Helical Crystallization of Proteins on Carbon Nanotubes: A First Step towards the Development of New Biosensors**

Fabrice Balavoine, Patrick Schultz, Cyrille Richard, Véronique Mallouh, Thomas W. Ebbesen, and Charles Mioskowski*

Since their discovery in 1991[1] carbon nanotubes have attracted considerable interest because of their peculiar shape: they are several micrometer long tubes with diameters as small as a few nanometers. Their outstanding mechanical and electronic properties[2] have made them very promising nanomaterials for new applications in chemistry and physics, particularly for the development of new nanotechnologies. However, few applications have so far been reported in biology. Small proteins have been introduced into the inside hollow of opened nanotubes thus forming natural nano test tubes.[3] It should also be of great interest to decorate the outer surface of these carbon nanotubes with biological macromolecules, such as oligonucleotides[4] or proteins. Such devices could promote the development of new biosensors and bioelectronic nanomaterials, which could take advantage of the specific biomolecular recognition properties associated with the bound macromolecules.

For such purposes the specific recognition function has to be densely packed on the outer surface of the carbon nanotubes and the protein has to be functional. A good criterion for the conservation of the functional properties of the protein is its ability to form ordered arrays. In fact, the cylindrical shape, the perfect straightness, and the exceptional rigidity[5] of the carbon nanotubes make their surfaces good candidates for the growth of helical crystals of proteins (Figure 1).

These nanotubes are easy to handle and can be prepared in gram quantities by the arc-discharge method.[2] This method leads to the formation of multi-walled nanotubes (MWNTs) that are made of several coaxial graphite layers. The tube diameter shows a broad distribution ranging from 2 to 30 nm. Such a polydisperse material could increase the chance of crystallization since the protein has the opportunity to select the nanotube of suitable diameter for helical self-organization.

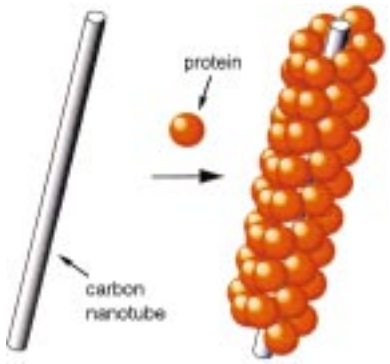


Figure 1. Schematic representation of the helical crystallization of proteins on the outer surface of a carbon nanotube.

Streptavidin, a water-soluble protein synthesized by *Streptomyces avidinii*,[6] was chosen to study the interaction of proteins with carbon nanotubes. Streptavidin has become very useful in many biochemical assays, such as labeling and affinity chromatography because of its high affinity for (+)-biotin ($K_a \approx 10^{15}$).[7] Upon incubation of streptavidin with MWNTs the protein molecules were found to strongly interact with the carbon tubes. In appropriate conditions the tubes were almost completely covered by streptavidin whereas only a few free molecules could be seen in the background of the electron microscope grid. This observation was not surprising since streptavidin can be purified on hydrophobic resins such as benzyl-DC beads of cellulose,[8] can form 2D crystals on nonspecific hydrophobic lipid films,[9] and binds strongly to plain carbon-coated electron microscopy grids. These properties probably reflect the fact that streptavidin displays hydrophobic domains within its structure. While most of the streptavidin molecules bound stochastically to the nanotubes and did not form any ordered arrangements (Figure 2a) we noticed that in some instances the nanotubes showed lateral striations that were regularly spaced at 6.4 nm with an angle of 71° to the tube axis (Figure 2b). Perpendicular striations were also visible, which suggests that the streptavidin molecules were organized in a square lattice.

The helical organization of the streptavidin molecules on the nanotube surface was evidenced by the optical diffraction pattern, which exhibited a characteristic layer line distribution (Figure 3a). Direct observation of the images and the analysis of the optical diffraction pattern, which showed a layer line spacing of 1/12.8 nm^{-1}, indicated that the helical repeat consisted of two striations. This distance between the layer lines is in good agreement with the size of the streptavidin molecule as revealed by X-ray analysis.[10] A noise-free view of the helical repeat was obtained by averaging 25 sub-images of the streptavidin-coated nanotube. The sub-images were extracted at regular spacings that corresponded to the repeat distance along a 300-nm long nanotube image (Figure 3b). The indexation of the Fourier transform revealed that the order of the Bessel functions were multiples of two, which indicated a two-start helical organization formed by two protein helixes that wind around the nanotubes and that each

[*] Dr. C. Mioskowski, Dr. F. Balavoine, C. Richard
CEA Saclay-DSV/DBCM/SMM
91191 Gif sur Yvette cedex (France)
Fax: (+33) 1-69-08-79-91
E-mail: mioskow@aspirine.u-strasbg.fr
Dr. P. Schultz, V. Mallouh
Institut de Génétique et de Biologie Moléculaire et Cellulaire
1 rue Laurent Fries, BP163, 67404 Illkirch cedex (France)
Dr. T. W. Ebbesen
ISIS, Louis Pasteur University, 4 rue B. Pascal
67000 Strasbourg (France) and
NEC Research Institute, 4 Independence Way
Princeton, New Jersey 08540 (USA)

[**] This work was supported by funds from the Commissariat à l'Energie Atomique, the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, and the NEC Research Institute. We are also very grateful to Dr. Paulette Vignais for providing us with pure HupR protein.

  1433-7851/99/3813-1912 $ 17.50+.50/0

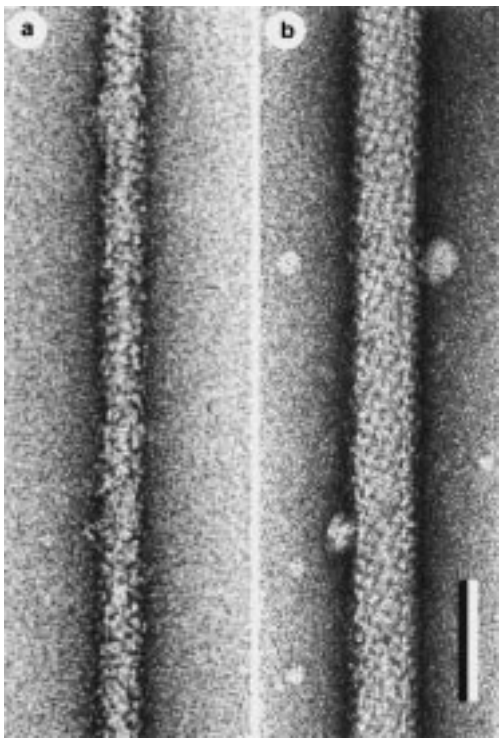


Figure 2. Electron micrographs showing multi-walled nanotubes (MWNTs) coated with streptavidin molecules and negatively stained with uranyl acetate. a) Stochastic binding of streptavidin molecules on a MWNT with a diameter smaller than 15 nm. b) Helical organization of streptavidin molecules on a carbon nanotube with a suitable diameter of 16 nm. The bar represents 50 nm.

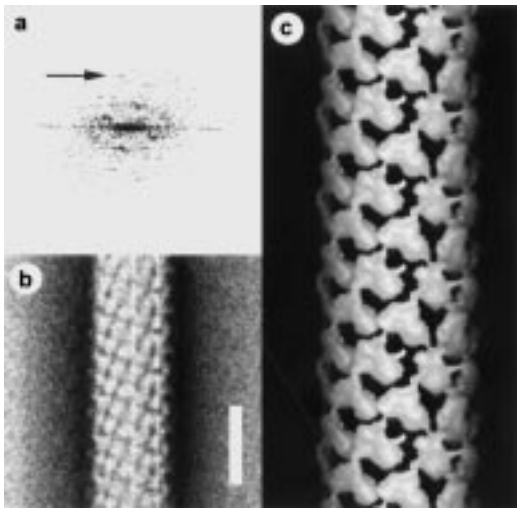


Figure 3. Analysis of the streptavidin helices formed on MWNTs. a) Computed power spectrum of the Fourier transform of a helical array of streptavidin molecules. The helical repeat is 12.8 nm and the arrow indicates 1/2.5 nm^{-1}. b) Noise-free view of the helical repeat obtained by correlation. c) Three-dimensional model of streptavidin assemblies calculated by retroprojecting the noise-free helical repeat shown in (b) along the Euler angles deduced from the analysis of the diffraction pattern. The bar represents 25 nm in (b) and 12.5 nm in (c).

helix consisted of eight streptavidin molecules per turn. The diffraction pattern showed structural information down to a resolution of 1/2.5 nm^{-1} on the fifth layer line (arrow in Figure 3a). The observation of a large number of striated tubes indicated that the outer diameter of the structure was constant at 27 ± 2 nm, which showed that the size of the tube was an essential parameter for helical organization. The diameter of the underlying carbon nanotube, which could occasionally be measured, was about 16–17 nm , which indicated that the protein layer had a thickness of 5 nm. This thickness is consistent with the size of a streptavidin molecule and demonstrates that a single protein layer coats the nanotubes. We further noticed that all the observed crystalline arrays exhibited the same handedness and the same helical repeat unit.

The helical parameters determined from the analysis of the optical diffraction pattern were used to reconstruct a 3D model of the assemblies by retroprojection (Figure 3c). The structure indicates that the orientation and the molecular contacts are similar to those found in 2D crystals with *C*222 symmetry.[11] The streptavidin-coated nanotubes should thus show biotin-binding properties since it was demonstrated for 2D crystals that two biotin binding sites face the aqueous phase and are accessible for interaction with biotinylated molecules. It was previously shown that crystals of streptavidin formed on biotinylated lipid monolayers[11] or tubules[12] can be decorated with polybiotinylated ferritin molecules from horse spleen or biotinylated Fab-fragments, respectively. Thus carbon nanotubes covered with streptavidin molecules could be used as a bioreactive docking matrix. These results provide carbon nanotubes with new and very powerful applications in biotechnology, since many biotinylated compounds, such as proteins or DNA fragment, are now available.

The binding of streptavidin to the carbon nanotubes was efficient in a wide range of conditions tested including various temperatures, incubation times, pH values, and ionic strengths. However, the formation of highly ordered helical arrangements in a quality suitable for structural investigations was poorly reproducible. The best results were obtained in the presence of a buffer containing 20% of methanol and we hypothesize that methanol reduces the strength of the hydrophobic interactions involved in the binding of the protein on to the carbon nanotubes and thus favors protein–protein interactions that lead to helical arrangements.

To further demonstrate the potential of carbon nanotubes in structural biology and biotechnology we also investigated the interaction and crystallization of HupR, another water-soluble protein. In the photosynthetic bacterium *Rhodobacter capsulatus* the DNA binding protein HupR is involved in the cell response to hydrogen and belongs to the subfamily of nitrogen-regulatory proteins (NtrC).[13] Like streptavidin, HupR was found to interact strongly with the carbon surface of MWNT and some nanotubes presented striations that made an angle close to 81° with the tube axis. The helical organization of HupR was more reproducible than for streptavidin and importantly occurred in the absence of methanol. We noticed that, unlike streptavidin, HupR protein formed ordered arrays on a wider range of MWNT diameters although a minimum value of about 12 nm was required (Figure 4). The optical diffraction pattern showed a lattice line spacing of 1/5.4 nm^{-1} and revealed structural details down to a resolution of 1/2.7 nm^{-1} (Figure 5a). The distance between the layer lines is in good agreement with the size of

  1433-7851/99/3813-1913 $ 17.50+.50/0

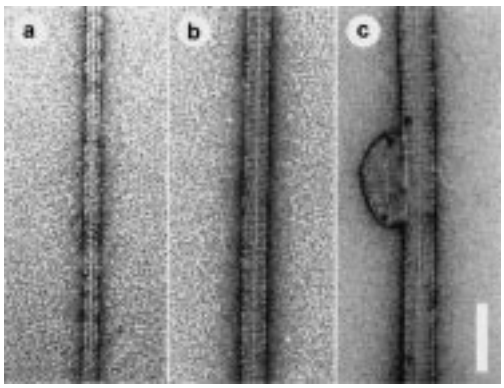


Figure 4. Electron micrograph showing MWNTs incubated with HupR protein and negatively stained with uranyl acetate. a) Stochastic binding of HupR proteins on a MWNT whose diameter of 8 nm is too small to accommodate a helical organization. b) and c) Helical organization of HupR proteins on carbon nanotubes with a diameter of 13 and 18 nm, respectively. The protrusion on the MWNT shown in (c) arises from contaminating amorphous carbon particles stacked on the tube. The bar represents 50 nm.

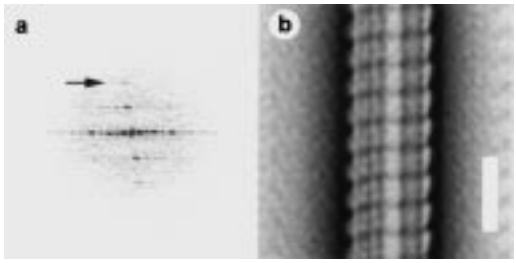


Figure 5. Analysis of the helices formed by the HupR protein on MWNTs. a) Computed power spectrum of the Fourier transform of a helical crystalline array. The repeat is 5.4 nm and the arrow indicates the second layer line at 1/2.7 nm^{-1}. b) Noise-free view of the helical organization obtained by a correlation over an average of 40 repeated units. The bar represents 15 nm.

the HupR molecule as revealed by recent electron microscopy observations of 2D crystals (Figure 5).[14]

Although the interaction and self-organization of proteins on the carbon tubes are still poorly understood, we have demonstrated the feasibility of organizing soluble proteins on the surface of multi-walled carbon nanotubes. These nanotubes thus appear capable of acting as new supports for protein crystallization and which are suitable for structural investigations by electron microscopy. In fact, analysis of self-organized protein arrays by electron microscopy and image processing has become a powerful method for such a purpose since a periodic signal can be readily extracted from noise. The natural propensity of membrane proteins to form 2D crystals allowed their structure determination by electron crystallography close to atomic resolution. To extend this concept to soluble proteins, functionalized lipid molecules were designed to allow the 2D crystallization of various systems.[15] However, a major drawback of the structure determination of 2D crystals is the limited possibility of tilting the specimen, a feature that is required to access the third dimension.[16] In this respect supramolecular protein assemblies with helical symmetry are of particular interest since several molecular views can be recorded in a single image[17] thus giving direct access to information on the three-dimensional structure without the need for tilting the specimen. Several biological macromolecules, such as viruses,[18] membrane proteins,[19] or filamentous components of the cytoskeleton such as microtubules,[20] exhibit helical symmetry suitable for studies by electron microscopy. However, naturally occurring helical assemblies of biological macromolecules present one or more of the following drawbacks that limit their general use or the attainable resolution: they may contain a limited number of coherently ordered repeating units, they may flatten themselves during specimen preparation, or their formation may be very specific. It is therefore of interest to control the formation of perfectly ordered helical arrangements of a sufficiently large size for a large variety of proteins.

Assemblies of lipids were shown to form tubular structures upon hydration and to induce the helical crystallization of proteins.[12] Multi-walled carbon nanotubes appear now as general and promising alternative supports for the helical crystallization of biological macromolecules. Carbon nanotubes can be prepared on a large scale and display properties such as straightness, rigidity, and conductivity that are not shared by lipid tubes. We anticipate that these supports will flatten to a lesser extent and will improve the attainable resolution when observed under appropriate preservation conditions such as in a frozen hydrated state.

The use of protein coated carbon nanotubes should extend well beyond the described applications in structural biology. Carbon nanotubes are likely to be amenable to industrial production and progresses in related fields will soon allow the manipulation of these nanostructures. A step towards this direction was witnessed by the recent application of carbon nanotubes as tips for scanning probe microscopes.[21] Our experiments demonstrate that it is possible to functionalize the nanotubes in order to modify their recognition properties. New applications of such nanomaterials now appear conceivable in the emerging fields of bio-nanotechnology.

Experimental Section

Multi-walled carbon nanotubes were prepared by the arc-discharge method[2] and stored as a suspension in methanol (2 $mg\,mL^{-1}$). In the case of the incubation with streptavidin molecules, 100 μL of the MWNT suspension were dried under an ethane gas flow and resuspended in 20 μL of a 40% aqueous solution of methanol. This suspension was sonicated to disperse the MWNTs prior to the addition of 20 μL streptavidin solution at a concentration of 10 $\mu g\,mL^{-1}$ in a buffer containing 10 mM Tris (pH 8) and 50 mM NaCl, and allowed to stand at room temperature for 45 minutes. The HupR protein was stored in a buffer containing 10 mM Tris (pH 8) and 350 mM NaCl. The same protocol was followed for the interaction with the MWNT except that the nanotubes were resuspended in pure water and in the absence of methanol prior to the addition of the protein. Five microliters of these preparations were placed on a 10 nm thick carbon film previously treated by a glow discharge in air. After two minutes of adsorption, the grid was negatively stained with a 2% uranyl acetate solution.

The images were recorded at a magnification of 45 000 times with a Philips CM120 transmission electron microscope operating at 100 kV. Three times enlarged prints of the electron micrographs were digitized using a Umax powerlook II scanner at a resolution of 375 μm, which corresponds to 5 Å at the specimen level. The images were processed with the IMAGIC software package to calculate Fourier transforms, self- and cross-correla-

  1433-7851/99/3813-1914 $ 17.50+.50/0

tion functions, to average images of the helical repeat, and to reconstruct a three-dimensional model from the weighted back projection algorithm.[22]

Received: December 30, 1998 [Z12857IE]
German version: *Angew. Chem.* **1999**, *111*, 2036 – 2039

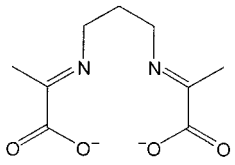
Keywords: electron microscopy · helical structures · nanotubes · proteins · self-organization

[1] S. Iijima, *Nature* **1991**, *354*, 56 – 58.
[2] T. W. Ebbesen in *Carbon Nanotubes—Preparation and Properties* (Ed.: T. W. Ebbesen), CRC, Boca Raton, FL, **1997**.
[3] J. J. Davis, M. L. H. Green, O. A. H. Hill, Y. C. Leung, P. J. Sadler, J. Sloan, A. V. Xavier, S. C. Tsang, *Inorg. Chim.* Acta **1998**, *272*, 261 – 266.
[4] a) S. S. Tsang, Z. Guo, Y. K. Chen, M. L. H. Green, H. A. O. Hill, T. W. Hambley, P. J. Sadler, *Angew. Chem.* **1997**, *109*, 2291 – 2294; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 2198 – 2200; b) Z. Guo, P. J. Sadler, S. C. Tsang, *Adv. Mater.* **1998**, *10*, 701 – 703.
[5] M. M. J. Treacy, T. W. Ebbesen, J. M. Gibson, *Nature* **1996**, *381*, 678 – 680.
[6] L. Chaiet, F. J. Wolf, *Arch. Biochem. Biophys.* **1964**, *106*, 1 – 5.
[7] E. A. Bayer, H. Ben-Hur, M. Wilchek, *Methods Enzymol.* **1990**, *184*, 80 – 89.
[8] W. D. Schwidop, P. Klossek, R. Muller, R. Claus, *J. Chromatogr.* **1990**, *520*, 325 – 331.
[9] T. Furuno, H. Sasabe, *Biophys. J.* **1993**, *65*, 1714 – 1717.
[10] W. A. Hendrickson, A. Pähler, J. L. Smith, Y. Satow, E. A. Merritt, R. P. Phizacker, *Proc. Natl. Acad. Sci. USA.* **1989**, *86*, 2190 – 2194.
[11] S. A. Darst, M. Ahlers, P. H. Meller, E. W. Kubalek, R. Blankenburg, H. O. Ribi, H. Ringsdorf, R. D. Kornberg, *Biophys. J.* **1991**, *59*, 387 – 396.
[12] a) P. Ringler, W. Müller, H. Ringsdorf, A. Brisson, *Chem. Eur. J.* **1997**, *3*, 620 – 625; b) E. M. Wilson-Kubalek, R. E. Brown, H. Célia, R. A. Milligan, *Proc. Natl. Acad. Sci. USA.* **1998**, *95*, 8040 – 8045.
[13] P. Richaud, A. Colbeau, B. Toussaint, P. M. Vignais, *J. Bacteriol.* **1991**, *173*, 5928 – 5932.
[14] C. Vénien-Bryan, F. Balavoine, B. Toussaint, C. Mioskowski, E. A. Hewat, B. Helme, P. M. Vignais, *J. Mol. Biol.* **1997**, *274*, 687 – 692.
[15] L. Lebeau, P. Schultz, H. Célia, P. Mésini, S. Nuss, C. Klinger, S. Olland, P. Oudet, C. Mioskowski in *Handbook of nonmedical applications of liposomes*, *Vol. II* (Eds.: Y. Barenholz, D. D. Lasic), CRC, Boca Raton, FL, **1996**, pp. 155 – 188.
[16] L. A. Amos, R. Henderson, P. N. T. Unwin, *Prog. Biophys. Mol. Biol.* **1982**, *39*, 183 – 231.
[17] D. J. DeRosier, P. B. Moore, *J. Mol. Biol.* **1970**, *52*, 355 – 369.
[18] T. W. Jeng, R. A. Crowther, G. Stubbs, W. Chiu, *J. Mol. Biol.* **1989**, *205*, 251 – 257.
[19] N. Unwin, *J. Mol. Biol.* **1993**, *229*, 1101 – 1124.
[20] A. Hoenger, E. P. Sablin, R. D. Vale, R. J. Fletterick, R. A. Milligan, *Nature* **1995**, *376*, 271 – 274.
[21] a) H. Dai, J. H. Hafner, A. G. Rinzler, D. T. Colbert, R. E. Smalley, *Nature* **1996**, *384*, 147 – 150; b) S. S. Wong, E. Joselevich, A. T. Wooley, C. L. Cheung, C. M. Lieber, *Nature* **1998**, *394*, 52 – 55.
[22] M. Van Heel, W. Keegstra, *Ultramicroscopy* **1981**, *7*, 113 – 130.

Template Assembly of Metal Aggregates by Imino-Carboxylate Ligands**

Dan M. J. Doble, Colin H. Benison, Alexander J. Blake, Dieter Fenske, Matthew S. Jackson, Richard D. Kay, Wan-Sheung Li, and Martin Schröder*

The aggregation of soft metal centers to form covalently bonded clusters is well known,[1–3] and there are well-established theories regarding the structure and bonding in these polynuclear complexes, for example Ru– and Os – carbonyl[2] and Au clusters.[3] In contrast, hard metal cations do not readily form metal – metal bonds, and usually require bridging ligands such as carboxylate,[4] oxide,[5] or hydroxide ligands[5] to induce aggregation. These ligands have enabled one-pot syntheses of some spectacular high-nuclearity aggregates, often with interesting magnetic and electronic properties.[4, 5] However, such ligands can adopt a variety of coordination modes, which, together with the often unpredictable coordination geometries of the metals concerned, makes it very difficult to rationalize the structures of the resulting aggregates. Recent developments in self-assembly have shown how metal – ligand frameworks can be sucessfully templated about a substrate anion[6] or cation.[7] Our approach has been to use the tetradentate ligand $(L)^{2-}$ [8] to chelate the four equatorial sites of a potentially octahedral metal ion M^{II} ($M^{II} = Ni^{II}$, Mn^{II}), leaving two free axial sites at the metal center for aggregation by interaction with the carboxylate oxygen donors of neighboring [M(L)] units.[9] Thus, each planar [M(L)] moiety has available two acceptor sites on M^{II} to form a 180° junction, and two carboxylates donors forming 90° junctions to one another and to the acceptor sites on M^{II}.



$(L)^{2-}$

Slow codiffusion of methanolic solutions of [Ni(L)][10a] and $La(ClO_4)_3 \cdot 6H_2O$ results in the growth of brown, columnar crystals of **1**. A single-crystal X-ray structure determination[11]

$[\{Ni(L)\}_6La](ClO_4)_3$ **1**

reveals a highly unusual[12] heptanuclear $[\{Ni(L)\}_6La]^{3+}$ cluster (Figure 1) in which a La^{III} center is located at the center of the

[*] Prof. Dr. M. Schröder, D. M. J. Doble, C. H. Benison, Dr. A. J. Blake, M. S. Jackson, R. D. Kay, Dr. W.-S. Li
School of Chemistry
The University of Nottingham
University Park, Nottingham, NG7 2RD (UK)
Fax: (+44) 115-951-3563
E-mail: m.schroder@nott.ac.uk
Prof. Dr. D. Fenske
Institut für Anorganische Chemie der Universität Karlsruhe (Germany)

[**] This work was supported by the Engineering and Physical Sciences Research Council (UK), Nycomed-Amersham (to D.M.J.D.), and the University of Nottingham.

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/home/angewandte/ or from the author.

  1433-7851/99/3813-1915 $ 17.50+.50/0