5. Immobile Crust Made of Tyrosine, Norepinephrine, Cellobiose and Other Rigid Edge Amphiphiles on the Hydrophobic Walls of Yoctowell

5.1 Introduction

A few years ago our group discovered an astonishing immobilizing effect in small, water-filled gaps ("yoctowells") in lipid monolayers,[23-25] it was found that water-soluble cyclic edge amphiphiles with a rigid carbon skeleton (benzene, cyclohexane) were entrapped irreversibly and did not equilibrate with bulk water volumes over periods of months. In the first experiments we looked for a recognition process between the hydrophobic surface of a steroid on the bottom of the well with the hydrophobic edge of D-glucose, which was dissolved in the water volume above. For this purpose the steroid islands were attached flatly to a gold electrode and surrounded by fluid or rigid alkyl monolayers. Such electrodes with a holey lipid coating were then applied for cyclovoltammetry (CV) of ferricyanide ions in bulk water. It was found that the coated electrode was active in CV, but was blocked after being plunged into 0.1 M glucose or 1,2-*trans*-cyclohexanediol or glucose solutions.[1] It was quickly shown that this blockade had nothing to do with stereoselective recognition between D-glucose and steroid surfaces, since D- and L-glucose solutes had the same blocking effect and the exchange of the bottom steroid by a porphyrin also remained without consequences. The blockade effect of several cyclic edge amphiphiles, however, was real and extremely long-lived. Cellobiose, for example, did not leave the yoctowells for several months, although it was in direct contact with bulk water.[29] A list of active edge blocking molecules was established, which contained phenolic (tyrosine, *o*-hydroquinone), carbohydrate (glucose, cellobiose, ascorbic acid) and cyclohexane (1,2-trans-diol, -diamine, dicarboxylate) derivatives.[25] Furthermore it was shown that stirring with dimethyl viologen by cyclic voltammetry removed the immobile solutes from the yoctowells on gold electrodes.[29,30] It is the lack of mobility, which differentiates the yoctowells from open synapses between membrane surfaces.

The experimental findings on the properties of the yoctowells and its water-soluble blockers, extend the list of efficient blocking molecules by two neurotransmitters and

then report on attempts to quantify the amount of tyrosine which is quasi-irreversibly entrapped in the yoctowells on gold electrodes.[29] Time dependent measurements of the radioactivity of ^{14}C-labelled tyrosine solid state ^{13}C- and ^{1}H-NMR spectroscopy on smooth silicate particles were applied to quantify the amount of tyrosine entrapped in the yoctowells. Furthermore we used infrared spectroscopy and solid state ^{1}H-NMR experiments to characterize the water volume in the yoctowells. A “crust model” will then be introduced, which is in agreement with the observed stereochemical selectivities of the blocking agents in comparison to inactive compounds, the determined ratio of monolayer and entrapped tyrosine material as well as the findings relating to the infrared and ^{1}H NMR-spectra of the water volumes within the yoctowells.

5.2 Blocking effect of neurotransmitters

Neurotransmitters are water-soluble compounds which migrate over synaptic clefts between two membrane proteins. This cleft can be as narrow as 10 nm and is thus comparable to the yoctowells discussed. There are a few γ-aminobutyric acid (GABA) -analogues, which are phenol derivatives and also active as neurotransmitters in the brain.[103] We tested four of them, namely serotonin, dopamine, norepinephrine and tyramine. All four are biologically active and recognized selectively by specific receptor proteins: the aromatic part presumably slips into a hydrophobic pocket on a protein surface and the amine group interacts with some acidic side-chain on the protein surface. In the case of our yoctowells, dopamine, norepinephrine and tyramine were almost 100% effective as blockers, whereas serotonin had no effect (Figure 5.1).

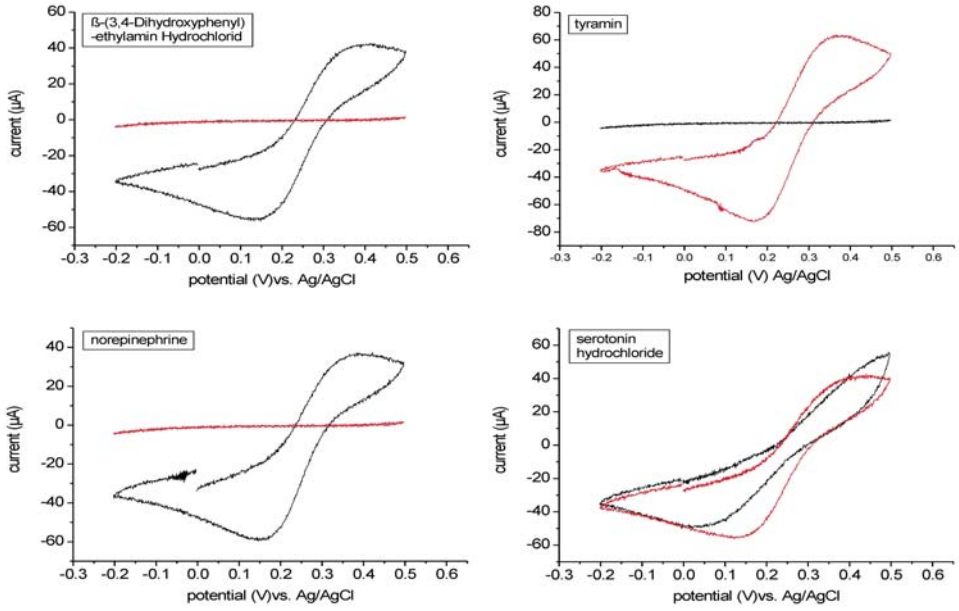
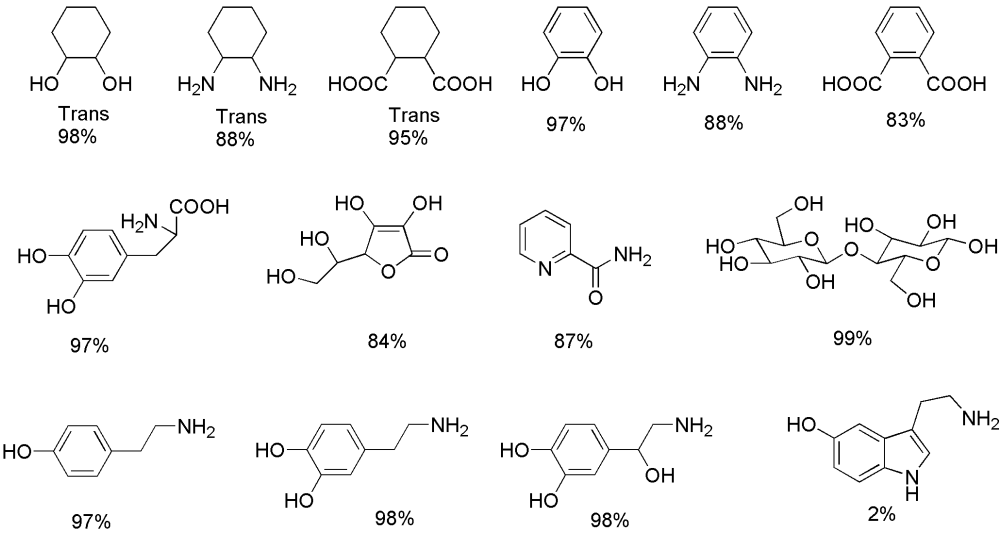


Figure 5.1

The order of blocking power for aqueous 0.1 M solutions of each of the active compounds, which have been discovered so far is as follows: cellobiose > tyrosine > 1,2-trans-dicarboxy-cyclohexane > brenzcatechol > ascorbic acid > 1,2-diamino-benzene > dopamine ~ norepinephrine > tyramine> serotonin (Scheme 1). A large variety of cyclopentane- and open-chain 1,2-diols and –diamines was also tested. None of them produced a significant blocking effect.



Scheme 1: Structural formula and blocking efficiency in percent for the transport of ferricyanide through yoctowells.

A model of the long-lasting blockade of two-nanometer gaps in rigid and fluid monolayers, which are in direct contact with a bulk water volume has to "explain" the following experimental findings:

(i) Cyclohexane-diols, -diamines and –dicarboxylates are only active, if both substituents are neighbouring each other and are in equatorial positions. The diasteromeric cis-compounds and 1,3 regio isomers have no effect. In the case of phenols *para*- and *ortho*-disubstituted compounds are effective, *meta*-isomers are not. Bicyclic, *para* – substituted serotonin is ineffective.

(ii) Cellobiose with equatorial OH-groups is a very potent blocker, and mixture of 1% of maltose removes the blocking effect. All carbohydrates with axial OH-groups are ineffective blockers.

(iii) Formation of a barrier for ferricyanide transport in 0.1 M solutions takes a long time. Two hours were a minimum standing overnight was standard.

5.3 Radioactive measurements of labelled tyrosine with in the yoctowells

With the help of Dr Ludwig and Dr. Li series of experiments with radioactive tyrosine were performed on the gold electrode. First a gold electrode was covered first with *meso*-tetra(phenyl-3,5-dicarboxy) porphyrin (~ 50 %) and then with octadecylthiol. Such a monolayer is fluid, dissolves tyrosine and partly covers the yoctowell with octadecyl-chains of neighbouring molecules. The system is thus similar to Sagiv's early monolayers containing dissolved dyes.[79] It has, however, been shown that such fluid gaps are blocked by tyrosine with the same efficiency observed for rigid yoctowells made of stiff diamido amphiphiles.[106] Since a closed fluid monolayer is much easier to prepare reproducibly and routinely, because eventual roughness on the gold surface are not so critical here, with the help of Dr Ludwig and Dr. Li applied them in a series of experiments with radioactive tyrosine. A control experiment with a rigid monolayer gave similar results for the adsorption of radioactive tyrosine. 0.1 M tyrosine solutions in aqueous NaOH (pH = 10.5 were applied and mixed with commercial radioactive ^{14}C - tyrosine (Amersham, 86 MBq/mg). The kinetics of the tyrosine release (see experimental section) from the loaded electrode surface into the bulk water volume of the supernatant is given in Figure 5.2. About 90 % of the total radioactivity was released within the first four days. Only the remaining 10 % were taken as membrane-integrated tyrosine and its release was studied systematically.

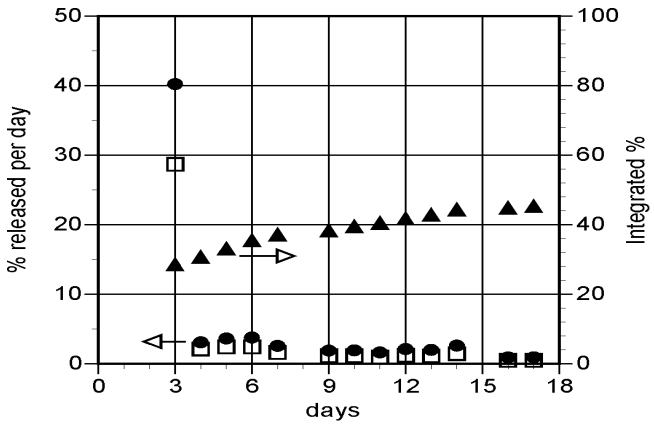


Figure 5.2: Percentage of the tyrosine released from the gold electrode into the bulk aqueous supernatant with time. □ : the total radioactivity of the gold platelet is set to 100 %; • : % of released tyrosine per day, when the remaining tyrosine is set to 100%; ▲ : integrated percentage of released tyrosine.

After two weeks about 30 % of the tightly adsorbed tyrosine had been dissolved away from the electrodes´ surface. Extrapolation to 100 % release yields a period of about 3 years (Figure 5.2), which would be needed to dissolve all the membrane-integrated tyrosine in the supernatant. This time course is similar to that observed in the cyclovoltammetry measurements of ferricyanide currents.

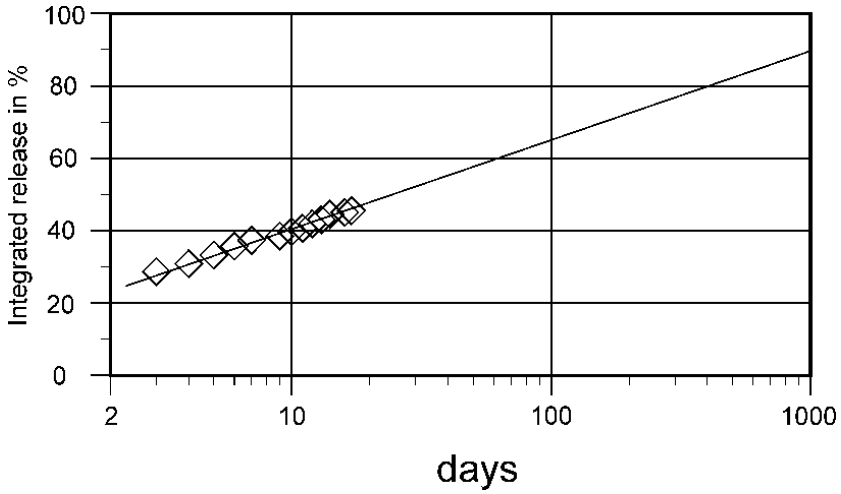


Figure 5.3 Spontaneous release of tyrosine from fluid monolayers containing porphyrin-based 2x2 nm gaps from day 4 to 17.

We then repeated the ferricyanide current and the radioactivity release experiments with the viologen stirrer driven by cyclic voltammetry. No stirring effect on the ferricyanide current was observed for the closed monolayer, although the monolayer without yoctowells also adsorbed and released large amounts of tyrosine (Figure 5.4a). The electrode with the holey surface monolayer showed already some leakage of ferricyanide ions after three days and opened completely within minutes after 20 CV-cycles from +1.0 V to –2.0 V. in the presence of viologen (Figure 5.4b). It is thus evident that the yoctowells allow ferricyanide transport, but not the smaller, irregular gaps in closed fluid membranes. Nevertheless it takes months to release tyrosine from the wells by spontaneous diffusion without stirring. Cyclic voltammetry of ferricyanide showed that the pores had been opened there after.

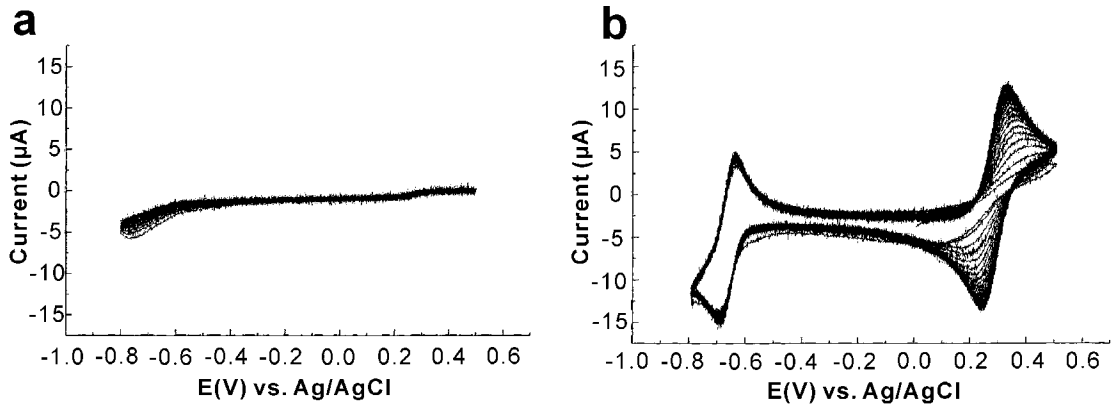


Figure 5.4 a): Cyclic voltammogram (= CV) of ferricyanide using a C_{18}SH-coated gold electrode blocked by 0.1 M tyrosine and a viologen stirrer in solution.[29] No current was observed after 20 CV-cycles. b) An electrode with tyrosine-filled yoctowells with a porphyrin bottom released the tyrosine upon CV – stirring with viologen and the yoctowell was opened for ferricyanide transport after 20 CV – cycles.

We then tried to quantify the amount of tyrosine in the monolayers by radioactivity measurements of labelled tyrosine. Surface concentrations of isotope-labelled adsorbates such as ^{14}C-labelled β-lactoglobulin have previously been determined on monolayers[105] and we used similar techniques for the yoctowells entrapments. The fluid monolayers' release of ^{14}C-tyrosine radioactivity from the fluid monolayers with and without porphyrin gaps and with stirring was studied with freshly-loaded monolayers. It was anticipated that tyrosine molecules which were dissolved in integer C_{18}-monolayers would not be released upon stirring, because the viologen could not enter them. Monolayers with gaps, on the other hand, should release about 50 % of their tyrosine if one assumes that about 50 % of the gold surface is covered by porphyrins. This was checked by measurement of the released radioactivity. At the end of the stirring experiment the gold coating was dissolved by sodium cyanide and the released radioactivity again measured. The number of tyrosine molecules remaining on the gold surface was thus determined. The results are summarized in Table 1. The probe without gaps released a total of 3200 cpm (~ 1.8×10^{15} molecules), the probe with gaps 4400 cpm (~ 2.5×10^{15} molecules). This points to about 25 % more tyrosine in the holey membrane. The ratio of water : membrane dissolved molecules is 6:10 in the C_{18}-monolayer and 8.3:10 in the holey monolayer.

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

	tyrosine radioactivity in supernatant after stirring (CPM)	molecules removed by CV stirring	tyrosine radioactivity on platelet after stirring (CPM)	molecules remaining on platelet
closed $C_{18}SH$-monolayer	1197	$76.8x10^{14}$	1991	$11.3x10^{14}$
$C_{18}SH$ monolayer with nanowells	2018	$11.5x10^{14}$	2415	$13.7x10^{14}$

The area of the monolayer was 2.4 cm^2 or $2.4x10^{14}$ nm^2, the height of the monolayer 2.3 nm. The volume of the monolayers was thus $5.5x10^{14}$ nm^3 and there were $25.2x10^{14}$ tyrosine molecules in the holey monolayer (~5 molecules/nm^3) and $18.1x10^{14}$ tyrosine molecules in the C_{18}-SH monolayer (~ 3.5 molecules/nm^3). This compares to 0.06 molecules/nm^3 in the applied aqueous solution. The holey monolayer thus enriched the tyrosine by a factor of 76, the "closed" monolayer by a factor of 55. A yoctowell with a volume of about 8 nm^3 could contain about 40 molecules of tyrosine, which would be enough to fill up the yoctowells with crystalline tyrosine or cellobiose see below model fig. 5.5.

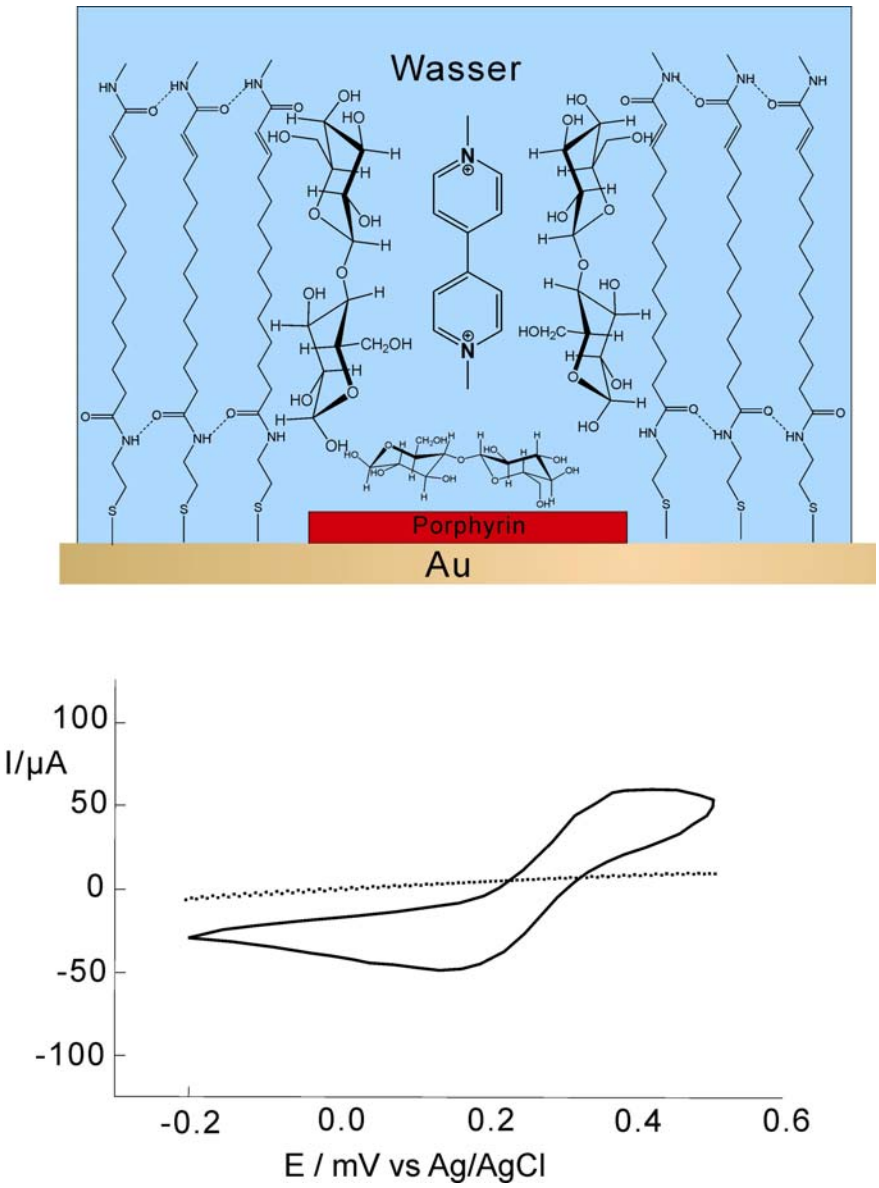


Figure 5.5 Model of cyclic voltammetry stirring with viologen: dark line = shows yoctowell on gold electrode transport of ferricyanide; dotted lines= of ferricyanide using a C_{18}SH-coated gold electrode blocked by 0.1 M cellobiose and a viologen stirrer in solution.[62] No current was observed after 20 CV-cycles.

5.4 Characterization of a D_2O dimer by infrared spectroscopy.

The yoctowells on gold electrodes as described above were also soaked with 0.1 M D_2O-solutions of tyrosine and infrared spectra were measured in the region between 2600 and 2800 cm^{-1}. A strong and narrow D_2O-signal at 2721 cm^{-1} was found (Figure 5.6), which disappeared after 1 h and was found again after a second D_2O-soaking 0.1 M tyrosine. If this wave number is multiplied by √2 a corresponding OH-wave number of 3841 cm^{-1}, results, which is far beyond the normal water valency vibrations in solution (3710 cm^{-1}) or in crystal water (3100-3600 cm^{-1}). Similar D_2O-stretching vibrations were, however, observed in argon and nitrogen matrices for isolated D_2O species.[106] 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.

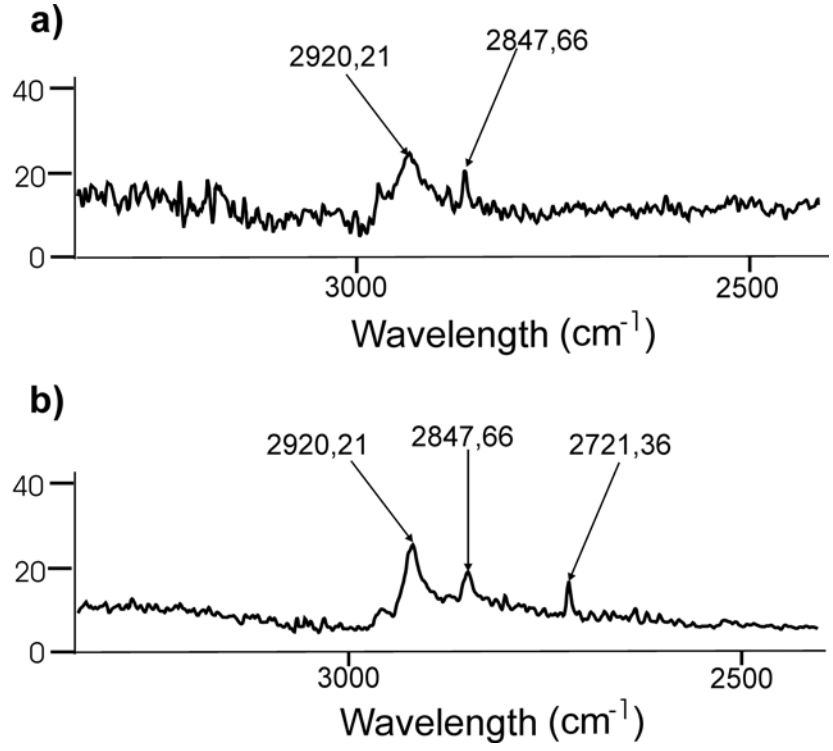


Figure 5.6 IR spectrum of yoctowells (a) before treatment with D_2O-soaking and 0.1 M tyrosine (b) after treatment with D_2O-soaking with 0.1 M tyrosine, visual band at 2721 shows D_2O-Dimers within the yoctowells.

5.5 Solid State ^{13}C- and ^{1}H-NMR Spectroscopy

5.5.1 Solid State NMR measurements.

The data was obtained from the group of Dr G. Buntkowsky by Thomas Emmler, 'Physical Chemistry Department FU- Berlin', I thanks them for allowing me to extract few spectra from a common publication which is in preparation. The details experiments concerning characterization of immobile solutes and entrapped water will be appear in Thomas Emmler thesis also.

All ^{1}H spectra were referenced against TSP (tetramethylsilyle propionic acid, Na salt), all ^{13}C NMR spectra against ^{13}C-glycine as external chemical shift standards. The repetition times of the experiments were chosen in such a way that all spectra were fully relaxed.

To suppress the considerable proton background signal of the probes, all spectra were recorded employing a rotor synchronized Hahn-Echo sequence with delay times between 800us and 3ms. The delay times were chosen as an optimized compromise between the signal decay owing to relaxation and the resolution gain owing to longer delay times. The monolayers on planar surfaces of solid materials are not accessible to NMR spectroscopy. We therefore replaced the electrode by colloidal silica particles. Smooth surfaces with negligible curvature were obtained with amine-coated silica gel particles with a diameter of 100 ± 10 nm. They were prepared from tetraethoxysilane (TEOS) and (3-aminopropyl)-triethoxy-silane.[105,107] 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 *meso*-tetra(phenyl-3-carboxyl) porphyrinate **26b** activated with ethylchloroformate, the rigid wall was made of diamido bolaamphiphile **10** (1.0 nm) as described earlier.[105] The surface of these particles consisted of the OEG-headgroups of the walls and of water-filled yoctowells with a porphyrin bottom. It was found that only about 5 % of the silicagel particle's surface was covered with yoctowells, which is about ten times less than observed for the gold electrode's surface. 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 140ppm was found for the bottom porphyrin units, none of the other membrane component gave signals which were significantly raised above the noise level.

Upon application of 98 % ^{13}C-enriched tyrosine its signals of the aromatic carbon atoms appeared between 100 and 150ppm, and weak signals for the $(O\text{-}CH_2)_m$ and $(CH_2\text{-}CH_2)_n$ carbons at 60 and 1ppm were now also detectable (Figure 5.7). If it is assumed that about 93 % of the silica gel particles are covered by the OEG-bola **10** (surface ratio yoctowells: walls ~ 1:13) and that the alkyl $(CH_2)_{12}$-groups contain about the same the number of carbon atoms as the benzene rings of two tightly packed tyrosine molecules in the well, one obtains approximately a ratio of 14 :1 between the alkyl and aromatic carbon atoms. The tyrosine's ^{13}C-enrichment at a ratio of 100:1 should then lead to a signal intensity ratio of 14:100 or 1:7. The measured signal ratio of the tyrosine signal in the region from 100 to160ppm to that at 17.8ppm in arbitrary units was 16 : 1. The order of magnitude around 10 as obtained by radioactivity measurements is thus also established by solid state ^{13}C-NMR spectroscopy.

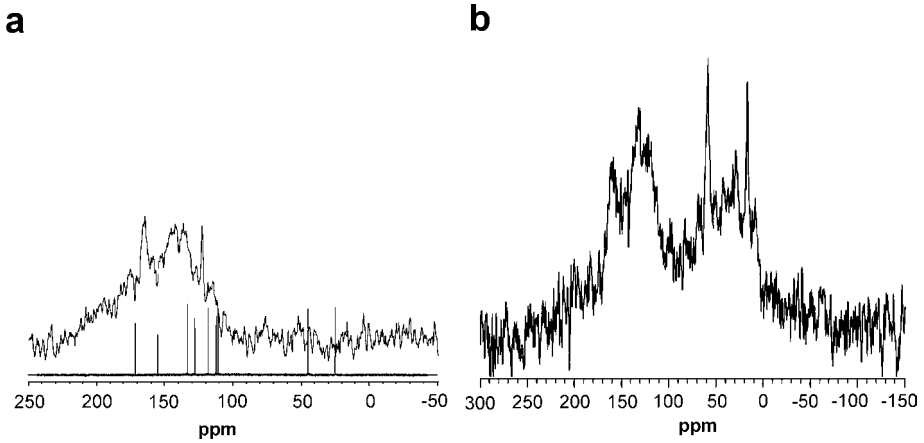


Figure 5.7 Solid state ^{13}C-NMR spectra of the silicate particles containing a monolayer of bola **10** with porphyrin **26b** as a bottom and ^{13}C-tyrosine filled yoctowells. Only the ^{13}C-enriched tyrosine is detected.

For characterization of the adsorbed water within the yoctowells on the silica particles, we applied ^{1}H MAS NMR spectroscopy. Since the sensitivity for protons is much higher, we anticipated information location and dynamic effects of entrapped water. In order to improve the resolution of the spectra we applied a Hahn-echo sequence with very long delay times (between 800µs and 3ms).[108] These echo sequences do unfortunately not allow a comparison of signal intensities, because different proton environments cause different losses of signal intensity. The high rotation speed also raised the temperature of

the sample to 55°C, but control experiments at room temperature did not show any changes of the chemical shifts.

The echo sequence applied in the ^{1}H-MAS-NMR spectra did thus not allow analysis of the monolayer or of tyrosine entrapment. No tyrosine proton signals were detectable. The strongest signals came from the CH_2-groups of the alkyl chain at 1.3ppm. The OCH_2 at 3.9ppm was negligibly small and could be caused, together with the 5.4ppm signal, which may be caused by the methane protons neighbouring the amide group or by adsorbed water (Figure 5.8). The bolaamphiphile consists of several ethylene glycol units, resonating at 3.8ppm, a double bond, which is to be seen at 5.4ppm, and a long –CH_2 chain that can be found in the spectrum at 1.3ppm. The signal arising at 0.9ppm is suspected to be ethanol or ethanolate that is used in the formation process as a solvent and seemingly still is present in the sample. The proton signal of porphyrine is not to be seen here, because its concentration on the sample is too low compared to the protons of the amphiphile used for coating the silica colloids. Even at 24 kHz rotation speed it was not possible to resolve the signals of the tyrosine. The signals cover the area between -5ppm and 15ppm with a very broad signal (originating from the large aromatic system that usually can not be resolved very good) and so the intensity of the signal is very low: This makes the detection of the tyrosine with ^{1}H MAS NMR on the colloid surface impossible.

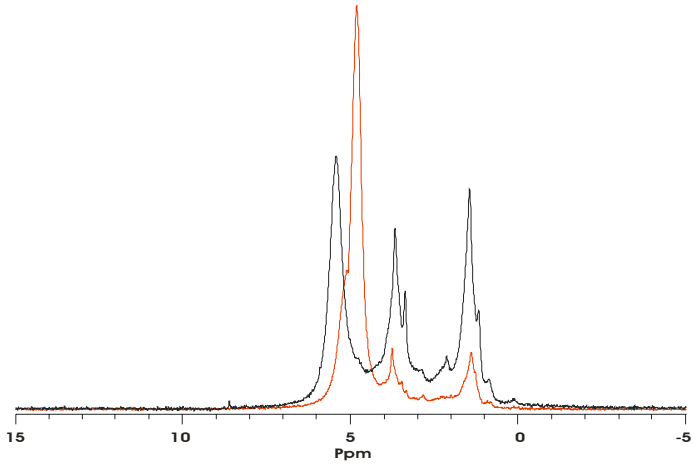


Figure 5.8 Comparison between the ^{1}H MAS NMR spectra (24 kHz rotation) surface coated silica colloids treated with tyrosine/water (black) and cellobiose/water solutions (red). Both samples were freshly prepared and directly measured after preparation. The spectra are scaled to a similar noise level.

The spectrum of the samples treated with the tyrosin/water solution reveals a complex structure of the sample. At 5.5ppm the typical "bulk" phase of water in the sample is to be seen. The large signal at 1.3ppm is the signal of the bolaamphiphile. It can be used as an approximate reference for the quantification of water molecules on the surface. In the case of cellobiose in the nanopores ("yoctowells") only one signal at 5.0ppm was observed for water, which was much stronger than that of the $-CH_2$ groups of the alkyl chains. In case of the tyrosine-filled yoctowells the water signal was much more complicated and considerably shifted to higher fields. This can be interpreted as being consistent with the assumption that all the entrapped water in the yoctowell is essentially hydration water of the solutes. Cellobiose has no effect on the chemical shift and its hydration water produces roughly the same signal as that on the amphiphile's surface.

5.5.2 H/D exchange experiments

Compared to the spectrum of the tyrosine/H_2O treated sample all exchangeable protons are not visible in the ^1H spectrum of the tyrosine/D_2O treated sample. The quality of the H/D exchange can be seen at the signal for the "bulk" water. This signal around 5.5ppm has nearly disappeared and only the signal of the double bond at 5.4ppm is visible (not shown).

The comparison in figure 5.9 reveals the differences between the two spectra. On the one hand all exchangeable protons are not visible anymore in the red spectrum (as mentioned above this is best viewed in the absence of the bulk water signal at 5.5ppm), on the other hand some signals have an enhanced intensity like the signal at 3.0ppm. Upon deuteration of a sample the proton dipolar coupling is disturbed and signals that were not to be seen (because of the broadening that arises from dipolar HH coupling) become visible when e.g. H_2O is replaced by D_2O since the coupling in this case is diminished by a factor of 6.5. This is obviously the case for the signal at 3ppm. That region of the ^1H NMR spectrum can be assumed as a region where some –SiOH groups that are left in or on the silica are to be found. The OEG unit of the amphiphiles is to be seen at 3.8ppm and the small signal for double bond can be found at 5.4ppm. The signal of the $-CH_2$ protons neighboring the double bond is seen at 2.1ppm (according to simulations of the OEG-bolaamphiphile using ACD Labs HNMR Predictor). The signal at 8.6ppm should be the

signal of a –ONH-CO- group. Some signals cannot be associated with the coating of the sample. There are sharp signals at 3.4ppm (normally this signal is attributed to the final –CH_3 group of the PEG chain, but than it should not vanish upon deuteration) and 1.2ppm that do not have anything to do with the coating and vanish upon deuteration. It is clear that these signals belong to exchangeable protons and can be interpreted as different water sites on the sample. The signal at 3.7ppm in the tyrosin/H_2O treated sample experiences a small shift to 3.8ppm in the Tyrosine/D_2O treated sample. This gives rise to the assumption that it is a superposition of at least two signals. This hypothesis is consistent with the width of the foot of the signal at 3.7ppm in the tyrosine/H_2O treated sample, which is very broad and covers the signal at 3.8ppm fully. The next interesting question is which effect additional water in the system has. There are two possibilities: water is distributed randomly over the whole sample or is there a build-up of a water pool that can be measured on a longer time scale (e.g. the trapping of water in the nano holes of the surface coating). By adding a certain amount of water (~10%Vol.) to the samples a possibility was given to investigate the dynamics of the water/SiO_2 colloidal system. The fate of the entrapped water was followed by time dependent measurements. As it is nicely to be seen within the first hour only small changes were observed, after three to four hours the signal at 3.2ppm has drastically decreased and was nearly replaced by the signal at 3.7ppm. This leads to the conclusion, that the water is trapped inside of a special cavity of the colloid coating.

This behavior can be interpreted only when using the results gained by cyclic voltammetry, radioactivity measurements and infrared spectroscopy. It points to a rearrangement of the crystalline tyrosine layers within the yoctowells. The water molecules may be first located on the surface of a monolayer of flat lying tyrosine molecules, which than stand upright on their hydrophilic edge and entrap water dimmers in a sandwich between two tyrosine benzene rings what explains the differences in the chemical shifts.

5.6 Discussion

It was shown, that by using isotopic labeling of the guest molecules of the colloids the presence of (in this case) tyrosine can be detected by ^{13}C VACP MAS NMR, despite its low concentration in the sample. Deuteration experiments revealed the coupling of some components to nearby protons, showing the location of a water site on the coated colloids. Different charges of the colloids revealed slightly different characteristics when they were freshly prepared. These difference however did not affect the special characteristics summarized below. Upon addition of water one very special site was detected that traps the water with a slow kinetic. One can speculate that this points to a rearrangement of the crystalline tyrosine layers within the yoctowells.

These experimental results point to a "nanocrystallization" process on the hydrophobic walls of the yoctowells. The hydrophobic edges of the rigid amphiphiles are first adsorbed by the hydrophobic walls and form a crystalline layer there which is stabilized by cooperative hydrogen bonds and by the fact, that hexagonal ice structures made of water tetrameters fit perfectly onto the hydroxyl patterns made of the OH-groups opposite to the hydrophobic membrane. Bent or conformationally labile edge amphiphiles obviously do not integrate with both, the lipid wall and an immobile water structure. Nanocrystallization takes only place, when the molecules have only one conformation and when the hydrophilic groups form cooperative hydrogen bonds with neighbours as well as with hexagonal water assemblies.

(i) Estimates of tyrosine radioactivity within the gaps point to about 30±20 molecules within each 8 nm^3 yoctowell. This means, that the well could be totally filled with ordered tyrosine crystallites and some hydration water.

(ii) ^{13}C-NMR spectra suggest, that the number of tyrosine molecules within the yoctowells on silica particles is roughly the same as the numbers of methylene carbons of the C_{12} chains of the monolayer.

(iii) ^{1}H-NMR spectra show an upfield shift of ~2ppm for all of the entrapped water molecules with respect to the adsorbed surface water. When cellobiose was entrapped no such shift occurred.

(iv) Infrared spectra point to deuteriumoxide dimers and no larger D_2O clusters in tyrosine – filled nanogaps.

All quantitative experiments led to have been repeated at least for times and gave consistent results within an error margin of less than ±30%. This limit can hardly be improved, because the number of yoctowells on a silica particles cannot be determined exactly. Furthermore the conditions under which "comparisons" of quantities were made had to be selected quite arbitrarily. Radioactivity values were only considered after a time lapse of 3-4 days, when most of the tyrosine had already been washed off. This proceeding is only justified, if one agrees with the assumption, that most of the "quickly released" tyrosine was adsorbed on the surface, whereas only the last 10% or so was entrapped in the yoctowells. We also found it disturbing, that the solid state ^{1}H-NMR spectra did not show any tyrosine and that the OEG-carbons were often missing in the ^{13}C-NMR spectra. Having summed – up all the uncertainties, one should also state, we provide the first NMR data of water entrapped in yoctowells and of nanocrystals, that are smaller by a factor of 100 than printed yoctowells and nanocrystals. reported before and that the obtained data on them are new. all fit: the entrapped water showed very different chemical shifts in the cases of cellobiose and tyrosine filled yoctowells; the time tables of tyrosine release in the radioactivity and water evaporation in the NMR experiments is very similar to that of the opening of the wells in the cyclic voltammetry experiments in water; the fact, that no infrared spectrum of bulk D_2O was found also indicated, that the well is almost completely filled with tyrosine. Crystalline tyrosine (γ=1.3; mw=181) contains 4.3 molecules per cubic nanometer. The yoctowells have a volume of about 8 nm^3 and are filled with about 30±20 molecules of tyrosine and a similar number of water molecules. These densities of molecules are so close to each other, that the assumption of highly hydrated crystallites, which stick as a crust to the walls of the yoctowells, seems inevitable.

From independent experiments with chloroform or ethanol filled yoctowells,[109] we know, that the water structure is not necessary for the formation of the immobile crystallites. The rigidity of the walls is also not necessary as shown earlier and also in the series of radioactivity measurements described above. The rigidity is presumably enforced on to the walls of the yoctowells by the adsorption of the rigid edge amphiphiles. All what is

needed to induce nanocrystallization is summarized in the molecular structures of table 1 and in the molecular narrowness of the monolayer gaps, in the constriction of three dim-tensional mobility, once the molecules have entered the gap. The molecules of table 1 are all water-soluble, but their mobility has become so low, that they cannot travel a few nanometers in a week. A big crystal in a large water volume dissolves within seconds, a nanocrystal in a small water volume, which is in contact with bulk water, does not dissolve within weeks. The surprising insolubility of the nanocrystal is obviously caused by its integration into the walls of the yoctowell. The calculated number of 35 tyrosine molecules per 8 cubicnanometer (nm^3) well brings practically all tyrosine molecules in contact with the walls. It is not necessary to consider specific interactions between tyrosine and porphyrin units, which are known to be strong, because other edge amphiphiles without polarizable π-systems show a similar behaviour under the same conditions (Table 1). We assume only that the a polar edge points to a non-polar wall, namely the porphyrin bottom or the CH_2-chains, and that the polar group is strongly hydrated. We arbitrarily orient the phenol groups perpendicular to the porphyrin bottom, the aromatic ring parallel to the CH_2-walls and the amino acid substituents in an up- and downward direction (Figure 5.12 and model 5.13).

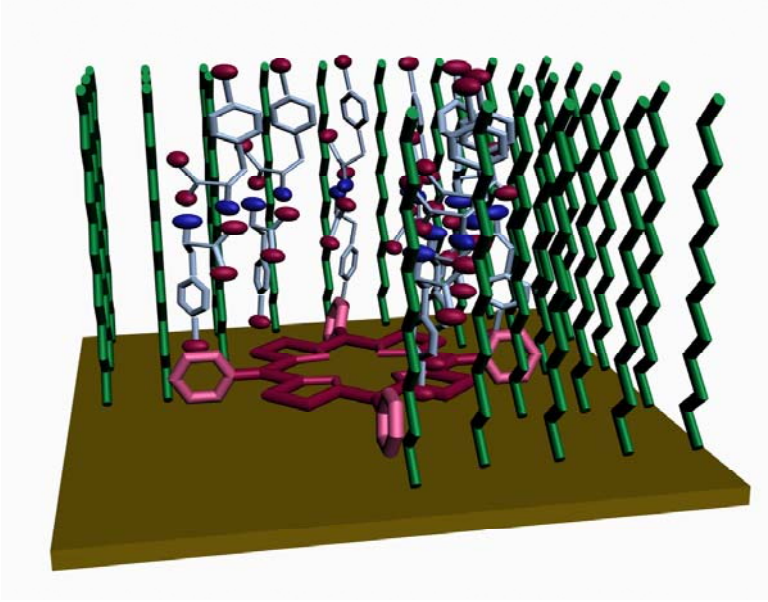


Figure 5.13 Model of the tyrosine crust (blue) in yoctowells (green) with a volume of about eight cubic nanometers.

5.7 Conclusion

Sixteen tyrosine molecules can thus be placed on the walls, which means about half of the molecules in the yoctowell. Since the thickness of the π-system is 4 Å, there remains an inner tube with a diameter of 12 Å or a volume of 3 nm^3 remain. This can take up 12 molecules of tyrosine and some water. We arranged six molecules in each plane in the same orientation as the wall tyrosines with the aromatic rings perpendicular to each other. These 28 molecules correspond nicely with the experimentally determined number of 30. It could also be only 16 or 36 if only the crust was considered or another layer was added to the inside. It is always the rigid crust, which makes the entrapment of tyrosine irreversible, which prevents the dissolution by bulk water. The phenomenon is very similar to that of the irreversible attachment of a second and third porphyrin to the bottom of the well, which has been quantified earlier. From experimental part we find following concepts.

(i) Cyclohexane-diols, -diamines and –dicarboxylates are only active, if both substituents are neighboring each other and are in equatorial positions. The diasteromeric cis-compounds and 1,3 regio isomers have no effect. In the case of phenols *para*- and *ortho*-disubstituted coumpounds are effective.

(ii) Infrared spectra point to deuteriumoxide dimers

(iii) Estimates of tyrosine radioactivity within the gaps point to about 30±20 molecules within each 8 nm^3 yoctowell.

(iv) ^{1}H-NMR spectra show an upfield shift of ~2ppm for all of the entrapped water molecules with respect to the adsorbed surface water.

These experimental results point to a “nanocrystallization” process on the hydrophobic walls of the yoctowells. Nanocrystallization takes only place, when the molecules have only on confirmation and when the hydrophilic groups form cooperative hydrogen bonds with neighbours as well as with hexagonal water assemblies.