hydrolytic enzymes. The assay couples product formation to the release of a fluorescent signal, achieved via periodate oxidation and albumin-catalyzed β-elimination, and uses nonactivated, chiral substrates.

We recently reported an enantioselective fluorogenic assay for alcohol dehydrogenases based on the detection of carbonyl oxidation products, such as **7** (Scheme 1), by β-elimination of the fluorescent product umbelliferone (**8**) catalysed by bovine serum albumin (BSA).[9, 10] Aldehyde **7** can also be produced from diol **5** or aminoalcohol **6** by oxidation with

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[**] We would like to thank Prof. R. Furstoss and Dr. A. Archelas at the Faculté des Sciences de Luminy, Marseille (France) for providing a sample of *Aspergillus niger* EH and Dr. C. Weijers at the Department of Food Technology and Nutrition Sciences, Wageningen University (the Netherlands) for providing a sample of *Rhodotorula glutinis* EH. This work was supported by the University of Bern, the European COST program (Action D12), the Swiss Office Fédéral de l'Education et de la Science, the Wander Stiftung, the Ministero della Università e della Ricerca Scientifica e Tecnologica (MURST), and Consiglio Nazionale delle Ricerche (CNR) (Roma). P.C. gratefully acknowledges Merck for generous financial support from the 1999 ADP Chemistry Award.