The development of yoctowells as a basis for modeling biological systems

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Yoctolitre-sized vessels (1 yL = 10^{-24} L) defined by encompassing porphyrin islands by rigid molecular monolayers of diamido bolaamphiphiles (with different characteristics) on smooth surfaces—the so-called yoctowells—have the ability to store (house) molecules in ways reminiscent of a molecular container. Such containers fill by kinetic or thermodynamic trapping processes allowing in some instances for the sorting and spatial positioning of molecular entities within the container. In this Emerging Area we describe the usefulness and versatility of yoctowells as the basis for modeling biological systems with a view to addressing some of the challenges in chemistry, molecular biology and biochemistry.

1. Introduction

The term 'supramolecular' was first applied by Jean-Marie Lehn in the late 1970's as a way of succinctly describing the chemistry of association, in the broadest sense.[1] Since this time, chemists have attained an astonishing degree of control over molecular interactions and have used the design techniques learnt to synthesise beautiful and intricate functional structures with dimensions on the nanometre scale.[2] This ability to construct nanoscale architectures with precision has provided impetus in the field of nanoscience, where preparation and manipulation of molecular structures occurs on the 1–500 nm scale.[3] The challenge is in the transition between solution and condensed phases and the ramifications for function and ordering.[4] Condensed phase molecular assemblies are, of course, ubiquitous in biological systems, underlying both the wide variety of complex biological structures and functions these systems possess. Understanding the nature of molecular assembly and the associated non-covalent interactions that connect complementary interacting molecular surfaces in biological ensembles is of central concern to structural biology and biochemistry. By way of example, energy conversion and molecular recognition processes usually occur in the non-solution environment of various cell membranes and proteins, in which are embedded the biological ensembles designed to carry out specific functions.[5–7] Photosynthesis, arguably the most important biological process, is one such example. The bacterial photosynthetic reaction center (PRC) formed for long-lived light-induced charge separation shows several membrane proteins, which fixate the functional components—one bacteriochlorophyll molecule (D_L, as a special pair), two bacteriopheophytins and two quinones (Q_A and Q_B)—along an axis through a membrane

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Steven Langford (left) and Sheshanath Bhosale (right)

Steven Langford was born in Sydney, Australia, in 1967. He received his BSc with first class Honours from the University of Sydney in 1990 and PhD under the supervision of Professor M. J. Crossley in 1993 at that same institution. After a successful two years' work with Professor J. F. Stoddart in Birmingham, UK, as a Ramsay Memorial Fellow (1993–1995), he moved to the University of New South Wales to further his interest in electron and energy transfer processes under the mentorship of Professor M. N. Paddon-Row (1996–1998). He was appointed as Lecturer within the School of Chemistry at Monash University, Australia, in 1998 and he currently holds the Chair of Organic Chemistry at that same institution. In 2006 he was awarded a Young Investigator Award by the Society of Porphyrins and Phthalocyanines. His research interests are at the interface between synthetic organic chemistry and biology, applying supramolecular chemistry to mimic photosynthetic processes, molecular devices and studying motor neuron disease.

within a pseudo-C_2 symmetry *i.e.* a 12 yoctolitre volume. These regiospecifically well-defined functional components are the key in the initial stages of photosynthetic reactions in bacteria and green plants (Fig. 1a).[8,9] Of interest to an overall appreciation of the reaction centre is the role of proteins and associated ions and water molecules, which can only be made within models that mimic the specific environment.

Chemists have been successful with the isolation and analysis, but not with the reproduction of the working molecular apparatus, of the PRC, which was devised using long-term evolutionary processes. A small step towards the development of reaction centers of yoctolitre size are membrane gaps, which replace the protein helices.[10] One such approach is highlighted in Fig. 1b. Here the light-induced phenomena are mimicked by a helical channel superstructure comprising core-substituted naphthalene diimides and contained within a lipid bilayer membrane.[11] The photosystem was prepared by self-assembly of four *p*-octiphenyl components each bearing eight amino-core-substituted naphthalene diimides along the rigid-rod scaffold and assembled using hydrophobic and van der Waals interactions. The photosystem ensemble was characterised in vesicles equipped with external electron donors (ethylenediaminetetraacetate, EDTA), internal electron acceptors (1,4-naphthoquinone-2-sulfonate, Q), and HPTS as an internal fluorescent pH meter (Fig. 1b). In this system, transmembrane charge separation in response to the irradiation of the helix is translated into external EDTA oxidation [$E_{1/2}$ (NHE) +430 mV] and internal quinone reduction [$E_{1/2}$ (NHE) −60 mV] for the fluorometric detection of photoactivity as intravesicular deacidification with light was detected intramembrane by utilising HPTS dye. Despite this success, few artificial systems have been specifically designed for the entrapment and ordering of molecules, in particular dyes, within well-defined cavities. One of the first reported examples was of a "pea in a pod" (Fig. 2a) in which continuous rows of C_{60} molecules were aligned within carbon nanotubes as observed by transmission electron microscopy (TEM).[12] In another example, the hydrophilic pore of zeolite L, with a pore diameter of 0.75 nm, took up thousands of hydrated, electron-conducting methyl viologen molecules or energy-transporting oligophenyl derivatives, which were thin enough to enter the tunnel together with solvent molecules. Entrapped diphenyl hexatriene

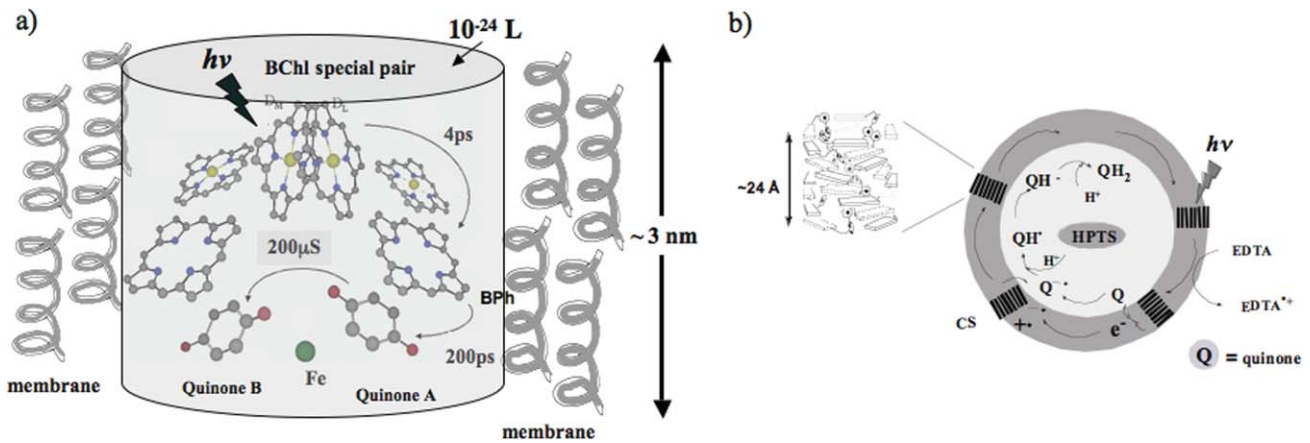

Fig. 1 (a) Schematic of the 12 yoctolitre-sized bacterial photosynthetic center for light-induced charge separation. It consists of a sorted sequence of bacteriochlorophyll derivatives inside the cell membrane and a quinone on its outside. (b) Transmembrane photoinduced electron transfer from EDTA donors to quinone acceptors Q is measured as formal proton pumping with light across lipid bilayers using a rigid-rod π-*M*-helix as a supramolecular photosystem. HPTS (1-hydroxypyrene-3,6,8-trisulfonate) is used to measure intravesicular deacidification with light.

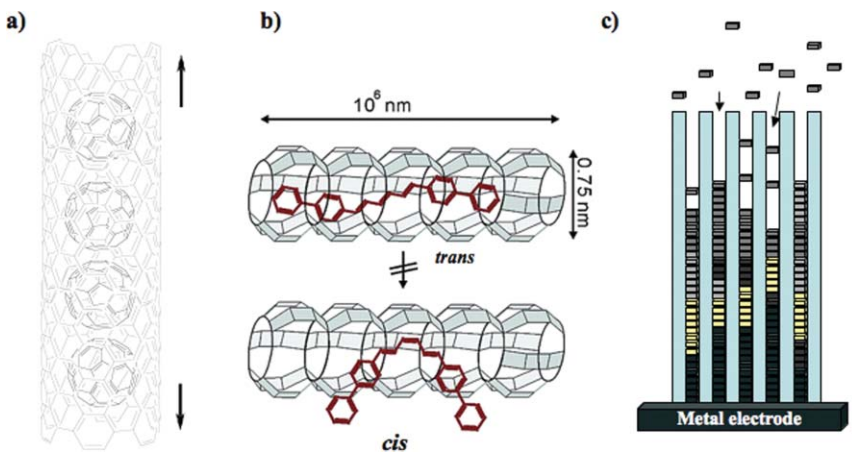

Fig. 2 (a) Schematic representation of the packing arrangement of C_{60} inside carbon nanotubes, like peas in a pod. (b) *trans*-Diphenyl hexatriene in a zeolite L channel. There is not enough space to allow for the formation of the *cis* isomers by light-induced isomerisation. (c) Ordering of dye molecules in zeolite L channels by consecutive addition. Molecules cannot pass each other in the narrow pores, leading to molecular sorting.

fitted only as a *trans* isomer, and upon irradiation did not isomerise to the broader *cis* isomer within the pores (Fig. 2b). This same zeolite, shown schematically in Fig. 2c, showed molecular sorting based on hydrophilic/hydrophobic effects of the ensemble in response to the addition of water. Because dye molecules did not pass each other in the pore, they could be ordered by stepwise addition. However, the number of molecules in each individual domain and intermolecular distances remain unknown (Fig. 2c).[13] Naturally, the entrapment of molecules in nanospaces also leads to unusual properties. Water molecules inside hydrophobic nanotubes, for example, manifest phases of ice that are not found under bulk conditions.[14] Condensation-drying isotherms of water within zeolites bearing hydrophobic and hydrophilic pores showed that hydrophilic surfaces lead to an enhanced density of 1.2 g mL^{-1} of quasi-liquid water layers, whereas hydrophobic pores had no measurable effect at room temperature.[15]

While much has been learnt in confining molecules in molecular capsules and efforts to extrapolate to condensed phases are underway, much is still to be undertaken before pragmatic molecular devices, highly functional sensors and complex biological models can be formed with confidence. Designer yoctowells which may act as tiny chemical reactors or sensors by manipulating the interactions between guest molecules and the walls of the yoctowell gap could become a useful supramolecular tool for studying intermolecular interactions. Such studies could also advance our understanding of phase transitions of dimensionally confined environments, hydrophobic and hydrophilic interactions and the mechanisms of chemical reactions. Gaps filled with biologically active molecules may be used as vehicles for drug delivery, for separation science or sensors in living organisms. Encapsulation of electron spin or optically active compounds have also been proposed as an approach for the assembly of electronic nanodevices suitable for quantum information processing. In this short review, we will give an up-to-date view of the progress in the field of yoctowell chemistry and its implications in biological and supramolecular chemistry, including distance-dependent electron transfer, receptor and host–guest chemistry.

2. Gaps on solid surfaces

Three general methods exist for preparing gaps on surfaces: lithographic (photo and electron beam), embossing, and chemical deposition methods such as self-assembled monolayers or direct surface functionalisation, in which discrimination is possible. Of these methods, lithography is a destructive rather than a constructive technique, which still suffers at the low nm level in terms of spatial resolution and so will not be included in further discussion.[16] Laser-assisted embossing with transparent molds of 100 nm silica spheres has been used to generate hemispherical nanowells in amorphous silicon of zeptolitre (10^{-21} L) volume. The size and depth of the wells were readily manipulated by changing the fluence of the laser and by applying variable pressure. These nano-scale wells, which were as small as 50 nm in diameter, were used as reaction vessels for the growth of inorganic salts and semiconducting nanocrystals with controlled sizes (Fig. 3).[17]

Relevant to the following discussions are Sagiv's studies of gaps in covalently bound molecular monolayers on glass.[18,19] Here, glass plates were coated using trichlorooctadecylsilane (TCODS) punctuated by cyanine dyes with long alkyl chain substituents

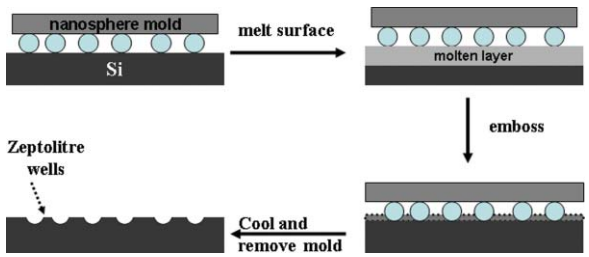

Fig. 3 Scheme for fabricating nanoscale wells in silicon by laser-assisted embossing (1 zeptolitre (zL) = 10^{-21} L).

(Fig. 4). The physisorbed dye was extracted with chloroform after the fixation of the monolayer and then added again in the same solvent. The cyanine dye template was re-adsorbed within 30–60 min. Larger chromophores, also bearing long alkyl chains, were adsorbed into the cavities made by smaller dyes at a slower rate but in equal amounts. The fact that the monolayer "remembered" molecules that were present in its formation period indicated that the glass surface was smooth enough to make the formation of molecular monolayers with empty areas of several square micrometres possible (Fig. 4).[19]

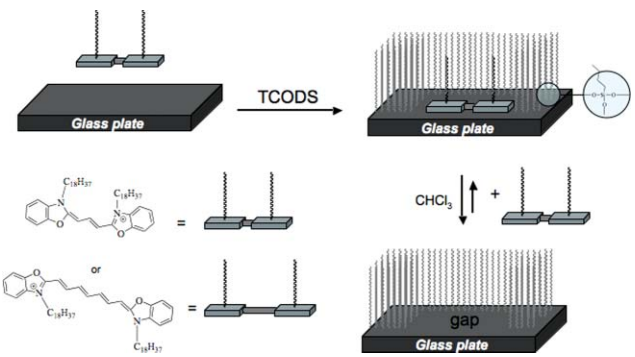

Fig. 4 Functionalisation of glass plates with a silyl chloride, TCODS, in the presence of cyanine dyes leads to alkyl monolayers with dye-filled holes. The gap remains if the dye is simply washed out with $CHCl_3$. Readsorption of the dye into the gaps occurs, but is neither size-selective nor orientation-discriminating.

The ability to bind into the gaps in a cooperative manner was explored a decade ago by the group of Fuhrhop. Their approach was based on developing hydrophobic gaps of steroid dimensions on gold as a means of actively sequestering 1,2-*trans*-cyclohexanediols and glucose from bulk water (Fig. 5).[20] The membrane gaps were produced by chemisorption of thiol-functionalised steroid molecules and subsequent self-assembly of alkyl (hydrophobic) thiols on gold surfaces. We should note that steroids alone had negligible effects. Although it was difficult to quantify the amount of D-glucose or cyclohexanediol that was physisorbed, there was a strong differentiation between 1,2-*cis*- and 1,2-*trans*-cyclohexanediols.

Recently, the group of Forster has prepared interfacial self-assembled monolayers (SAMs) of γ-cyclodextrins functionalised with two aminomethylpyridine groups on platinum electrodes (Fig. 6). Electrochemistry studies revealed that the layers are permeable, a phenomenon restricted when 1-nonanethiol is added within the layer. SERS (surface-enhanced Raman scattering) spectra of the backfilled layers showed both pyridine and alkanethiol

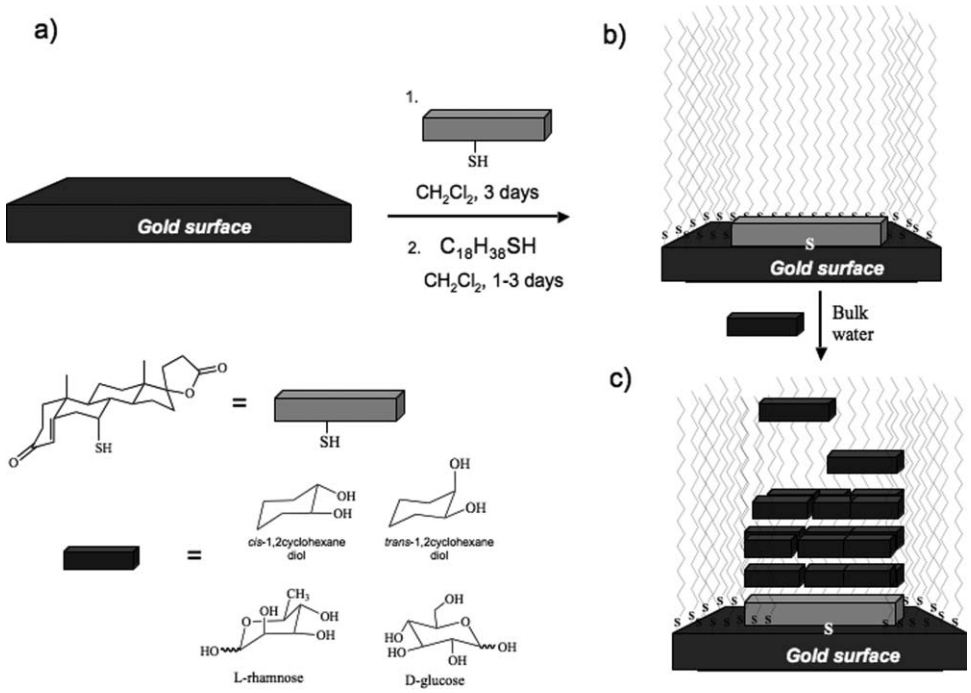

Fig. 5 Schematics showing: (a) a gold electrode surface; (b) a mixed monolayer of octadecanethiol and steroid; and (c) a nanosized hydrophobic gap with flexible walls and a representation of sequestration of D-glucose from the bulk phase into the gaps.

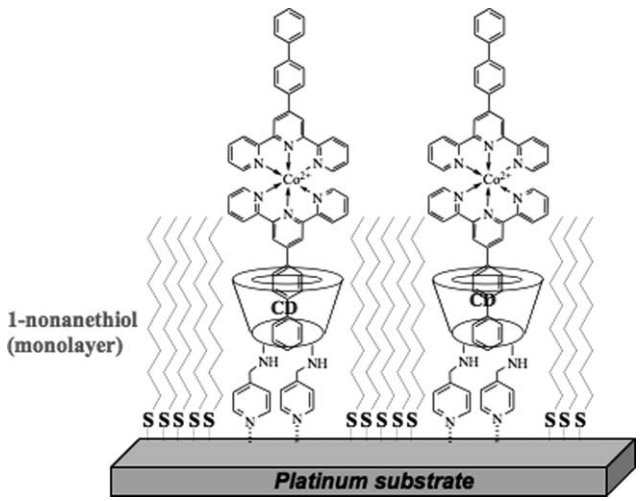

Fig. 6 Schematic illustrating a possible well-like orientation of the γ-CD-$(py)_2$ molecules on the platinum surface after backfilling with 1-nonanethiol.

bands, indicating that backfilling with alkanethiols did not displace the cyclodextrins (CDs). This backfilling appears to dramatically affect the layer structure however, and the electrochemical data obtained is consistent with CDs undergoing reorientation.[21]

3. Further criteria for functional rigid and fluid monolayers

Fluid or rigid monolayers covered with reactive end groups were later applied to yield electron-conducting materials or machinery based on molecular recognition processes. The reactivity and structure of bolaamphiphiles (compounds in which two or more functionalities, which are usually hydrophilic, are connected by a hydrophobic section (the bola))[22] open easy and accessible ways to change the properties of surfaces with a minute amount of material. Fluid and rigid bolas were also applied on gold and silicon electrode surfaces to fixate redox systems and to introduce an insulating monolayer.[23] Colloids, proteins,[24] quinones,[25] fullerenes,[26] and porphyrins[27] have so far been attached and reduced or oxidised from a distance. Examples are shown in Fig. 7.

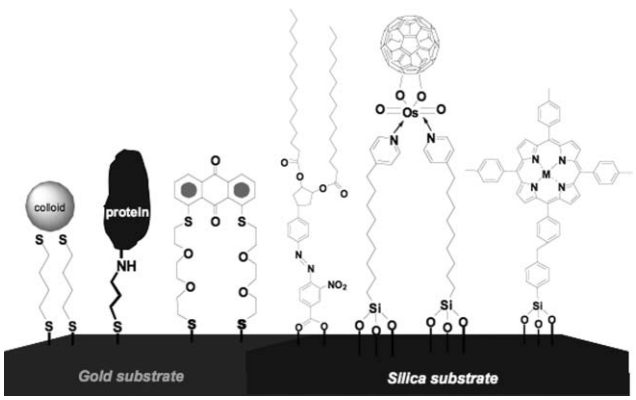

Fig. 7 Fluid and rigid bolas applied on gold and silicon electrodes to fixate redox molecules.

The stiffness (and order) of the bola monolayer can be increased through judicious choice of functionalisation. For example, by incorporating two secondary amide groups at the end of the hydrophobic section of the bola, two parallel running hydrogen-bond motifs act cooperatively to reduce flexibility and conformational changes (Fig. 8).[28] The interactivity and hence stiffness of the monolayer depends strongly on the scant angle

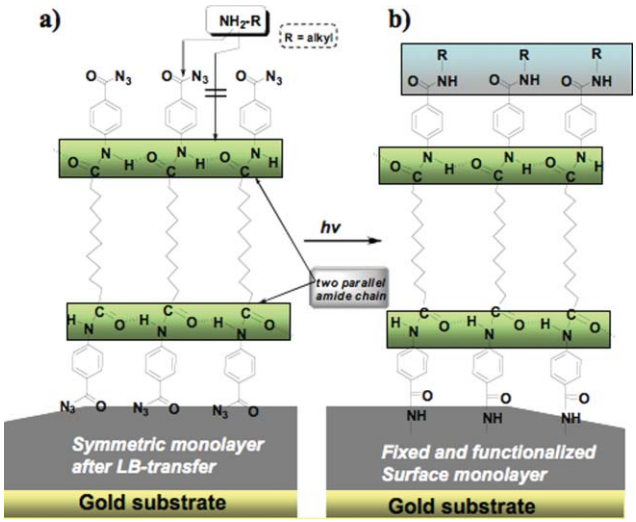

Fig. 8 Schematic of a section of the crystalline monolayer of two *p*-amidobenzoyl azides: (a) before the surface reaction with amine; and (b) after amination and photolinking to the polyacrylonitrile (PAN) surface. In each case, hydrogen bonding provides order and stiffness to the layers.

(*ca.* 20° required) and an even number of methylene groups in between both secondary amide groups—a result demonstrated in other work.[28,29] Upright-standing and odd-numbered diamide bolas form fluid monolayers as a result of geometry constraints. The impermeability for water and other small molecules, such as methylamine, also depends upon an even-numbered spacer between the two amide groups.[29]

4. Construction of yoctowells on gold

meso-Tetraphenylporphyrins bearing four or eight carboxylic acid groups on the four *meso* phenyl rings, *e.g.* **1**, bind strongly to smooth gold at pH 12 (Fig. 9a).[29] The binding of the requisite four carboxylate groups of the porphyrin to gold under the conditions used is faster than the steroid-hydrosulfide shown in Fig. 5, which requires extended exposure times (*i.e.* >8 h). The unwanted aggregation of porphyrins in either a stacking or lateral mode, which would fill up potential yoctowells or lead to domains and large wells of ill-defined diameter, is circumvented by thoroughly washing with potassium hydroxide and water. These newly functionalised gold surfaces, with approximately 20–40% of their surface covered with porphyrins, are then immersed in a solution of mercaptodiamido bola (10^{-3} M) for several hours, completing the surface coverage (Fig. 9).

The monolayer of flat-lying, separated porphyrin monomers on gold produced a residual fluorescence spectrum upon excitation with an argon laser. Fluorescence enhancement by small holes between gold atoms was assumed to explain the small quenching effect of the metal surface.[30,31] Visible spectroscopy proved that the amount of porphyrin on the gold surface had not changed after the second self-assembly of dimido bolaamphiphile. The rigidity of the monolayer (and hence the gap walls) was assured in this case by the introduction of the two intermolecular (but intraensemble) hydrogen bond chains formed by the specific functionalisation of the bola (Fig. 9b) without affecting the porphyrin fluorescence.[29] The yoctowell made up of porphyrin **1** and octadecanethiol walls

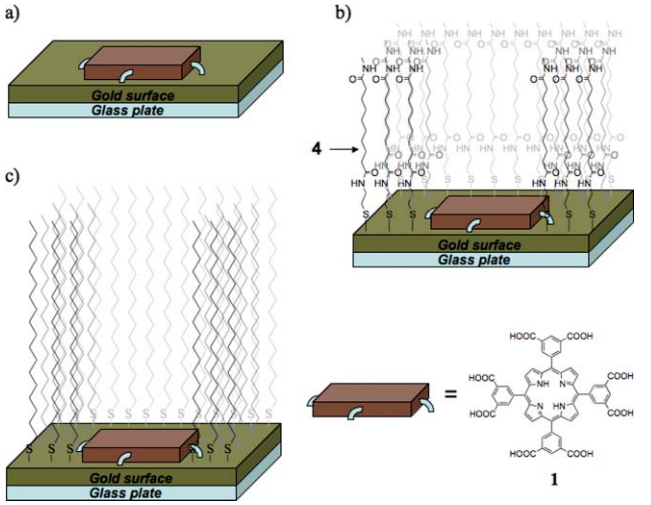

Fig. 9 Schematics of: (a) octacarboxyporphyrin **1** bound to the gold plate (b) a yoctowell made up of basal porphyrin **1** and walls of diimido bolaaphiphile **4**, which form a rigid membrane gap, and (c) fluid gaps made up of porphyrin **1** and octadecanethiol.

(which are unable to hydrogen bond) forms more fluid membrane gaps (Fig. 9c).

The rigid nature of the monolayer formed using the diamido bolaamphiphile allows for size exclusion studies to be performed (Fig. 10). Under conditions of yoctowell formation, porphyrin **1** exists in an anionic state (as the carboxylate). Hence, electrostatic interactions (possible through the introduction of pyridinium porphyrins **2** and **3**) can be employed as a stabilising feature for inclusion into the yoctowell. The difference in overall size between **2** and **3** (porphyrin diameters of 2.4 and 3.3 nm, respectively) and their ability to be included in the gap (of *ca.* 2–3 nm diameter) should differ if the gap's geometry is conserved by hydrogen bonding. Moreover, fluorescence quenching techniques can be used to monitor inclusion because of the redox and photophysical differences between the porphyrin base **1** and the manganese porphyrins **2** and **3**. Ultimately, while fluorescence quenching was observed between **1** and **2** or **3** in the absence of the bola (Fig. 10a), only **2** exhibited quenching when **1** was incorporated in a yoctowell (Fig. 10b–d). In contrast to the gaps in rigid monolayers made from the diamide bola, a gap in a monolayer made of octadecanethiol should have only very limited discriminating power, since the edges of the membrane pores are not defined. Indeed, addition of the large manganese(III) porphyrin **3** led to total fluorescence quenching after few minutes (not shown).[29]

5. Functionalising and filling yoctowells

Two general approaches to functionalising and filling this class of yoctowell can be realised. The first relates to the judicious choice of base porphyrin (*e.g.* **1**) in which its overall dimensions dictate, in part, the potential volume of the yoctowell and its functionalisation, which leads to stability in chemisorption, ability for recognition events through axial ligation (in the case of metalloporphyrins) and in the defined photophysical properties of the yoctowell as an optical readout of inclusion. The second relates to the functionalisation of the walls, which, as we have already

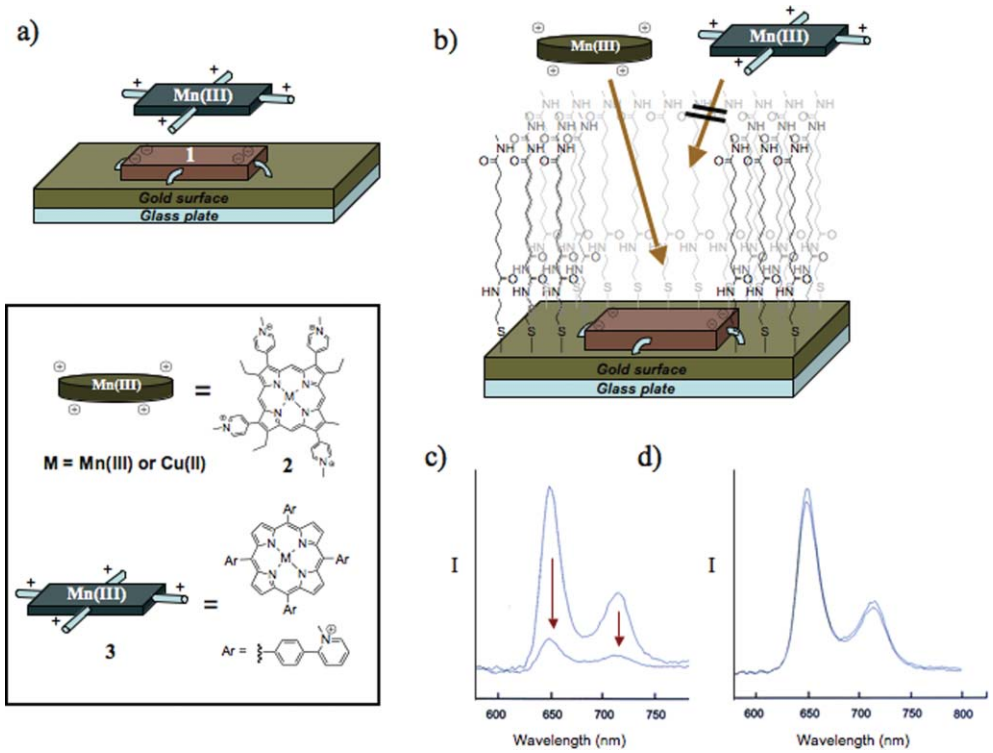

Fig. 10 Schematics of: (a) heterodimerisation of porphyrin **1** (2.4 nm) with manganese porphyrinate **3** (3.3 nm) through non-covalent interactions on a naked gold surface leads to fluorescence quenching; and (b) the yoctowell bearing porphyrin **1** surrounded by rigid walls shows size discrimination. (c) Fluorescence spectra of **1** (in yoctowell) before and after the addition of Mn(III) porphyrin **2** (2.4 nm diameter) show significant quenching. (d) The larger manganese(III) porphyrin **3** (3.3 nm diameter) has no effect on the fluorescence quantum yield of **1**.

discussed, can impart hydrophobic/hydrophilic properties as well as providing rigidity to the monolayers defining the yoctowell geometry. As we will now discuss, further functionalisation of the bolaamphiphiles (*e.g.* **4**) can lead to novel closed structures (Fig. 11a–c).

Long-chain sulfide-functionalised bolas containing carbon–carbon double bonds were self-assembled on gold surfaces around a flat-lying octaanionic porphyrin (Fig. 11a).[29] Rigid and reactive, surface monolayers with 2 nm wide yoctowell gaps were thus obtained (Fig. 11a). Only the double bonds at the border of the gaps were accessible to methylamine dissolved in the bulk water and thus able to react. Experiments using ferricyanide ion as the electrolyte (10^{-4} M) showed electrochemical activity as the ferricyanide entered the yoctowell (Fig. 11c (i)–(iii)). Functionalisation of the bola **5** with methylamine yielded a new yoctowell in which the charge could be manipulated based on pH (Fig. 11b). At pH < 7, the yoctowells contained enough positive charge as a result of generating ammonium groups to electrostatically hold a Mn(III) tetraanionic porphyrin above the surface, inhibiting the inclusion of ferricyanide as indicated by a complete loss of the CV current (Fig. 11b,c (iv)–(v)). The distance between the porphyrins (estimated at 8–10 Å) was such that the inclusion did not lead to a quenching of the base porphyrins' fluorescence. The porphyrin cap could be removed and replaced by adjusting the pH between 12 and 7, leading to the flow and inhibition of ferricyanide ions (Fig. 11c (v)–(vii)).[32] The cover effect only occurred when the gap had been treated with methylamine. The original gaps containing only unreacted double bonds were not blocked by the tetrasulfonated porphyrin.

Similar electrochemical experiments can be performed to monitor the irreversible adsorption of cellobiose, ascorbic acid and tyrosine to the yoctowells shown in Fig. 9.[29,32–34] The results of CV experiments showed: 1) that tyrosine, ascorbic acid, cellobiose, and related molecules block the yoctowell for several days in contact with bulk water so that no electron transport at all takes place from the electrode to ferricyanide in bulk water; 2) that the blocking effect is abolished when 1% of maltose is added to the cellobiose; 3) that the yoctowell, closed by the cellobiose, will re-open in the presence of dimethyl viologen (10^{-3} M) after CV cycling between +0.5 and −0.8 V (against Ag/AgCl).[33] The main interest in the phenomenon of solute fixation comes first from the astonishing non-equilibrium behavior and second from the possibility that similar effects may also be responsible for glycoprotein recognition processes on cell surfaces.

6. Yoctowells on colloidal gold particles

A significant development in the science behind yoctowell inclusion phenomena came in changing from flat gold (electrode) surfaces to colloidal gold particles. This selection allowed the application of most of the chemistry developed on the solid gold electrodes, but the larger surface areas possible allowed photochemical experiments to be conducted. Small, water-soluble particles (diameter <4 nm), such as the Brust gold particles, were found to be ineffective in forming well-organised membrane gaps (Fig. 12a), probably as a result of the high curvature of these particles which does not allow the preparation of wall-like domains around the adsorbed dyes. Experiments with 20 nm citrate gold

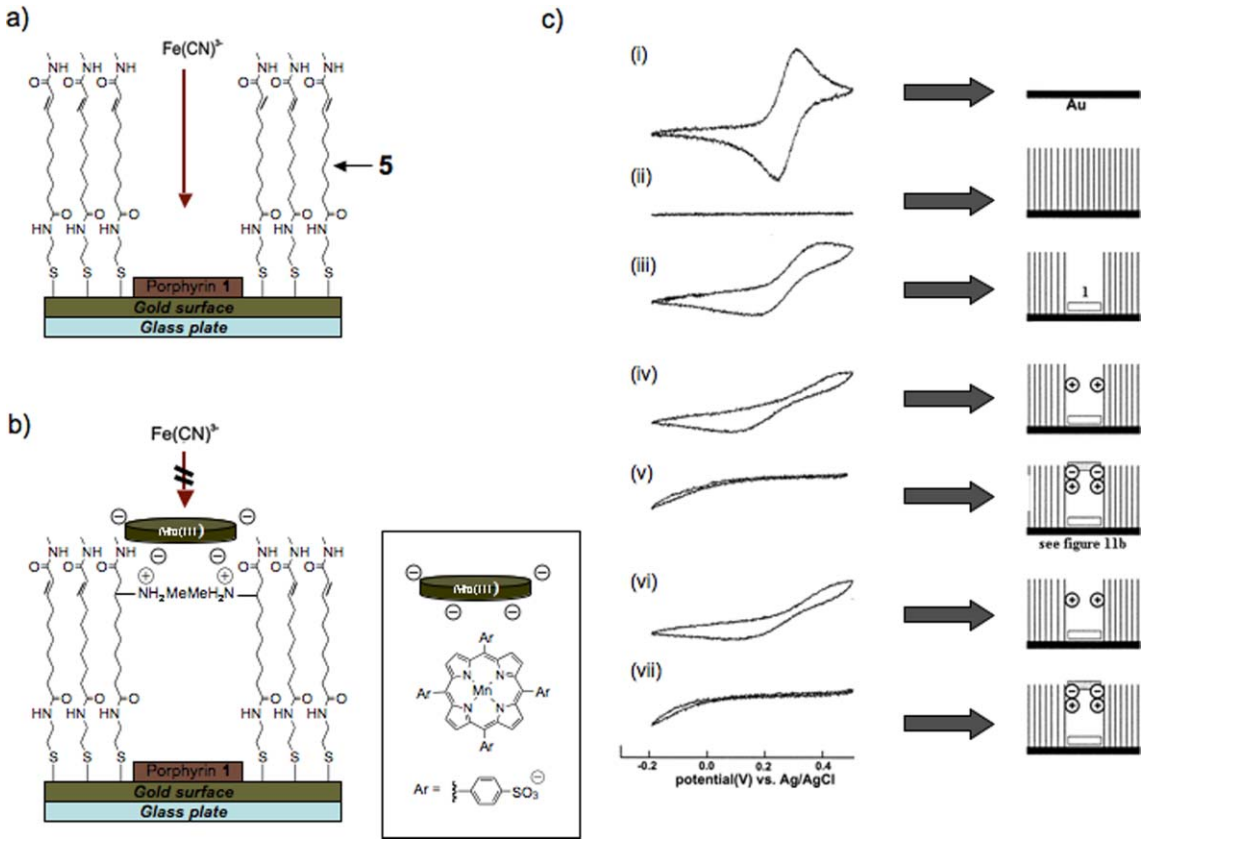

Fig. 11 Schematics of: (a) a functionalised yoctowell allowing ferricyanide to diffuse into the gap, leading to an electrochemical response; and (b) addition of protonated methylammonium groups leading to a closed yoctowell upon the addition of a tetraanionic porphyrin. (c) Cyclic voltammetry curves and pictograms of ferricyanide ions (1 mM) in bulk water depending on the coating of the gold electrode: (i) bare gold, (ii) bola **5**, (iii) bola **5** + porphyrin **1** (approx. 30% coverage) (iv) after methylamination, (v) after addition of tetrasulfonated porphyrin, (vi, vii) removal and placement of porphyrin cover using pH manipulation.

particles proved more successful, resulting in size-selective rigid gaps (Fig. 12b) and more practically, fluorescence quenching experiments could now be performed in aqueous solution using a routine spectrophotometer.[34] Importantly, the tightness and impermeability of the hybrid monolayer could be confirmed by monitoring the effect of cyanide ion on the gold surface,[33] and surface functionalisation did not lead to particle aggregation effects.[35]

7. Developments in yoctowells formed on smooth aminated silica particles and their applications

Despite the significant advances made using gold, applications requiring higher fluorescent quantum yields and different sizes, stabilities, analyses and chemical compositions of the yoctowell components and carriers led to the development of smooth amine-coated silica gel particles[36] as the carrier of choice. Their modestly curved, reactive, and photochemically inactive surface and ability to be prepared under a variety of conditions with different coatings provides a perfect basis for the establishment of closed a monolayer with yoctowells. The main aims in moving in this direction were based on whether the yoctowell could be successfully applied to: (1) flash photolysis experiments in water and organic solvents, (2) enhancing the lifetime of base porphyrin triplet states by a factor of 10, (3) studying 2D diffusion of fluorescence quenching molecules on a variety of surfaces and 1D diffusion within the pores using a standard spectrometer, (4) the analysis of reversible particle aggregation, (5) the establishment of nanometre-sized containers that can be closed and opened by pH changes, and most interestingly, (6) for the establishment of long-distance redox pairs in aqueous medium as well as their advantages with respect to assembled polymer capsules.[37]

Preparation of yoctowells on silica

The silicate particles developed by van Blaaderen, produced by hydrolysis of tetraethoxysilane (TEOS) with aqueous ammonia in ethanol and further stabilised by treatment with 3-aminopropyltriethoxysilicate (Fig. 13a), were used as the carrier.[38] Spherical particles with a diameter between 20 and 150 nm (depending on the concentration and hydrolysis time) can be prepared by this method. The smaller particles showed, however, a rough surface in TEMs which was not appropriate for the self-assembly of rigid membranes and defined nanometre gaps.[29,32–34] The smallest uniform particles with a perfectly smooth surface had a diameter of 100 ± 10 nm. These particles, reproducibly made in 1–10 g quantities and purified by repeated precipitation at pH 2 and centrifugation followed by redissolution at pH 11, could be stored indefinitely as a moist powder.[38]

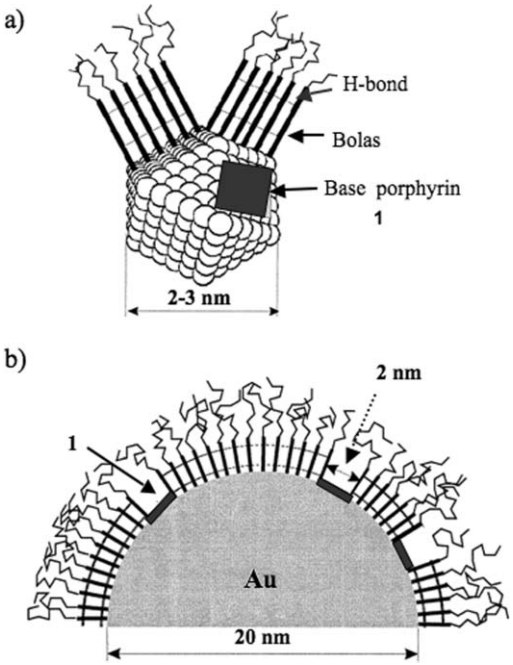

Fig. 12 (a) Schematic of a Brust gold particle (2–3 nm) partially covered with a porphyrin base, which is about the size of a surface plane, and small blocks of isolated hybrid monolayers. (b) Schematic of a citrate gold particle (20 nm) coated with a closed hybrid lipid monolayer and porphyrin-based yoctowells.

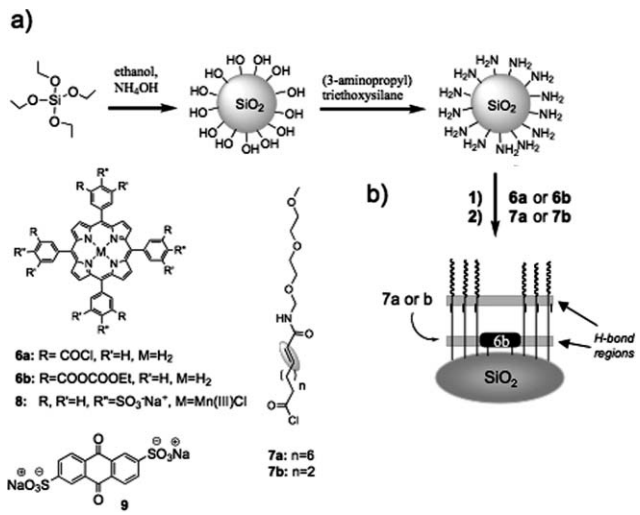

Fig. 13 (a) Preparation of 100 nm smooth aminated silica colloidal particles. (b) Formation of yoctowells on aminated particles by two-step self-assembly of the flat-lying porphyin **6b** and the bolas **7a** or **7b** around it.

The yoctowells were formed using *meso*-(tetra-*m*-benzoyl-chloride)porphyrin **6a** or the mixed tetraanhydride **6b** as the base porphyrin, which stops the aggregation associated with **1** under the conditions used. After centrifugation and redispersion in dichloromethane, bolaamphiphile **7a** or **7b** was added.[38] The more stable mixed anhydrides **6b** made with ethyl chloroformate were much more reliable for yoctowell formation than **6a**, which forms domains on the silicate surface rather than spots of monomeric porphyrins. The results using **6b** clearly indicated that no measurable domain formation of the bottom porphyrin had taken place and that the walls of the gaps were neither fluid nor contained any irregular bends.

Electron transfer within heterodimers

The yoctowells defined by the base porphyrin derived from **6b** and surrounded by bolaamphiphiles derived from **7a** or **7b** walls were functionalised at the double bond with methylamine (Fig. 14). The manganese(III) tetraphenylsulfonate porphyrin **8**, used as a cap in previous studies (Fig. 11), bound at the rim of the gap (Fig. 14a), thus impeding further inclusion of excess **8**, or more importantly quinone **9** (Fig. 14b). Two long-distance (0.5 nm

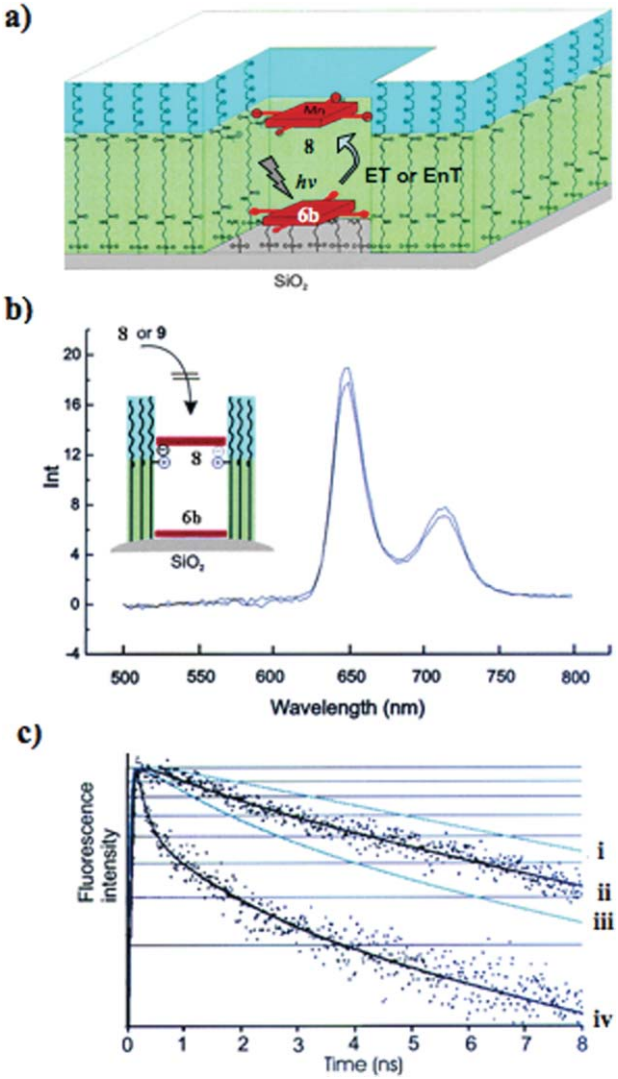

Fig. 14 (a) Schematic of porphyrin **8** covering the yoctowell gap forming a heterodimer within the yoctowell. (b) After functionalisation with methylamine, addition of large excesses of **8** or **9** to the porphyrin-covered yoctowell does not lead to fluorescence quenching of the bottom porphyrin, demonstrating exclusivity. (c) Decay profiles after photoexcitation. Trace (i) shows the decay of **6b** on aminated silicate particles; trace (iii) is the whole system with **6b** and bola **7a**. Traces (ii) and (iv) were obtained after fixation of Mn(III) TPPS at a distance of 10 Å (using bola **7a**) and 5 Å (using bola **7b**), respectively. Only trace (iv) indicates an effect of Mn(III) TPPS, namely 60% decay after 0.16 ns.

and 1.0 nm) redox pairs of **6b** with metalloporphyrin **8** were thus established using **7a** and **7b**, respectively. The behavior of the excited singlet state of the base porphyrin in the absence or presence of Mn(III) TPPS **8** at both distances has been studied (Fig. 14c). The excited singlet state of the base porphyrin was produced with 200 fs pulses at 420 nm, and its decay profile was measured over time. Experiments conducted in the absence of **8** or at the extreme distance (1 nm) in the presence of **8** showed little difference in the decay profile (Fig. 14c, plots (i)–(iii)). With **8** at a distance of 0.5 nm, however, the decay rate increased, leading to a decay time of 0.16 ns (Fig. 14c).[38]

The mechanism of quenching is thought to be energy transfer, which is expected in non-organised, aqueous systems, though more detail is not forthcoming.[39]

Molecular sorting in yoctowells

Silica particles bearing a 4% yoctowell coverage of on the surface and comprising amphiphilic walls of three different heights (6, 10, and 15 Å) have been prepared to investigate solvent and sorting effects.[34,35,38,39] For example, manganese(III) tetra(2-methylpyridinium)porphyrinate **10**, with an external diameter of 3.6 Å, doesn't exhibit quenching phenomena in chloroform–ethanol solution (5 : 1 v/v) because this diameter is larger than the diagonal of the yoctowell (2.4 nm) (for similar results on gold see Fig. 10b). Titrations with size-complementary porphyrins **10a**, **12** or **12a** (in water) or **12b** (in chloroform) on the other hand, caused time-dependent quenching of the base porphyrin's fluorescence (Fig. 15b). The half-life of this quenching process was extremely long ($\sim 10^3$ s), indicating the stability of the well-filled ensemble in water, ethanol, and chloroform solutions. The observed slowness of diffusion (and hence the quenching effect) is presumably dominated by interactions of the porphyrin and solvent with amphiphilic walls, the former finally sticking on the walls by van der Waals interactions. Desorption is exceedingly slow, because of desolvation about the polyether portion of the bolamphiphile (Fig. 15a).[39]

An advantage of the observed slow, and somewhat irreversible, adsorption process is the ability to develop a procedure for the "sorting" of porphyrin stacks within the yoctowells (Fig. 15c)

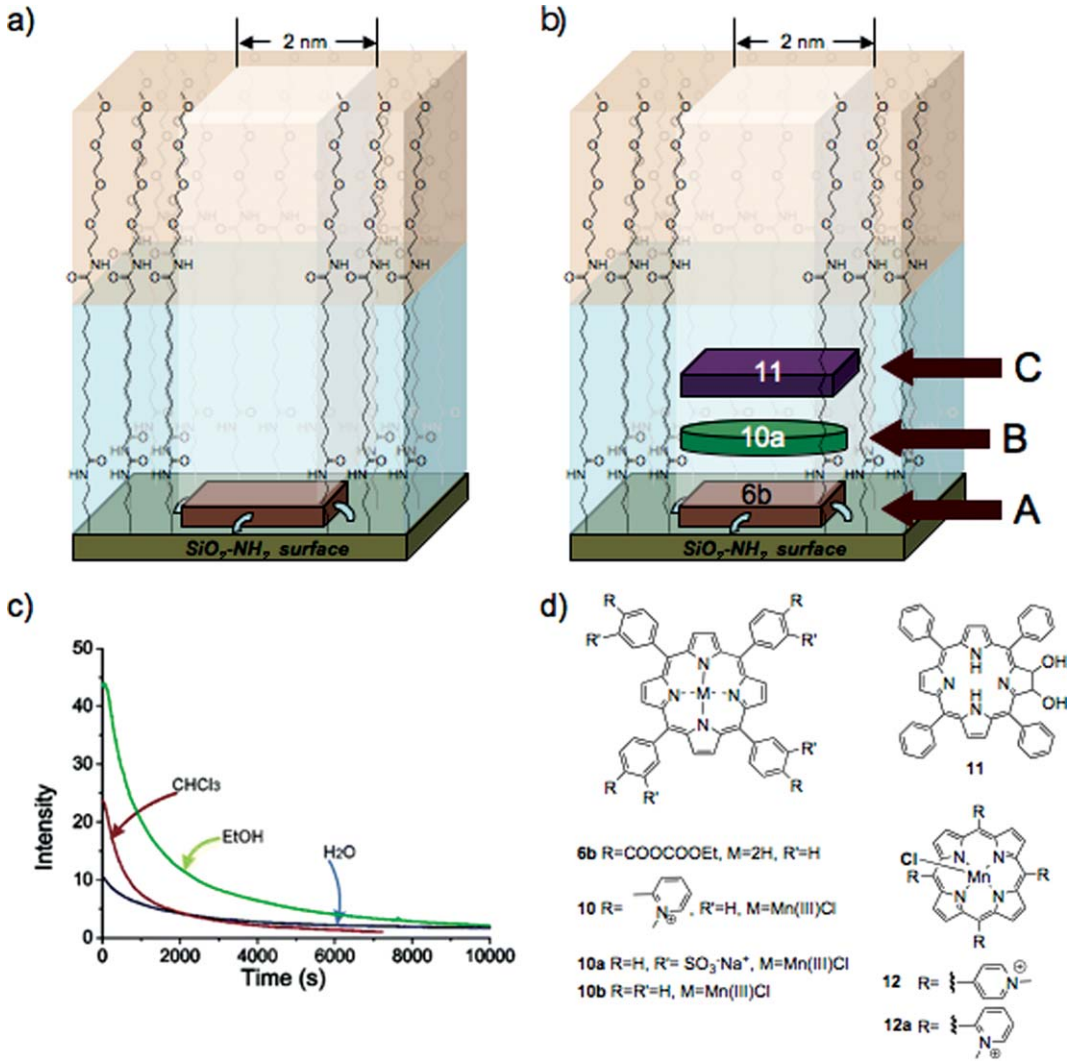

Fig. 15 (a) Schematic of the yoctowell showing hydrophilic (blue) and hydrophobic (cream) sections of the bola, (b) Schematic of the stacking of the three porphyrins **6b**, **10b**, and **11** (A, B, and C, respectively) in a 2 nm yoctowell. (b) Quenching of the base porphyrin's fluorescence by the addition of **10a** and **12** applied in water or ethanol, and **10b** applied in chloroform. (d) Structures of the porphyrins used in these studies.

in a desired fashion, *e.g.* **A**··**B**··**C** or **A**··**C**··**B**. For example, the chloroform-soluble *meso*-tetraphenyl-2,3-dihydroxychlorin (**11**, designated **B**) was deposited within the wells already containing the base porphyrin (**6b**, designated **A**) (**A**). After filtering the silica particles through a 100 nm steel frit in combination with a 200 nm pore filter, manganese(III) porphyrinate (**10a**, designated **C**) was then added, and the particles centrifuged. All three components were then detectable by UV/vis spectroscopy in the expected ratio of 1 : 1 : 1, or by electrochemical methods. The procedure was also inverted: first manganese porphyrinate **10a** (desigated **B**), and then chlorin **11** (designated **C**) were added as shown in Fig. 15b.[39,40] This is, to the best of our knowledge, the only method to produce isolated and defined stacks of three or four different molecules at a defined distance in linear order, and opens the possibility for the introduction of all sorts of redox-active or -inactive groups between the porphyrin–porphyrin–chlorin or chlorin–porphyrin–chlorin heterotrimers.

Characterisation of immobile solutes by NMR and IR techniques

One of the most interesting properties exhibited by the yoctowells on silica or gold is their ability to entrap specific solutes from dilute (0.1 M) aqueous solutions.[33,34,38] A range of techniques are open to use, including ^{14}C-radioactivity and solid-state ^{13}C-NMR spectroscopy to determine the extent of capture (and subsequent release), and IR, ^{1}H NMR and ^{2}H NMR techniques, which also yield information on the types of water environments possible as a result of anisotropic effects of the base porphyrin. As an example of capture and release, a 0.1 M tyrosine solution prepared in aqueous NaOH (pH = 10.5) and mixed with commercial radioactive ^{14}C-tyrosine (Amersham, 86 MBq mg^{-1}) leads to the rapid uptake of radiolabeled tyrosine into the yoctowell. The ensemble is believed to be stabilised by hydrophobic interactions between the tyrosines and the yoctowell walls, as well as intramolecularly. Further stabilisation is gained by curious water dimers which are also immobilised within the well (Fig. 16a). The immobility of these interconnected molecules is the assumed reason for the formation and entrapment of the nanocrystallites. The nanocrystal model, which requires specific conformational constraints, agrees with the estimated 35 ± 10 molecules within each 8 nm^3 yoctowell. The kinetics of the tyrosine release from the loaded surface into the bulk water volume was then measured (Fig. 16b). About 90% of the total radioactivity was released from the surface within three days, the remaining 10% being retained as entrapped tyrosine. The release of this final 10% was followed further. Extrapolation of the results indicated complete release after a period of about 3 years![41] Similar experiments with 0.1 M solutions of ^{12}C-tyrosine in D_2O could be monitored by IR spectroscopy. In particular, a strong, narrow and diagnostic D_2O signal at 2721 cm^{-1} was found, indicative of the presence of D_2O-dimers within the yoctowell.[42,43] Displacement of the included water and tyrosine molecules was shown to be possible by the addition of viologen through a molecular stirring process.

Replacing the bola with glycol and peptide walls

The hydrophobic yoctowells act, in general, as size-selective kinetic traps for various solutes in water and provide a unique means to study water-soluble molecules in confined systems. But what about the reverse case, that is yoctowells containing hydrophilic walls? One such example replaces the hydrophobic oligomethylene walls with functional walls of tetraethyleneglycol (TEG, **13**; Fig. 17a), which resembles a three-dimensional crown ether,[38,41,44] or diglycinyl triamide (**14**; Fig. 17c).[44] The form and stability of the new wells was demonstrated again by size-selective fluorescence quenching experiments using porphyrins **8** and **12**, porphyrin **8** being too large to fit in the yoctowell.

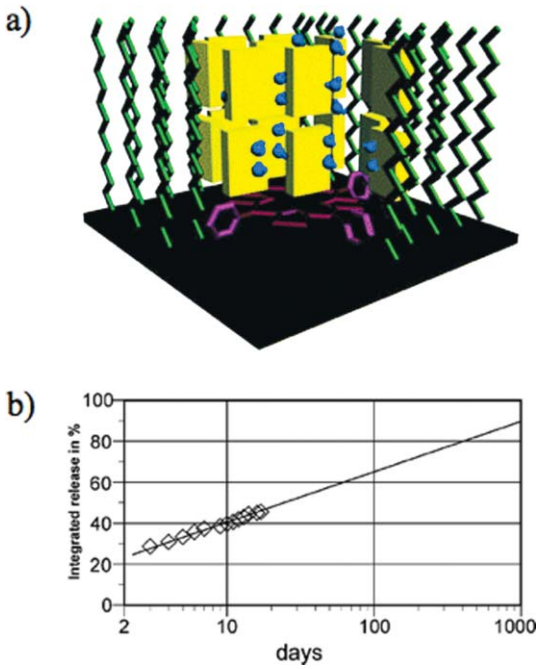

Fig. 16 (a) Schematic illustrating the organisation of 20 tyrosine molecules (represented by yellow blocks) onto the yoctowell walls and 16 more within the *ca.* 8 yL volume. Water dimers (shown in blue) which orientate at the amino acid end are also given. (b) Release of tyrosine from yoctowells from day 4 to 17.

The new hydrophilic TEG yoctowells (Fig. 17a), bearing a similarity to crown ethers, tightly bind oligoamines (*e.g.* spermine, polylysine, and the rigid tricyclic tetraamine tobramycin) at around physiological pH (Fig. 17b). Titration of the filled yoctowell with naphthoquinone 2-sulfonate **15** failed to displace the oligoamines, suggesting binding constants in the order of $>10^3$ M^{-1} in water or ethanol. This differed substantially from the hydrophobic yoctowells containing only flexible TEG headgroups on the outer surface (*e.g.* **7a**, **7b**); neither spermine, polylysine nor tobramycin had any measurable blocking effect in water at the concentrations which were used with the hydrophilic well (**13**).[44]

The use of diglycinyl triamide bola **14** allowed the testing of amide hydrogen bonding as a stabilising feature of yoctowell inclusion. Addition of the triglycine derivative of fluorescein **16** as a guest molecule (Fig. 17c and d) displaced the naphthoquinone **15**, restoring the base porphyrin fluorescence, leading to concomitant fluorescence quenching of the fluorescein moiety. This arrangement allowed, for the first time, counting of the number of entrapped molecules in each well by fluorescence comparisons of the base porphyrin and the guest **16**. Comparisons consistently pointed to a fluorescein:porphyrin molecular ratio of 2 : 1 and a binding constant $K > 10^{13}$ M^{-2} for inclusion into the well.[44]

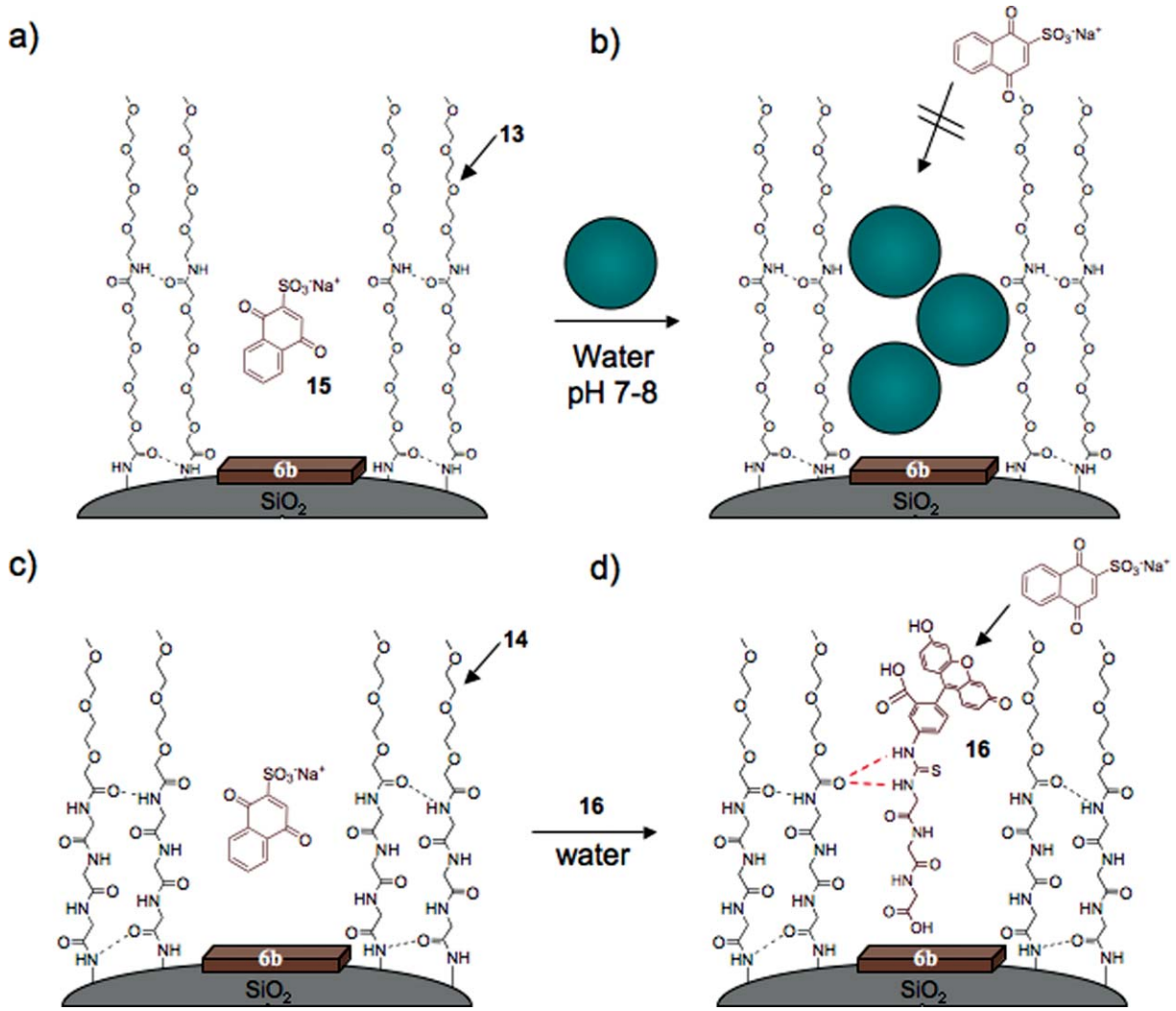

Fig. 17 Schematics showing: (a) a yoctowell made up of TEG bolas **13** and including a fluorophore **15** that induces quenching of the base porphyrin's fluorescence; (b) blocking of **15** by spermin, polylysine and tobramycin (all symbolised by the green sphere); (c) a yoctowell made up of diglycinyl triamide bola **14**; (d) that binding of triglycine derivative **16** of fluorescein as a guest molecule impedes base porphyrin quenching, but leads to fluorescein quenching.

8. Conclusions and perspectives

Yoctowells bearing a variety of side walls were precisely constructed through two-step self-assembly of a porphyrin and a diamido bolaamphiphile on gold plates, gold particles and aminated silica particles. The nature of the gaps and their size-exclusivity properties as confirmed by fluorescence quenching experiments indicate their well-defined nature, the propensity for structure–function relationships, and indeed the dimensions of the gaps consistent with a volume of a few yoctolitres (1 yL = 10^{-24} L).

The most characteristic property lies in the inclusion phenomena they exhibit—from the formation of well-filling "nanocrystals" in dilute (10^{-1} M) aqueous solutions such as cellobiose or tyrosine to the molecular sorting exhibited by three different components. Yoctowells are easy to prepare and can have hydrophobic as well as hydrophilic side walls, providing simple models of biological systems. Some significant experimental opportunities have thus been established with yoctowells, which are not accessible with the zeolite pores and dye-loaded multilayers mentioned in the introduction, with vesicular and micellar[45] assemblies or with printed attolitre containers.[46] The apparently non-swelling walls of the yoctowells allow for dissolution of molecules exclusively in the entrapped water, ethanol, or chloroform volumes. The use of porphyrins as the base by which the yoctowell is templated allows both electrochemical and photophysical handles with which to study inclusion phenomena. Modification of the wall properties (hydrophobic to hydrophilic *etc.*) within the yoctowells with oligomethylene or triglycinyl yoctowells allow new phenomena with biological implications or biological mimicry to be investigated. For example, the phenomenon of "irreversible kinetic trapping" of amphiphiles within the hydrophobic yoctowells may help to provide an understanding of the detachment of steroidal hormones from the carrier systems in blood by glycoproteins on membrane surfaces. Future directions for these yoctowell models may be related to the recognition process between steroid hormones and branched oligoglycosides on the surface of biological membranes, and further monomolecular distance-dependent electron transfer may be achievable. Whatever the approach, it is

clear that yoctowells like the ones described herein provide new opportunities in biology, materials science and medicine.

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