Yoctowells as a simple model system for the encapsulation and controlled release of bioactive molecules

Sheshanath V. Bhosale & Sidhanath V. Bhosale

School of Applied Sciences, RMIT University, GPO Box 2476, Melbourne, VIC-3001, Australia, Polymers and Functional Material Division, Indian Institute of Chemical Technology, Hyderabad-500607, Andhra Pradesh, India.

The development of nanosized drug delivery systems to transport drugs to target cells, are promising tools to improve the drug therapeutic index. Transport systems should have a simple design to control the release of loaded drug to the target areas, thereby increasing concentration and prolonging retention. Herein, we demonstrate the use of yoctoliter wells (1 yL = 10^{-24} L) as simple model systems for the encapsulation and release of biologically active molecules, by manipulating pH. The drug molecule employed here is doxorubicin, which diffuses into the bottom of yoctowells from a bulk solution at pH 7. Capping of the yoctowells is achieved by addition of an anionic-porphyrin by electrostatic interaction. Furthermore, controlled release of the Doxorubicin and capping agent from the yoctowells is achieved by pH control. The effectiveness of the sustain release of the bioactive molecule from yoctowells, provides potential for development of a new generation of drug-delivery system for practical application.

Drug delivery systems which medically-transport active molecules, in a controlled manner, to diseased cells, have gained much attention in the past decade. Various drug delivery systems have been employed such as polymers, dendrimers, micelles, vesicles, nanoparticles and vesicular supported particles. However, although drug delivery systems have their own advantages, they also possess limitations, such as poor thermal and chemical elimination, difficulties in controlling targeting and the well-organised release of the drug, as well as rapid elimination by the immune system. Ideally, the transport system should be designed to control the release of loaded drug to the target areas, thereby increasing its local concentration, bioavailability and prolonging its retention. In recent years, silica nanoparticles have generated a significant amount of interest because of their inherent properties as biocompatible alternatives. Several sophisticated drug delivery systems have been developed and employed using mesoporous silica nanoparticles. These controlled drug release systems are designed to react to cells with internal stimuli, such as pH, or specific enzymes, or to external stimuli such as light, redox properties or temperature. Dual-release mechanisms triggered by pH and reduction have also been proposed.

Supramolecular self-assembly and self-organisation plays an important role in nanotechnology, biotechnology and in regenerative medicine. The self-assembly is driven by non-covalent and reversible interactions, such as hydrophobic, electrostatic, van der Waals, metal-ligand interactions, hydrogen bonds and π-π stacking, with a wide range of possible applications. Yoctowells represent a self-assembled porous material in which a useful volume of the pore is in the yoctolitre scale (10^{-24} L). Yoctowells are generally prepared using a porphyrin template as a base, which allows for both electrochemical and photophysical handles with which to study inclusion and release phenomena. Yoctowells with various kinds of walls can be precisely constructed through a range of techniques depending upon the walls of the wells employed. The integrity of yoctowells can be confirmed by size-exclusion fluorescence quenching experiments such that, large molecules over 2.5 nm in diameter cannot enter into the gaps whereas molecules with size commensurate with that of the gap are able to enter and quench the fluorescence of the base porphyrin, whose role is both as a structural element and as a fluorescence reporter. The chemical versatility offered by the fabrication technique means that the walls of yoctowells can be designed with different phobicities which can provide simple models for biological systems. Yoctowells with hydrophobic, hydrophilic and peptidic side walls are easy to prepare and characterize. The non-swelling behaviour of the walls of the yoctowell allows for dissolution of molecules in a range of solvents. Modification of the walls has also enabled inclusion phenomena, typically with high association constants. The most characteristic property of yoctowells is their ability to induce the formation of well-filling “nanocrystals” in dilute (10^{-14} M) aqueous
solutions using cyclic and rigid edge amphiphiles and to allow molecular sorting. Rigid yoctowells have outstanding abilities to selectively bind small guests and provide a useful platform for the construction of various interesting supramolecular systems. In particular, yoctowells can be functionalized with molecular as well as supramolecular stimuli in order to control the encapsulation and release of bioactive molecules. 'Designer' yoctowells may act as tiny chemical reactors or alternative in-vivo drug-delivery systems, and manipulation of the interactions between reactant molecules and the walls of yoctowell gaps and/or the base porphyrin, provide a useful supramolecular tool for real world application.

Results
Herein, we demonstrate the use of yoctoliter wells (1 yL = 10^-24 L) as simple model systems for the encapsulation and release of bio-logically active molecules (Fig. 1), facilitated by manipulating pH, in which doxorubicin (DOX) and Mn(III)TPPS are loaded on the yoctowells.

Initially, DOX diffuses into the wells and is stabilized by hydrophobic and van der Waals interactions. This is followed by capping of the wells by 3, which is held in place by electrostatic interaction. Upon confirmation of the presence of the molecules within the wells, release of both molecules from the wells was achieved by altering the pH of the solution. At lower pH (4.5) only 3 were released, whereas at higher pH (7.2–9.4), slow release of 2 was observed. Thus both molecules were released from the wells by manipulating naturally occurring stimuli in vivo, i.e. pH.

Of prime importance to all of the cases described herein, is the use of porphyrin based wells, which possess useful optical properties (emission and absorption), and which can be used to monitor the encapsulation and release processes.

Figure 1 | Schematic design of the construction and release of bioactive molecules from the porphyrin based yoctowells. A new efficient strategy of a porphyrin based yoctowell as a receptor for the formation of inclusion complexes of doxorubicin and anionic porphyrin molecules via hydrophobic, van der Waals interactions and electrostatic interactions respectively, followed by release of both the molecules (DOX and Mn(III)TPPS) from the yoctowells by manipulating naturally occurring stimuli in vivo i.e. pH.

Figure 2 | Schematic illustration of the release process by pH stimuli. First, the capping molecule is released by lowering the pH (4.5), followed by release of the bioactive molecule at pH 7.8 (above). Compounds employed in this study (below).
Construction of the yoctowell system. Yoctowells were constructed by a two-step self-assembly procedure on smooth, aminated silica particles. Firstly the flat-lying porphyrins were attached, followed by reaction of the diazide bolaamphiphile 4 around the porphyrin islands 1, leading to small wells of yoctoliter (10⁻²⁴ L) size (see Supplementary Fig. S1). The colloidal carrier of choice consists of smooth amine-coated silica gel particles with a diameter of 100 nm with no pores, such as those introduced by van Blaaderen et al.¹. Their modestly curved, reactive, and photochemically inactive surface provides a perfect basis for the establishment of a closed monolayer⁵. The absorption and fluorescence spectra of porphyrin 1 bound to the well surface, hardly changes after the two step self-assembly of bolaamphiphiles around the porphyrin, in water or organic solvents²⁶,²⁹. Discriminative fluorescence spectroscopy showed that the yoctowell gaps formed are rigid and that the gaps are of the same size as the porphyrin (~2.2 nm), (see Supplementary Fig. S2). The aminated silica particles covered with porphyrin 1 and surrounded by bolaamphiphile 4 walls, were then functionalized at the bolaamphiphile double bond by the Michael addition of methyamine. A positive ring of methyloxolaniumium groups being formed directly below the triethylene glycol chain (TEG) headgroups⁴⁴.

Concept and design. The use of porphyrins as base for the yoctowell template, allows the introduction of both electrochemical and photophysical handles through which the study of inclusion and release phenomena can be pursued. DOX 2, and Mn(III)TPPS 3 molecules were chosen to monitor the independent release processes due to their unique absorption and emission properties, and their presence and concentration can be judged by spectroscopy (UV-vis absorption and fluorescence). DOX is widely used in chemotherapy for the treatment of Kaposi's sarcoma⁴⁰, ovarian carcinoma³⁸ and breast cancer³⁹. However, it is highly toxic to the heart and kidneys, thereby limiting its application. Recently, a report of the release of DOX from mesoporous silica films has been described in cellular studies⁴⁰. Therefore, systems which are able to administer such drug molecules in a controlled and sustained fashion would be very beneficial.

Ordered molecules and structural studies. We explore the trapping of DOX 2 via hydrophobic interactions, and Mn(III)TPPS 3 by electrostatic interactions, with the positive rim of the wells (Fig. 3a–c). DOX 2 (2.5 × 10⁻³ M) was added as an aqueous solution, and the decrease of fluorescence of well porphyrin 1 monitored over time at a 650 nm wavelength. The fluorescence quenching of 1 by 2 occurs almost immediately (< 1 s) if porphyrin 1 is bound to the aminated silicate without a surrounding wall of bolaamphiphiles (Fig. 3d). However, when porphyrin 1 within the yoctowell i.e. surrounded by bolaamphiphile 4, maximal quenching occurs after ~50 min. We took advantage of this slow immersion process to develop a procedure for the encapsulation of 2 within the yoctowells (Fig. 3e). To ensure complete and homogenous binding of DOX 2, fresh sample of the yoctowell material were suspended in Milli-Q water and the solution was treated with DOX 2 solution overnight. The excess DOX was removed by centrifugation, dispersion and repeated washing with Milli-Q water. Indeed, the inclusion of 2 into the pre-formed well was confirmed by the absence of an emission band at 650 nm due to 1, and the appearance of a new band at 630 nm associated to DOX 2 (Fig. 3e). Fig. 3f shows UV-vis absorption spectra of 1 and 2. A ratio of ~2 : 0.1 was obtained for the height of peaks, which is consistent with the 1 : 1 ± 10% stoichiometry expected based on molar extinction coefficients (1, 200,000 M⁻¹ cm⁻¹ and DOX 2, 11,300 M⁻¹ cm⁻¹).

Following quantitative encapsulation of 2 within the pores, the potential for sequential addition of a third molecule into the yoctowell was explored. It was demonstrated that the walls of the wells bear enough positive charge at the rim to bind an anionic species, such as Mn(III)TPPS 3, via electrostatic interactions⁵⁰. Mn(III)TPPS 3 (2 × 10⁻⁷ M) was then added to the bulk water solution. Interestingly, the fluorescence spectrum of 1 remained quenched, implying that the encapsulated 2 remained intact within the pores. Upon addition of 3, about 95% fluorescence quenching of the 2 was observed (Fig. 3e). After removal of excess 3 by centrifugation and washing with Milli-Q water, all three components 1, 2 and 3 were detectable in the UV-vis spectra of the aqueous solution of the re-suspended particles. The fluorescence spectrum of 2 (Fig. 3) showed complete quenching of fluorescence by manganese porphyrin 3. The UV-vis absorption spectrum of re-suspended particles was also measured in deionised water (Fig. 3f, red line), which clearly indicated an absorption maximum corresponding to 1 (420 nm) and a combined band of 2 and 3 at 450–470 nm. The ratio of the areas of the Soret band of well 1 at 420 nm, and the combined band of 2 and 3 at 440–470 nm in the UV-vis spectrum, was about the same as that in a 10⁻⁷ molar water solution of all three components. Importantly, capping of the pores in 1 by 3, prevents the addition of 2. Encapsulation not only depends on the sequential addition of the final adsorption process, but also on the limitation to only one molecule in each well at a time. These limitations allow the establishment of a 1 : 1 : 1 ≥ 10% ratio between the number of yoctowells and accessible molecules in aqueous solutions. We believe that a 20-fold excess of solute molecules are adsorbed on the outer surface of the particles or on the walls of the cuvettes, and is later removed quantitatively in the centrifugation and washing procedures. To evaluate the efficiency of encapsulation of DOX 2 within the yoctowells, loading experiments, investigating the attachment of DOX 2 to native silica particles, were performed. Typically, amino silica particles were titrated with aqueous DOX 2 solution (10⁻² M) overnight. However, UV-Vis absorption spectra of the resultant redispersed silica particles (after centrifugation and washing) revealed no DOX 2 absorption peak, and thus the DOX had washed out.

Encapsulation of bioactive molecules in the yoctowell on gold surfaces. The encapsulation of DOX 2, and the capping of the wells by Mn(III)TPPS 3, was confirmed by an independent electrochemical method (Fig. 3g). Following an earlier protocol, yoctowells prepared on gold electrodes were constructed by two step-self-assemblies (see Supplementary Fig. S3), followed by functionalization of the double bond of the bolaamphiphiles⁵⁵. Upon confirmation of a positive rim around the wells of the yoctowell, electrodes were then immersed in DOX 2 solution (10⁻³ M) overnight, followed by washing with MilliQ water. Cyclic-voltammetry confirmed the encapsulation of 2 within the wells. Finally, the electrode was immersed into an aqueous solution of tetrasulphanato-porphyrin 3 (10⁻³ M) for 2 h. Complete closing of the gaps with 3 was confirmed by cyclic voltammetry using ferricyanide as redox-active compound.

Release of bioactive molecules by pH stimuli. Yoctowell loaded with DOX 2 and capped with Mn(III)TPPS 3 was prepared, and the pH stimulated control release process subsequently investigated (Fig. 4a–c). It is worth mentioning here that it is most important to wash the nanoparticles with Milli-Q water (pH 7) after each step, as excess molecules (2 and 3) can adsorb onto the outer surface, and may be released before the operating pH is attained. Yoctowells bearing 2 and 3 molecules on silica particles were placed in a cuvette and water was added. The release of 2 and 3 from yoctowells was monitored via the recovery of fluorescence of 2 and 1 at 650 nm (λex = 430 nm) respectively, which was measured as a function of time. A flat baseline showed that 2 and 3 were held within the yoctowells at neutral pH without premature release. The pH of the solution was then lowered to 4.5 by addition of 0.1 M HCl, and recovery of the fluorescence emission of 2 (which was quenched by 3) was observed within less than an hour (Fig. 4d). Mn(III)TPPS 3 was released simply by lowering the pH, the protonated (SO₃⁻ ᵃ степени 3
into SO3H form of dye being expelled from the wells. Thereafter, increasing the pH by addition of NaOH (0.1 M), enabled the release of DOX 2 from the yoctowell cavities. Upon release of DOX 2, a rapid increase of fluorescence intensity around 650 nm vs time was detected. This was attributed to the recovery of fluorescence of the base porphyrin 1 (Fig. 4d). Fluorescence intensity measurements were used to calculate the % release of molecules from the yoctowells. To confirm pH dependent release, yoctowell bearing only DOX 2 was employed in the release process - a flat baseline at pH 7 indicated that DOX 2 were held within the yoctowells, and thereafter no release was observed upon reducing the pH to 4.5. However, upon increasing the pH of the solution to 7.8, release of DOX 2 was observed as a function of time, and approximately 15% of 2 were released from 3 mg of yoctowells after ~6 h (Fig. 4d, dash line).

In order to evaluate the pH dependency for release of the encapsulated molecules, we have performed two independent protocols. The first to determine the exact time of release of 3, which was only held within the wells by electrostatic interactions, and the second, to...

Figure 3 | Schematic diagrams for the encapsulation of bioactive molecules. a, Vacant yoctowells on the silica/Au surface. b, Encapsulation of DOX 2. c, Capping of the yoctowells by Mn(III)TPPS 3 held in place by electrostatic interactions. d, Fluorescence quenching of bound porphyrin 1 upon addition of DOX 2, with or without bolaamphphiles surrounding the porphyrin 1 (λex = 430 nm). Fluorescence quenching with water-soluble DOX 2 occurs immediately (black line) if the only porphyrin 1 attached to the silicate particles (see model), whereas maximal quenching is only reached after ~50 min when porphyrin 1 is within the yoctowells. e, Fluorescence spectra of: well porphyrin 1 (black solid line) alone; well porphyrin 1 + DOX 2 (blue dotted line), well porphyrin 1 + DOX 2 + Mn(III)TPPS 3 (red solid line) and spectra’s of pure 10−6 M solution of DOX 2 (pink line) and Mn(III)TPPS 3 (green line) in water respectively. f, The UV-Vis absorption spectra of well porphyrin 1 (black solid line); well porphyrin 1 + DOX 2 (dark red), well porphyrin 1 + DOX 2 + Mn(III)TPPS 3 (blue) and well porphyrin 1 + Mn(III)TPPS 3 (green) in water, respectively. g, Cyclic-voltammograms of an aqueous solution of ferricyanide ions (10−3 M, 1 M KCl), measured at 100 mV s−1 in water at pH 7: forms yoctowell (curve A), which shows open clefts; the blocking effect after treatment with 10−3 M DOX 2 (curve B), where the yoctowells contain sufficient positive charge, as a result of the generated ammonium groups, to hold a Mn(III)TPPS 3 molecule electrostatically above the surface, thus inhibiting the inclusion of ferricyanide as indicated by a complete loss of the CV current (curve C).
test whether Mn(III)TPPS 3 interferes in the second release process, by independent release of Mn(III)TPPS 3 (Fig. 4e). At pH 7, a flat baseline indicated that the base porphyrin 1 was quenched when 3 was bound to the positive rim of the wells, mainly due to electron transfer from porphyrin 1 to 3. The fluorescence of porphyrin 1 remained quenched at pH 7 indicating that no premature release of 3 occurred. The pH of the solution was adjusted to 4.5 by adding HCl (0.1 M) and a rapid increase in the emission intensity at 650 nm.
(λex = 430 nm) was observed i.e. the recovering fluorescence of 1 indicated that Mn(III)TPPS 3 was released immediately from the yoctowells upon lowering the pH (> 20 minutes). However, yoctowell loaded with 2 and 3 at pH 4.5 only released 3 i.e. DOX 2 remained intact within the cavity at pH 4.5. pH dependency for the release of 2 was evaluated by varying pH from 7 to 9.4 (Fig. 4f). The flat baseline indicated that 2 remains held within the wells at pH 7 even after 6 h in aqueous solution. As the pH of the solution was increased (pH 7.2–9.4) gradually, we observed an overall increase in fluorescence intensity (λem = 650 nm) as a function of time with increasing pH. At pH 8.4 and 9.4, a rapid increase in the fluorescence of 1 was observed; however, pH in the range of 7.4–7.8 gave a slow and sustained release of 2. The release of DOX 2 from the yoctowells was further investigated at physiological salt concentration, using Phosphate Buffered Saline (PBS, pH 7.2), between pH 7.2–9.5 (see Supplementary Fig. S4). The results obtained were similar to the release of DOX 2 from the wells in Milli-Q water.

Independent electrochemical methods were also used to study the pH dependent release process (Fig. 4g). Cyclic voltammetric curves of ferricyanide (10−5 M) were recorded in the presence of aqueous KCl solution. Yoctowell bearing 2 and 3 showed a complete loss of CV current, thereby confirming that the encapsulated guest remained intact. Upon immersing the electrode in a solution of pH 4.5 for 60 minutes, a cyclic voltammogram with a current regeneration of about 20% was recorded, thus confirming the re-opening of the wells. When the electrode was immersed in an aqueous medium of pH 7.8 for 3 days and the CV measured, about 50% of the current was observed which was similar to the empty yoctowells (Fig. 3g). Therefore, cyclic voltammetry also supports the pH dependence of the release process of 2 and 3 by pH control.

The results suggested a good capping efficiency of Mn(III)TPPS 3, via electrostatic interaction of the DOX 2 molecule, versus the undesired leaching problem at neutral pH. Addition of HCl to the aqueous suspension of Mn(III)TPPS-capped yoctowells triggered a rapid release of the Mn(III)TPPS 3 (< 20 minutes) and the release reached 100%. Interestingly, only ~15% of the encapsulated DOX was released after 6 h, and thus the total release of DOX 2 within 3 days. This large difference between the portion of 2 and 3 released from the yoctowells implies that Mn(III)TPPS 3 was only electrostatically attached within the wells, whereas DOX molecules were more strongly physisorbed to the walls of the yoctowell via hydrophobic interactions. These results were in agreement with our earlier hypothesis that the blockage of small molecules is caused by the “immobile hydration water” of solutes, which binds to the hydrophobic walls. Upon increasing to pH > 7, DOX 2 disperses in the yoctowell water and is released from the cavity. There appears to be a critical point in the rate of release, as at pH > 7.8 the DOX 2 is released quickly, whilst at pH’s which are very slightly basic but less than pH7.8, the release of DOX 2 takes place in a slower fashion. This critical pH point may coincide with a critical point in the deprotonation-protonation equilibrium, where at pH < 7.8 the hydroxyl groups are probably in the deprotonated oxide form.

The absorption and fluorescence spectra of the solution, which were examined before and after the release of 2, were totally different and comparable to those of pure DOX 2 in aqueous solution (Fig. 5). The particles (5 mg) were dispersed in water (5 mL) and the pH of the solution lowered to 4.5 by addition of 0.1 M HCl (25 μL). After 1 h, the particles were isolated by centrifugation and washing with Milli-Q water. Complete release of 3 was achieved by dispersion of the particles in water at pH 7, as confirmed by the fluorescence recovery of 2 in (Fig. 5a). On increasing the pH of the solution by addition of 0.1 M NaOH solution (25 μL), the release of 2 was observed. After recovering the particles by centrifugation, the supernatant showed only emission of 2 and redissolving the particles in water only gave the fluorescence emission of 1. Furthermore, absorption spectroscopy was used to estimate the amount of 2 after its release from the wells (Fig. 5b). The absorption band for the encapsulated DOX 2 within the yoctowell appears at ~460 nm at neutral pH, whereas released DOX 2 at pH 7.8 gave absorption bands at 490 and 570 nm, and was comparable to that of pure DOX 2 in solution. However, the UV-vis absorption bands of 2 at pH >8 appeared at 520 and 620 nm. UV-vis absorption spectroscopy therefore confirmed that DOX 2 (10−6 M) was completely released from yoctowells after 3 days.

For the preparation aminated silica colloidal particles in 1–10 g quantities we followed Blaaderen’s protocol with slight modified procedure for achieving 100 nm size with smooth surface. The smoothness, size, and chemical self-assembly procedures were optimized in order to establish a yoctowell with modest curvature and containing functional gaps. Furthermore, the smoothness and size of particles have been determined by transmission electron microscopy (TEM) and observed ill-defined networks of 100-nm spheres with a smooth surface (Fig. 6a). TEM always showed perfectly smooth particles even after the self-assembly of porphyrin 1 and the bolaamphiphile 4.
Our results demonstrate a new approach to the use of yoctoliter wells and 6d, respectively. Thus, silica based yoctowell system for drug release of biologically active molecules, by employing a yoctowell system in conjunction with naturally occurring stimuli in vivo. This drug delivery yoctowell system has the potential to change radically the practice of therapy for a variety of diseases and disorders. The success of such a simple yoctowell systems based on this strategy promises well for future, more sophisticated designs that employ for a similar approach.

**Methods**

**Spectroscopic measurements.** UV/vis absorption spectra of membrane-coated silica colloids were acquired using a Varian Cary 1 Bio spectrophotometer at 25°C. Fluorescence measurements and quenching experiments were performed on a FluoroMax-4 equipped with an injector port and stirrer 25°C. Cyclic voltammetric experiments were performed in a one-compartment three-electrode cell in a PG310 equipped with a potentiostat (HEKA, Dr Schulz GmbH, Germany). The working electrode was a circular bar gold electrode or monolayer-coated gold electrode with a surface area of 0.5 cm². A Pt wire counter electrode and aqueous Scanning Calomel Electrode reference electrode were used. An aqueous solution containing 0.1 M KCl and 1 mM K3[Fe(CN)6] were used as electrolyte.

Fluorescence Quenching Decay Measurements Experiments. Colloidal silica decorated with yoctowells (3 mg) was dispersed in 3 mL of water and placed in a quartz cuvette. A 30 µL aliquot of an aqueous solution of Doxorubicin (2 × 10^-5 M) was added. The fluorescence spectrum of the well base porphyrin 1 was continuously monitored. The same quenching procedure was applied for attachments of anionic Mn(III)TPPS 3 (ref. 26).

**Encapsulation of Doxorubicin 2 and Mn(III)TPPS 3.** Yoctowells with functionalised walls, with a positive rim, on silica particles (20 mg) were dispersed in 14 mL of Milli-Q water and placed in a clean glass tube and soaked with Doxorubicin 2 (3 mL of 10^-3 M) at pH 7; overnight at room temperature, to allow the Doxorubicin 2 to diffuse into the wells of the silica nanoparticles. Excess doxorubicin was then removed by centrifugation, dispersion and ultrasonication. This was followed by addition of Mn(III)TPPS 3 (3 mL, 10^-5 M of pH 7.0). After 30 min, and washing with Milli-Q water (pH 7.0) three times to remove excess adsorbed molecules 3, silica particles with a trimeric system were obtained. The obtained particles in heterotrimer form were confirmed by UV–vis and fluorescence spectroscopy thereby indicating the presence of a strong inclusion complex. Most importantly, the yoctowell obtained in trimeric form can be stored in a dried form for more than three months without release of any of the molecules from the cavities. In each step, UV-visible and fluorescence spectroscopy were used to confirm the encapsulation and stability of guest molecules.

**Release of the molecules from yoctowells on silica particles.** Release of the encapsulated molecules from yoctowells was examined by fluorescence spectroscopy. A silica nanoparticle system (3 mg) containing yoctowells along with Doxorubicin 2 and Mn(III)TPPS 3 were placed in a fluorescence cuvette (t = 0 sec). Milli-Q water (2 mL) was then added and a 1 mm magnetic stirrer bar was placed into the bottom of the cuvette. Upon stirring of the mixture for 2 minutes, the fluorescence emission (λ_em = 650 nm) intensity was measured using an excitation wavelength, (λ_ex = 430 nm, as a function of time during addition: after a flat baseline at pH 7 had been recorded, the cuvette was continuously stirred and washes with Milli-Q water at pH 7 had been removed by centrifugation, dispersion and ultrasonication. This was followed by addition of the obtained particles in heterotrimer form were confirmed by UV–vis and fluorescence spectroscopy thereby indicating the presence of a strong inclusion complex. Most importantly, the yoctowell obtained in trimeric form can be stored in a dried form for more than three months without release of any of the molecules from the cavities. In each step, UV-visible and fluorescence spectroscopy were used to confirm the encapsulation and stability of guest molecules.

**Transmission Electron Microscopy (TEM).** TEM measurements were performed on an electron microscopy Igor 1200EX, operating 80 kV of an accelerating voltage. Freshly prepared samples were prepared by dropping 5 µL aliquots of silica colloidal particle solution (H2O) onto a TEM grid (400-mesh copper grid coated with carbon). After a few minutes, remaining solution was blotted off with a filter paper and images were collected.

**Discussion**

The particles examined by transmission electron microscopy (TEM) and observed ill-defined networks of 100-nm spheres with a smooth surface after two step self-assembly (Fig. 6b). TEMs of silica colloidal particles after loading and release of DOX 2 and Mn(III)TPPS 3 shows ill-defined 100 nm spheres, see figures 6c and 6d, respectively. Thus, silica based yoctowell system for drug delivery in vivo will be biocompatible.

**Figure 6 | Transmission electron micrographs (TEM). a, Synthetic aminated silica particles. b, after the self-assembly of porphyrin 1 and the bolaamphiphile 4 monolayers. c, Upon encapsulation of DOX 2 and capping of the yoctowells by Mn(III)TPPS 3. d, After release of DOX 2 and Mn(III)TPPS 3 from the yoctowells.
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Author contributions
RMIT S.V.B. designed the experiment and performed synthesis of bolaamphiphiles, particle
preparation, encapsulation and release of bioactive molecules using fluorescence, UV-Vis
absorption spectroscopic measurements. IUCT S.V.B. performed synthesis of porphyrins
and electrochemical experiment. RMIT S.V.B. analysed the data. RMIT S.V.B. has written
the manuscript and both co-authors reviewed the manuscript.

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