Counting of labelled tyrosine molecules in hydrophobic yoctolitre wells filled with water†

Sheshanath Bhosale,[a] Guangtao Li,[b] Fengting Li,[c] Tianyu Wang,[a] Rainer Ludwig,[a] Thomas Emmler,[a] Gerd Buntkowsky[a] and Jürgen-Hinrich Fuhrhop*[a]

Received (in Cambridge, UK) 3rd March 2005, Accepted 12th May 2005
First published as an Advance Article on the web 1st June 2005
DOI: 10.1039/b503141a

Time-dependent radioactivity and solid-state ^{13}C-NMR measurements of tyrosine entrapped in water-filled yoctolitre (10^{-24} L) wells with hydrophobic walls are reported; the results indicate that such wells induce the formation of quasi solid tyrosine if they are brought in contact with 0.1 M solutions of this edge amphiphile.

The successive self-assembly of substituted, flat-lying *meso*-tetraphenylporphyrins **3** or **4** followed by amphiphile **1** or bolaamphiphile **2** produces lipid monolayers on smooth surfaces with holes above a porphyrin bottom (Fig. 1). In an aqueous environment such hydrophobic membrane gaps enclose a few yoctolitres (10^{-24} L) of water. Such "yoctowells" have been established on gold electrodes[1–5] as well as on smooth aminated silica particles.[6,7]

The most interesting property of these hydrophobic wells in an aqueous environment consists of their ability to entrap specific solutes from 0.1 M aqueous solutions. **The rigid edge amphiphiles of Scheme 1, for example, migrate from the dilute solutions into the yoctowells and render them inaccessible to water-soluble ions. This blocking effect of the solutes is stereoselective.** *trans*-1,2-Cyclohexanediol or cellobiose with equatorial OH-groups are, for example, very efficient blockers, *cis*-1,2-cyclohexanediol or maltose with axial substituents have no blocking effect (Scheme 1).

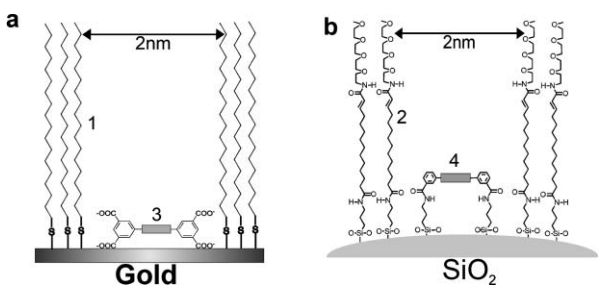
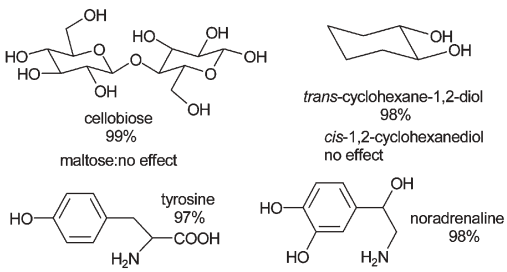


Fig. 1 Models of porphyrin-based yoctowells on a) gold electrodes and b) silica particles.



Scheme 1

With respect to the blocking mechanism we speculated the cause to be "immobile hydration water" of solutes, which sticks to hydrophobic walls. In this paper we describe ^{14}C-radioactivity and solid-state ^{13}C-NMR experiments, which determine the number of molecules within one yoctowell, characterize the water volume in the yoctowells by infrared as well as by ^{1}H- and ^{2}H-NMR spectroscopy, and lead to a consistent model of the solute entrapment.

Labelling with radioactive ^{14}C-tyrosine. The system of Fig. 1a is similar to Sagiv's early monolayers containing dissolved dyes.[8,9] Such fluid gaps are blocked by tyrosine with the same efficiency observed for rigid yoctowells made of stiff diamido amphiphiles.[10] A control experiment with rigid monolayers, such as the one sketched in Fig. 1b, gave similar results for the adsorption of radioactive tyrosine. We applied 0.1 M tyrosine solution in aqueous NaOH (pH = 10.5) and mixed it with commercial radioactive ^{14}C-tyrosine (Amersham, 86 MBq mg^{-1}). The kinetics of the tyrosine release from the loaded electrode surface into the bulk water volume of the supernatant was then measured. About 90% of the total radioactivity was released from the surface within three days, only the remaining 10% was taken as "entrapped" tyrosine. Its release was then followed for another 60 days. After two weeks about 30% of it had been dissolved away from the electrodes' surface. Extrapolation to 100% release yields a period of about 3 years (Fig. 2), which would be needed to empty all yoctowells or dissolve all of the membrane-integrated tyrosine in the supernatant.

We then plunged the gold electrode into a 0.1 M solution of 4,4′-*N*,*N*′-dimethyldipyridinium bromide ("viologen") and subjected it to 20 voltammetry cycles from +0.5 V to −0.8 V. These potential changes moved the linear dication within the yoctowells ("molecular stirring")[5] and the entrapped tyrosine was removed quantitatively from the yoctowells. At the end of the stirring experiment the gold coating was dissolved by sodium cyanide and

† Electronic supplementary information (ESI) available: experimental section. See http://www.rsc.org/suppdata/cc/b5/b503141a/index.sht

[a]*Freie Universität Berlin, FB Biologie, Chemie, Pharmazie, Institut für Chemie/Organische Chemie, Takustr. 3, D-14195 Berlin, Germany. E-mail: fuhrhop@chemie.fu-berlin.de*
[b]*Department of Chemistry, Tsinghua University, Beijing 100084, P. R. China*
[c]*School of Environmental Science and Engineering, Tongji University, Shanghai 200092, P. R. China*
*fuhrhop@chemie.fu-berlin.de

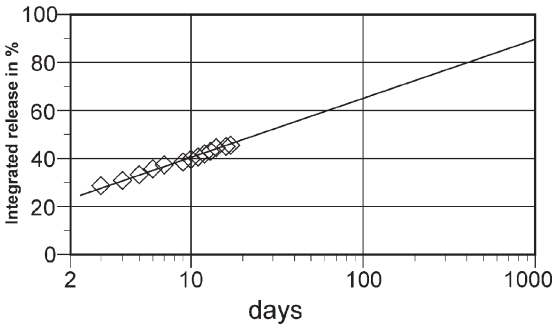


Fig. 2 Spontaneous release of tyrosine from fluid monolayers containing porphyrin-based 2 × 2 × 2 nm gaps from day 4 to 17.

the rest radioactivity again measured. The number of tyrosine molecules within the wells was thus determined. The area of the monolayer was 2.4 cm^2 or 2.4 × 10^{14} nm^2, the height of the monolayer 2.3 nm. The volume of the monolayers was thus 5.5 × 10^{14} nm^3 and there were 24.9 × 10^{14} tyrosine molecules in the holey monolayer (~5 molecules nm^{-3}) and 19.1 × 10^{14} tyrosine molecules in the C_{18}-SH monolayer (~3.5 molecules nm^{-3}; Table 1). A yoctowell with a volume of about 8 nm^3 should therefore contain about 35 molecules of tyrosine, which would be enough to fill up the yoctowells with crystalline tyrosine. Crystalline tyrosine (γ = 1.3°; M = 181) contains 4.3 molecules per yoctolitre. The yoctowell is three orders of magnitude smaller than the zeptowells (10^{-21} L) of 50 nm dimensions, which were obtained by laser embossing.[11]

Infrared spectroscopy. The same yoctowell-coated gold electrodes were also soaked with 0.1 M solutions of tyrosine in D_2O and infrared spectra were measured. A strong and narrow D_2O signal at 2721 cm^{-1} was found, which disappeared after 1 h and was found again after a second soaking with D_2O containing 0.1 M tyrosine. If this wave number is multiplied by $\sqrt{2}$ a corresponding OH wavenumber of 3841 cm^{-1} results, which is far beyond the normal water valency vibrations in solution (3710 cm^{-1}) or in crystalline water (3100–3600 cm^{-1}). Similar D_2O stretching vibrations were, however, observed in argon and nitrogen matrices for isolated D_2O species.[12–14] The D_2O-monomer in Ar-matrices absorbed at 2771 cm^{-1}, the dimer at 2746 cm^{-1} and at 2725 cm^{-1} in N_2-matrices. Our spectrum thus indicates the presence of D_2O-dimers. No signal at 2623 cm^{-1} was observed, which would correspond to the 3710 cm^{-1} water monomer band.

Solid-state ^{13}C- and ^{1}H-NMR spectroscopy. For NMR-measurements we replaced the electrode by colloidal silica particles.[15] Smooth surfaces with negligible curvature were obtained on amine-coated silica gel particles with a diameter of 100 ± 10 nm. They were prepared from tetraethoxysilane (TEOS) and 3-aminopropyl triethoxysilicate.[7,15] The covalent self-assembly of single, well-separated porphyrin molecules on the amino coating of the silica particles was executed with the mixed anhydride of zinc *meso*-tetra(phenyl-3-carboxyl)porphyrinate and chloroethyl formiate. The rigid wall was made of diamido bolaamphiphile **4** with OEG head groups as described earlier.[7] Semiquantitative spectra of the Soret band of the particle bound porphyrin indicated that about 93% of the silica gel was covered by the OEG-bola. First attempts to characterize the particles by ^{13}C-NMR with the wells and adsorbed tyrosine in natural abundance failed. Only a broad peak around 140 ppm was found for the tyrosine and porphyrin units and none of the other membrane components gave significant signals above the noise level . Upon application of 98% ^{13}C-enriched tyrosine, the signals of its aromatic carbon atoms appeared between 100 and 150 ppm, and weak signals for the $(OCH_2)_m$ and $(CH_2CH_2)_n$ carbons at 60 and 1 ppm were now also detectable (Fig. 3). The tyrosine's ^{13}C-enrichment at a ratio of 100:1 should then lead to a signal which is about 4 times more intense than that of the CH_2-carbons. The measured area under the signals was 3 times larger for the entrapped tyrosine than for the membrane CH_2-carbons. The radioactivity measurements on gold electrodes were thus confirmed by the solid-state ^{13}C-NMR spectra of silica particles. All quantitative experiments have been repeated at least four times and gave consistent results within an error margin of less than ±30%.

^{1}H-NMR spectra using a Hahn-echo sequence with very long delay times (between 800 μs and 3 ms) produced water signals. Signal intensities were now insignificant, because signals arising from protons in different environments show very different losses of signal intensity. No tyrosine proton signals were, for example, detectable, the strongest signals came from the CH_2-groups of the alkyl chain at 1.3 ppm and the OCH_2 proton signal at 3.9 ppm was negligibly small (Fig. 4). The remaining signals clearly originated from water, because the absolute and relative and intensities of the signals changed massively with time, indicating evaporation from the surface or migration within the yoctowells. The large signal at

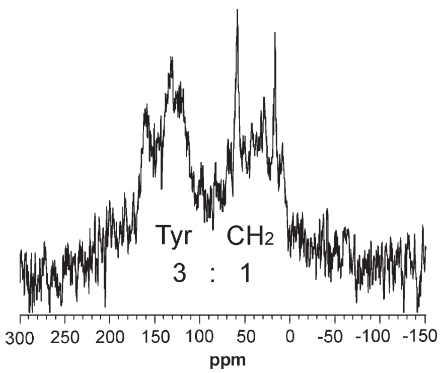


Fig. 3 Silica particles containing a monolayer of the oligoethylene bolaamphiphile shown in Fig. 1b, yoctowells with *meso*-tetra(3-carboxyphenyl)porphyrin as a bottom and dissolved ^{13}C-tyrosine. From this spectrum and similar ones it is concluded, that the number of CH_2-carbons and phenyl carbons of tyrosine is approximately the same.

Table 1 Measured radioactivity of ^{14}C-tyrosine in closed and holey surface monolayers after cyclic voltammetry stirring with dimethyl viologen

	Tyrosine radioactivity in supernatant after cyclic voltammetry stirring (CPM)	Molecules removed by CV stirring	Tyrosine radioactivity on platelet after stirring (CPM)	Molecules remaining on platelet
Closed C_{18}-SH monolayer	1197	6.7 × 10^{14}	1991	12 × 10^{14}
With yoctowells	2018	11 × 10^{14}	2415	14 × 10^{14}

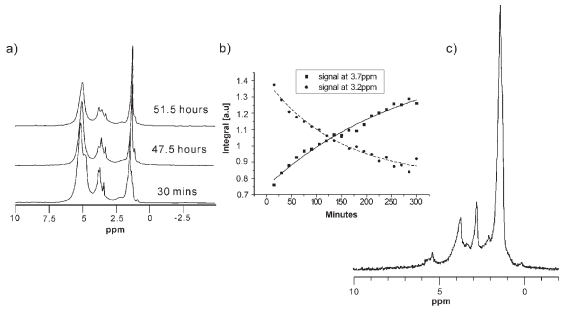


Fig. 4 Time dependence of ^{1}H-MAS-NMR echo spectra of coated silica nanoparticles treated with a tyrosine–water solution. a) The whole spectra; b) only the signals at 3.2 and 3.7 ppm; c) after treatment with a tyrosine solution in D_2O.

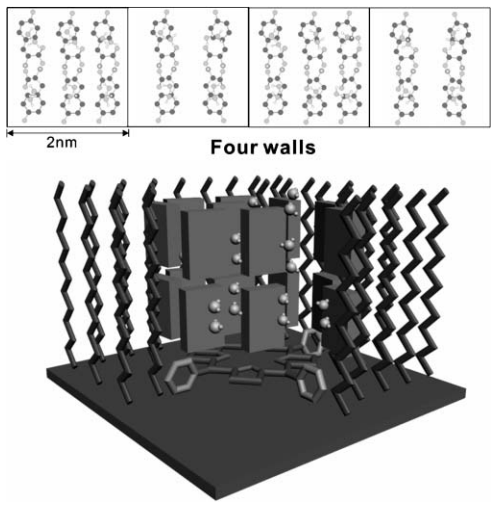


Fig. 5 Model of 20 tyrosine molecules on the yoctowell's walls and 16 within its volume of about eight yoctolitres. Water dimers at the amino acid groups above the phenol are also given. They should be quite mobile. Water above the porphyrin planes is not shown.

5.2 ppm was assigned to water adsorbed by the particles on the OEG surface and disappeared quickly. Three smaller, more stable signals at higher field are probably caused by water molecules within the yoctowells. They are shifted upfield by adsorption to the entrapped tyrosine molecules (3.7 ppm) or the porphyrin bottom (3.2 ppm, Fig. 4a). The 3.2 ppm signal raises in intensity with time, because the bottom water is most immobile (Fig. 4b). Water in cellobiose-filled yoctowells gave a signal at 3.9 ppm. H/D exchange experiments were then performed by addition of D_2O. The large extent of the H/D exchange becomes obvious with the signal for the bulk-adsorbed water at 5.2 ppm which disappeared (Fig. 4c). The weak signal at 5.4 ppm is probably caused by the CH=CH protons of the monolayer.

The model in Fig. 5 assumes that the solutes are first immobilized by their attachment to the hydrophobic walls and formation of planar tetramers and hexamers occurs there. The immobility of these interconnected molecules is the assumed reason for the formation and entrapment of the nanocrystallites. The remaining volume is then filled-up with 16 more solute molecules. These "inner molecules" can be displaced by viologen and allow for molecular stirring, which removes the molecules from the walls. The "nanocrystallization" process on the hydrophobic walls of the yoctowells, starts and continues only with molecules, which have only one stable conformation and which can form hydrogen bond chains in a plane. The nanocrystal model agrees with the estimated 35 $\pm$ 10 molecules within each 8 nm^3 yoctowell. The applied methods were at their detection limits, linear signal–tyrosine quantity relationships are not certain. Nevertheless, all data are in agreement with yoctowells which are totally filled with partially ordered, hydrated tyrosine molecules. The absence of isolated water clusters was indicated by both NMR and infrared spectra.

The model can be related to the recognition process between steroid hormones and branched oligoglycosides on the surface of biological membranes. This has, however, to occur in the other direction: hydrophobic steroids are entrapped by ordered sugar dendrimers and it is the dendrimer crystallite, whose conformational mobility is blocked by two hydrophobic surfaces of the steroid, which is localized in its center. The steroid then may diffuse into the membrane, but is not in equilibrium with the carrier systems in the aqueous phase. In both cases kinetic effects dominate over thermodynamically controlled molecular recognition.

This work was supported by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg "Wasserstoffbrû¥cken", SFB 448 "Mesoscopic Systems, Normalverfahren Fu 29/2), the CARBONA network of the Human Resources program, Brussels, the Förderungskommission FNK of the Freie Universität Berlin and the Fonds der Chemischen Industrie.

Additional material containing details of all physical measurements is available.

Notes and references

1 J.-H. Fuhrhop, T. Bedurke, M. Gnade, J. Schneider and K. Doblhofer, *Langmuir*, 1997, **13**, 455.
2 W. Fudickar, J. Zimmermann, L. Ruhlmann, B. Roeder, U. Siggel and J.-H. Fuhrhop, *J. Am. Chem. Soc.*, 1999, **121**, 9539.
3 M. Skupin, G. Li, W. Fudickar, J. Zimmermann and B. Roeder, *J. Am. Chem. Soc.*, 2001, **123**, 3454.
4 G. Li, W. Fudickar, M. Skupin, A. Klyszcz, C. Draeger, M. Lauer and J.-H. Fuhrhop, *Angew. Chem.*, 2002, **114**, 1906, *Angew. Chem. Int. Ed.*, 2002, **41**, 1828.
5 G. Li, K. Doblhofer and J.-H. Fuhrhop, *Angew. Chem.*, 2002, **114**, 1855, *Angew. Chem. Int. Ed.*, 2002, **41**, 2730.
6 G. Li, Sh. Bhosale, T. Wang, S. Hackbarth, B. Roeder, U. Siggel and J.-H. Fuhrhop, *J. Am. Chem. Soc.*, 2003, **125**, 10693.
7 Sh. Bhosale, S. Bhosale, T. Wang, G. Li, U. Siggel and J.-H. Fuhrhop, *J. Am. Chem. Soc.*, 2004, **126**, 13111.
8 J. Sagiv, *J. Am. Chem. Soc.*, 1980, **112**, 92.
9 J. Sagiv, *Isr. J. Chem.*, 1979, **18**, 346.
10 M. Gnade, PhD Thesis, Freie Universität, Berlin, 1999.
11 J. E. Barton and T. W. Odom, *Nano Lett.*, 2004, **8**, 1525.
12 G. P. Ayers and A. D. E. Pullin, *Spectrochim. Acta, Part A*, 1976, **32**, 1629; G. P. Ayers and A. D. E. Pullin, *Spectrochim. Acta, Part A*, 1976, **32**, 1695.
13 A. J. Tursi and E. R. Nixon, *J. Chem. Phys.*, 1970, **52**, 1521.12.
14 F. Huisken, M. Kaloudis and A. Kulcke, *J. Chem. Phys.*, 1996, **104**, 17.
15 A. van Blaaderen and A. J. Vrij, *J. Colloid Interface Sci.*, 1993, **156**, 1.