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ACTIVE INCLUSION BODIES: THE UNEXPECTED JOURNEY

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ABSTRACT

The demand for large-scale production of pure and functional proteins via cost-effective and simple methods is highly emerging at present. In biotechnology, proteins are synthesized in heterologous systems, since natural sources do not always allow satisfactory yields and purity of desired proteins. Heterologous systems are focused to produce a high amount of an expressed protein, often leading to translation mechanism overload. As a result, insoluble aggregates of the protein called inclusion bodies (IBs) are formed. Since the beginning of recombinant protein production, the formation of IBs has been seen as an obstacle and great afford has been made to prevent their presence. On the contrary, many independent studies in the recent decade challenged this wide-accepted opinion and proved the huge potential of IBs. This review focuses on the benefits of tailored-made production of IBs and their emerging use as self-immobilized catalysts used in the synthesis of several industrially interesting products, as well as on their utilization in different areas.

Keywords: active inclusion bodies, biocatalysis, immobilization, biotechnology

INTRODUCTION

Bacterial inclusion bodies are commonly known as insoluble protein aggregates, originally developed during recombinant proteosynthesis. In *Escherichia coli* cells, they are observed since the beginning of recombinant technologies **(Garcia-Fruitós *et al.*, 2012)**. As they have been seen as a major obstacle in the production of soluble proteins, many protein-specific molecular and biochemical strategies have been employed to avoid their formation. Nonetheless, yet around 80 % of overexpressed recombinant proteins are packed into IBs **(Jürgen *et al.*, 2010)**. Consequently, when protein itself cannot be expressed in a high amount in soluble form, IBs are considered the main source of recombinant protein. However, time-consuming and expensive refolding strategies are required to obtain an active and properly folded form. Over the years, understanding the relation between their origin, function, and properties opened new possibilities for direct IBs applications in a wide range of areas. Furthermore, many types of research proved, that even partially or incorrectly folded proteins within IBs are capable of maintaining their functional or catalytic activity. Thus, IBs, previously viewed as undesirable waste have become a subject of interest in fields of medicine, biotechnology, pharmacy, material science, and more.

SHAPE, STRUCTURE AND COMPOSITION

IBs are mostly located on the poles of *E. coli* cells and range in size from 50 to 800 nm. Their amount depends on the produced target protein, the genetic background of the host, and the collection time of IBs **(Jäger *et al.*, 2020).** Bacterial inclusion bodies are pseudo-spherical to rod-like shaped, with a rough surface and high density (1.3 mg/ ml). Despite that, they have a high level of porousness and hydration **(Singh and Panda, 2005)**. According to morphological properties, IBs have a more complex origin than they seemed at first. Besides primarily synthesized protein, IBs contain also a small spectrum of cellular proteins, which include chaperones, cellular polypeptides, and remnants of nucleic acids. During cell lysis, the residues of the cell wall are partially incorporated into a fraction of IBs. The chemical composition of IBs is variable and mostly depends on the characteristics of recombinant protein, cultivation conditions, the genetic background of the host, and IBs purification protocol **(Jürgen *et al.*, 2010)**. The IBs studied so far share the structure of the amyloid-like β-folded sheet regardless of the original structure of the target protein **(Paraskevopoulou and Falcone, 2018)**. This structure supports mechanical and chemical stability **(Rinas *et al.*, 2017)** and even provides the advantage of resistance to cellular proteases **(Upadhyay *et al.*, 2012)**.

ORIGIN AND FORMATION

The exact physiological and biological origin of IBs has been overlooked for a long time, as they were not considered a point of interest. In the past, it was not easy to assume, whether the expressed protein would naturally result in IBs production. Over time, current knowledge and computational methods have cleared the evaluation of the aggregation behaviour of proteins. Accordingly, not only the primary structure of the protein but also its length, conformation, location, function, and abundance may influence IBs production in positive or negative ways **(De Groot and Ventura, 2010)**. IBs origin is mostly the result of a high local concentration of polypeptides emerging from ribosomes during translation when the translation mechanism in the cell is overloaded. Cross interactions between polypeptides prevent folding of the chain into standard conformation when the hydrophobic parts are oriented inside of the protein and the hydrophilic parts are exposed to the aquatic environment. Thus, these hydrophobic surface areas are the driving force of interaction, followed by aggregation **(Hrabárová *et al.*, 2015)**. Other important factors supporting protein folding into IBs are lack of side-modifications, incorrect interaction with chaperones, protein translation kinetics related to rare codons and cytoplasmic reducing environment, preventing the disulphide bonds formation **(Idicula-Thomas and Balaji, 2007)**. In recombinant bacteria, the formation of IBs begins immediately after the induction of gene expression. Subsequently, the volume of particles increases along with the continuous protein expression, resulting in a gradual aggregation of polypeptide chains within a crowded cell environment **(Chebotareva *et al.*, 2013)**. However, the question remains, whether the formation of IBs is merely a passive process dependent only on physical interactions of protein chains, or it is an energy-driven process that requires active cell proteome collaboration **(Oliveira *et al.*, 2016)**. According to kinetic-related studies of *in vitro* protein aggregation **(Kurganov, 2002; Kurganov et al., 2002; Wang and Fersht, 2012)** and *in vivo* monitoring **(Hoffmann *et al.*, 2001)**, protein aggregation into IBs involves two major strategies. In the first one, the accumulation starts from one or a limited number of molecules, that act as nucleation sites and peptides aggregate directly into IBs. In the latter, smaller aggregates are collected and delivered to form larger aggregates by cell transport or by diffusion **(Upadhyay *et al.*, 2012**). It is possible, that this transport is energy-driven **(Govers *et al.*, 2014)**, nevertheless several reports showed, that exclusion of aggregates from the centre of the cell to the poles is an energy-free process **(Oliveira *et al.*, 2016; Winkler *et al.*, 2010; Coquel *et al.*, 2013; Neeli- Venkata *et al.*, 2016; Gupta *et al.*, 2014)**. Transport and formation of insoluble aggregates within the cell depend on another contributing factor cytoplasmic composition. If the cytoplasm is less fluid, movement of particles larger than 30 nm is restrained, resulting in decreased IBs production. A more fluid environment in the cell allows motion of larger particles above 30 nm, however, ATP hydrolysis is required for this movement **(Amen and Kaganovich, 2015)**.

The quality control system of the cell can deal with partially and poorly folded peptides in both active and passive ways. Even misfolded protein can be reassessed within the cell or be proteolytically cleaved **(Rinas *et al.*, 2007)**. IBs are targeted by cell chaperones during *in vivo* aggregation, therefore degradation of IBs takes place simultaneously **(Carrió and Villaverde, 2002)**. Refolding is mediated by ATP- driven interaction with the chaperones DnaK/DnaJ/GrpE and GroEL **(de Marco *et al.*, 2019)** and heat shock proteins IbpA and IbpB, as they stabilize and decrease the size of aggregates **(Ratajczak *et al.*, 2009)**. Along with interaction with ClpP family proteases **(Carrió and Villaverde, 2002)** aggregates can proceed to ATP-depended proteolytic cleavage.

TAILORED PRODUCTION OF IBS

To increase the production of physiologically active IBs as a source of a biocatalyst or for other direct applications, it is necessary to set the cultivation conditions in favour of their formation. Several parameters should be followed, such as non-regulated pH during both, cultivation at 37 °C and expression **(Castellanos-Mendoza *et al.*, 2014)** at 15 °C. The temperature shift benefits the optimal yield of active IBs. Molecular conditions for IBs production are high concentration of inductor, usually isopropyl β-D-1 thiogalactopyranoside (IPTG), which increases the overall production of active IBs **(Lamm *et al.*, 2020)**, use of high-copy cloning vector and use of strong promotor, usually lac, tac, and T7, which naturally overloads the translation mechanism **(Carrió and Villaverde, 2005)**.

Nowadays, a novel strategy for creating IBs is introduced. This procedure represents a fusion of target protein with aggregation-supporting short peptide tags or domains. These "pull-down" domains ensure that protein is purposefully folded into the form of IBs. The design of fusion proteins for the production of catalytically active IBs is still a series of trials and errors, and examination of many combinations of constructs is required. Currently, there is no common strategy for tag selection. Generally, they must support protein aggregation, leading to active IBs in sufficient quantities **(Jäger *et al.*, 2019a)**. The variability of fusion tags differs in several properties, like their origin, which may be bacterial, viral or synthetic; and their length, varying from 8 to 600 amino acids. The complexity of tags differs from simple short peptide chains to *β*- sheet, *α*-helix structures, coiled domains, or even whole proteins **(Jäger *et al.*, 2020)**. Small synthetic peptides such as *β*-sheet structure ELK16 and GFIL8 **(Wang *et al.*, 2015; Jiang *et al.*, 2019; Wu *et al.*, 2011)**, surfactant-like L6KD **(Zhou *et al.*, 2012),** or amphipathic helical 18A peptide variants **(Lin et al., 2013)**, affected the efficiency of different oxidases, hydrolases and oxidoreductases IBs production between 61-120 %, with high residual activity of IBs. Also, small poly-His or poly-Lys tails attached to the N- and/ or C-terminal end of lipase B (CALB) (*Candida antarctica*) successfully produced active IBs suitable for catalytic hydrolysis with enhanced enantioselective ratio compared to commercial catalyst **(Zhou *et al.*, 2017)**. A well-studied group of coiled-coil tags fused with several target enzymes and proteins **(Kloss *et al.*, 2018a; Jäger *et al.*, 2018; Diener *et al.*, 2016; Jäger *et al.*, 2019a, b; Kloss *et al.*, 2018b; Lamm *et al.*, 2020)** are 3HAMP of Aer2 protein (*Pseudomonas aeruginosa*) **(Airola *et al.*, 2010)** and TDoT of tetrabrachion surface protein (*Staphylothermus marinus*) **(Stetefeld, *et al*, 2000).** The use of the TDoT domain showed higher efficiency in the production of IBs with higher purity, but the use of 3HAMP helped to maintain higher residual activity compared to TDoT. In addition, 3HAMP IBs had a higher lipid content, and more permeable structure, and compared to TDoT were less firmly folded. All of this may be the cause of the higher residual activity of 3HAMP IBs **(Jäger *et al.*, 2019a)**. Further advantages of embedding coiled-coil domains into IBs production are summarized in **Gil-Garcia and Ventura, 2021** review. In addition, even larger proteins and protein domains were applied as fusion tags, due to their natural tendency to cause aggregation, as cellulose-binding domain (CBD) with 108 amino acids (*Cellulomonas fimi*) **(Choi *et al.*, 2011)** respectively with 256 amino acids (*Clostridium cellulovorans*) **(Nahálka, 2008; Köszagová *et al.*, 2018; Nahálka and Nidetzky, 2007; Nahálka and Pätoprstý, 2009; Nahálka *et al.*, 2008)**. As reviewed in **Levy and Shoseyov, 2002**, the CBD domain has various applications as an immobilization tag, an affinity tag, or purification tag in bioprocessing, targeting, diagnostics, and others. Although, a highly emerging field since the 1990s is protein engineering, where the target protein is fused with the CBD domain in a specific pET vector (34-38), resulting in high protein expression levels **(Kim *et al.*, 1998; Otomo *et al.*, 1999)**. Also, other proteins with a tendency to aggregate were used as pull-down tags, as variants of human Aβ-amyloid peptide Aβ42 (F19D) and capsid viral protein (VP1). Their use leads only to the average formation of IBs, however, the activity of IBs VP1 *β*-galactosidase increased 1.6 times compared to cell lysate **(García-Fruitós, *et al.*, 2005)**. Another type of aggregation promoting fusion tag is green fluorescence protein (GFP) (*Aequorea victoria*), while not altering the function or localization of the target protein. By fusion of GFP and alkaline phosphatase (phoA) (*Enterobacter aerogenes)*, IBs retained 48-58 % of their activity **(Huang *et al.*, 2013)**. The largest proteins with an aggregating tendency comprise a variant of maltose-binding protein (MalE31) (*E. coli*) **(Arie *et al.*, 2006)** and pyruvate oxidase with 574 aminoacids (poxB) (*Paenibacillus polymyxa*) **(Park *et al.*, 2012)**, which are far larger than target proteins. Fusion of amylase with poxB and GFP produced active IBs, showing twice as much residual activity as a soluble form of amylase **(Park *et al.*, 2012)**. Properties of target protein such as size, oligomerization, or presence of cofactors do not appear to be limiting factors to IBs production **(Jäger *et al.*, 2020)**. The only common feature known to date is, that proteins with larger hydrophobic sites on the surface generally have higher efficiency in IBs formation **(Jäger *et al.*, 2019a)**. Choice of fusion tag that does not alter the correct composition of the catalytic site of enzyme or active site of protein is necessary. For this reason, when designing a fusion tag sequence, not only the primary but also the quaternary structure of the protein must be considered. As an example, lysine decarboxylase (CadA) (*E. coli*) is convenient. The N-end of CadA is embedded in the decameric structure, and the C-end is exposed on its surface. The difference in CadA IBs activity was substantial, when TDoT fused at C-end, activity was 6-times higher than when fused at N-end **(Jäger *et al.*, 2019a)**. Another factor that can influence the production of active IBs is the presence and composition of polypeptide linker, which bonds the fusion tag with the target protein on its C or N end **(Jäger, *et al.*, 2020)**. Often, they also play role in the stabilization of protein structure **(Reddy Chichili *et al.*, 2013)**. Linkers usually consist of threonine and proline combination, providing a rigid linker **(Receveur *et al.*, 2002)**, whereas the presence of glycine or serine preserves the flexibility of the linker and allows proper orientation and connection of protein domains without interfering with their function **(Sammond *et al.*, 2012)**. They can also differ in length, which can affect both IBs production and activity. Usually, when fused with artificial pull-down peptides, linkers share the same length as tags **(Wang e*t al.*, 2015)**. The combination of various linkers and aggregation tags was compared in the work of **Küsters *et al.*, 2021**, which was desired to pull down the activity of CadA into IBs. In the final, 10 different variants were constructed: a combination of flexible serine/glycine (SG) or rigid proline/threonine (PT) linkers with artificial peptides (18AWT, L6KD, and GFIL8) or coiled-coil domains (TDoT and 3HAMP). As the most productive variant was identified as PT linker and L6KD peptide with the highest conversion rate (93 % after 3 minutes) of l-lysine to 1,5-diaminopeptane (DAP or cadaverine). However, the variant of L6KD with SG linker showed the exact opposite result, while having the same morphology and same amount of IBs per cell. **(Küsters *et al.*, 2021)**. In the work of **Huang *et al.*, 2013**, when replacing the flexible GGGS 5-linker in GFP IBs with a fixed AAAKE 5-linker, the total activity of IBs increased by 10 % **(Huang *et al.*, 2013)**. On the other hand, when the GGGS 5-linker was completely removed from TDoT IBs, the efficiency of IBs production increased by 30 % **(Jäger *et al.*, 2019a)**. As shown in mentioned studies, each combination of linker and fusion tag must be designed individually and followed by examinations. An alternative, linker may also serve as a cleavage site for proteases in order to analyse the fusion tag and target IBs enzyme individually **(Nahálka, 2008; Nahálka and Nidetzky, 2007; Nahálka and Pätoprstý, 2009; Nahálka, *et al.*, 2008; Köszagová *et al.*, 2018)**. Last, but not least, the choice of cloning strategy may also influence the successful production and effectiveness of IBs. The most common approach is traditional cloning with regular restriction endonucleases, however, due to complex procedural steps and to create a larger database of IBs variants, it is preferable to use less time-consuming methods such as ligation independent cloning, Gibson method, or "Golden Gate" cloning **(Nahálka 2008; Nahálka and Nidetzky 2007; Nahálka and Pätoprstý 2009; Engler, *et al.*, 2008)**.

ISOLATION AND PURIFICATION OF IBS

Due to their high density, simple isolation by centrifugation from host cells presents one of the main advantages of IBs. However, it is necessary to keep in mind, that the isolation process should neither disrupt IBs structure nor denature proteins trapped inside IBs. Although, optimal isolation protocol can vary for each protein. There are several commonly used lysis methods, such as sonication, high-pressure homogenization, enzymatic lysis, and the use of freeze-thawing cycles. In their study, **Peternel and Komel, 2010** compared three methods for bacterial cell disruption, non-mechanical enzymatic lysis, and two mechanical methods, sonication and high-pressure homogenization. Granulocyte colony-stimulating factor (G-CSF) and GFP were used as model proteins. They concluded that, while non-mechanical lysis is gentler to IBs, cell disruption is less effective compared to mechanical methods. The solution could be a combination of the lysis method with one of the mechanical cell disruption methods. **Rodríguez-Carmona *et. al* (2010)**, also published similar results. By a suitable combination of chemical and mechanical cell disruption methods, they were able to achieve undetectable levels of viable cell contamination in isolated IBs. Moreover, for affinity separation and purification of IBs, magnetics particles of Fe_3O_4@$NiSiO_3$ and Fe_3O_4@Ni_xSiO_y can be used, as an alternative to nickel -nitrilotriacetic acid (Ni-NTA) resins. Under both native and denaturation conditions, IBs-Fe_3O_4@$NiSiO_3$/Fe_3O_4@Ni_xSiO_y complexes were collected by external magnetic force yielding up to 80 % purity of IBs streptokinase **(Seyedinkhorasani *et al.*, 2020)**.

ENHANCING THE SELF-IMMOBILIZATION

The current trend in the application of recombinant enzymes in biocatalysis is to prepare cost-effective robust biocatalysts with high operational stability and effectivity. Immobilized catalysts have several advantages over soluble catalysts, such as reutilization, often increased stability, easy handling, and simple separation from reaction mixtures **(Liese and Hilterhaus, 2013)**.

Generally, soluble enzymes are immobilized by adsorption/covalent binding to insoluble carriers or by chemical/physical approaches in carrier-free mode. However, in some cases, the adsorption or physical bond between carrier and enzyme is not very strong, which may lead to leakage of enzyme and a decrease in catalytic activity. Moreover, the active site of the enzyme may randomly interact with the carrier and may lead to a decrease in the activity or complete inactivation **(Santos *et al.*, 2015)**. Therefore, immobilization approaches such as entrapment or encapsulation can be more preferred in some cases. However, a covalent bond is usually rigid enough to minimize the leaking of the enzyme from carrier support **(Cao, 2006)**. On the other hand, chemical immobilization by covalent bonding on carrier substrates and self-immobilization may alter the structure of the enzyme **(Velasco-Lozano *et al.*, 2016)**. In carrier immobilization, the cost of the carrier can be the most expensive, limiting the initial use of enzymes in industrial applications. This issue can be circumvented by self-immobilization, usually by glutaraldehyde crosslinking **(Migneault *et al.*, 2004)**, ending up in crosslinked IBs (CLIBs), or their alternatives crosslinked enzymes (CLEs), cross-linked enzyme aggregates (CLEA), or crosslinked enzyme crystals (CLEC). By using crosslinking approach favourable properties such as increased specific activity, easier and cost-effective production, higher volumetric activity per biocatalyst mass, higher purity, and lower mass transfer limitations can be achieved in some cases **(Sheldon, 2007; Cao *et al.*, 2003)**. However, IBs as aggregated proteins in insoluble clusters can be directly considered as *in vivo* self- immobilization, without any further need for an additional carrier or cross-linking. Besides, the protein fraction from inside the IBs is released into the environment only in small amounts **(Nahálka, 2008)**. Carrier-free catalysts are requested in processes, where high yields and catalytic productivity are required and standard carrier support cannot be approached **(Illanes *et al.*, 2009)**. As matter of fact, commercial biocatalytic reactions often require harsher conditions, such as high temperature, high alkaline or acidic pH values, or the use of organic solvents **(Sheldon and Brady, 2018)**. CLIBs, CLEA and CLEC have their thermal and mechanical stability improved and can tolerate such conditions while maintaining their activity. Although, when using CLEs, low mechanical stability, poor reproducibility, and less than 50 % of activity retention are usually achieved **(Cao *et al.*, 2003)**. As long as CLEC seems a promising catalytic alternative, protein crystallization requires several arduous steps, highly increasing production costs **(Brady *et al.*, 2004)**. On the other hand, preparation of CLEA is a cheaper option, as long as enzyme molecule aggregates are based on protein precipitation step in the presence of salts, non-ionic polymers and organic solvents bounded by non-covalent bond **(Sheldon, 2007; Cao *et al.*, 2000)**. After covalent crosslinking, CLEA remains permanently insoluble **(Sheldon, 2007)**. The main factor affecting the sensitivity of both CLEC and CLEA is the conformation of aggregates or crystals. In CLEA, the conformation and structure of the active site resulting in catalytic activity may be affected by several different precipitating agents, enzyme concentration, temperature and pH **(Cao *et al.*, 2000)**. Simultaneously, the concentration of glutaraldehyde should be considered. Low concentration is insufficient to form tight cross-linkage **(Broun, 1976)**, however higher concentration supports a high level of crosslinking, improves thermal stability and a more rigid structure of CLEA is achieved by excluded water molecules. Nevertheless, an extensive concentration of crosslinking agents may result in distortion of the active site of the enzyme, thus decreasing the accessibility for substrate and catalytic activity **(Majumder *et al.*, 2008; Chui and Wan, 1997)**. Both CLEC and CLEA protocols, present the disadvantage of requiring protein isolation and purification, which happens before the critical precipitation step **(Nahálka and Nidetzky, 2007)**. Although the design of crosslinking and aggregation protocol for CLEA and CLIBs/IBs must be developed for each enzyme individually, therefore significant optimization procedures are required **(Roessl *et al.*, 2010; Jäger *et al.*, 2019a)**. However, easy handling, cost-effective production, eluting the step of tedious enzyme purification, and the possibility to achieve aggregation due to pull-down tags, give IBs an advantageous potential in the application as biocatalysts instead of CLEC and CLEA **(Nahálka *et al.*, 2008)**.

Meanwhile, crosslinking is not the only supporting method for self-immobilized IBs. Modification by magnetic particles by natural ionic strength not only enhances their mechanical and operational stability but also allows a high level of reusability of catalytic IBs **(Han *et al.*, 2021; Köszagová *et al.*, 2018)**. Besides, low toxicity, easy and cost-effective production, and hardly unfavourable effects on enzymatic activity ensure, that magnetic particles are widely used in immobilization processes (**Su *et al.*, 2018; Badoei- Dalfard *et al.*, 2019; Zhou *et al.*, 2018)**. Their use simplifies the separation and recovery step of catalytic IBs by natural magnetic strength, therefore no further centrifugation or filtration is necessary. Compared to separation by centrifugation, when UDP-pyrophosphorylase was inactivated only after 10 reaction rounds, the use of magnetic particles of $FeSO_4$ prolonged and even increased catalytic activity of IBs for 50 reaction rounds. Moreover, no protein leakage from IBs was found in the used reaction mixture **(Köszagová *et al.*, 2018)**. Within the same work, even N-acetyl-D-neuraminic acid aldolase (SAA) *(E. coli)* was magnetized by iron oxides. High operational stability with around a 60 % level of conversion was achieved in 10 reaction rounds **(Köszagová *et al.*, 2018)**. Magnetically modified IBs of CALB lipase by oxidized dextran and Fe_3O_4 particles showed an excellent increase of reusability, thermal, storage, and pH stability while remaining around 85 % of its initial activity when compared to unmodified CLIBs CALB, uncrosslinked IBs CALB, or commercial CALB **(Han *et al.*, 2021)**.

Entrapment or encapsulation is another immobilization approach suitable for IBs. Entrapment represents a gentle technique when IBs are entrapped into insoluble gelatinous beads or microcapsules. The structure and active site of catalytic IBs are not altered by covalent binding or chemical reactions. Compared to soluble immobilized enzymes, the native structure of IBs also prevents leaking from the matrix, **(Hrabárová *et al.*, 2015)**. CLIBs particles entrapped in alginate beads were successfully used in the synthesis of sialic acid while showing long operational stability **(Nahálka *et al.*, 2008)**. Undoubtedly, handling and sedimentation with alginate beads are easier than with unmodified gelatinous IBs. However, alginate gel tends to destabilize in presence of Ca^{2+} chelating agents. To prevent these circumstances, a low constant concentration of Ca^{2+} ions or the absolute absence of mentioned agents must be ensured during biotransformation **(Hrabárová *et al.*, 2015)**.

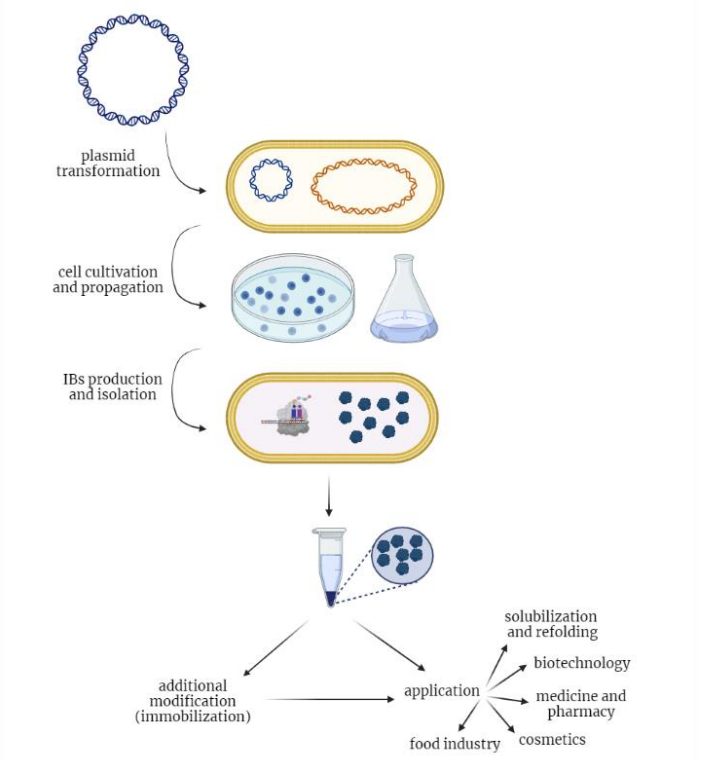

Figure 1 Production and applications of IBs (created with BioRender.com)

IBS IN BIOCATALYTIC REACTIONS

Currently, active IBs as purposely tailored self-immobilized enzymes can be directly applied and recycled in biotechnological processes. In this regard, catalytic reactions and synthesis with substrates/intermediates to final products are catalyzed by various enzymes, which have been studied as IBs with encouraging results.

From the pharmaceutical industry's point of view, the most valued are catalytic reactions, where production costs of therapeutics are decreased. D-amino acid oxidase (TvDAO) (*Trigonopsis variabilis*) above other reactions catalyses O_2 dependent conversion of cephalosporin C into glutaryl-7-aminocephalosporanic acid, one of the most economically significant intermediates in antibiotics production. Successful *in vivo* immobilization of this enzyme via the CBDClos pull-down domain showed enhanced operational stability compared to its soluble form. Therefore, it represents a valid replacement for the traditional and economically demanding process of immobilization of TvDAO into CLECs or CLEAs **(Nahálka and Nidetzky, 2007)**. Another pharmaceutically interesting reaction is a breakdown of hyaluronan. This hydrolysis not only allows better permeation of cell membrane for drug absorption **(Menzel and Farr, 1998)** but also hyaluronan oligosaccharides as a product of breakdown, help to suppress the growth of tumours and stimulate angiogenesis **(Guo *et al.*, 2009)**. For this purpose, an active IBs hyaluronidase (*Apis mellifera*) was prepared in specific conditions for IBs formation, however, only a low concentration of IBs was achieved **(Schwaighofer *et al.*, 2020)**. Besides the role of N-neuraminic acid in cellular metabolism, it is also an interesting product included not only in therapeutics pathways of antivirals **(Liese *et al.*, 2006)** but also in the nutrition and cosmetics industry. Therefore, its cost-effective and smart synthesis is highly demanded. SAA is an industrial enzyme condensing N-acetylmannosamine and pyruvate into sialic acid and its analogues. Successful production of SAA as *in vivo* immobilized IBs linked with CBD domain at the N-terminus resulted in the high final activity of 0.35 U/ mg **(Nahálka *et al.*, 2008)**. In similar work, SAA was fused with GFP protein instead of CBD domain with examined stability across a wide range of temperatures, pH and storage conditions, where SAA IBs showed persistent performance. Different variants of SAA fused with GFP were constructed and

showed conversion from 22 % to 75 % after 48 hours of the reaction round. However, when testing the reusability of IBs SAA, the yield decreased approximately by 50 % after each cycle **(Venning-Slater *et al.*, 2014)**.

α-D-glucose-1-phosphate production by α-glucan phosphorylases is achieved by reversible cleavage at the *α*1-4 bond in starch. This glucose derivative is the starting substrate for various biochemical synthesises. Maltodextrin phosphorylases from *E. coli* or potatoes are commonly used in this synthesis. Thermostatic maltodextrin phosphorylase (*Pf*MP) (*Pyrococcus furiosus*) could be a better alternative due to starch's low solubility. *In vivo* immobilized IBs *Pf*MP fused with CBD domain demonstrated improved technological parameters in conversion with only 10 % of activity loss in ten repeated rounds **(Nahálka, 2008)**. Another enzyme that is an important part of carbohydrate metabolic pathways and oligosaccharide synthesis is UTP-glucose-1-phosphate uridylyltransferase (GalU) (*E. coli)*. It synthesizes UDP-glucose from glucose-1-phosphate and UTP while releasing pyrophosphate. UDP-glucose, an activated form of glucose, is a precursor in the synthesis of glycogen for example. GalU can be successfully *in vivo* immobilized by the N-bound CBD domain. An excellent performance of active IBs modified by magnetics particles was achieved in 10 reaction rounds, with a high level of conversion (60-70 %), followed by 50 1-hour long reaction rounds, where operational activity increased from 30 % to 50 % substrate conversion. Even unmodified GalU IBs performed well with 35-45 % conversion degree in the first 4 cycles, however, the centrifugation step after each of 10 reactions round observably caused their inactivation (**Köszagová *et al.*, 2018)**.

Stable and reusable catalysts are valued not only in biomedicine and pharmacy but also in the petrochemical industry. Via catalytically active IBs of CadA fused with the TDoT coiled-coil domain on the C-terminal end, an alternative way was created to produce DAP **(Kloss *et al.*, 2018b)**, which is an important monomer for bio-based polyamides used in various chemical applications **(Weichao *et al.*, 2017)**. The final conversion of synthetized DAP was 87-100 % in repetitive batches in a total reaction time of 69 hours **(Kloss *et al.*, 2018b)**. Enantioselective hydrolysis of racemic (*R, S*)-*N*-(2-ethyl-6-methyl phenyl) alanine methyl ester (*R, S*-NEMPA-ME) to *N*-2-ethyl-6-methyl phenylalanine (S-NEMPA) by CALB is used as a key step for the synthesis of most widely used herbicides **(Zheng *et al.*, 2004)**. Attachment of poly- amino acid tail (*n*His or *n*Lys) on N/C terminal ends of lipase B (*Candida antarctica*) produced catalytically active IBs with strong specificity towards substrate **(Zhou *et al.*, 2017; Han *et al.*, 2021)**. Another excellent example of IBs application in industry is the production of 1-butanol. 1-butanol as solvent is widely used in a wide spectrum of products starting from cosmetics products, flavourings, supplementary vitamins, and vegetable oils production to medical agents manufacturing like antibiotics or hormones **(Green, 2011)**. Multienzymatic cascade reaction for 1-butanol production was designed, as four catalysts were fused with leucine zipper on N-terminus along with antiparallel leucine zipper fused to CBD gene **(Han *et al.*, 2017)**. This peptide-peptide interaction assembled IBs composed of all four enzymes in the 1-butanol pathway. The creation of this artificial metabolome shows the efficient way how to assemble productive metabolic tools for the production of valuable products. Multienzymatic reactions are becoming a part of new approaches, where several enzymes are organized *in vitro* or *in vivo* to stimulate metabolic pathways. In addition, not only regular and well-known but also purposely-designed metabolic pathways can be now constructed. The design of such synthetic pathways represents a new challenge, mostly in industrial and therapeutic applications. Achieving such a chemical complexity requires cofactors since they often contain ATP-dependent enzymatic reactions. Such energy can be provided by polyphosphate kinases (PPK). In the work of **Nahálka and Pätoprstý, 2009**, a purposely designed multi-enzymatic pathway for the synthesis of CMP-sialic acid and 3´sialyllactose was constructed, as they both are expensive and important substrates for sialylation of pharmaceutical glycoproteins. Homologues of polyphosphate kinases (SpPPK) (*Silicibacter pomeroyi*) and cytidilate kinase (CMK) *(E. coli)* were individually N-terminally fused with the CBD domain and formed active IBs. The other three enzymes that were part of the cascade (SAA, CMP- sialic acid synthetase (CSAS), and α-2,3-sialyltransferase (ST)), were co-expressed in whole cells. IBs of CMK and SpPPK catalysed cyclic synthesis of CTP. The IBs SpPPK homologue had increased sensitivity to pyrimidine base co-substrates, which was successfully used to add a phosphate group to the CDP that was synthesized by IBs CMK (**Nahálka and Pätoprstý, 2009)**. Another great example of a multi enzymatic cascade catalysed by IBs is the production of vicinal 1,2-diol (1*R*,2*R*)-1-phenylpropane-1,2-diol (PPD), where the stability of soluble enzymes is a limiting factor. This cofactor-dependent cascade involved thiamine diphosphate (ThDP)-dependent benzaldehyde lyase (*Pf*BAL) (*Pseudomonas fluorescens)* and alcohol dehydrogenase (*R*ADH) (*Ralstonia sp*.). Both proteins were fused with TDoT and/or 3HAMP domains by linker combined of G/S with Factor XA protease cleavage site, creating single or co-immobilized IBs. Main substrates, benzaldehyde, and acetaldehyde were converted by *Pf*BAL *to* (*R*)-2-hydroxy-1-phenylpropanone (HPP) followed by conversion to PPD. To achieve a recycling cascade, co-substrate benzyl alcohol was added for utilization of the nicotinamide cofactor of the *R*ADH, which was oxidized by *R*ADH to benzaldehyde. By employing catalytic single or co-immobilized IBs, increased stability was achieved, compared to the soluble, purified enzymes. Almost full conversion (>90 %) was achieved by IBs *R*ADH/*Pf*BAL, while soluble enzymes yielded at most >50 % conversion under the same conditions (**Jäger *et al.*, 2019b)**. From this point, so-called multi enzymatic "one-pot" synthesis requires an accurate balance of enzymatic activity of several catalysts under certain reaction conditions, and certainly, numerous optimization processes are necessary. However, promising results are in favour of further use, nevertheless definitely challenging.

OTHER APPLICATIONS OF IBS

Until the beginning of the new millennium, IBs were seen only as poorly folded inactive protein waste that aggregates into malfunctioning clusters and is removed by the quality control mechanism of the cell. However, the new perception of IBs in terms of their functionality and mechanical stability structure extended their application in several areas. Nevertheless, their presence in heterologous expression was highly undesirable and their main application in biotechnology mostly remained as protein renaturation, which involves solubilization and refolding of protein aggregates **(Rinas *et al.*, 2017)**. The solubilization approach is still used on daily basis, in a more aggressive or mild form, depending on the nature of IBs. More aggressive solubilization by high concentrations of denaturants such as urea or guanidinium chloride causes complete denaturation of the protein in IBs. Using more moderate solubilization, protein remains partially folded in its native conformation, thus avoiding re-aggregation during the refolding process. Mild solubilization includes the use of alkaline pH buffer solutions and low urea concentration combined with n-propanol or *β*-mercaptoethanol **(Singh *et al.*, 2012)**. By removing the solubilizing agent, proteins are refolded in refolding buffer. These solutions are a commercially available mixture of sugars, amino acids, alcohols, chaotropic salts, and reducing agents. In short, these ingredients help to stabilize the emerging protein and prevent possible reaggregation **(Yamaguchi and Miyazaki, 2014)**. One of many examples, horseradish peroxidase (HRP) (*Armoracia rusticana*), an exceptionally relevant enzyme in industrial and medical applications, was prepared in form of IBs, followed by solubilization and refolding **(Humer *et al.*, 2020)**. By recombinant production and refolding of its glycosylated form in *E. coli*, high final yield and purity of soluble HRP were achieved. This process can be an alternative to extraction from plant roots which is time-consuming, ends in low yields and it is not suitable for direct use in therapeutics and diagnostics applications. Denaturing conditions followed by refolding of target protein from IBs also serve as an alternative to produce proteins, which cannot be expressed in their natural soluble state in *E.coli* cells due to their toxicity. Protein toxicity within the cells often originates from an active soluble state, therefore the protein aggregates appear to be non-toxic **(Invernizzi *et al.*, 2012)**. However, this view only applies to the production of toxic recombinant proteins, because in several pathologies, the presence of protein amyloid aggregates and IBs correlates with increased cytotoxicity and has a direct impact on disease state and development. Also, IBs offer new challenging opportunities in biomedicine, such as *in vivo* studying and monitoring amyloidosis and prion diseases, targeted and untargeted drug delivery systems, therapeutic proteins, and even in tissue engineering, where they can replace existing standard materials and also serve as scaffolds. An interesting application is the production of small microbial peptides in IBs, which may serve as an alternative to common antibiotic treatment. This topic is sufficiently reviewed in **Köszagová and Nahálka, 2020**. In addition to mentioned development, IBs could also be used as biosensors for monitoring metal ions and (poly-) phosphates (**Hrabárová *et al.*, 2022**).

CONCLUSION

To sum up this review, IBs are no longer considered inactive protein waste, that needs to be discarded. On the contrary, they are gradually becoming a viable alternative to common assays in various areas of industry, science, or medicine. There are several advantages of working with IBs, such as high yield of protein amount within IBs, cost-effective and easy production, simple isolation and purification protocols, and the possibility to predict successful aggregation by the tailored design of IBs. Under favourable conditions, IBs preserve their catalytic or innate activity. Thus, they offer valued properties of biocatalysts as cheap self *in vivo* immobilization, high operational, mechanical, and thermal stability, and reusability. Last but not least, they still serve as an excellent source of recombinant protein with difficult production or cytotoxic traits. Within the cell, folded in an amyloid structure, they have the advantage of being resistant to proteases. However, even disadvantages occur when working with IBs, as not every protein can be purposefully folded into IBs while maintaining its biological activity. Along this correlates also the fact, that tailoring is often difficult and requires multiple combinations of fusion tags with linkers followed by arduous examinations for every IBs protein individually. In short, the composition of IB-proteins must be considered individually in developing their design and isolation/purification protocols.

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