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Enzymatic reactions in confined environments

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Within each biological cell, surface- and volume-confined enzymes control a highly complex network of chemical reactions. These reactions are efficient, timely, and spatially defined. Efforts to transfer such appealing features to *in vitro* systems have led to several successful examples of chemical reactions catalysed by isolated and immobilized enzymes. In most cases, these enzymes are either bound or adsorbed to an insoluble support, physically trapped in a macromolecular network, or encapsulated within compartments. Advanced applications of enzymatic cascade reactions with immobilized enzymes include enzymatic fuel cells and enzymatic nanoreactors, both for *in vitro* and possible *in vivo* applications. In this Review, we discuss some of the general principles of enzymatic reactions confined on surfaces, at interfaces, and inside small volumes. We also highlight the similarities and differences between the *in vivo* and *in vitro* cases and attempt to critically evaluate some of the necessary future steps to improve our fundamental understanding of these systems.

For many years, the structure, stability, and catalytic properties of water-soluble enzymes have been studied by analysing their crystal structures and by investigating their catalytic properties when dissolved at a certain concentration in a buffered aqueous solution of defined composition and temperature. In this way, characteristic *in vitro* features of individual, purified and dissolved enzymes could be elaborated and mechanisms formulated for explaining the enzymes' ability of catalysing a particular type of chemical reaction[1,2]. Such studies have shown that substrate binding and an intact active site are essential for the proper functioning of enzymes as dynamic globular macromolecules[3]. However, a simple buffer solution does not reflect the compositional complexity of the biological medium in which enzymes normally perform. Most *in vivo* enzyme-catalysed reactions occur in a molecularly crowded environment[4] and/or in a confined environment, such as on a surface, at an interface, or inside a small volume[5–7]. These factors — among others — have to be taken into account if the *in vivo* environment of the enzymes of interest is to be synthetically imitated, or if the altered behaviour of isolated enzymes *in vitro* (for example, stability) is to be understood.

In this Review, we will focus on a few aspects of confined enzymatic reactions both *in vivo* and *in vitro*. We will refer to examples of enzymes that perform in confined environments *in vivo*, and we will present some general features and selected examples of *in vitro* reactions catalysed by volume- and surface-confined (immobilized) enzymes. Particular attention will be paid to *in vitro* enzymatic cascade reactions with different types of enzyme that catalyse sequential multi-step reactions[6,8–10]. Furthermore, selected applications of immobilized enzymes will be discussed, with particular emphasis on applications where the defined confinement of enzymatic reactions to either a surface or a volume appears advantageous.

In vitro surface- and volume-confined enzymatic reactions with isolated, immobilized enzymes are often carried out not only for understanding *in vivo* behaviour, but also for elaborating the possibilities for *in vitro* applications. Indeed, analytical and biotechnological applications of immobilized enzymes exist for the preparative modification, degradation, or synthesis of organic molecules[1,11–14]. Immobilizing enzymes on surfaces or confining them in small volumes often allows for a facile separation of the enzymes from the reaction products[3], a key advantage with respect to reactions with dissolved enzymes. More sophisticated systems can involve enzymatic cascade reactions, in which the relative spatial localization of the enzymes is a prominent aspect, both for *in vitro* applications and for characterizing *in vivo* systems[6,13].

For surface-confined enzymatic reactions, the enzymes are either adsorbed or bound to a support via non-covalent or covalent bonds. For volume-confined enzymatic reactions, the enzymes are physically entrapped either within a macromolecular network or within compartments. In the latter case, the substrate molecules have to be able to access the enzymes from the external medium or from other compartments, unless the substrate molecules are already present within the compartment from the beginning.

Conceptually, there are obvious similarities between confined *in vivo* and *in vitro* enzymatic reactions. However, there are also noticeable differences. One significant difference is that in biological systems, new enzymes are constantly synthesized to replace the ones that have been released, inactivated, or degraded[15], whereas in non-living *in vitro* systems, there is no such continuous *de novo* synthesis. Furthermore, the efficiency of multi-enzyme complexes[6,13] with a spatially defined localization of different types of enzyme with their specific substrate channelling is difficult to achieve outside cells. Hence, enzymes extracted from biological samples and applied *in vitro* cannot compete with the *in vivo* situation when long-term performance and efficiency of enzymatic cascade reactions are considered. Consequently, any type of application of immobilized enzymes requires not only an optimal enzyme localization but also an optimization of the enzymes' storage and operational stabilities[1,11,16].

Despite these limitations, and the fact that enzymes are intrinsically unstable, confined enzymes can still be powerful *in vitro* catalysts for the following two seemingly contradictory reasons. First, enzymes often catalyse chemical reactions regio- and stereoselectively with high substrate specificity[1,2]. Thus, a high selectivity can be achieved, which is hard to attain using traditional organic chemistry approaches. On the other hand, many enzymes (for example, lipases, oxidative enzymes) exhibit low specificity, which also allows them to be used for catalysing transformations of completely synthetic, non-natural substrates[17–21]. Moreover, enzymes from

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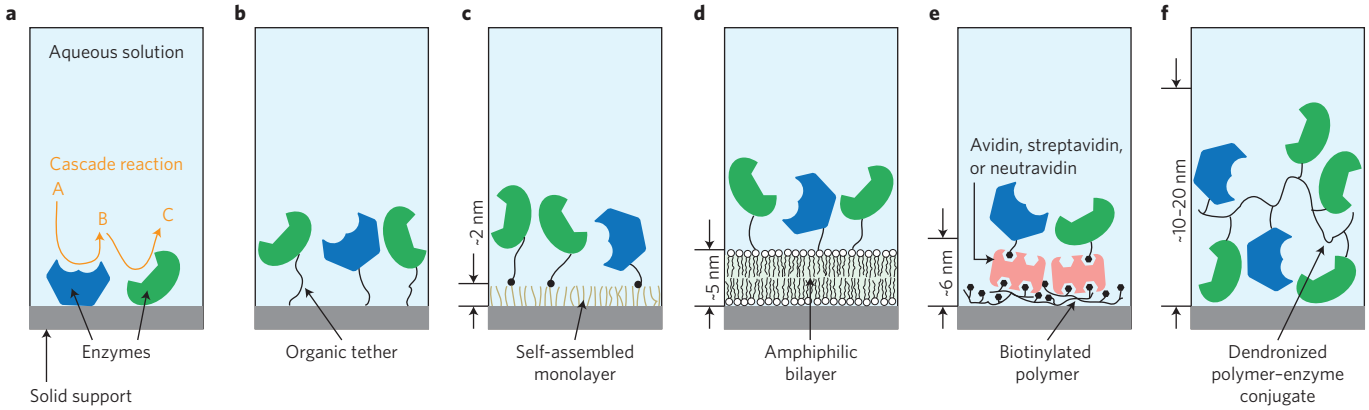

Figure 1 | Enzyme immobilization on solid supports for surface-confined enzymatic reactions in an aqueous environment. The green and blue objects represent two different types of enzyme with their active sites indicated as indentations. For enzymatic cascade reactions to occur efficiently, immobilization of the different enzymes involved should occur in a defined way by placing them at specific positions and relative ratio. The enzymes' activity must be retained, and the system must have acceptable storage and operational stabilities. Different strategies have been developed to immobilize enzymes on solid supports. **a**, By direct enzyme adsorption via non-covalent interactions between the enzymes and the support[36]. **b**, By one or more organic linker molecules that allows for covalent bonding between the support and the enzymes[36] ensuring that the enzyme is kept at a distance from the surface. **c**, By adsorbed or covalently bound self-assembled monolayers. **d**, By adsorbed or covalently bound bilayers of amphiphiles. **e**, By non-covalently adsorbed organic polymers[32,38] or proteins (the pink object denotes the protein avidin (or streptavidin or neutravidin) with its four biotin-binding sites)[36]. **f**, By non-covalently adsorbed dendronized polymer–enzyme conjugates[33,39]. The solid support can be inorganic and smooth (planar silicate glass[33,38,39], dispersed graphene oxides[107], or carbon nanotubes[108]), inorganic and rough (mesoporous silicates[109,110]), or organic (polystyrene particles[111], DNA origami tiles[42], vesicles[58,112–114], or cells[115]).

different host organisms can be combined *in vitro*, which makes it possible to create enzymatic cascade reactions that do not occur in biological systems[22].

Surface- or interface-confined enzymatic reactions

Many enzymatic reactions in living systems take place in biological membranes[5,6,23]. The study of these surface- or interface-confined enzymatic reactions *in vivo* has inspired the use of various *in vitro* systems that mimic the lipid matrix of biomembranes in the form of lipid vesicles[24–26], reversed micelles[27–29], or solid supported lipid bilayers[30,31].

Apart from these biomimicking approaches, enzymatic reactions occurring on non-natural surfaces have been studied and applied for many years. Figure 1 schematically illustrates some of the possibilities of immobilizing isolated enzymes on solid or soft supports to conduct surface-confined enzymatic reactions *in vitro*. In all cases, the immobilized enzymes are exposed to the bulk aqueous solution in which the substrate molecules are dissolved. The substrate accessibility to the active site of an enzyme may be restricted by physical constraints, for example, when the enzymes are adsorbed in the pores of mesoporous particles or in the inner part of a hydrogel, or when the active site faces the surface of the support instead of the bulk solution. One of the main advantages of immobilizing enzymes on insoluble solid supports is that the products at the end of the reaction are easy to collect, provided they are still soluble[1,2,11]. This permits the construction of surface-confined enzymatic reactions in flow reactor systems (for example, microfluidic chips[32,33] or nanochannels[34]). The immobilization of enzymes on solid supports often results in a lower catalytic activity than in bulk solution, because immobilization leads to a decrease in conformational flexibility[35], but frequently in a higher operational stability than in bulk solution[11].

Conceptually, there are different ways of immobilizing enzymes — or any other types of protein — on solid supports[1,11,12,14,36,37]. Apart from simple adsorption (Fig. 1a), often, the support is first modified with small organic linkers with reactive functional groups (Fig. 1b). The linkers are exposed to the bulk solution for a direct, covalent attachment of the enzymes to the surface. Alternatively, the solid support is first coated with an organic layer to which the enzymes are covalently bound (Fig. 1c–e), again through linker molecules. Such soft organic coating prevents enzyme inactivation that might occur in case of direct contact with the solid support (Fig. 1a). The coating may consist of adsorbed or covalently bound self-assembled monolayers (Fig. 1c) or bilayers (Fig. 1d) of amphiphilic molecules, globular proteins (bovine serum albumin, avidin)[36], or polymers[32,38] (Fig. 1e). Another possibility is to adsorb large dendronized polymer–enzyme conjugates, previously prepared in solution[33,39] (Fig. 1f). In some cases, enzymes immobilized on a surface can be more stable than in bulk solution[11]. However, it is still unclear how to quantitatively describe enzymatic reactions with surface-bound enzymes[1,2], as the precise concentration of bound enzymes is difficult to determine, and as the enzymes are fixed on a solid support (no three-dimensional diffusion) while the substrate molecules diffuse in the entire volume.

One exciting perspective is that different types of enzyme can be immobilized in a precise and sequential way to design multi-step cascade reactions (specific examples are shown in Fig. 2)[6,8,9,13,38,39]. A fine spatial control may speed up the reaction, reduce unwanted side reactions, and decrease the accumulation of inhibitory or reactive intermediates[6,13]. To do so, one particular concept is illustrated in Fig. 2a. Biotinylated glucose oxidase (GOD) and horseradish peroxidase (HRP) are bound to biotinylated DNA origami building blocks with the help of neutravidin to form a dimer nanoreactor[40].

Based on geometrical considerations, the active sites of two co-immobilized enzymes that catalyse two consecutive reactions should not be farther away from each other than 0.1–1.0 nm to make direct substrate chanelling between the two enzymes possible[41]. However, this prediction appears to contradict experimental results in that it was found that such a close proximity of two enzymes is not necessary for an increase in the reaction efficiency compared with the free enzymes (Fig. 2b)[42]. This apparent discrepancy can be explained by considering that substrate channelling between enzymes positioned farther away from each other than 1.0 nm is possible if the local density of the two (or more) enzymes involved in the cascade reaction is over a certain threshold ('enzyme cluster-mediated channelling')[41]. Placing GOD and HRP

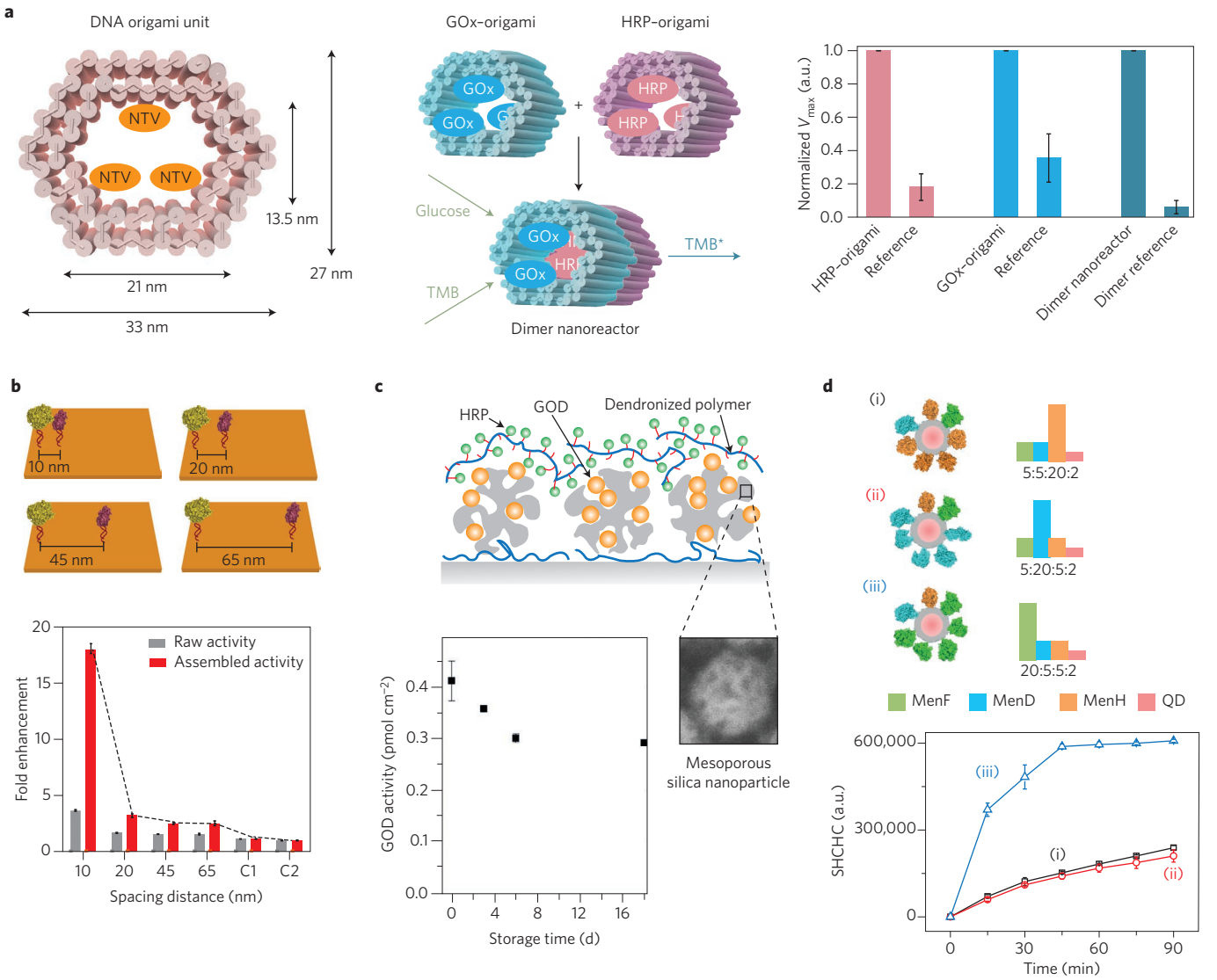

Figure 2 | Examples of surface-confined enzymatic cascade reactions. a, Localization of biotinylated glucose oxidase (GOD = GOx) and horseradish peroxidase (HRP) using tubular DNA origami building blocks and neutravidin (NTV). A catalytically active 'dimer nanoreactor' containing the two enzymes was obtained, as determined by analysing the transformation of D-glucose and 3,3',5,5'-tetramethylbenzidine (TMB) at pH 5.0 in the presence of O_2 into TMB imine (TMB*). The activity was determined as rate of formation of TMB*, whereby for each case, the NTV-containing nanoreactor was normalized to 1, defined as V_{max}. In the 'reference' measurements, NTV was omitted, which decreased the amount of bound enzymes[40]. **b**, Localization of GOD (yellow) and HRP (purple) at defined distances on the surface of DNA origami tiles. An increased catalytic activity was observed when the inter-enzyme distance was 10 nm, as analysed with D-glucose and $ABTS^{2-}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)) and O_2 as substrates at pH 7.2. No enhanced activity was found when the inter-enzyme distance was 65 nm, while at a distance of 20 nm, the increase in activity was only small[42]. C1 and C2 refer to tiles without nucleic acid and free enzymes, respectively. **c**, Localization of GOD (orange) and HRP (green) on silicate surfaces with the help of a dendronized polymer (blue) and mesoporous silica nanoparticles (grey). GOD was positioned inside the pores of the particles and HRP was spatially separated on the particles through covalently linking HRP along the polymer chain, followed by simple adsorption of the obtained polymer–enzyme conjugate. The enzymes remained active for at least 18 d when stored at 4 °C, as analysed with D-glucose, *o*-phenylenediamine, and O_2 as substrates (expressed as GOD activity of the cascade reaction, that is, as determined without admixture of HRP)[45]. Diameter of silica nanoparticle in image is ~60 nm. **d**, Localization of the three enzymes MenF, MenD, and MenH of the menaquinone biosynthetic pathway on CdSe–ZnS core–shell quantum dots (QDs). About 16–20 enzyme molecules were bound to each particle. The reaction was more efficient when each particle contained a mixture of the three enzymes than when each particle contained only one type of enzyme. The activity was highest when MenF was in excess over the other two enzymes (case iii), as determined with chorismate as substrate at pH 7.0 to yield 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) as product[46]. Figures adapted with permission from: **a**, ref. 40, RSC; **b**, ref. 42, American Chemical Society; **c**, ref. 45, RSC; **d**, ref. 46, American Chemical Society.

via DNA origami tiles at a distance of 10 nm from each other leads to a significant activity increase compared with the free enzymes and compared with being placed 20, 45, or 65 nm apart (Fig. 2b)[42]. This activity increase could be due to an efficient migration (channelling) of the reaction intermediate (H_2O_2) from the active site of GOD to the active site of HRP through the hydration layer on the surface of the two enzymes. Substrate channelling occurs in living systems too, specifically in membrane-bound multi-enzyme complexes (also called enzyme supercomplexes or metabolons[6]), for example, in the case of the eight-enzyme complex responsible for

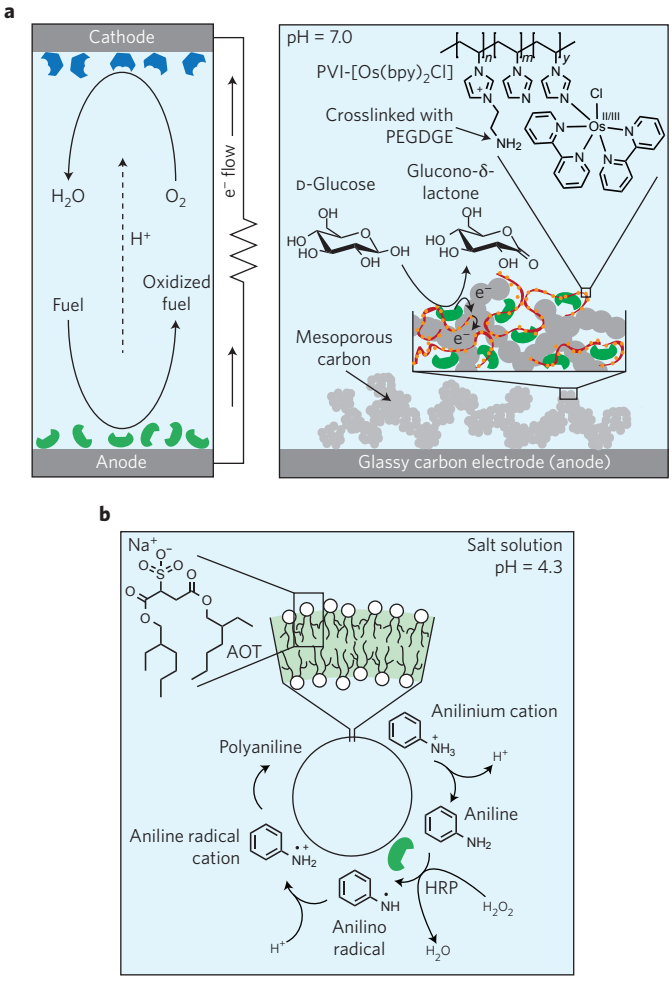

Figure 3 | Two examples of the application of surface-confined enzymatic reactions. a, Enzymatic fuel cell. Left: Illustration of a membraneless enzymatic fuel cell[51,116] in which the fuel is oxidized at the anode by an immobilized oxidative enzyme (green). The electrons (e^-) released during the oxidation move through the external wire to the cathode at which O_2 is reduced by the immobilized reductive enzyme (blue). Right: A specific example of an anode that was coated with mesoporous carbon (average pore diameter of 38 nm and a surface roughness of several tens of micrometres) to which the enzyme d-FAD-DGH (deglycosylated flavin adenine dinucleotide-dependent glucose dehydrogenase, green) was immobilized within a hydrogel formed from poly(1-vinyl-imidazole) that was crosslinked with PEGDGE (poly(ethylene glycol) diglycidyl ether) and complexed to $[Os(2,2'\text{-bipyridine})_2Cl]^{+/2+}$ for efficient electron transfer. D-glucose was used as fuel, yielding glucono-δ-lactone (oxidized fuel)[56]. **b**, Enzymatic polymerization of aniline on the surface of anionic vesicles catalysed by a redox enzyme that is localized on the vesicle membrane surface[58]. Unilamellar vesicles with a diameter of about 100 nm were prepared from AOT (sodium bis(2-ethylhexyl)sulfosuccinate) in an aqueous salt solution of pH 4.3. After HRP and the aniline monomers (mainly present as anilinium cation) had associated with the vesicle surface, the aniline oxidation was triggered by adding H_2O_2. Polymerization of the obtained aniline radial cation into the emeraldine salt form of polyaniline (PANI-ES) occurred on the vesicle surface. PANI-ES did not form in the absence of the vesicles.

the citric acid cycle[43]. In this particular case, substrate channelling between the active sites is likely to be due to electrostatic interactions on the surface of the enzymes[43]. Apart from this sequestration mechanism, covalent tethering, that is, the covalent binding of substrates and intermediates to the enzymes, is an alternative way of *in vivo* substrate channelling[44].

The example illustrated in Fig. 2c shows a completely different way of co-localizing GOD and HRP, but with much lower positional precision and a much less sophisticated approach than in the case of the DNA origami systems of Fig. 2a,b. GOD was adsorbed inside mesoporous silicate particles, and HRP was placed on top of the particles via a HRP–polymer conjugate to form a two-enzyme system of high storage stability[45].

Another example of an *in vitro* cascade reaction involves three enzymes implicated in the menaquinone biosynthetic pathway (Fig. 2d)[46]. The enzymes were randomly immobilized on CdSe–ZnS core–shell quantum dots with a diameter of 3.5 nm. The efficiency of the cascade reaction depended on (i) the total number of enzymes per particle, and (ii) the relative ratio of the three enzymes per particle[46]. This study demonstrates the importance of co-localizing the three enzymes as well as the importance of the inter-enzyme distance. As the enzymes were tightly packed on the quantum dot surface, it is unlikely that the surface itself had an effect on the behaviour of the enzymes and only served as a scaffold for bringing the different enzymes in close proximity. Interestingly, however, nanoparticle-confined enzymes may show enhanced activity compared with freely diffusing enzymes, even if only one type of enzyme is used (no cascade reaction)[35]. For example, chymotrypsin immobilized on modified gold nanoparticles showed enhanced catalysis depending on the charge of the substrates, indicating the influence of the microenvironment of the immobilized enzyme on the reaction[47].

Using surface-confined enzymes force cascade reactions to occur close to the surface of the support, thus enabling applications in which the surface itself plays an active role, as in the case of enzymatic fuel cells[48–53] or electrochemical biosensor devices, both involving redox enzymes. Examples include *in vivo* power generators that use glucose in the blood as a fuel[54] and sensors for measuring the glucose concentration in blood[55]. In these devices[48–55], a steady flow of electrons occurs between the supporting electrode and the immobilized redox enzymes. The reactions must take place close to the electrode surface, and the active site of the enzymes must have the correct orientation. The electron exchange between enzyme and electrode can occur either directly or through a conductive small molecule, polymer, or particle[54]. To achieve appreciable current densities, nanostructured electrodes with a high surface area are usually used. Such electrodes can be prepared from conductive carbon-based materials (carbon nanotubes or graphene) or from conductive polymers.

One recent example of an enzymatic fuel cell comprised an oxidative enzyme (deglycosylated flavin adenine dinucleotide-dependent glucose dehydrogenase, d-FAD-GDH) immobilized on a nanostructured anode surface (magnesium oxide-templated mesoporous carbon)[56] (Fig. 3a). The adsorption of d-FAD-GDH on the electrode surface was achieved by adding the enzyme to a hydrogel coating consisting of an electrically conductive polymer containing an osmium complex that can undergo a redox reaction[57] and a crosslinker poly(ethylene glycol) diglycidyl ether. Using this configuration, current densities as high as 100 mA cm^{-2} were obtained at the anode as a result of the oxidation of 0.5 M glucose at pH 7.0 (ref. 56).

Another example of a confined enzymatic reaction *in vitro* is the polymerization of aniline on the surface of anionic vesicles (Fig. 3b). HRP and H_2O_2 react with aniline on the vesicle surface[58] to form the emeraldine salt form of polyaniline (PANI-ES). This is an example that relies on the fact that the peroxidase can oxidize non-natural substrates (aniline). Aniline monomers adsorb from the bulk aqueous solution onto the vesicle membranes, and during the course of the reaction, the intermediates and products bind to the vesicle surface, where the enzyme is also localized[58]. The

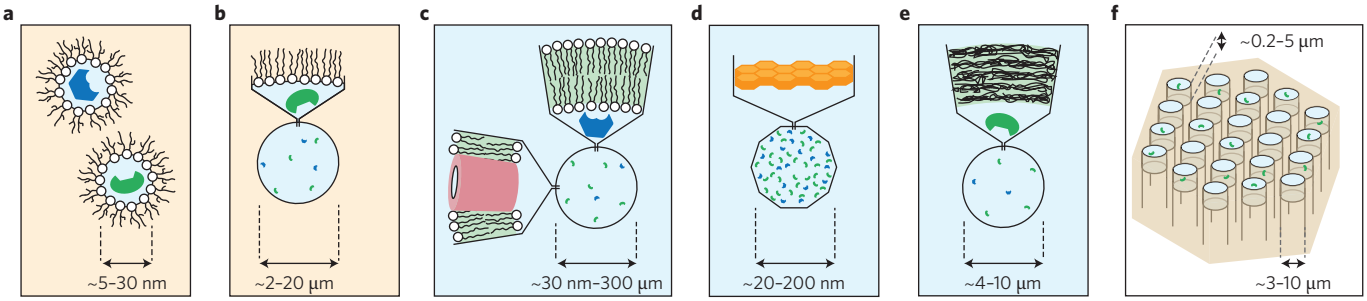

Figure 4 | Volume-confined enzymatic reactions. a, Reverse (or inverted) micellar solutions or water-in-oil microemulsions[27–29,80,117], that is, submicrometre-sized aqueous droplets that are stabilized in a water-immiscible organic solvent — or an ionic liquid[83] — with the help of amphiphilic molecules (surfactants). In the case of reverse micelles, one type of amphiphile stabilizes the water droplets, while for water-in-oil microemulsions, a cosurfactant (often a long-chain alcohol) is also used. Typical sizes of the internal aqueous volumes range from 5 nm (6.5×10^{-23} l = 65 yl) to 30 nm (1.4×10^{-20} l = 14 zl), including the space occupied by the enzymes. For enzymatic cascade reactions with different types of enzyme, different enzyme-containing micellar solutions have to be used. **b**, Micrometre-sized aqueous droplets that are dispersed in a water-immiscible solvent with the help of a shell of amphiphilic molecules that form the boundary layer[70,118]. Water-soluble enzymes are localized in the aqueous volume, which is separated from the bulk organic solvent. Typical droplet sizes vary between 2 μm (4.2×10^{-15} l = 4.2 fl) and 20 μm (4.2×10^{-12} l = 4.2 pl), usually prepared by microfluidic devices to achieve monodispersity in droplet size[70]. For enzymatic cascade reaction in which different types of enzyme are involved, each water droplet contains the different enzymes in the desired amounts. **c**, Spherical artificial vesicles (called lipid vesicles[68] or liposomes when prepared from biological bilayer-forming amphiphilic phospholipids; or polymeric vesicles (polymersomes)[119–121] when prepared from amphiphilic block copolymers). The internal size of spherical vesicles (*D*) may vary from about 30 nm (small, 1.4×10^{-20} l = 14 zl) to 100 nm (large, 5.2×10^{-19} l = 0.52 al) to several hundred micrometres (giant, 1.4×10^{-8} l = 14 nl, for *D* = 300 μm), depending on the method of preparation[68,69]. Although efficient loading of vesicles with water-soluble enzymes may be a challenge[68], once entrapped, the enzymes remain inside the vesicle's aqueous volume due to their macromolecular sizes, separated from the bulk aqueous medium by one or several lamellae of amphiphilic molecules. The schematic shows a unilamellar vesicle with a single lamella. The permeability of the vesicle shells depends on temperature and can be modified by varying the chemical structure of the amphiphiles, by using mixtures of amphiphiles, or by inserting pore- or channel-forming peptides and proteins[90,119,122]. **d**, Protein cages, submicrometre-sized compartments (40–80 nm) with a boundary that is composed of proteins[77,93,95,123], such as virus capsids or prokaryotic microcompartments[62–64,96]. Here, efficient entrapment of enzymes inside protein cages is yet to be achieved, unless the enzyme is part of the inner surface of the shell. The permeability of the shell is determined by the shell structure. **e**, Polymer capsules[124–127] obtained by a layer-by-layer deposition method involving polyelectrolytes and a core structure template that is dissolved and removed after capsule formation[125,127]. The typical size range is 4–10 μm. The layer permeability depends on the polyelectrolyte used and the details of the layer structure. **f**, Arrays of small reaction vessels obtained through chemical etching of glass fibres (vessel diameters between 3 and 10 μm and depths between 0.2 and 5 μm)[128,129]. There is no exchange of matter between the individual, physically separated reaction vessels. The green and blue objects represent two different types of enzyme with their active sites indicated as indentations. The pink object in **c** denotes a channel-forming peptide or protein, the orange objects in **d** are capsule shell-forming proteins, and the black chains in **e** represent polyelectrolytes. Aqueous solutions are indicated by light blue boxes and organic solutions (or ionic liquids), which do not mix with water, are indicated by yellow boxes.

vesicles act as a reaction regulator in that the outcome of the reaction is influenced by the vesicles in a positive way (formation of the desired PANI-ES)[58,59].

Volume-confined enzymatic reactions

These enzymatic reactions are also common in biological systems[5,23,60]. In eukaryotes, for example, endosomes (typically 100–500 nm in diameter[61]) host degradative reactions carried out by about 40 different hydrolytic enzymes, which in turn are contained inside smaller lysosome vesicles[5]. The membrane of endosomes consists of amphiphilic lipids and various embedded proteins that separate the inside environment with a pH of ~5, where degradative enzymes work best, from the cytoplasm, at pH 7.2. Other examples of volume-confined enzymatic reactions in eukaryotes can be found in mitochondria (~0.5–5.0 μm in diameter) and peroxisomes (~500 nm in diameter[5,61]). A particularly useful and much investigated system is the carboxysome, a confined environment found in certain types of prokaryote where carbon fixation from CO_2 is carried out[23,60,62–64].

Carboxysomes are icosahedral compartments of 100–200 nm in diameter separated from the cytoplasm by a membrane consisting only of proteins, with a thickness of about 2–3 nm (ref. 65). They contain only two types of enzyme: CsoSCA (carboxysome shell carbonic anhydrase) and RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase)[65–67] that are specifically localized to optimize a two-step cascade reaction[63] — conversion of bicarbonate (HCO_3^-) to CO_2 and H_2O, catalysed by CsoSCA, and subsequent carboxylation of ribulose-1,5-bisphosphate with the formed CO_2, catalysed by RuBisCO, to yield two molecules of 3-phosphoglycolate, that is, products in which the C atom of CO_2 is incorporated (corresponding to the first major step in carbon fixation)[63]. In *Halothiobacillus neapolitanus*, for example, each carboxysome contains about 40 copies of CsoSCA[65,66], attached to the inner surface of the protein shell, and about 270 copies of RuBisCO, in the interior of the compartment[23,63,65,67]. This example illustrates that the localization and the number of enzymes in each compartment are key parameters that need to be taken into account to ensure optimal reaction efficiency in volume-confined systems.

Figure 4 shows a schematic representation of various types of confined environment in which enzymes can be localized for catalysing reactions *in vitro*. They include reverse micelles (Fig. 4a), water-in-oil droplets (Fig. 4b), vesicles (Fig. 4c), protein cages (Fig. 4d), polymer capsules (Fig. 4e), and arrays of small reaction vessels (Fig. 4f). A characteristic feature of all these compartments is the high ratio of interfacial (surface) area to volume, which may vary from 10^9 m^{-1} for a 5 nm water pool in a reverse micelle (Fig. 4a) to 10^5 m^{-1} for a 50 μm giant vesicle (Fig. 4c). Therefore, possible boundary effects arising from interactions with the inner surface of the compartment become more pronounced the smaller the volume is. Volume-confined reactions of the type discussed here (Fig. 4) can occur in three conceptually different ways.

First, the enzymes and all reacting molecules are placed into the confined volume from the outset. In this case, the enzymatic reactions are expected to start during the preparation of

the compartment systems, for example, during the formation of vesicles[68,69] (Fig. 4c) or during the formation of water-in-oil droplets[70] (Fig. 4b).

Second, substrate molecules are delivered to the enzyme by merging or transiently fusing compartments loaded with either component. Merging compartments, as in the case of vesicles[71] or water-in-oil droplets[70], leads to an increase in the size of the confined volumes and at the same time to a decrease in the number of separated volumes. In the case of reverse micelle droplets (Fig. 4a), fusion and fission occurs continuously and without any significant change in the average size and number of water pools over time (provided that the volumes of the colliding compartments are the same)[27,28]. The kinetics of the enzymatic reactions in such dynamic systems is influenced by the collision and fusion kinetics; a robust, quantitative kinetic model for measuring reaction rates in these systems remains to be developed[27,28,72,73].

Third, water-soluble substrates can be delivered to a volume-confined enzyme across the compartment boundary. This is how enzymes inside cells and inside their organellar subcompartments receive their substrates[5]. The reaction is dependent on the rate of substrate permeation across the boundary, which is determined by the chemical structures of the substrate and the boundary. Thus, the specificity of an enzymatic reaction can be controlled by the activity of the entrapped enzyme and by the vesicle shell permeability[74,75] (Fig. 4c), as is the case of protein capsules[76,77] (Fig. 4d).

Sophisticated examples of volume-entrapped enzymatic reactions include a biochemical oscillator confined to water-in-oil droplets of 2–40 μm diameters[78], and a cell-free gene expression system confined in 3-μm-deep poly(dimethylsiloxane) wells with a volume of 20 fl (ref. 79). Most of the volume-confined reactions investigated so far are much simpler and have been localized in either reverse micelles, water-in-oil microemulsions, or in vesicles. Enzymatic reactions in reverse micelles are an interesting case because the number of water molecules within the core of a reverse micelle is very small. As a result, enzymes in reverse micelles can behave differently than in bulk aqueous solutions, although choosing proper conditions for a correct comparison of the two systems is not trivial[28]. Several reports indicate that some enzymes — for example, chymotrypsin[80,81] or HRP[82,83] — appear to act more efficiently when confined in reverse micelles than in bulk solution[27,80]. However, there still is no clear and general understanding of this enzyme 'superactivity'. It has been suggested that it may be due to conformational changes[27,80], to the particular local concentrations of enzyme and substrates[73,80], or to the thermodynamic and kinetic properties of the confined water[82] that may lead to an altered hydration state of the active site of the enzyme[83]. It is likely that different effects play a role, depending on the type of enzyme, the chemical structures of the substrate, and the amphiphiles forming the reverse micelle.

A simple but important geometric consideration arises in the case of enzymatic reactions inside vesicles. The larger the volume of the vesicle, the more the enzyme approaches bulk behaviour. This is simply because the volume-to-surface ratio of the vesicles increases with increasing volume. Consider a large unilamellar vesicle (LUV) with a diameter of 100 nm and a bilayer membrane thickness of 5 nm, as well as a giant unilamellar vesicle (GUV) with a diameter of 50 μm and a bilayer membrane thickness of 5 nm. If the LUV is scaled up to a sphere with a diameter of 10 cm, it will have a sphere shell thickness of 5 mm. Conversely, if the GUV is scaled up by the same amount, it will also have a sphere shell thickness of 5 mm but its diameter will be 50 m. In comparison, if a small monomeric enzyme with a diameter of 5 nm is also scaled up by the same amount, it will have a size of 5 mm. This exercise shows that from the point of view of the enzyme, the situation in a GUV is nearly identical to the situation in a bulk solution. Nevertheless, if complex enzymatic cascade reactions with different types of enzyme and substrate at low concentrations are considered, then a volume-confinement as large as a few micrometres can still have significant effects due to stochastic fluctuations in the volume composition (extrinsic stochasticity), and therefore in the volume properties (that is, the local concentrations of the different enzymes and substrates). Extrinsic stochasticity may result in significant differences between individual enzyme-containing compartments with respect to enzymatic reaction efficiency (that is, rate of product formation and product distribution). Such stochastic effects are expected to be more substantial the smaller the vesicles are and the lower the solute concentration is[84]. Simple calculations show that spherical vesicles with a diameter of 10 μm (corresponding to an internal volume of 5.2×10^{-13} l) loaded with an enzyme at a concentration of 10 μM, contain on average 3.2×10^{6} enzymes. On the other hand, 100 nm spherical vesicles (internal volume of about 5.2×10^{-19} l) loaded with the same 10 μM enzyme solution will on average contain only three enzymes. This means that under loading conditions that lead to a Poisson distribution of the enzymes among a population of monodispersed vesicles, stochastic fluctuations are particularly relevant for populations of small vesicles[84]. It is worthwhile to remark that a Poisson distribution is theoretically expected for equally sized compartments only when the entrapment of molecules is solely driven by chance, that is, in the ideal case where solute–solute and solute–compartment boundary interactions are negligible. However, the difference becomes clearly more pronounced when different types of enzyme are loaded within such small vesicles, as both the total amount of enzyme molecules present in one vesicle and their relative ratio vary. This stochastic effect is expected to have significant consequences in the case of enzymatic cascade reactions, as there will be a large vesicle-to-vesicle variation[84]. For micrometre-sized volumes, one may expect that stochastic effects due to different enzyme loadings are less likely, although they have been observed experimentally in giant lipid vesicle-confined protein expression experiments involving more than 30 different enzymes[84–86]. These experiments indicate that the Poisson distribution based on an ideal solute behaviour is too simple for accurately describing more complex systems[87].

In spite of experimental difficulties with respect to the entrapment of enzymes in vesicles, a number of potential applications have been reported. One study is illustrated in Fig. 5a. Unilamellar phospholipid vesicles with a diameter of about 100 nm containing the degrading enzyme phosphotriesterase were prepared *in vitro* for *in vivo* application as a nanoreactor system that could circulate in the bloodstream after appropriate injection[88] and hydrolyse neurotoxic organophosphorous compounds. These partially hydrophobic organophosphorous compounds permeate into the vesicles where the enzymatic hydrolysis into non-toxic products takes place. The vesicles protect the enzyme from inactivation by blood components. The hydrolysis products may accumulate inside the vesicles or leak out into the blood circulation. The residence time of the vesicles in the blood circulation depends on the vesicle membrane composition. Clearance of the vesicles by the immune system is expected to be slowed down by the presence of poly(ethylene glycol) on the vesicle surface[89].

In another example, illustrated in Fig. 5b, GOD and catalase were co-entrapped inside 100-nm-diameter unilamellar phospholipid vesicles in which the membrane contained a porin transport protein[90]. The vesicles were covalently bound to chitosan gel beads and were used as an *in vitro* reactor for the conversion of D-glucose into glucono-δ-lactone, followed by the non-enzymatic hydrolysis into gluconic acid. The migration of D-glucose from the bulk aqueous solution into the vesicles was promoted by the channel protein OmpF. The catalase protected the activity of the oxidase as it catalysed the degradation of H_2O_2, a side product of the oxidation reaction that inactivates the oxidase[90].

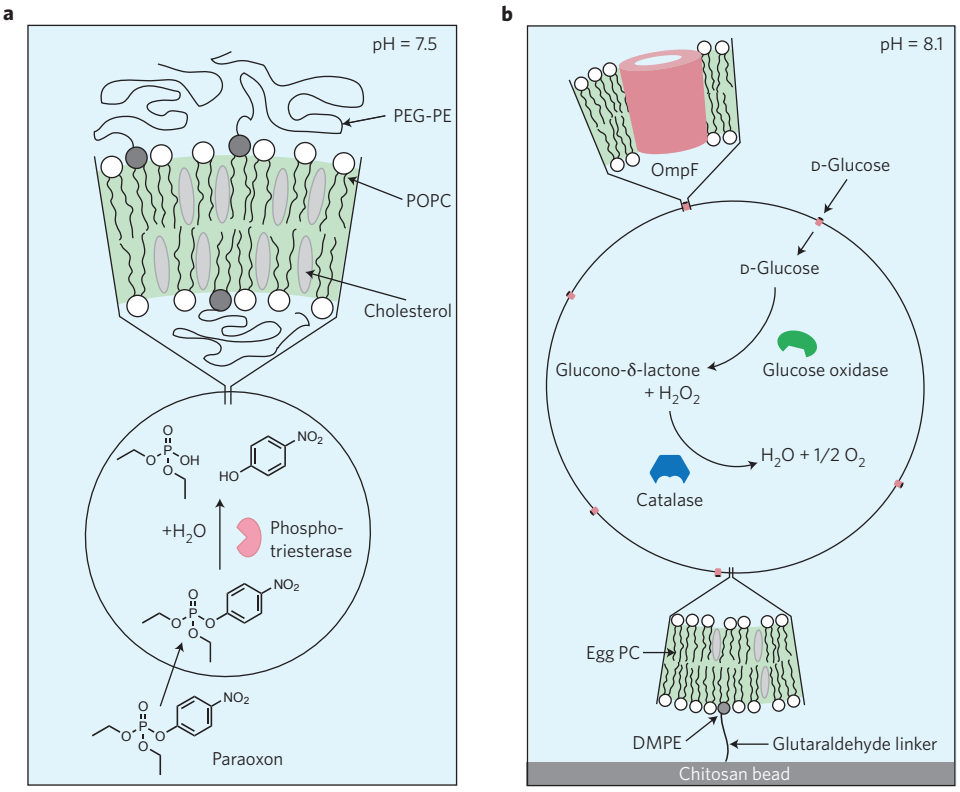

Figure 5 | Two examples of enzymatic reactions inside vesicles. a, Phospholipid-based vesicles containing an encapsulated enzyme for possible *in vivo* applications as detoxifying nanoreactors that circulate in the bloodstream[88]. Unilamellar vesicles with a diameter of about 100 nm were prepared from a mixture of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), the PEGylated phospholipid PEG-PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(poly(ethylene glycol)-2000)), and cholesterol. Cholesterol was included for increasing the stability of the vesicles; PEG served as a steric stabilizer for preventing rapid clearance by the immune system after intravenous injection. The vesicles contained in their aqueous interior a phosphotriesterase that catalysed the hydrolysis of paraoxon (a metabolite of parathion that is used as insecticide) into diethylphosphate and *p*-nitrophenol. Paraoxon is expected to translocate from the bulk medium into the vesicles' interior as it is partially hydrophobic and a relatively small molecule. **b**, Immobilized phospholipid-based vesicles containing two encapsulated enzymes for *in vitro* applications as enzymatic nanoreactors for the oxidation of D-glucose[90]. Unilamellar vesicles with a diameter of about 100 nm were prepared from a mixture of phosphatidylcholines from egg yolk (egg PC), DMPE (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine), and cholesterol. The vesicles contained the two enzymes glucose oxidase (GOD) and catalase and were immobilized on chitosan beads via glutaraldehyde linker molecules. The entrapped GOD catalysed the oxidation of D-glucose to glucono-δ-lactone and H_2O_2; H_2O_2 disproportionation to O_2 and H_2O was catalysed by the co-entrapped catalase. The transport of D-glucose from the bulk medium into the vesicles' interior was facilitated by the incorporation of the channel-forming protein OmpF into the membrane.

Vesicles are attractive systems to study enzymatic reactions mainly for two reasons: the large variability of their size and the possibility to design multiple vesicles systems. More specifically, depending on the preparation procedure, vesicle diameters can vary between ~30 nm (corresponding to a volume of 1.4×10^{-20} l) to more than 300 μm (volume = 1.4×10^{-8} l). Moreover, because vesicles do not spontaneously fuse or exchange their aqueous interiors, it is possible to create multi-vesicular systems in which large vesicles contain smaller vesicles in their interior. In principle, the chemical composition of any membrane in the system can also be changed by design. This large variability allows investigations of cascade reactions with enzymes that are located in different internal vesicles mimicking eukaryotic cells and their enzyme-specific organelles[91].

Potential drawbacks arising from volume-to-volume variations like in the case of vesicles, for example, two-enzyme-containing polymersomes (Fig. 6a)[92], are also expected for other compartment systems. In contrast, however, with specially designed protein capsules and mutant enzymes (Figs 6b and 7)[77,93], or viral capsid-like cages[94,95], a higher and/or more defined enzyme loading can be achieved. In these systems, compartment-to-compartment variations in terms of composition (extrinsic stochastic effects) would be minimal, even though the compartment size is small. An important consideration concerns how substrate molecules can reach the interior of the capsules from the exterior. Recent studies of proteinaceous prokaryotic microcompartments have shown that selective substrate permeability across a lipid-free compartment shell occurs through pore proteins[76,96]. If these pore proteins could be modified to make them selective to specific solutes, it would be possible to combine the intrinsic advantage of proteinaceous capsules created *in vivo* (a high or defined enzyme loading and little stochastic effects) with a selective shell permeability to make efficient nanoreactors for *in vitro* applications. For example, a 27-nm-sized phosphatase-containing protein capsule in which the substrate permeability across the capsule shell was controlled by the structure of the shell-forming proteins has been reported (Fig. 6b)[77]. It has also been shown that 58-nm-sized protein cages containing three different types of enzyme — at well-defined amounts and ratios — can be prepared through an elegant *in vivo* protein synthesis and assembly approach (Fig. 7)[93] that minimizes stochastic variations, a result difficult to achieve by *in vitro* methods. Such types of protein capsule can also be used for *in vivo* applications, in which compartmentalized enzymatic cascade reactions are designed to operate inside cells[94].

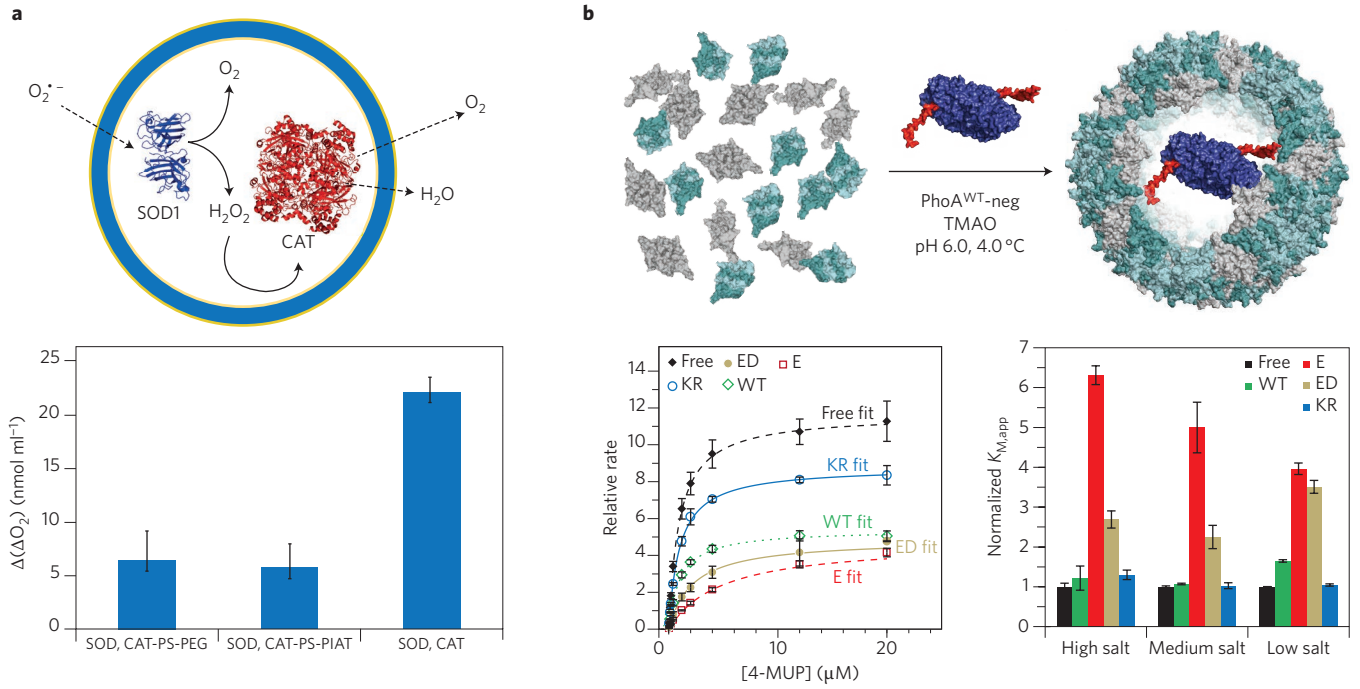

Figure 6 | Examples of volume-confined enzymatic reactions. a, Polymersome-confined enzymatic cascade reaction for the elimination of superoxide radical anions ($O_2^{\bullet-}$) inside the polymersomes by co-entrapped Cu/Zn superoxide dismutase (SOD1) and catalase (CAT)[92]. Top: Schematic of one single polymersome encapsulating SOD1 and CAT that cooperatively catalyse the conversion of $O_2^{\bullet-}$ into dioxygen and water. The superoxide anions, as well as O_2 and H_2O permeate the polymersome membrane. The membranes of the polymersome (diameter 120 nm) were composed of either poly(styrene)$_{40}$-b-poly-(L-isocyanoalanine(2-thiophen-3-yl-ethyl)amide)$_{50}$ (PS-PIAT), or poly(styrene)$_{160}$-b-poly(ethylene glycol)$_{24}$ (PS-PEG). Each polymersome was loaded with 58 ± 8 SOD1 and 1,270 ± 200 CAT molecules (PS-PEG), or 60 ± 10 SOD1 and 623 ± 186 CAT molecules (PS-PIAT), respectively. This exceptionally high entrapment yield indicates at least a partial binding of the enzymes to the block copolymers. Bottom: Experimental evidence for the functioning of the polymersome-confined SOD1–CAT systems, compared with the free enzymes at the same overall enzyme concentrations. The formation of O_2 was measured during the first 400 s after chemical $O_2^{\bullet-}$ production in the bulk solution. Although the free enzymes were more efficient, the experiments indicate that the polymersome-confined enzymatic cascade reactions also occurred. **b**, Protein capsule-confined enzymatic reaction, whereby the kinetic properties of the reaction are determined by the structure of the capsule shell[77]. Top: Schematic of the preparation of the protein capsules. The capsule (diameter 27 nm) was assembled *in vitro* from 180 proteins in the presence of trimethylamine oxide (TMAO) and *Escherichia coli* alkaline phosphatase, which carried a C-terminal sequence of 16 negatively charged amino acids (PhoA^{WT}-neg). This C-terminal sequence was used for the localization of the phosphatase inside the capsid on the basis of electrostatic interactions with positively charged residues on the capsid interior surface. The capsid shell contained pores with diameters of ~1.8 nm, allowing small molecules but not folded proteins to migrate inside the capsid. Bottom: The activity of the entrapped PhoA^{WT}-neg was measured for the wild-type (WT) capsid as well as for different capsid mutants (KR, ED, E) at pH = 8.0 with negatively charged 4-methylumbelliferyl phosphate (4-MUP). The determined apparent Michaelis constant, $K_{M,app}$, and the catalytic constant, k_{cat}, that is, the value of the relative rate at high substrate concentration, varied with the electrostatic properties of the capsid shell. The WT capsid had a negative charge around the pore periphery, but not inside the pore, resulting in a lower k_{cat} value than the free enzyme, but the $K_{M,app}$ values were about the same. The mutants E and ED had significant negative charge throughout the pores. This led to k_{cat} values of the enzyme inside the capsids of E and ED that were similar to — or slightly lower than — k_{cat} values of the enzyme inside the WT capsid; and to significantly higher $K_{M,app}$ values, independent of the salt concentration. For the mutants with positively charged pores (KR), k_{cat} of the entrapped enzyme was higher than for the enzyme inside the WT capsid; $K_{M,app}$ was about the same. Figures adapted with permission from: **a**, ref. 92, American Chemical Society; **b**, ref. 77, American Chemical Society.

Conclusions and perspective

Although the idea of immobilizing enzymes for *in vitro* applications is not new[1,11,97], challenges remain for a stable, efficient, and spatially controlled localization of active enzymes participating in cascade reactions on surfaces or within compartments. If one claims that a co-localization of enzymes involved in cascade reactions has a better performance than a proper reference system, convincing quantitative experimental evidence is required. This is, however, particularly challenging, for example, due to the difficulty in determining the exact amount of confined enzymes, either per surface area or per volume. It may well be that efficient substrate channelling may not be possible for *in vitro* enzymatic cascade reactions with enzymes that do not operate together *in vivo*.

Enzymatic fuel cells, devices that transform chemical energy in the form of organic waste into electrical energy through biochemical transformations, are a promising application for surface-confined enzymatic cascade reactions[98]. For such devices, the development of stable electrode-surface-confined sequential multi-step enzymatic reactions is one approach for obtaining high current and power densities. To achieve this goal, the preparation of an optimally nanostructured electrode, for example, a porous electrically conductive material with a large surface area[56], has to be combined with an optimal enzyme immobilization on this particular material, for example, by using DNA as a structural scaffold[42,98]. Whether enzymatic fuel cells will ever be available commercially depends on the general performances of the device and on the fabrication costs. Therefore, the development of simple, cheap, and reproducible enzyme immobilization methods remains an important goal, in addition to the large-scale production of cheap and stable enzymes, possibly optimized via *in vitro* evolution approaches[21].

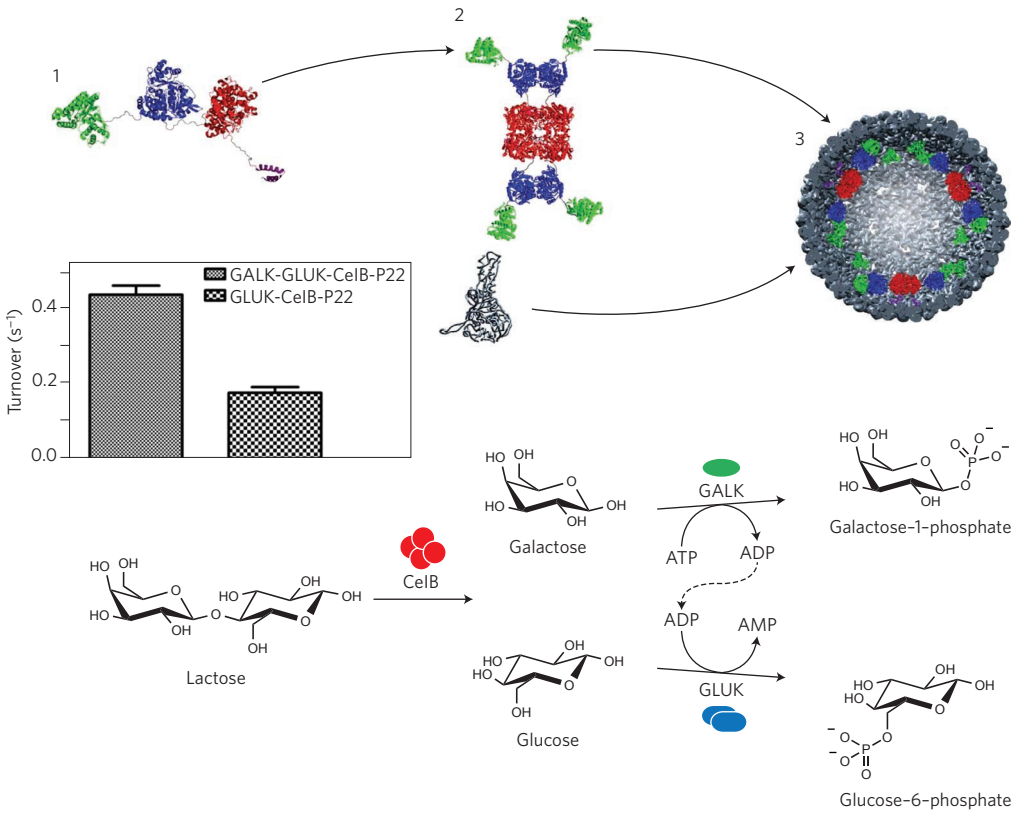

Figure 7 | Protein capsule-confined enzymatic reaction, whereby the three enzymes of a cascade reaction were encapsulated at a defined ratio. Top: Schematic representation of the preparation of a bacteriophage P22 capsid (diameter 58 nm) containing the three enzymes CelB (red, a tetrameric galactosidase), GLUK (blue, a dimeric ADP-dependant β-glucokinase), GALK (green, a monomeric ATP-dependant galactokinase), and a scaffold protein domain (SP, purple). (1) Genes were constructed for the expression of fusion proteins containing the three enzymes that were linked together through flexible spacer peptides. The coat protein (CP, grey) was expressed as well. (2) Assembly of the three covalently linked enzymes to satisfy the properties of the native enzymes as tetramer (CelB) or dimer (GLUK). (3) The capsid formation is facilitated by the interaction of the SP domains and CP subunits, leading to the encapsulation of the multienzyme gene product. The capsid consisted of 420 CP monomers that assembled with the aid of ~300 SP monomers. The three enzymes catalyse a three-step cascade reaction (bottom): (i) hydrolysis of lactose to galactose and glucose (catalysed by CelB); (ii) phosphorylation of galactose in the presence of ATP by GALK to yield galactose-1-phosphate and ADP; (iii) phosphorylation of glucose by GLUK with the formed ADP to yield glucose-6-phosphate and AMP. The graph shows experimental evidence for the successful co-encapsulation of the three enzymes by analysing the turnover of lactose to glucose-6-phosphate and galactose-1-phosphate on addition of lactose and ATP. The turnover with all three enzymes was significantly higher than with CelB and GLUK only. Figure adapted with permission from ref. 93, American Chemical Society.

With respect to applications of volume-confined enzymatic cascade reactions, vesicular compartments offer unique opportunities *in vitro* as well as *in vivo* as compartmentalized enzymatic nanoreactors. Artificial vesicles (formed from natural phospholipids or from fully synthetic block copolymers), lipidic bilayer-based organelles, and biological cells have obvious structural similarities. One may even think of using polymersomes containing entrapped enzymes as artificial organelles for incorporation into living cells[99–101]. At the moment, it is too early to conclude whether such a futuristic idea will ever lead to successful real applications. Critical research should be devoted to this goal. One specific challenge in this respect is the efficient loading of vesicles with enzymes, independent of whether the vesicles are prepared from amphiphilic block copolymers or phospholipids. One possible alternative approach could be the use of protein capsules as enzyme-containing compartment systems, characterized by a high, or well defined, and non-stochastic enzyme entrapment. An immediate need here is to develop methods to control the capsule shell permeability.

With respect to biological cells viewed as highly complex, dynamic, molecularly crowded, and evolvable compartment systems[4], in which all chemical transformations are driven by surface- and volume-confined enzymatic reactions, one active and fascinating field of research deals with the synthesis of cell-like model systems to study the key principles of biological cells[85,102]. This may also lead to the development of reasonable models of the possible precursors that are thought to have preceded the first cells at the origin of life ('protocells')[103].

In general, the majority of the often rather sophisticated systems involving confined enzymes are based on a large number of previous experiments from various extensive basic research studies in seemingly independent fields. This includes, but is not limited to, investigations of: (i) the self-assembly and guided assembly of amphiphiles to form vesicles, micelles, reverse micelles, or supported bilayers; (ii) the synthesis of fluorescent or fluorogenic molecules and the concomitant improvement of fluorescent detection systems, which enable the investigation of single-enzyme kinetics[104–106] and quantification at low substrate conversion; and (iii) kinetics and structural analysis of isolated enzymes. Basic research has created and will continue to create the ideal foundation for the development of new artificial systems, with important technological implications.

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Competing financial interests

The authors declare no competing financial interests.