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Enzyme co-localisation: Mechanisms and benefits

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ABSTRACT

Co-localising enzymes can drastically affect their properties, such as stability, specificity, and activity, thus influencing reaction kinetics. In this review, we present a brief overview of the main methods developed for enzyme immobilisation, co-localisation, and conjugation and discuss how and why they affect the enzyme properties. We also describe the effects emerging from bringing two sequential enzymes of a cascade reaction together, particularly if and when it speeds up reaction velocity. Furthermore, we discuss enzyme compartmentalisation, or clustering of several enzymes of a cascade, and present theoretical approaches developed to optimise synthetic enzyme clusters. We also point out the plenitude of open questions, which exist despite the enormous research effort channelled into understanding enzyme co-localisation.

1. Introduction

Enzyme-catalysed reactions are probably the most elegant and ubiquitous reactions on earth. Enzymes catalyse virtually all life-sustaining reactions and are of utmost importance in modern technology (Singh et al., 2016; Bhatia, 2018). In billions of years of life history, nature optimised enzymes and enzymatic cascades in the framework of metabolic pathways to function effectively and efficiently. Comprehending the mechanisms of how natural enzymatic cascades are organised and maintained in living organisms is vital for understanding life and for building and optimising synthetic enzymatic cascades in state-of-the-art biotechnological applications.

Since the last century, experimental evidence has been accumulated demonstrating that enzymes are not randomly distributed in living cells but locate themselves at specific places, on or within membranes, or form functionally relevant assemblies. In 1857, Bernard analysed sugar production by the liver and isolated a "glucose-forming material" that was later named glycogen (Bernard, 1857; Rybicka, 1996). A hundred years later, metabolic experiments suggested that glycogens are directly associated with enzymes involved in their metabolism, forming dynamic organelles or enzyme clusters, called by Scott and Still glycosomes (Rybicka, 1996; Scott and Still, 1968). Since then, several other enzyme assemblies have been identified and investigated (Chen and Silver, 2012; Schmitt and An, 2017), motivating theoretical and experimental work on understanding and optimising enzyme clusters (Castellana et al., 2014; Tsitkov and Hess, 2019; Gopich, 2021).

In the early 1950s, kinetic experiments suggested a direct transfer of intermediary metabolites between two sequential enzymes of a cascade (Cori et al., 1950). This and other observations (Srivastava and Bernhard, 1984, 1986) led to the concept of substrate channelling, occurring either due to enzyme proximity or within static or dynamic enzymatic complexes, called multifunctional enzymes (Miles et al., 1999) and metabolons (Srere, 1985), respectively. These works caused heated discussions in the literature (Srivastava and Bernhard, 1987; Chock and Gutfreund, 1988; Srivastava et al., 1989; Wu et al., 1991) on the benefits and significance of substrate channelling and enzyme proximity *in vivo*, which is still a subject of debates (Idan and Hess, 2012, 2013a; Wheeldon et al., 2016; Poshyvailo et al., 2017; Sweetlove and Fernie, 2018; Kuzmak et al., 2019). In biotechnology, much effort has been directed towards developing novel methods to bring and keep enzymes together, hoping to speed up the reactions by decreasing the diffusion path of intermediates (Wheeldon et al., 2016; Zhang, 2011; Fu et al., 2012, 2014).

Already in the early 1970s, it had become clear (Katchalski et al., 1971) that localising or immobilising single (*i.e.*, not-conjugated) enzymes could alter their properties, suggesting convenient tools to control and enhance enzymatic reactions. Since that time and until now, methods have been

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developed to localize, immobilise and conjugate enzymes with the overarching goal to optimise single and cascade reactions. In this review, we provide a brief overview of relevant (co)-immobilisation methods (Section 2) and discuss how and why immobilising enzymes affects reaction kinetics (Section 3). We then examine the effects and benefits of the spatial proximity of two sequential enzymes (Section 4), paying particular attention to reaction velocity, a subject of recent controversies (Idan and Hess, 2012, 2013a; Wheeldon et al., 2016; Poshyvailo et al., 2017; Sweetlove and Fernie, 2018; Kuzmak et al., 2019). We also discuss enzyme clustering or compartmentalisation, focusing on natural enzyme clusters found in living cells and analysing models introduced to optimise clusters of synthetic enzymatic cascades (Section 5). We finish by pointing out the abundance of open questions (Section 6).

2. Methods of enzyme (co)-immobilisation and conjugation

In biocatalysis and synthetic chemistry, enzymes are often immobilised to yield enzyme formulations that can be more conveniently handled, stored and reused. Conventionally, enzyme immobilisates are prepared by adsorption or covalent coupling of purified enzymes to organic or inorganic carrier materials (Sheldon et al., 2021; Sheldon and van Pelt, 2013). In addition to these more conventional methods, also different nanomaterials like DNA nanocarriers, carbon nanotubes, nanoparticles and nanofibres have more recently been used for the immobilisation of enzymes (Bilal et al., 2019a, 2019b, 2021), often employing different chemistries for target conjugation (Bilal et al., 2018, 2020). While these methods are commonly performed *in vitro*, with enzyme production and immobilisation being decoupled, recent additions to the enzyme immobilisation toolbox utilised synthetic biology to generate enzyme immobilisates *in vivo* using molecular biological means, facilitating enzyme production and immobilisation in one step (Sheldon et al., 2021; Bilal et al., 2021; Bilal et al., 2019a; Ölçücü et al., 2021). Moreover, due to the increasing importance of cascade reactions, more and more methods were developed that facilitate the co-immobilisation of enzymes on or within suitable organic, inorganic or even biologically produced supports/carrier materials (Ren et al., 2019; Xu et al., 2020). In addition, spatial co-localisation (without immobilisation in a strict sense) can also be achieved by molecular biological fusion of multiple enzymes using suitable linkers (Ren et al., 2019). This sections will describe selected methods appropriate for enzyme co-immobilisation. A complete review of all available methods or even all alternatives to the discussed methods exceeds the scope of the present contribution. An excellent overview, though, is provided by a number of recent reviews (Bilal et al., 2021; Bilal et al., 2019a; Ölçücü et al., 2021; Ren et al., 2019; Xu et al., 2020; Bilal and Iqbal, 2019; Bi é et al., 2227).

2.1. Multi-enzyme cascades by gene fusion

Arguably the simplest way of bringing multiple enzymes together in spatial proximity is genetic fusion (Aalbers and Fraaije, 2019). To generate such artificial fusion enzymes, the genes encoding for the corresponding enzymes are fused using molecular biological methods, by in-frame fusion of the corresponding target genes and by omitting a stop codon between them. To enable correct folding of the fused enzymes,

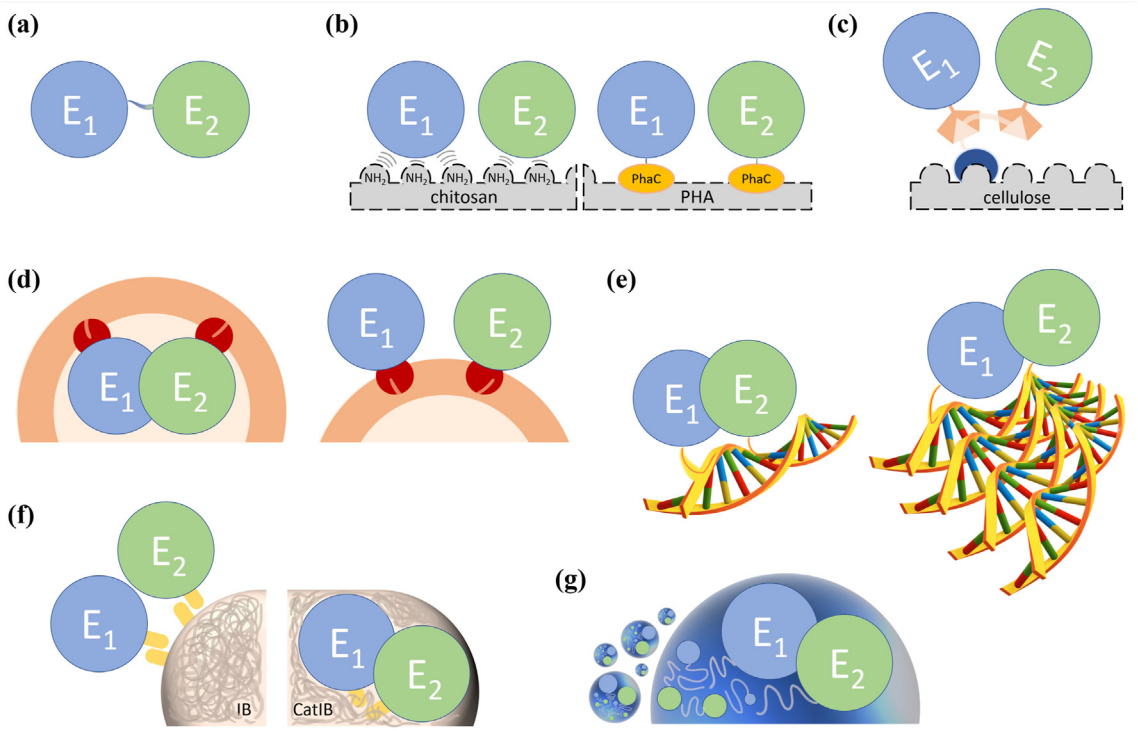

Fig. 1. Methods for enzyme co-immobilisation. a, Enzyme cascade setup by fusion of two enzyme encoding genes via a linker segment. **b**, Co-immobilisation on chitosan and polyhydroxyalkanoates(PHAs) *e.g.* via enzymes fused with PhaC. **c**, Co-immobilisation of enzymes on cellulose using a scaffoldin consisting of a cellulose-binding domain (blue) fused with several cohesin domains (light salmon triangles). Enzymes are co-localised to the scaffoldin using specific dockerin domains fused to the enzymes. **d**, Co-immobilisation of enzymes within (left side) and on the surface (right side) of virus-like particles (VLPs). **e**, Enzyme co-immobilisation on a linear DNA strand (left side) and a DNA origami structure (right side). Immobilisation is achieved *e.g.* by attaching suitable single-stranded oligonucleotides to the enzymes, which enables hybridisation with the corresponding DNA-scaffold. **f**, Enzyme co-immobilisation using inclusion body (IB) display and catalytically-active inclusion bodies (CatIB). IB-display is achieved using complementary coiled-coil domains as interaction modules (yellow). CatIB-formation is induced by molecular biological fusion of CatIB-inducing tags (yellow), which facilitate the entrapment of correctly-folded target protein within the largely unfolded IB matrix. **g**, Enzyme co-immobilisation by liquid-liquid phase separation (LLPS). LLPS droplet formation is *e.g.* achieved by directly fusing intrinsically disordered proteins modules (light grey) to the target enzymes, which allows sequestration to synthetic membraneless organelles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

linker sequences are commonly added between the fusion partners (Fig. 1a). When multiple genes are fused to produce multi-enzyme gene fusions, both the order in which the genes are present in the genetic fusion as well as the type of linker sequence that links the partners, needs to be considered. The selection of suitable linkers can be a complicated process and is often guided by intuition and trial and error. Nevertheless, linker elements are indispensable for the generation of fusion proteins, since the omission of linkers may result in misfolding of the fusion protein, low expression levels or loss or reduction of activity (Chen et al., 2013). In the past, general building principles for linker design have been derived from the analysis of interdomain linkers of naturally occurring multi-domain proteins (Argos, 1990; George and Heringa, 2002; Williamson, 1994). Similarly, various empirically derived linkers with different lengths, sequences, and structures were designed and utilised, encompassing, *e.g.*, flexible, rigid and cleavable linkers (excellently reviewed by Chen et al. (2013)). In addition, also a couple of bioinformatics tools and databases exist that can ease the design of linkers for fusion proteins. These include, for example, the LINKER tool (Crasto and Feng, 2000) and the linker database (www.ibi.vu.nl/programs/linkerdbwww/), which both rely on identifying loop structures in X-ray or NMR solution structures. For the design of fusion proteins for co-immobilisation purposes, rigid and flexible linkers appear better suited than cleavable linkers. In addition, some enzymes, due to structural reasons, might not accept the fusion of another partner at either N- or C-terminus (Aalbers and Fraaije, 2017), while in other cases the order of conjugation does not play a significant role (Torres Pazmino et al., 2008). Since the invention of the concept in the 70ies and 80ies (Bülow and Mosbach, 1987; Yourno et al., 1970), many successful examples of artificial fusion enzymes have been described (reviewed by Aalbers and Fraaije (2017); Conrado et al. (2008); Yang et al. (2016)), and the method seems particularly well suited to multi-enzyme cascades that require effective channeling of cofactors or co-substrates (Aalbers and Fraaije, 2019).

2.2. Scaffolding of multiple enzymes

In nature, (multiple) enzymes are often co-localized, or brought in spatial proximity, with the help of so-called scaffolding biomolecules, which, from the application point of view, can be classified into synthetic and natural macromolecular scaffolds (Cavalcante et al., 2021; Gad and Ayakar, 2021). Binding of the target enzymes to the scaffold often relies on non-covalent specific interactions utilizing specific binding domains, but can also be realized by using systems/methods for covalent conjugation using Halotag and Spytag systems (Gad and Ayakar, 2021; Hoelzel and Zhang, 2020) (Fig. 1b). Synthetic scaffolds that have been used for enzyme immobilisation include, *e.g.*, polystyrene nanospheres, metal-organic frameworks, multi-walled carbon nanotubes and chitosan magnetic particles (Cavalcante et al., 2021; Gad and Ayakar, 2021). Given the diversity of polymeric bio-macromolecules, it is not surprising that a multitude of different biological supramolecular scaffolds have been used for (multi)enzyme immobilisation. In the following, we describe a few of the more widely employed “natural” scaffolding systems.

2.2.1. Sugar-based polymers and polyhydroxyalkanoates

Many polysaccharides have traditionally been employed for enzyme immobilisation, including cellulose, chitosan, alginate and derivatives thereof (Xingyi et al., 2021). In particular cellulose and chitosan, the two most abundant biopolymeric materials, have been used extensively. While for the immobilisation on cellulose, often cellulose binding domains (CBDs) are employed (Kopka et al., 2015), the immobilisation on chitosan is possible via different mechanisms, relying on covalent coupling, hydrogen-bonding, electrostatic and Van der-Waals interactions mostly mediated by the primary amino groups of the chitosan monomer (Tapdigov, 2021) (Fig. 1b). In addition to those highly polar sugar-based polymers, also less polar biopolyesters, such as polyhydroxyalkanoates (PHAs), which are synthesized by certain Gram-positive and Gram-negative bacteria (Schlegel et al., 1961), can be used for enzyme immobilisation (Ölçücü et al., 2021; Rehm et al., 2016). Under suitable cultivation conditions, PHAs can make up a significant fraction of the cell dry weight accumulating in the cytoplasm as spherical hydrophobic particles, which are surrounded by a protein layer. The latter is constituted by PHA-associated proteins like the polyhydroxybutyrate (PHB) synthase PhaC, different phasins and other proteins (Barnard and Sanders, 1989; Gerngross et al., 1993). Target proteins are then immobilised to the PHA core by fusion to PHA-associated proteins such as PhaC and PhaF (Ölçücü et al., 2021; Rehm et al., 2016) (Fig. 1b). While these carriers have been used more often for the immobilisation of single enzymes, examples for enzyme co-immobilisation have also been reported (Gur et al., 2018; Li et al., 2019).

2.2.2. Synthetic cellulosomes and virus-like particles

Apart from natural biopolymers, also proteins have been used for enzyme (co)-immobilisation. One approach is to use so-called protein scaffolds to which modified proteins can bind orthogonally using specific binding domains. In the first report by You et al. (2012), cohesin and dockerin domains, derived from a naturally occurring scaffold protein of the cellulosome, were utilised for the co-immobilisation of multiple enzymes. Three enzymes were bound via attached dockerin domains to a mini scaffoldin consisting of a CBD and three cohesion domains utilizing specific dockerin/cohesin interactions. In turn, the CBD domain was then utilised to localize the scaffolded multi-enzyme cascade to regenerated amorphous cellulose (You et al., 2012) (Fig. 1c). Another multi-protein complex that can be used for enzyme (co)-immobilisation are virus-like particles (VLP), which facilitates enzyme immobilisation within or on the surface of the VLP particle (Fig. 1d). VLPs consist of one or more viral capsid proteins and self-assemble into structures resembling empty virus envelopes (Ölçücü et al., 2021; Seo and Schmidt-Dannert, 2021), devoid of genetic material, and hence are non-replicating and non-infectious. Immobilisation is possible by relying on various strategies, with the target protein(s) being bound non-covalently or covalently to either the surface of the virus capsid or within its interior (Ölçücü et al., 2021). Non-covalent encapsulation of protein(s) within VLPs is hereby possible by using interacting domains like E and K coils, which, when fused to the viral capsid protein and the target suitably, allow target encapsulation within VLPs (Minten et al., 2009). In addition to relying on non-covalent interactions, target proteins can also be directly fused to the viral capsid protein to allow for VLP encapsulation (Rurup et al., 2014). The decoration of the VLP surface, in principle, is possible using the same methods. Target proteins have been displayed on the surface of VLPs by relying on non-covalent E and K coil interactions (Servid et al., 2013), by direct fusion to a viral capsid protein (Servid et al., 2013) or by using sortase-catalysed ligation (Patterson et al., 2017). VLPs have been used for the *in vivo* and *in vitro* co-immobilisation of enzymes (Giessen and Silver, 2016; Patterson et al., 2014).

2.2.3. DNA and DNA origami

Already since the 1990ies, proteins have been immobilised to DNA-macromolecules producing supramolecular protein-DNA bioconjugates (Niemeyer et al., 1994) or linear protein-DNA-assemblies (Niemeyer et al., 2002). More recently, designer DNA-macromolecules, self--assembling into defined 2- and 3-dimensional shapes (DNA origamis (Rothemund, 2006)), have been used for the purpose of enzyme (co)-immobilisation (Fu et al., 2012, 2014; Ke et al., 2016; Ngo et al., 2016; Timm and Niemeyer, 2015; Wilner et al., 2009) (Fig. 1e). Hereby, a variety of methods have been reported for loading cargo, to enable binding of proteins to DNA. Traditionally, DNA hybridisation was utilised, where the target cargo is chemically functionalised with single-stranded oligonucleotides that can directly interact with the DNA molecule. Further examples include the use of reversible antibody-antigen interactions, aptamer binding, utilization of

biotin–streptavidin interactions, self-labeling protein tags or the use of DNA-binding proteins (Hernandez-Garcia, 2021).

2.3. Inclusion bodies and catalytically-active inclusion bodies

Another biomaterial that has in recent years been utilised for enzyme (co)-immobilisation is bacterial inclusion bodies (IBs). Commonly, IBs are known as inactive protein waste materials that accumulate in, *e.g.*, bacteria, due to the strong heterologous overexpression of recombinant genes. They are typically assumed to represent unfolded polypeptides of the recombinant protein, which aggregate into dense water-insoluble particles inside the bacterial cytoplasm (Baneyx and Mujacic, 2004; Rinas et al., 2017). In the past, IBs have been utilised as protein scaffolds to display single or multiple enzymes to realise enzyme cascades. Cargo proteins can hereby be loaded to the IBs by using scaffold and target fused coiled-coils (E/K coiled-coil, leucine zippers) (Choi et al., 2011; Han et al., 2017; Spieler et al., 2016) (Fig. 1f). More recently, so-called catalytically-active inclusion bodies (CatIBs), which, in contrast to conventional IBs, retain a certain degree of catalytic activity, have been used for the enzyme (co)-immobilisation (Ölçücü et al., 2021; Rinas et al., 2017; Jäger et al., 2018, 2019, 2020; Krauss et al., 2017). CatIB formation is hereby achieved by fusion of CatIB-formation inducing tags, which can have variable structure (Krauss et al., 2017) to a given target enzyme. Co-immobilisation is then achieved by co-expressing multiple CatIB-tag tagged target enzymes within the same expression host yield Co-CatIBs (Jäger et al., 2018).

2.4. Liquid-liquid phase separated synthetic organelles

The most recent addition to the toolbox of systems for enzyme co-immobilisation are liquid-liquid phase separated (LLPS) synthetic membraneless organelles or biomolecular condensates (Ölçücü et al., 2021; Krauss, 2021). They usually form from highly concentrated and enriched biomolecules such as proteins and nucleic acids, yielding micron-sized liquid-like droplets within the cytoplasm (Alberti et al., 2019), whereby condensation is often mediated either by direct fusion of intrinsically disordered proteins/protein domains (IDP modules) or by fusion of the IDP modules with suitable interaction tags, to which the cargo proteins are then recruited (Fig. 1g). While still in its infancy for metabolic engineering and biocatalysis, LLPS-based methods have already been successfully used for the sequestration of multiple metabolic enzymes or for the dynamic rewiring of metabolic pathways (Peeples and Rosen, 2021; Zhao et al., 2019).

2.5. Limitations of the co-immobilisation methods

In the following, we briefly outline some potential limitations of each method and highlight the advantages important for the application. The direct fusion of genes requires only minimal protein engineering effort and is relatively straightforward to implement, despite some freedom regarding the choice of the fusion site and the employed linker. Another critical factor that limits the applicability of this strategy is the oligomerisation state of the target enzymes. While monomeric enzymes should be easier to fuse, dimers or higher-order functional oligomers, due to structural constraints, might be more complicated to work with (Bülow, 1991). Since this method does not directly yield enzyme immobilisates, further carrier-based approaches are needed when enzyme reuse is necessary for application. All methods relying on the scaffolding of enzymes on different carrier materials, like sugar-, polyhydroxyalkanoate-, protein- or DNA-based carriers face similar limitations, where the methods that rely on non-covalent coupling might suffer from leaching of the bound enzymes (Sheldon and van Pelt, 2013).

Similarly, certain carrier materials, like chitosan, might be more prone to mechanical destruction, *i.e.*, due to shearing forces in a bioreactor (Szymańska and Winnicka, 2015), while others, like DNA (nanocarriers), might be too expensive to produce on a large scale and to meet the requirements of large-scale industrial applications (Xu et al., 2020). In addition, VLPs, due to their small size, are more challenging to prepare, requiring ultracentrifugation or size-exclusion chromatography (Steppert et al., 2017), which is hardly applicable on a large industrial scale. While CatIBs are very easy to prepare by simple centrifugation, they often suffer from low activities since a sizeable fraction of the immobilisate consists of unfolded target protein (Jäger et al., 2019). Along the same lines, for CatIBs, as also for all immobilisates where the target enzymes are immobilised within a carrier matrix, the diffusional limitation is an issue since substrates and products have to cross a phase boundary to reach the enzymes or be released after turnover (Ölçücü et al., 2021).

Thus, like virtually any method, the outlined methods for the enzyme (co)-immobilisation have advantages and disadvantages. Their realisation often requires a case-to-case optimisation, making it challenging to formulate generic rules for the selection of the best-suited immobilisation method.

3. How co-localisation affects an enzyme and its environment

Understanding had crystallised in the 70ies (Katchalski et al., 1971; Douzou and Maurel, 1977; Klibanov, 1983) that immobilising or co-localising enzymes could lead to changes in enzymes' local environment and conformation that affect their kinetics. The immobilisation can have various consequences for enzymes ranging from inhibition and inactivation to enhanced stability and increased activity. Here, we describe the main (or most frequent) effects related to enzyme immobilisation and refer the interested readers to several more detailed biochemical reviews (Secundo, 2013; Rodrigues et al., 2013; Zhang et al., 2015; Lancaster et al., 2018; Wahab et al., 2020; Abdallah et al., 2022).

3.1. Enzyme activity

Enzyme co-localisation can affect the enzyme activity positively or negatively, depending on the changes in the local environment and enzyme conformation. The enhanced kinetics in the case of sequential enzymes have sometimes been mistakenly attributed to enzymes' proximity (Fu et al., 2012; Wilner et al., 2009) (cf. Section 4). A classic example is the speedup of the GOx-HRP reaction cascade by immobilising GOx and HRP on a DNA origami (Fu et al., 2012). Zhang et al. (2016) instead conjugated the two enzymes with a small molecular linker and observed no enhancement, even in the presence of catalase, an enzyme competing with HRP for hydrogen peroxide. These authors showed that the enhanced reaction velocity reported by Fu et al. (2012) could be due to a decreased pH at the DNA origami, creating favourable reaction conditions for HRP. Kuzmak et al. (2019) estimated that a significant enhancement could only be achieved for extremely high (millimolar) concentrations of catalase. Such high concentrations are unusual and have not been used in these experiments (cf. Section 4.2 and Fig. 4).

As in the above example, pH and ionic strength are frequently the main factors contributing to the altered enzyme activity (Fig. 2a). When an enzyme is localised on a charged material, such as a DNA nanostructure, the unscreened charge can alter the local pH. For an aqueous solution with no salt, pH at a charged surface can be estimated from the Boltzmann distribution of dissociated protons, $[H^+] = [H^+]_{bulk} e^{-e\psi/k_B T}$, where ψ is the electrostatic potential, e is the proton charge, k_B is the Boltzmann constant, T is temperature, and $[H^+]_{bulk}$ is the bulk concentration of protons corresponding to $\psi = 0$. Taking the logarithm of the Boltzmann distribution gives

$$pH_{surf} \approx pH_{bulk} + \frac{0.43 e \psi_{surf}}{k_B T}, \quad (1)$$

where pH_{surf} and pH_{bulk} are pH's at the surface and in the bulk, and ψ_{surf} the surface potential. Since DNA is negatively charged, it creates a negative surface potential that increases the concentration of H^+ at the surface and hence reduces the pH locally compared to its bulk value. By

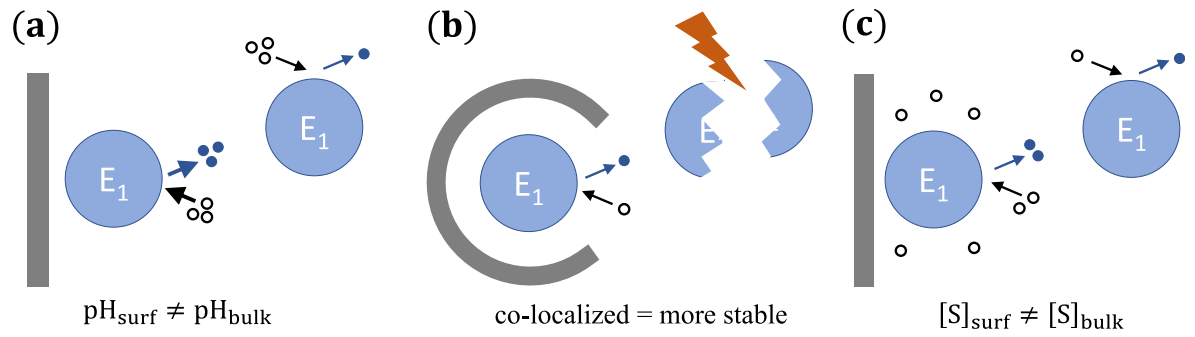

Fig. 2. Effect of co-localisation on enzyme's micro-environment. a, Enzyme immobilisation can change local pH or ionic strength, affecting the enzyme kinetics. **b**, Localising enzymes, *e.g.* in DNA nanocages, can protect them from degradation or attacks of destabilising agents from the bulk solutions. **c**, Enzyme localisation may increase or decrease the local concentration of substrates, altering the apparent reaction rate or/and affecting enzyme's specificity.

solving the Poisson-Boltzmann equation to find ψ_{surf} at a DNA origami, Zhang et al. (2016) estimated that pH could be one-two units lower than in the bulk, significant to increase the HRP activity few folds.

There are other reports in the literature documenting the enhanced kinetics of localised enzymes, often due to the altered pH (Abdallah et al., 2022; Zhang et al., 2017; Xiong et al., 2020). For instance, Zhang et al. (2017) conjugated Cytochrome C (CytC) and a negatively charged polyelectrolyte, lowering the local pH to values close to CytC's optimal acidic condition. The conjugation allowed the optimal operation of CytC together with D-amino acid oxidase enzyme, which is active under alkaline conditions.

In recent work, Lin et al. (2021) localised two different enzymes on DNA nanostructures and observed enhanced kinetics of both enzymes, despite their different optimal pHs. The origin of the enhancements remains unclear, but these experiments demonstrate that the change in the local pH is not the only or deciding factor affecting an enzyme's activity.

3.2. Enzyme stability

Experimental studies show that conjugating or encapsulating enzymes into DNA nanostructures often have a positive effect on their stability. The enhanced stability is due to the physical protection from destabilising agents or because of a favourable local environment (Fig. 2b). For instance, Zhao et al. (2016) demonstrated that nanocaging could protect enzymes from proteolysis. Encapsulating GOx-HRP complexes into nanocages increased their stability against trypsin digestion, retaining about 95% of the GOx-HRP initial activity after incubation with trypsin; free GOx/HRP enzymes retained only 50% of their activity after incubation (Zhao et al., 2016). Collins et al. (2017) grew long dsDNA 'hairs' on the surface of HRP and reported an enhanced stability against various denaturation conditions, such as freeze-thaw cycling and thermal incubations. For instance, the HRP-dsDNA complexes retained about 50% of their activity when stored for 10 weeks at room temperature, against only 20% of free HRPs (Collins et al., 2017).

3.3. Enzyme specificity

Similarly to enzymes' activity and stability, the immobilisation or conjugation of enzymes can affect their specificity if they operate on different types of substrates. Frequently, this effect is due to electrostatic interactions and occurs for differently charged substrates (You et al., 2006; Azuma et al., 2018). The specificity can be controlled, *e.g.*, by immobilising enzymes in polyelectrolyte matrices, protein cages, or onto DNA structures. For instance, Azuma et al. (2018) encapsulated a protease in a negatively charged protein and observed that the inherent protease specificity was inverted by about 480 times towards positively charged peptides. Recently, Coscolín et al. (2018) demonstrated how enzyme specificity could be tuned by immobilising enzymes in polymeric carriers with different pore sizes and chemical properties.

3.4. Enzyme conformation

Apart from changing an enzyme's environment, (co-)immobilisation may change the enzyme's conformation and dynamical properties, which also affects its stability and activity. For instance, immobilising an enzyme on a charged surface may cause the attraction with ionic groups of the enzyme's surface, leading to its distortion, rendering it inactive (Secundo, 2013; Malinin et al., 2011). Similarly, adsorbing an enzyme on a hydrophobic surface may lead to dehydration, causing changes in an enzyme's structure, often reducing the enzyme activity (Secundo, 2013; Czeslik et al., 2002). However, Koutsopoulos et al. (2005) observed increased heat stability of endo-β-1,3-glucanase when immobilised on a hydrophobic support (Teflon) despite some minor structural distortions.

Attaching an enzyme to a surface may limit its flexibility and hinder the movement of the backbone and/or side chains, often leading to reduced enzymatic activity (Secundo, 2013). The opposite is also possible, however. In particular, lipases showed enhanced activity on hydrophobic surfaces because such surfaces favour their open (activated) conformation (Mingarro et al., 1996).

3.5. Substrate concentration

Assuming the Michaelis-Menten kinetics, velocity of an enzyme-catalysed reaction is

$$v = \frac{k_{cat}[S]}{K_M + [S]} \approx \frac{k_{cat}}{K_M}[S], \tag{2}$$

where k_{cat} is the turnover number, K_M the Michaelis-Menten constant, and [S] the concentration of enzyme's substrates. The second equation is an approximation for low substrate concentrations, $[S] \ll K_M$, applicable, in particular, to initial rates. Localising enzymes on a surface may alter the local conditions for substrates, *e.g.*, due to surface-substrate interactions, so that their concentration at the surface, $[S]_{surf}$, differs from the bulk concentration, $[S]_{bulk}$ (Fig. 2c). Then the reaction velocity is

$$v \approx \frac{k_{cat}}{K_M^{app}}[S]_{bulk}, \tag{3}$$

where $K_M^{app} = K_M[S]_{bulk}/[S]_{surf}$ is the apparent Michaelis-Menten constant. Thus, the locally enhanced (reduced) substrate concentration can be viewed as the reduced (enhanced) apparent Michaelis-Menten constant.

Reduction of the apparent K_M has been achieved in several studies, reported in particular by the Wheeldon group. For instance, Gao et al. (2015) conjugated HRP with 20 base pair double-stranded DNA and observed a few-fold reduced K_M^{app}, arguably due to the locally increased concentration of substrates tetramethylbenzidine (TMB) and hydrogen peroxide. Molecular dynamic simulations showed that van der Waals interactions drove this enhancement. Indeed, simulating the same systems without van der Waals attraction (*i.e.*, only with steric repulsions)

led to no enhancement (Gao et al., 2015). In another work, Gao et al. (2016) conjugated aldo-keto reductase (AdhD) and horseradish peroxidase (HRP) with various DNA structures and also observed few-fold decreases in the apparent Michaelis-Menten constants. In more recent work, Lang et al. (2020) immobilised phosphotriesterase (PTE) on a DNA scaffold and observed an 11-fold decrease in the apparent Michaelis-Menten constant of organophosphates hydrolysis, which is critical to combat organophosphates poisoning, the cause of more than 100,000 deaths a year worldwide (Eddleston, 2019).

An interesting effect has recently been reported by Dinh et al. (2020). These authors co-localized several enzymes of the same type on a DNA scaffold and observed faster kinetics when enzymes were placed closer to each other. Dinh et al. (2020) explained this effect by entropic forces (also called depletion forces (Götzelmann et al., 1998)), which increased the local substrate concentration in the packed configuration, thus enhancing the overall reaction velocity.

4. Proximity of two sequential enzymes

This section discusses how the spatial proximity of sequential enzymes affects the overall kinetics of a cascade reaction. For simplicity, we consider a tandem reaction, $S \overset{E_1}{\rightarrow} I \overset{E_2}{\rightarrow} P$, and assume the standard Michaelis-Menten kinetics. We note that conjugation or immobilisation can generally change the reaction rates of both enzymes (Section 3), and these changes may depend on the method chosen for co-localisation. To focus on the effects due to enzyme proximity as such, we assume here that (hypothetically) the kinetics of both enzymes are not altered by bringing them together.

4.1. Enhancement of reaction velocity

In a steady-state, proximity of sequential enzymes cannot speed up a cascade reaction if the intermediates do not degrade or are not sequestered by competing enzymes (Idan and Hess, 2012, 2013a, 2013b; Wheeldon et al., 2016; Kuzmak et al., 2019). The rates of forming intermediates and their conversion into the product must be equal in steady-state; otherwise, the concentration of intermediates would change over time. This conservation law is mathematically rigorous and does not depend on enzymes' position, *i.e.*, bringing enzymes closer to each other can not affect the overall reaction velocity. This conclusion is independent of reaction rates, diffusion constants, and other factors. Nevertheless, we recall that enzyme proximity has been mistakenly used to explain enhanced kinetics of co-localised sequential enzymes (Fu et al., 2012; Wilner et al., 2009) (see Section 3.1 for an example).

However, enzyme proximity can lead to temporal speedup of a cascade reaction due to a locally enhanced concentration of intermediates (Fig. 3a). Consider, as an example, that initially, there are no intermediary metabolites in a system. As the reaction commences, the concentration of intermediates starts to increase. During this stage, clearly, the highest concentration is in the vicinity of the first enzyme of a cascade, hence bringing two sequential enzymes together should enhance reaction velocity (Fig. 3b). The enhancement can be estimated by using the following equation (Kuzmak et al., 2019)

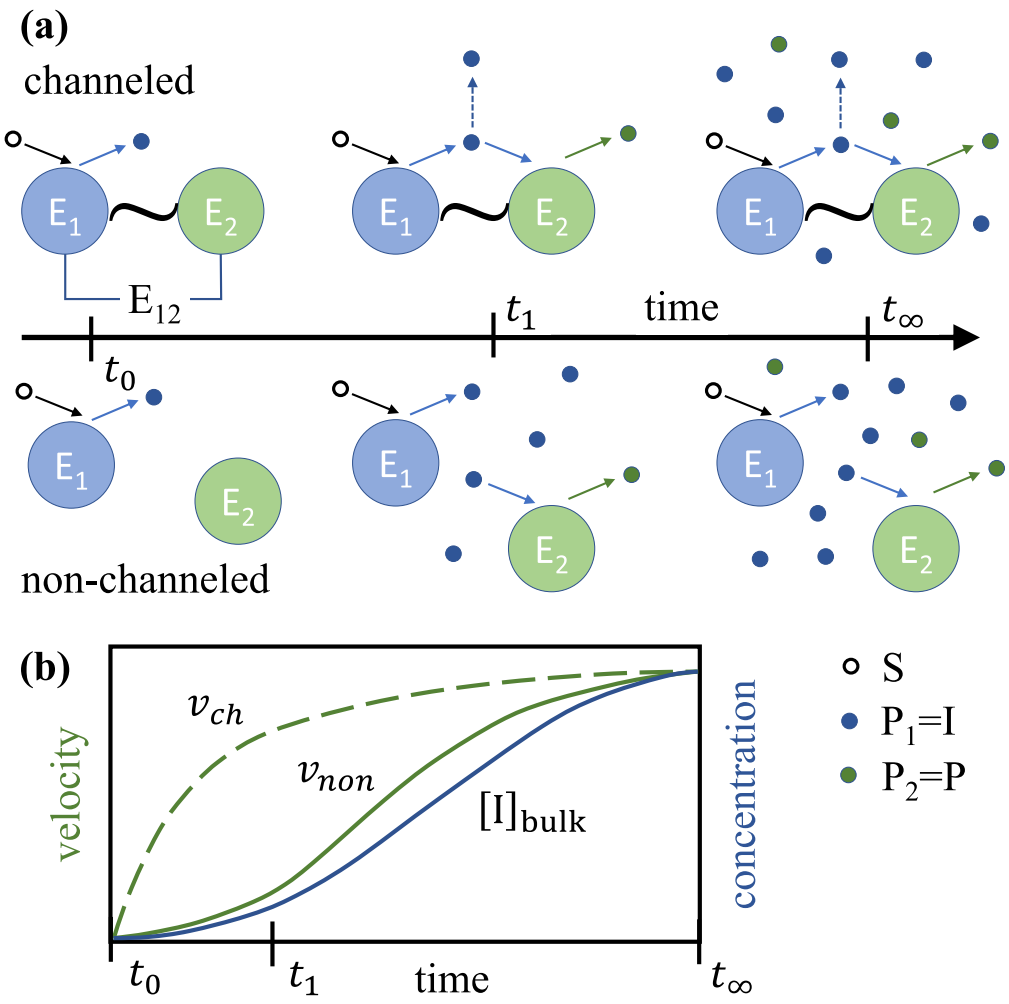

Fig. 3. Enzyme proximity and reaction velocity. a, Schematics of the evolution of metabolite concentrations in the channeled and non-channeled tandem reactions. $E_{12} = E_1 + E_2$ is an enzyme complex, $P_1 = I$ is an intermediary metabolite and $P_2 = P$ is the product. Note that proximity channeling is leaky. **b**, Enzyme proximity can enhance reaction velocity only temporary, before the bulk concentration of intermediates reaches its steady-state value. The enhancement v_{ch}/v_{non} is given by eq. (4) (Kuzmak et al., 2019).

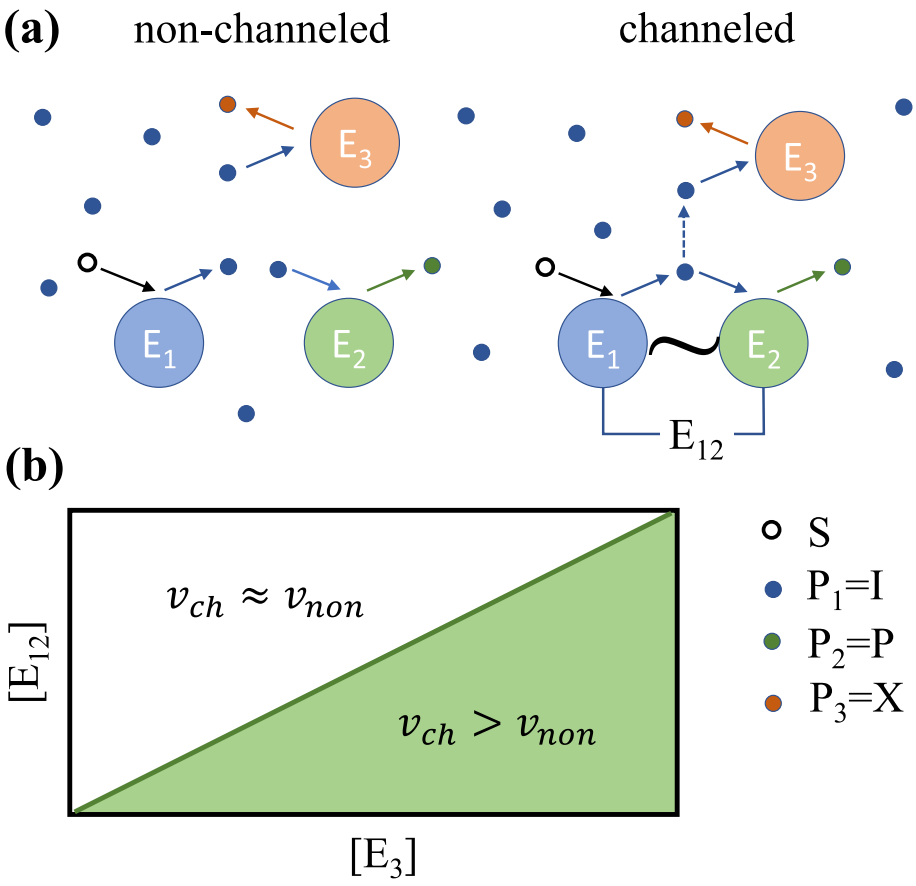

Fig. 4. Protection of intermediates by enzyme proximity. a, Schematics of channeled and non-channeled reaction cascades. $E_{12} = E_1 + E_2$ is an enzyme conjugate and E_3 is an enzyme competing with E_2 for the intermediary metabolites. **b**, Enzyme proximity is beneficial to reaction velocity at low concentrations of conjugates E_{12} or high concentrations of competing enzymes E_3 (green shaded area). The line marking this region is given by eq. (6) with $k_{seq} \approx k_{E_3}[E_3]$, where k_{E_3} is the rate and $[E_3]$ the concentration of competing enzymes (Kuzmak et al., 2019). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

$$v_{ch} \Big/ v_{non} \approx 1 + \frac{k_{E_1}}{k_{D\ell}[I]_{bulk}}. \tag{4}$$

Here $[I]_{bulk}$ is the current (time-dependent, Fig. 3b) bulk concentration of intermediates in the non-channeled systems (which is approximately the same as in the channeled system), $k_{E_1} \approx k_{cat}^{(1)}/K_M^{(1)}$ is reaction rate of the first enzyme ($k_{cat}^{(1)}$ and $K_M^{(1)}$ are the turnover number and the Michaelis-Menten constant, respectively) and $k_{D\ell} = 4\pi D\ell$ is the rate due to diffusion, where D is the mutual diffusion coefficient and ℓ the separation between the active sites of two enzymes. This equation shows that enzyme proximity increases reaction rate when $k_{E_1}/k_{D\ell} \gg [I]_{bulk}$. Under this condition, the local production of intermediates is faster than the rate at which they diffuse away, and hence the concentration of intermediates is enhanced locally at the first enzyme, leading to a faster rate.

Idan and Hess have estimated that proximity is beneficial to reaction velocity within a time frame $t_{ch} = (k_{D\ell}[E_{12}])^{-1}$ from the beginning of a reaction (Idan and Hess, 2012, 2013a). Notably, t_{ch} does not depend on enzyme activities and is determined solely by diffusive ($k_{D\ell}$) and geometric ($[E_{12}]$ and ℓ) properties. It is greater for slower intermediates and larger separations and decreases with increasing the enzyme concentration. As an example, for a GOx-HRP conjugate with a separation $\ell = 10$ nm and the hydrogen peroxide diffusion coefficient $D = 10^3 \mu m^2 s^{-1}$ (Fu et al., 2012), one finds $t_{ch} \approx 14$ ms for enzyme concentration $[E_{12}] = 1$ nM and just 14μs for $[E_{12}] = 1$ μM. For slower metabolites, this time is slightly larger; for instance, $t_{ch} \approx 23$ ms for $D = 600 \mu m^2 s^{-1}$ corresponding to glucose in water at room temperature ($[E_{12}] = 1$ nM). In the cytoplasm, diffusion is slowed down about an order of magnitude (Dauty and Verkman, 2004), giving $t_{ch} \approx 0.23$s. Although these times are relatively short, enzyme proximity may nevertheless be useful, *e.g.*, for enhancing the response of an enzymatic system to external stimuli, such as variations of substrate concentrations, or improving the stability of an enzymatic system to external perturbations (Kondrat et al., 2016). Such effects have not been studied in the context of proximity channeling, however.

4.2. Protection of intermediates

Enzyme proximity may play an essential role in protecting intermediary metabolites from sequestration due to degradation or competing reactions (Fig. 4a). Sequestration leads to the reduction of bulk concentration of intermediates, which slows down a cascade reaction. Using eq. (4) and estimating the bulk concentration of intermediates sequestered with rate k_{seq}, Kuzmak et al. have obtained an approximate equation for reaction enhancement (Kuzmak et al., 2019)

$$\frac{v_{ch}}{v_{non}} \approx 1 + \frac{k_{E_2}}{k_{D\ell}} \left(\frac{k_{seq}}{k_{E_2}[E]} + 1 \right), \tag{5}$$

where $k_{E_2} = k_{cat}^{(2)}/K_M^{(2)}$ is the rate constant of the second enzyme of a cascade and $[E]$ is the concentration of enzymes and enzyme complexes, assumed the same for a fair comparison (*i.e.*, $[E] = [E_1] = [E_2] = [E_{12}]$). If the sequestration is due to competing enzymes (say E_3), then $k_{seq} \approx k_{E_3}[E_3]$ where $[E_3]$ is their concentration and $k_{E_3} = k_{cat}^{(3)}/K_M^{(3)}$ the rate constant. Here $k_{cat}^{(i)}$ and $K_M^{(i)}$ ($i = 1, 2, 3$) are the turnover number and the Michaelis-Menten constant of i'th enzyme, respectively. For $k_{seq} \gg k_{E_2}[E]$, eq. (5) reduces to $v_{ch}/v_{non} \approx 1 + k_{seq}/(k_{D\ell}[E])$. Thus, the enhancement is determined by how fast the intermediates diffuse compared to their sequestration rate. This limit has been considered by Idan and Hess (2013a), who arrived at a similar equation.

Equation (5) implies that enzyme proximity provides a $100 \times \delta\nu$ percent enhancement when the following condition is satisfied (Kuzmak et al., 2019)

$$(k_{D\ell}\delta\nu - k_{E_2})[E_{12}] = k_{seq}. \quad (6)$$

For a given k_{seq}, this equation gives the concentration of enzyme complexes, $[E_{12}]$, below which enzyme proximity provides an enhancement $\delta\nu$ or higher. Fig. 4b demonstrates this schematically for $k_{seq} \approx k_{E_3}[E_3]$, *i.e.*, when the sequestration is due to competing enzymes. In this case, and assuming $[E_{12}] \approx [E_3]$, eq. (6) predicts that enzyme proximity can be advantageous to a cascade only for 'perfect' enzymes, catalysing reactions at rates above $\approx 10^9 M^{-1}s^{-1}$ (Kuzmak et al., 2019). However, higher concentrations of competing enzymes or decreased mutual diffusivity may make enzyme proximity beneficial also for slower enzymes. This is particularly important for crowded conditions *in vivo*, where the enzyme and metabolite diffusion is considerably reduced (Dauty and Verkman, 2004; Han and Herzfeld, 1993; Höfling and Franosch, 2013; Kondrat et al., 2015; Feig et al., 2017; Skóra et al., 2020).

4.3. Protection of surrounding

For metabolic channelling with molecular tunnels (Miles et al., 1999; Perham, 2000; Huang et al., 2001; Yon-Kahn and Hervé, 2009), a multifunctional enzyme providing such a channelling prevents the intermediates from diffusing away, hence their concentration in bulk solution vanishes. This is vital to cells when intermediates are toxic (Zhang, 2011; Jørgensen et al., 2005) or strongly hydrophobic (Miles et al., 1999; Yon-Kahn and Hervé, 2009; Hyde et al., 1988). Under certain conditions (Poshyvailo et al., 2017; Cornish-Bowden, 1991a, 1991b; Mendes et al., 1992; Cornish-Bowden and Cardenas, 1993; Menedes et al., 1996), the formation of enzyme complexes with direct channelling can reduce the concentration of intermediates in the bulk, lowering their potentially negative effects due to toxicity or increased viscosity.

For proximity channelling, continuum models show that in steady-state, the concentration of intermediates in the channelled and non-channelled systems are comparable (Idan and Hess, 2013a; Kuzmak et al., 2019). This means that enzyme proximity is ineffective in protecting the enzyme environment from intermediary metabolites. However, Brownian dynamics simulations of metabolons with electrostatic highways suggest that guided channelling may protect the surroundings to a certain degree (Elcock and McCammon, 1996; Huang et al., 2018). To our knowledge, however, such effects have not been quantified so far.

5. Clusters of enzymes

In some respects, enzyme compartmentalisation (or clustering of enzymes) is similar to proximity channelling discussed in the previous section, except that there are usually more than two enzymes in a cluster (Fig. 5a). Thus, some of the conclusions we have drawn for enzyme proximity also apply in this case. In particular, (i) in the absence of competing enzymes and for stable intermediates, the clustering of sequential enzymes can speed up a cascade reaction only transiently. The timeframe during which the clustering is beneficial can be longer than estimated for proximity channelling (Section 4.1) due to a larger number of enzymes, but this aspect has not been quantified so far. (ii) In a steady-state, enzyme clustering can enhance reaction velocity only in the case of sequestrated intermediates. Unlike proximity channelling, if there is a (semi-permeable) membrane enclosing the clustered enzymes, it may hinder the outflux of intermediates, potentially reducing their concentration in the bulk and protecting the surrounding from intermediates.

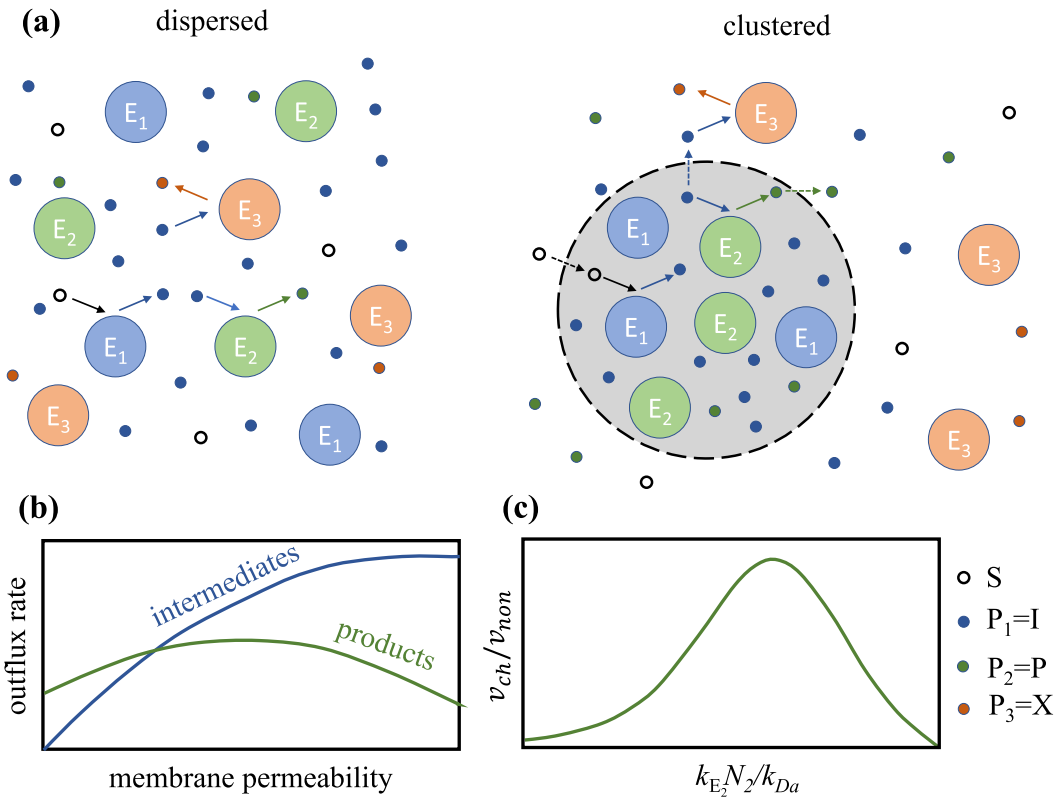

Fig. 5. Enzyme clustering. a, Systems with clustered and dispersed distribution of enzymes E_1 and E_2. Enzymes E_3 compete with E_2 for intermediates and are not clustered. **b**, Schematic drawing of the flux of intermediates (I) and products (P) out of the cluster depending on the membrane permeability. There is a trade-off between optimising the production rate and minimizing the flux of intermediates (Tsitkov and Hess, 2019). **b**, Schematic drawing of velocity enhancement as a function of $k_{E_2}N_2/k_{Da}$, where N_2 is the number of enzymes E_2, k_{E_2} is the reaction rate, and $k_{Da} = 4\pi Da$ is the diffusion rate constant, with a being the cluster radius. There is an optimal value of $k_{E_2}N_2/k_{Da}$ maximizing reaction velocity. The enhancement is given by eq. (7) (Gopich, 2021).

Clustering also adds new degrees of freedom, *viz.*, the size of a cluster, the number of enzymes and their spatial distribution inside the cluster, which one can use to optimise synthetic enzymatic cascades.

5.1. Natural enzyme clusters

In eukaryotic cells, enzyme clustering in microcompartments and membrane-bound organelles is common. However, prokaryotes also show some degree of compartmentalisation (Cannon and Shively, 1983; Badger, 2003; Penrod and Roth, 2006; Sampson and Bobik, 2008; Tanaka et al., 2008, 2009). For instance, carboxysomes of cyanobacteria encapsulate ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) and carbonic anhydrase, two enzymes involved in the rate-limiting step of the Calvin cycle (Cannon and Shively, 1983; Tanaka et al., 2008). Carboxysomes likely enhance the slow turnover rate of RubBisCO by providing a higher local concentration of carbon dioxide (Badger, 2003; Tanaka et al., 2009).

Two important examples of enzyme compartmentalisation in eukaryotes are glycosomes and purinosomes. Glycosomes are natural membrane-bound glycolytic clusters found in trypanosomatids. They contain enzymes of the first several steps of glycolysis (Opperdoes and Borst, 1977; Parsons et al., 2001; Haanstra et al., 2016). In addition, proteomics showed that glycosomes might contain enzymes from pyruvate, TCA, pentose phosphate and other pathways (Colasante et al., 2006). Thus, despite its name, glycosomes likely coordinate multiple metabolic pathways and not only glycolysis. Glycosomes are essential for trypanosomatids. Depleting trypanosomatids with peroxin, a protein required for the glycosome formation, ultimately led to their death even in the presence of glucose (Guerra-Giraldez et al., 2002).

Fluorescence microscopy studies indicated that purinosome macrobodies could contain six *de novo* purine biosynthesis enzymes and emerge under conditions of high purine demand (An et al., 2008; Pedley and Benkovic, 2017). Biochemical analyses further suggested that purinosomes consist of a core containing three enzymes of the first five steps, while the other three enzymes are dynamically associated with the core complex (Deng et al., 2012). In more recent work, Pareek et al. (2020) used metabolomics and mass spectrometry imaging and revealed that purinosomes of the *de novo* purine biosynthesis pathway consist of at least nine enzymes. Purinosome formation was linked to a higher production of purine compared to cells without purinosomes (Zhao et al., 2015). In particular, Pareek et al. found a seven-fold increase of the *de novo* pathway flux (Pareek et al., 2020).

5.2. Optimising enzyme clusters

To study optimal enzyme configurations, Buchner et al. (2013) considered a continuum reaction-diffusion model for a tandem reaction $S \overset{E_1}{\rightarrow} I \overset{E_2}{\rightarrow} P$, with boundary conditions corresponding to initial rates (*i.e.*, zero concentration of the intermediates at the cluster's boundary). Assuming a localised cluster of E_1 enzymes, they found that an optimal configuration for E_2 enzymes is a tightly packed co-cluster (at the same position), with a low-density tail whose extension increases with increasing the reaction rates. The tail is to pick up the intermediates that escape the core cluster. In follow-up work, Hinzpeter et al. (2017) showed that a specific enzyme arrangement inside clusters does not significantly affect the productivity of a tandem reaction. However, reaction velocity could be maximised by adjusting the number of enzymes inside a cluster and the cluster size. Assuming a homogeneous distribution inside clusters and a simplified version of the model, Tsitkov and Hess (2019) discussed general design principles and requirements under which clusterisation is beneficial from various perspectives. For instance, assuming that membrane permeability is the same for all metabolites, they demonstrated that there is a tradeoff between reaction velocity and the outflux of intermediates into the bulk solution (Fig. 5b).

Castellana et al. (2014) also used a continuum reaction-diffusion model, but considered sequestering intermediates and conditions in line with steady-state (*i.e.*, no flux of intermediates through the cluster's membrane). They found an optimal configuration consisting of a compact cluster of E_1 and E_2 enzymes surrounded by a halo of E_2 enzymes. They also saw that a specific spatial organisation of enzymes did not affect the cascade efficiency considerably. For enzymes uniformly distributed inside a cluster, the radius of a spherical cluster optimising the cascade efficiency was of the order of a few microns, in line with experimental observations (Castellana et al., 2014).

In more recent work, Gopich (2021) used a similar model and derived analytical formulas allowing one to quantify how enzyme clustering affects the production velocity. For a homogeneous distribution of enzymes inside clusters, she found (Gopich, 2021)

$$\frac{v_{\rm ch}}{v_{\rm non}} = \frac{k_{Da}(\tilde{k}_{\rm seq}+1)}{k_{\rm E_1}N_1 + k_{Da}} \quad \frac{k_{Da} + \tilde{k}_{\rm seq}k_{\rm E_2}N_2}{k_{Da} + \tilde{k}_{\rm seq}(k_{Da} + k_{\rm E_2}N_2)}, \tag{7}$$

where $\tilde{k}_{\rm seq} = k_{\rm seq}/(k_{\rm E_2}[{\rm E_2}])$, $N_i (i = 1, 2)$ is the number of enzymes E_i in a cluster, $k_{Da} = 4\pi Da$, with a being the cluster radius (compare with $k_{D\ell}$ below eq. (4)), and we used the same notations as before for reaction velocities in the clustered ($v_{\rm ch}$) and dispersed ($v_{\rm non}$) systems. This equation shows that for $k_{\rm seq} = 0$, reaction velocity of the clustered systems, $v_{\rm ch} = v_{\rm non}k_{Da}/(k_{Da} + k_{\rm E_1}N_1)$, is always smaller than $v_{\rm non}$. In other words, clustering cannot accelerate a cascade reaction for stable intermediates or in the absence of competing enzymes. Unlike proximity channeling, $v_{\rm ch}$ can be considerably smaller than $v_{\rm non}$, which occurs for dense clusters (Gopich, 2021). Equation (7) also shows that a significant enhancement can be obtained only when $k_{\rm seq} > k_{\rm E_2}[{\rm E_2}]$, that is, when sequestration rate is faster than the total production rate of the second enzyme. In Fig. 5c, we show schematically $v_{\rm ch}/v_{\rm non}$ as a functions of $k_{\rm E_2}N_2/k_{Da}$, assuming a constant N_1/N_2 ratio and constant total numbers of both enzymes (*i.e.*, we only consider how they redistribute into clusters). The reaction enhancement exhibits a single maximum at intermediate values of $N_2k_{\rm E_2}/k_{Da}$. For fast diffusion of intermediates compared to the production rate of E_2, the intermediates redistribute quickly in the system, making clustering inefficient. When the density of enzymes in the cluster increases, clustering becomes inefficient because a massive conversion of substrates S (of the first enzyme) decreases [S] locally in the clusters, slowing down the reaction compared to the dispersed system.

6. Concluding remarks

An enormous research effort has been channelled into developing novel tools and methods for enzyme immobilisation and co-localisation. With the plethora of available methods, encompassing novel chemistries and materials, and providing bottom-up strategies relying on synthetic biology, the field of enzyme (co)-immobilisation rapidly becomes too large to track comprehensively. Therefore, generic methods that work for any target enzyme without extensive optimisation and benchmarking studies aimed at, *e.g.*, industrial application become more and more important. Co-immobilisation methods hereby allow placing enzymes close to each other with precise separation control, encapsulating enzymes into DNA nanostructures, compartmentalising them in space, *etc.* Frequently, co-localisation leads to enhanced reaction velocity, improved enzyme stability and controlled selectivity. However, as this review shows, our understanding and the ability to predict and quantify the benefits of co-localisation are still in their infancy.

While solid evidence exists for enzyme clustering *in vivo*, its role and significance for living organisms are not quantitatively well understood. What are the mechanisms of metabolic regulation with enzyme compartmentalisation? Why and how do enzyme clusters form? What are their control mechanisms? Recent theories have investigated how clustering affects reaction velocity, the concentration of intermediary metabolites, *etc.* So far, however, no experimental validation has been

achieved. Catalytically active inclusion bodies (CatIBs) and liquid-liquid phase-separated organelles (LLPS) are good candidates to investigate enzyme clustering experimentally. However, research on CatIBs and LLPS is relatively scarce, and more work needs to be done to align theory and experiments.

Macromolecular crowding deserves particular attention. Experimental and theoretical work shows that crowding can drastically influence the diffusion of macromolecules and metabolites (Dauty and Verkman, 2004; Han and Herzfeld, 1993; Höfling and Franosch, 2013; Kondrat et al., 2015; Feig et al., 2017; Skóra et al., 2020), protein stability (Gomez et al., 2019), chemical equilibria (Ellis, 2001), *etc.* The effects of crowding on enzymatic reactions, proximity channelling and enzyme clustering have received much less attention (with a few notable exceptions (Kuzmak et al., 2019; Norris and Malys, 2011; Pastor et al., 2014; Skóra et al., 2021)). Important questions remain unanswered: How does macromolecular crowding affect the efficiency and formation of enzyme clusters? How does it affect proximity channelling? What is its role in metabolic regulations?

Thus, there are many open questions despite decades of intensive investigations. Answering these questions requires interdisciplinary research and collaborative work of biologists, chemists, physicists, engineers and mathematicians. We hope that our brief review will stimulate further work and particularly theoretical and simulation studies on these exciting topics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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