# Bacterial inclusion bodies: discovering their better half

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**ABSTRACT**

Bacterial inclusion bodies are functional, non-toxic amyloids occurring in recombinant bacteria that show analogies with secretory granules of the mammalian endocrine system. The scientific interest in these mesoscale protein aggregates has been historically masked by their status as a hurdle in recombinant protein production. However, insights into their molecular organization and the progressive understanding on how the cell handles the quality of recombinant polypeptides have stimulated the interest on inclusion bodies and opened ways for their usability in diverse technological fields. The engineering and tailoring of inclusion bodies as functional protein particles for materials science and biomedicine is a good example of how formerly undesired bacterial by-products can be re-discovered as promising functional materials for a broad spectrum of applications.

## BACKGROUND

Bacterial inclusion bodies (IBs) are submicron proteinaceous particles (usually ranging from 50 to about 800 nm (Figure 1 A, B) observed in recombinant bacteria. IB formation has been regularly reported during the forced expression of cloned genes as an intrinsic, often unavoidable idiosyncrasy of the recombinant 'bacterial factory'. Under transmission electron microscopy IBs are visualized as electrodense (Figure 1 A, bottom), dark areas in the cytoplasm (or sporadically in the periplasm, for secretory proteins), as the result of the aggregation and deposition as insoluble clusters of significant fractions of recombinant protein.

In this context, IBs are considered as a major drawback for high yield production of soluble proteins, either for research or as marketable goods in the biotechnological or pharmaceutical industry [1]. In an attempt to minimize IB formation and thus shift production towards soluble protein versions, many strategies have been proposed, including genetic approaches (e.g., reduction of gene dosage or gene expression rates), physical methods (e.g., lowering the culture temperature), physiological methodologies (e.g., co-producing chaperones and folding modulators) and nutrient availability (e.g., carbon source limitation) [2, 3]. The success in the application of these procedures is not predictable, since being variable and product-linked they have not led into a universally established protocol applicable to all IB-forming proteins. On the other hand, IBs are mechanically stable protein deposits. They can be easily isolated upon cell disruption by simple physical means and used as a relatively pure source of recombinant protein which can be transformed into the active soluble form by solubilisation and subsequent refolding. For example, the first recombinant protein approved by the FDA for human application was insulin, the two chains produced in separate *E. coli* strains as fusion proteins forming IBs. Still today, human insulin is produced using two major routes. One route involves the production of a precursor in form of IBs using *E. coli* as production host with subsequent solubilization and refolding [4]. The other route involves yeast-based expression systems, which secrete a soluble insulin precursor into the culture supernatant [5, 6]. Both routes are economically viable.

## CELLULAR FORMATION OF IBs

IB formation has been associated to conformational stress, similar to that occurring under heat shock. However, the physiology of IB formation as well as the biological

traits of these entities have been in general neglected, since aggregation of recombinant proteins has been regarded as a problem to solve rather than as an appealing research target. Recombinant protein aggregation leading to IB formation, occurs in a crowded cellular environment occupied by other macromolecules not directly involved in the aggregation process. Increased crowding favors the formation of high-molecular-weight associates at the cost of correct folding, but the presence of chaperones tend to reduce aggregation even in such environment [7]. *In vitro* studies on protein aggregation indicated variability in the mechanisms of aggregation even for a given protein [8]. Kinetic analysis of *in vitro* protein aggregation [8-10] but also *in vivo* monitoring of IB formation [11] revealed that aggregate formation can be described as a pseudo-first-order process. There are two possible mechanisms for IB formation, either IBs start growing from single or limited number of molecules acting as nucleation site or from smaller aggregates which assemble and form larger aggregates [12] . An initial lag phase and the subsequently following observable aggregation process is best described by nucleation models which are also used to describe the formation of amyloids [13]. It has also been proposed that a crowded environment accelerates the rate-limiting step in amyloid formation, namely the formation of the nucleus [14].

Bacterial cells are able to actively or passively deal with synthesized proteins that cannot fold into a soluble form either because of intrinsic protein properties or because of circumstances of recombinant production. There is still controversy whether IB formation is a passive process only depending on physically interacting protein chains or if it is an energy-driven process that requires active involvement of the cell [15-17]. Studies on the properties of the bacterial cytoplasm revealed that it can adopt an either glass-like or more fluid consistency [18]. To transform the cytoplasm from a glassy (macromolecular crowded) to a more fluidic environment allowing movement of larger particles (>30 nm) requires ATP-hydrolysis and active metabolism [18, 19]. Thus, it is possible that IB formation occurs passively but only when biochemical reactions and active metabolism are disturbing the glassy structure of the cytoplasm. On the other hand there is no doubt that protein disaggregation is an ATP-driven process [20]. For example, HtpG can interact with DnaK/DnaJ/GrpE to further promote refolding of aggregated proteins in an ATP-dependent manner [21]. Alternatively, ClpB can also assist in cooperation with DnaK/DnaJ/GrpE in ATP-driven refolding of aggregated proteins [20, 22, 23]. The small heat shock proteins (sHSPs) IbpA and IbpB stabilize and decrease the size of protein aggregates [24] thereby promoting DnaK/DnaJ/GrpE and ClpB mediated refolding [25] and further possible proteolysis. IB proteins are targets of these disaggregating chaperones, which remove IB material even during IB

formation in a steady IB construction/deconstruction process [26, 27]. During recombinant protein synthesis, this process is unbalanced towards IB volumetric growth, but during biosynthetic arrest or culture ageing IBs tend to disintegrate within recombinant cells.

IBs are found preferentially in the polar region either in both poles or in the older pole after cell division (Figure 1A). The polar presence is mainly attributed to macromolecular crowding in the nucleoid region. There are claims that transport of aggregates to the poles is energy-driven as studies on reassembly of pressure dissociated IBs revealed that confinement to the pole is caused by the presence of the nucleoid but reassembly of smaller aggregates into large IBs does not occur in energy and nutrient depleted cells [16]. However, there are also reports stating that exclusion of aggregates from mid-cell to the pole is energy-free, and simply results from nucleoid exclusion [17, 20, 28-30]. Restriction of aggregates to the pole was also observed during treatment with substances disrupting the protein motive force, suggesting an energy independent process of aggregation [20]. Energy-independent polar localization was also confirmed by others [30] but also convincing opposite results were obtained [15]. Thus, it is not absolutely clear if active ATP-dependent transport of smaller particles is required or if the fluidizing properties of active metabolism are responsible for the polar preference of protein aggregates [16]. IBs and aggregation foci are not only found at the polar sites but also in mid-cells presumably at the place of future septation sites [20, 30, 31]. The major disaggregating chaperones (DnaK, ClpB) also co-localize at the poles [20] and are required for disaggregation of polar aggregates [15]. Polar distribution of IBs or damaged proteins may reflect an evolutional benefit compared to unbiased dilution of misfolded and aggregated proteins as continued cell division leads to rejuvenation [20, 31].

**COMPOSITION OF IBs**

During their formation, IBs trap, in lower amounts, a spectrum of cell proteins. This includes interacting chaperones but in a more unspecific fashion, diverse cell polypeptides as well as traces of other macromolecules from the producing bacteria, namely nucleic acids and cell wall components. A fine compositional analysis of IBs remains elusive, and the chemical diversity of IBs seems to be variable, linked to the properties of the specific recombinant protein and deeply affected by the bacterial culture conditions, host genetic background and IB purification procedures ([32], and references therein).

## INNER IB ARCHITECTURE AND FUNCTIONALITY

Initially, IBs were perceived as “boiled eggs” consisting of denatured protein devoid of biological activity and with no chances for physiological refolding within the hosting cell (Box 1). However, first reports on the pseudo-spherical to rod-shaped morphology, surface roughness (Figure 1 B) and high porosity and hydration level of IBs already indicated a more complex nature [33]. In 2005, both the amyloidal structure of bacterial IBs as well as the presence of active proteins in IBs were reported [34-36]. The identification of these two coexisting but distinguishable protein populations within the IB (amyloid forms and native-(like) variants) were offering two novel possibilities for exploration; (i) IBs as a convenient, real time platform to study and monitor amyloidosis *in vivo* and (ii) IBs as a platform to package, as mechanically stable material, functional proteins, for example, enzymes as self-immobilized catalysts. These observations strongly revitalized the research on bacterial IBs in the formerly unexpected fields of anti-amyloidal drug research (related to neurodegenerative disorders and prion diseases), material science and drug delivery (Box 1).

**Box 1. Historical shift in the perception of bacterial inclusion bodies**

| | | BEFORE 2005 | Seminal references |
|---|---|---|---|
| Perception | | Inclusion bodies are exclusively formed by misfolded or unfolded proteins, which aggregate into non-functional protein clusters excluded from the activities of the cell quality control (boiled egg model). | [1] |
| Applications | Biotechnology | Protein renaturation | [37, 38] |
| | | AFTER 2005 | |
| Perception | | Inclusion bodies are formed by a mixture of amyloid protein (giving mechanical stability) and protein variants with native-(like) conformations (providing functionality). IB proteins are released in host bacterial cells (or in uptaking mammalian cells) by components of the quality control system (dynamic IB model) | [39] |
| Applications | Biotechnology | Protein renaturation | [37] |
| | | Protein extraction | [40] |
| | | Immobilized catalysts | [41-43] |

| | | |
|---|---|---|
| Material sciences | Scaffolds in tissue engineering | [44] |
| Medicine | Models for amyloidosis and prion propagation | [45] |
| | Functional materials in tissue engineering | [46] |
| | Targeted and non-targeted drug delivery systems | [47, 48] |
| | Implantable depots of therapeutic proteins | [49] |

The discovery of amyloidal proteins in bacterial IBs changed their early consideration as amorphous particles towards that of entities possessing an inner molecular organization. Amyloid formation requires self-complementary stretches found in most proteins [50], and protein homology has been considered critical for the *in vitro* formation of IB-like aggregates [51]. Since the co-production of different unrelated but aggregation-prone proteins leads to the formation of separate instead hybrid IBs [52], the specificity in the IB formation process was then verified. Also, it was shown that *in vitro* digestion of IBs with proteinase K resulted in remnant amyloidal fibers [52], representing around 20 % of the recombinant target protein [53]. Upon partial digestion, IBs show the same morphometry than the original material, but less dense, suggesting that the amyloidal network acts as a skeleton providing the notable mechanical and chemical stability of IBs (Figure 2). The proteinase K-sensitive subpopulation of the recombinant protein, the major fraction in fact, appears as non-amyloid material most likely composed of native-(like) structure variants being responsible for the biological activities often exhibited by IBs. Culture conditions have a profound impact on IB composition and organization [32, 54, 55].

## IBs AS RAW MATERIAL FOR PROTEIN RECOVERY

First attempts to recover active proteins from bacterial IBs were mainly based on solubilisation of IB proteins using strong chaotropic reagents followed by subsequent

renaturation procedures [37, 38] (Figure 2). Usually, IB solubilisation involves only urea or guanidinium hydrochloride and a reducing reagent. Renaturation, however, is multifaceted involving various procedures, physical conditions and supplemented chemicals. Best conditions depend on individual protein properties and still need to be established by trial-and-error; a universally applicable renaturation procedure appears inaccessible.

Apart from renaturation of IB proteins solubilised by structure-breaking chaotrops, some IB deposited proteins can be extracted in their biologically active form using mild extraction conditions (Figure 2) [40]. Although IBs are solid, mechanically stable entities, the functional fraction of IB protein appears to occur in a soluble or quasi-soluble state, and it can be released *in vitro* by means of mild extraction protocols [55], complementing the denaturation-based strategies for IB protein recovery (Figure 2). The extractable fraction can be enhanced by e.g. growing the bacterial cells during IB formation at low temperatures [55]. Moreover, this strategy simplifies the protein recovery from IBs by combining the former solubilization and renaturation steps into a single extraction step.

## IBs AS IMMOBILIZED CATALYSTS

In physiological buffers, the fraction of native-(like) IB protein remains embedded in the IBs and only a minor part is released into the environment. Thus, IBs formed by enzymes can act as mechanically and functionally stable porous catalysts, reusable, and having a high mass transfer capacity [42]. Reductases, oxidases, kinases, phosphorylases, aldolases, lyases, synthases and lipases [42, 43], in form of IBs, have been studied as self-immobilized particulate catalysts with encouraging results (Figure 3 A left, recombinant protein i). In a step further, enzyme-decorated IBs based on scaffold proteins (recombinant protein ii) to form IBs, followed by *in vivo* surface-immobilization of an enzyme (Figure 3 A left, recombinant protein iii) have been developed as promising modular IB-based particulate catalysts [56]. In both approaches, IBs are easily removed from the reaction mixture by centrifugation, and can be recycled in repeated-batch reactions for further use [42] (Figure 3 B).

For enzymes that require post-translational modifications, aggresomes produced in mammalian cells represent functional alternatives to bacterial IBs. In a recent study, a recombinant human α-galactosidase A (GLA) was produced in mammalian cells as enzymatically active, mechanically stable aggresomes [57] representing approximately

40 % of the total product. These functional aggresomes were more resistant to thermal inactivation than the soluble version of the enzyme, and were easily recovered and reused at least in five consecutive enzymatic reactions [57]. A comparative between the performance of aggresomes and IBs formed by enzymes, respective to soluble enzymes, is shown in Figure 3 C.

## IBs AS MECHANICALLY STABLE AND BIOCOMPATIBLE MATERIALS

The mechanical stability of bacterial IBs is notable. They resist harsh cell disruption conditions based on sonication or high pressure homogenization (as in French Press), as well as lyophilisation or long-term storage under different conditions [44, 58]. Moreover, the size of IBs is controllable by selecting the harvesting time after induction of protein synthesis as they progressively grow during synthesis. IBs complement the collection of other newly developed protein-based materials such as elastin-like polypeptides, silk proteins, mussel adhesive proteins, and other self-assembling peptides providing architecture and functionality [59]. In this line, IBs have also emerged as a promising novel biomaterial which supports adhesive growth of mammalian cells [60, 61]. They do not show any sign of toxicity despite their compositional heterogeneity and naturally high, intrinsic potential to cross cell membranes and internalize into the cytoplasm. Immobilized on 2D or 3D surfaces, IBs increase cell adhesion and provide mechanical stimuli for cell proliferation and differentiation. For example, human mesenchymal stem cells show differentiation towards osteoblasts when grown on GFP-based IBs produced in a ClpA$^-$ strain [62]. Also, topographical microscale patterns made of IBs impact on cell positioning, morphology and migration [63]. Interestingly, IB physical parameters such as size, wettability, stiffness and superficial charge are within the range of optimal values for surface cell colonization [64]. Moreover, the values of all these features vary between particles produced in bacteria with different genetic background, namely mutants in key genes of the quality control/protein folding machinery, including *dnaK*, *clpA*, and *clpP [64]*.

When IBs are formed by a biologically relevant protein, e.g. basic fibroblast growth factor 2, they exert a dual action onto growing cells, both as topographies but also as functional agents. Such combined effect of IBs, exhibiting biological activity but also

representing a scaffold, offer a promising approach to decorate surfaces which then mimic the natural extracellular matrix in artificial environments [65].

## IBs AS PROTEIN-RELEASING AGENTS

Active protein is released from IBs when adhered to substrate on which cells grow (of interest in tissue engineering) [65-67], but also, and more efficiently, upon spontaneous internalization into mammalian cells (Figure 4) [47, 68]. The high cell membrane avidity of bacterial IBs is probably supported by the amphiphilic nature of the material [64], that might promote an early anchorage to facilitate micropinocytosis. IBs are recognized by the mammalian cell sensing machinery and get closely attached to cell membranes quickly after addition of IBs to the culture media. This conduces to a high percentage of exposed cultured cells (up to 70 %) to internalize non-targeted IBs. *In vivo*, up to 75-80 % of macrophages isolated from the zebrafish spleen contained IBs 48 h after IB intraperitoneal injection [58].

IB protein release can be verified by the physiological effect that the IB-derived soluble protein exerts in different cellular models *in vitro* [47, 65], showing a biological impact such as reconstruction of the cytoskeleton (keratin 14) [69], cell growth recovery (dihydrofolate reductase, leukemia inhibitory factor, fibroblast growth factor) [47], reduced oxidative stress (catalase) [47] or anti-apoptotic rescuing (Hsp70) [47] and *in vivo* as immunostimulant (cytokines) [70]. Oral delivery of IBs in mice and intraperitoneal or intratumoral injection in zebrafish and in mice does not result in detectable signs of local or systemic toxicity [47, 70]. Tumor necrosis factor (TNF)-$\alpha$ IBs, orally administered to zebrafish, were able to establish close contact with relevant sections of the gut for further IB-derived immune protection from bacterial infections [70]. Functional IBs, when delivering intracellular proteins with potential healing value can be regarded as a novel type of nanostructured therapeutic agent or nanopill. The protein production process can be then tailored to package a given functional protein in form of biologically active IBs for delivery, and nanopills can be targeted to specific cell types by the chemical (downstream, [71]) or genetic (upstream, [48]) addition of a specific cell surface ligand. In the latter case, through the fusion of the CXCR4-binding peptides T22 and R9 to the amino terminus of an IB forming GFP, fluorescent IBs penetrate CXCR4+ cells *in vitro*, in a receptor-dependent specific way. An RGD domain, inserted in the central region of a chimeric IB protein also favors cell binding and penetration through cell surface integrins [68]. Furthermore, the release of the functional protein to the intracellular space may also be favored by tailoring the fusion protein that forms IBs with autocleavable domains, such as sortase A and intein, that are activated under physiological conditions [71, 72].

IBs appear to belong to the so-called group of non-toxic functional amyloids, to which secretory granules of the mammal endocrine system also belong. Protein hormones such as glucagon, bombesin, obestatin, prolactin, secretin and urotensin are delivered to the body for function by secretory granules showing an amyloidal organization, in which cross-β sheet contacts confer stability to the whole protein complex [73]. Acting as long term *in vivo* depots, these granules release monomeric hormone units through a poorly known mechanism that probably involves molecular chaperones [73]. The functional analogies observed between secretory granules and IBs used as nanopills has prompted to the recent testing of IBs as immobilized *in vivo* depots for functional proteins. Here, the intratumoral injection of IBs formed by a downregulator of CXCR4, the peptide T22, promotes local apoptosis in absence of systemic toxicity [48]. Experimental data suggest a sustained release from the IB-based depot of the IB protein component to surrounding tissues, while the depot itself contains functional protein at least one week after inoculation of the material [49].

## PROMOTING IB FORMATION

The enhancement of IB formation might be desirable when IBs are the intended product of a protein production process. Shifting protein production towards IB formation can be achieved, e.g. by growing cells at suboptimal elevated temperatures (40-42ºC) or more elegantly, by fusion of aggregation-prone peptides to the target protein. Attachment of pull-down peptides promote IB formation at physiological temperatures (Table 2) and can at the same time preserve the functionality of the non-amyloidal fraction of the IB protein.

**Table 2. Pull-down peptides recently developed to enhance protein aggregation or IB formation in *E. coli*.** Most tags have been designed to keep the functionality of the recombinant protein, resulting in biologically active aggregates or IBs.

| Type | Pull down peptide | Target IB protein | Comments | Reference |
|---|---|---|---|---|
| Viral peptide | VP1 protein (from Foot-and-Mouth Disease Virus) | *Escherichia coli* β-galactosidase | N-terminal tag | [74] |
| Cellulose-binding domain (CBD) | *Cellulomonas fimi* exo-glucanase CBD | *Escherichia coli* β-glucuronidase (GusA) and *Thermus caldophilus* β-glycosidase (BglA) | C-terminal tag | [75] |
| | *Clostridium cellulovorans* CBD | *Trigonopsis variabilis* D-amino acid oxidase | N-terminal tag | [76] |
| Bacterial β- | β-Barrel membrane | N-terminal region of human | N-terminal tag | [77] |

| | | | | |
|---|---|---|---|---|
| barrel membrane protein | protein PagP | cardiac troponin I (residues 1–71) | Protein purification from IBs | |
| All hydrophobic self-assembling peptides | GFIL8 (GFILGFIL) and GIFL16 (GFILGFILGFILGFIL) | *B. subtilis* lipase A (LipA) and *A. fumigates* amadoriase II (AMA) | C-terminal tag<br><br>GFIL8 with the hydrophobic residue Ala (GAIL8) showed negligible protein aggregating properties | [72] |
| Surfactant like peptides | $L_6KD$, $L_6K_2$, $DKL_6$ | *A. fumigatus* amadoriase II (AMA), *B. subtilis* lipase A (LipA), *B. pumilus* xylosidase (XynB), and green fluorescent protein (GFP) | C-terminal tag | [78] |
| Other peptides | Polypeptide derived from the C-terminal region of *Bacillus thuringiensis* Cry4Aa toxin (4AaCter) | Cysteine protease inhibitor (Cystatin C) | N-terminal tag | [79] |
| | Npro (EDDIE)-autoprotease fusion protein | Intrinsically disordered proteins (IDPs) | N-terminal tag<br><br>Protein purification from IBs | [80] |
| | Ketosteroid isomerase (Ksi) | Tandem repeats recombinants of *Conus lividus* Conotoxin LvIA (CTx LvIA) | N-terminal tag<br><br>Protein purification from IBs | [81] |
| | Tetramerization domain of the cell-surface protein tetrabrachion (TdoT) from *Staphylothermus marinus* | *Bacillus subtilis* the lipase A (BsLA), *Arabidopsis thaliana* hydroxynitrile lyase (AtHNL), and *E. coli* 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase MenD (EcMenD) | N-terminal tag | [82] |

## CONCLUSIONS AND FUTURE PERSPECTIVES

Applications of functional IBs in veterinary medicine, as food additives, in human cosmetics and for transdermal or transmucosal protein delivery might be highly realistic. However, a wider biomedical applicability of bacterial IBs in human medicine might pose, a priori, severe scientific and regulatory concerns (see Outstanding Questions). These are mainly associated to the intrinsic compositional heterogeneity and presence of contaminants from the bacterial cell factory, including endotoxins, whose complete removal looks technically unapproachable. In this regard, and although IBs have been traditionally associated to the enterobacterium *E. coli* as host for gene expression, IB-like materials had also been described in the Gram-positive bacterial species *Corynebacterium glutamicum* [83], *Lactococcus lactis* [84] and *Bacillus subitlis* [85]. Besides, the presence of functional aggregates had also been reported in the yeast species *Schizosaccharomyces pombe* [86] and *Saccharomyces cerevisiae [87]*. The

controlled production of functional IB analogues in *Pichia pastoris* [88] and in the food-grade bacterium *Lactococcus lactis* has just been demonstrated [89]. Together with the recent description of IB fabrication in endotoxin-free *E. coli [90, 91]*, the production of IBs in microorganisms other than Gram-negative, endotoxin-containing bacteria is expanding the catalogues of cell factories for biologically safer IB-related products, what would conduct to a smoother implementation of IB-based protein delivery technologies in different fields.

**Acknowledgments**: We are indebted to Alejandro Sánchez-Chardi for his assistance in electron microscopy. Also, we acknowledge MINECO (BIO2013-41019-P), AGAUR (2014SGR-132), Marató de TV3 foundation (TV32013-132031) and CIBER de Bioingeniería, Biomateriales y Nanomedicina (project NANOPROTHER) to AV, and Marató de TV3 foundation (TV32013-3930) and FIS (PI15/00272) to EV for funding research on protein-based therapeutic materials. UR gratefully acknowledges funding through the Cluster of Excellence “Rebirth” and German Research group 2180 “Graded Implants”. AV received an ICREA ACADEMIA award, JSF is a recipient of a post-doctoral fellowship from Asociación Española Contra el Cáncer (AECC), and EGF received a post-doctoral fellowship from INIA (DOC-INIA, MINECO).

## Legends

**Figure 1. General morphometric traits of bacterial inclusion bodies**. A. Polar distribution of fluorescent IBs formed by a GFP fusion in *Escherichia coli* cells, upon recombinant gene expression (top, reproduced with permission from [91]. Under transmission electron microscopy IBs are visualized as electrodense areas (bottom). B. Scanning electron microscopy of purified GFP-based IBs (top), and broad field confocal images and 3D reconstructions, showing the roughness of IB surface (bottom).

**Figure 2. IBs as raw material for protein recovery through renaturation or mild extraction.** Red lines in IBs represent the amyloidal scaffold in which proteins with native-(like) conformational states are embedded (green). **z** symbols represent solubilized unfolded polypeptides while **a** symbols represent protein in the native-(like) conformation.

**Figure 3. IBs as immobilized catalysts.** A. Functional IBs solely composed of enzymes encoded by gene i present in the insoluble cell fraction (I) or IBs formed by a scaffold protein (encoded by gene ii) decorated *in vivo* by a co-produced recombinant enzyme with binding affinities to the scaffold protein being present in the soluble cell fraction (S) and encoded by gene iii. B. Both types of IBs are easily reusable in repeated enzyme catalyzed reactions. C. Comparison of properties of soluble enzymes, bacterial IBs and related mammalian aggresomes formed by enzymes.

**Figure 4. Endocytosis of IBs into mammalian cells.** A. Deep interactions between membranes of cultured mammalian cells (red) and GFP based IBs attached to the culture surface (green). Reproduced from [44] with permission. B. Green fluorescent IBs suspended in culture media penetrating cultured mammalian cells (reproduced from [92]). C-D. Overview of the contacts between IBs in suspension and filopodia of cultured cells, showing some IB particles fully endocyted. Scale bar represents 1 μm. Panels E and F show IB-cell membrane contacts in more detail. Red arrows indicate points in which the particle seems to disrupt cell membrane and to get partially

embedded. Scale bar in panel E represents 200 nm while in panel F it indicates 100 nm. G. Scheme of IB interaction with mammalian cells at distinct stages.

References

1 Baneyx, F. and Mujacic, M. (2004) Recombinant protein folding and misfolding in Escherichia coli. *Nature biotechnology* 22, 1399-1408

2 Sorensen, H.P. and Mortensen, K.K. (2005) Advanced genetic strategies for recombinant protein expression in Escherichia coli. *Journal of biotechnology* 115, 113-128

3 Sorensen, H.P. and Mortensen, K.K. (2005) Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli. *Microbial cell factories* 4, 1

4 Nilsson, J.*, et al.* (1996) Integrated production of human insulin and its C-peptide. *Journal of biotechnology* 48, 241-250

5 Kjeldsen, T. (2000) Yeast secretory expression of insulin precursors. *Applied microbiology and biotechnology* 54, 277-286

6 Gurramkonda, C.*, et al.* (2010) Application of simple fed-batch technique to high-level secretory production of insulin precursor using Pichia pastoris with subsequent purification and conversion to human insulin. *Microbial cell factories* 9, 31

7 Chebotareva, N.A.*, et al.* (2013) Effect of crowding and chaperones on self-association, aggregation and reconstitution of apophosphorylase b. *International journal of biological macromolecules* 60, 69-76

8 Wang, G. and Fersht, A.R. (2012) First-order rate-determining aggregation mechanism of p53 and its implications. *Proceedings of the National Academy of Sciences of the United States of America* 109, 13590-13595

9 Kurganov, B.I. (2002) Kinetics of protein aggregation. Quantitative estimation of the chaperone-like activity in test-systems based on suppression of protein aggregation. *Biochemistry-Moscow+* 67, 409-422

10 Kurganov, B.I.*, et al.* (2002) Kinetics of thermal aggregation of tobacco mosaic virus coat protein. *Biochemistry-Moscow+* 67, 525-533

11 Hoffmann, F.*, et al.* (2001) Kinetic model of in vivo folding and inclusion body formation in recombinant Escherichia coli. *Biotechnology and bioengineering* 72, 315-322

12 Upadhyay, A.K.*, et al.* (2012) Kinetics of Inclusion Body Formation and Its Correlation with the Characteristics of Protein Aggregates in Escherichia coli. *PloS one* 7

13 Villar-Pique, A.*, et al.* (2016) In vivo amyloid aggregation kinetics tracked by time-lapse confocal microscopy in real-time. *Biotechnol J* 11, 172-177

14 Magno, A.*, et al.* (2010) Crowding Effects on Amyloid Aggregation Kinetics. *J Phys Chem Lett* 1, 3027-3032

15 Rokney, A.*, et al.* (2009) E. coli Transports Aggregated Proteins to the Poles by a Specific and Energy-Dependent Process. *Journal of molecular biology* 392, 589-601

16 Govers, S.K.*, et al.* (2014) In Vivo Disassembly and Reassembly of Protein Aggregates in Escherichia coli. *Journal of bacteriology* 196, 2325-2332

17 Oliveira, S.M.D.*, et al.* (2016) Increased cytoplasm viscosity hampers aggregate polar segregation in Escherichia coli. *Mol Microbiol* 99, 686-699

18 Amen, T. and Kaganovich, D. (2015) Dynamic droplets: the role of cytoplasmic inclusions in stress, function, and disease. *Cell Mol Life Sci* 72, 401-415

19 Parry, B.R.*, et al.* (2014) The Bacterial Cytoplasm Has Glass-like Properties and Is Fluidized by Metabolic Activity. *Cell* 156, 183-194

20 Winkler, J.*, et al.* (2010) Quantitative and spatio-temporal features of protein aggregation in Escherichia coli and consequences on protein quality control and cellular ageing. *Embo J* 29, 910-923

21 Genest, O.*, et al.* (2011) Heat shock protein 90 from Escherichia coli collaborates with the DnaK chaperone system in client protein remodeling. *Proceedings of the National Academy of Sciences of the United States of America* 108, 8206-8211

22 Ben-Zvi, A.*, et al.* (2004) Active solubilization and refolding of stable protein aggregates by cooperative unfolding action of individual Hsp70 chaperones. *Journal of Biological Chemistry* 279, 37298-37303

23 Acebron, S.P.*, et al.* (2009) DnaK-mediated association of ClpB to protein aggregates. A bichaperone network at the aggregate surface (vol 583, pg 2991, 2009). *FEBS letters* 583, 3301-3301

24 Ratajczak, E.*, et al.* (2009) Distinct Activities of Escherichia coli Small Heat Shock Proteins IbpA and IbpB Promote Efficient Protein Disaggregation. *Journal of molecular biology* 386, 178-189

25 Matuszewska, M.*, et al.* (2005) The small heat shock protein IbpA of Escherichia coli cooperates with IbpB in stabilization of thermally aggregated proteins in a disaggregation competent state. *Journal of Biological Chemistry* 280, 12292-12298

26 Carrio, M.M. and Villaverde, A. (2002) Construction and deconstruction of bacterial inclusion bodies. *Journal of biotechnology* 96, 3-12

27 Betiku, E. (2006) Molecular Chaperones involved in Heterologous Protein Folding in Escherichia coli. *Biotechnology and Molecular Biology Review* 1

28 Neeli-Venkata, R.*, et al.* (2016) Robustness of the Process of Nucleoid Exclusion of Protein Aggregates in Escherichia coli. *Journal of bacteriology* 198, 898-906

29 Gupta, A.*, et al.* (2014) In Vivo Kinetics of Segregation and Polar Retention of MS2-GFP-RNA Complexes in Escherichia coli. *Biophys J* 106, 1928-1937

30 Coquel, A.S.*, et al.* (2013) Localization of Protein Aggregation in Escherichia coli Is Governed by Diffusion and Nucleoid Macromolecular Crowding Effect. *PLoS computational biology* 9

31 Lindner, A.B.*, et al.* (2008) Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proceedings of the National Academy of Sciences of the United States of America* 105, 3076-3081

32 Jurgen, B.*, et al.* (2010) Quality control of inclusion bodies in Escherichia coli. *Microbial cell factories* 9, 41

33 Villaverde, A. and Carrio, M.M. (2003) Protein aggregation in recombinant bacteria: biological role of inclusion bodies. *Biotechnology letters* 25, 1385-1395

34 Garcia-Fruitos, E.*, et al.* (2005) Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. *Microbial cell factories* 4, 27

35 Jevsevar, S.*, et al.* (2005) Production of nonclassical inclusion bodies from which correctly folded protein can be extracted. *Biotechnology progress* 21, 632-639

36 Carrio, M.*, et al.* (2005) Amyloid-like properties of bacterial inclusion bodies. *Journal of molecular biology* 347, 1025-1037

37 Vallejo, L.F. and Rinas, U. (2004) Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. *Microbial cell factories* 3, 11

38 Singh, S.M. and Panda, A.K. (2005) Solubilization and refolding of bacterial inclusion body proteins. *J Biosci Bioeng* 99, 303-310

39 Gonzalez-Montalban, N.*, et al.* (2007) Recombinant protein solubility - does more mean better? *Nature biotechnology* 25, 718-720

40 Singh, A.*, et al.* (2015) Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. *Microbial cell factories* 14, 41

41 Garcia-Fruitos, E.*, et al.* (2012) Bacterial inclusion bodies: making gold from waste. *Trends in biotechnology* 30, 65-70

42 Hrabarova, E.*, et al.* (2015) Insoluble Protein Applications: The Use of Bacterial Inclusion Bodies as Biocatalysts. *Insoluble Proteins: Methods and Protocols* 1258, 411-422

43 Garcia-Fruitos, E. and Villaverde, A. (2010) Friendly production of bacterial inclusion bodies. *Korean J Chem Eng* 27, 385-389

44 Garcia-Fruitos, E.*, et al.* (2009) Surface Cell Growth Engineering Assisted by a Novel Bacterial Nanomaterial. *Advanced materials* 21, 4249-+

45 Espargaro, A.*, et al.* (2012) Yeast prions form infectious amyloid inclusion bodies in bacteria. *Microbial cell factories* 11, 89

46 Seras-Franzoso, J.*, et al.* (2015) Integrating mechanical and biological control of cell proliferation through bioinspired multieffector materials. *Nanomedicine* 10, 873-891

47 Vazquez, E.*, et al.* (2012) Functional inclusion bodies produced in bacteria as naturally occurring nanopills for advanced cell therapies. *Advanced materials* 24, 1742-1747

48 Unzueta, U et al Engineering tumor cell targeting in nanoscale amyloidal materials. *Nanotechnology*. In press.

49 Cespedes, M.V.*, et al.* (2016) Bacterial mimetics of endocrine secretory granules as immobilized in vivo depots for functional protein drugs. *Scientific reports* 6, 35765

50 Goldschmidt, L.*, et al.* (2010) Identifying the amylome, proteins capable of forming amyloid-like fibrils. *Proceedings of the National Academy of Sciences of the United States of America* 107, 3487-3492

51 Speed, M.A.*, et al.* (1996) Specific aggregation of partially folded polypeptide chains: The molecular basis of inclusion body composition. *Nature biotechnology* 14, 1283-1287

52 Morell, M.*, et al.* (2008) Inclusion bodies: specificity in their aggregation process and amyloid-like structure. *Biochimica et biophysica acta* 1783, 1815-1825

53 Cano-Garrido, O.*, et al.* (2013) Supramolecular organization of protein-releasing functional amyloids solved in bacterial inclusion bodies. *Acta biomaterialia* 9, 6134-6142

54 Castellanos-Mendoza, A.*, et al.* (2014) Influence of pH control in the formation of inclusion bodies during production of recombinant sphingomyelinase-D in Escherichia coli. *Microbial cell factories* 13, 137

55 Peternel, S.*, et al.* (2008) Engineering inclusion bodies for non denaturing extraction of functional proteins. *Microbial cell factories* 7

56 Steinmann, B.*, et al.* (2010) In vivo enzyme immobilization by inclusion body display. *Applied and environmental microbiology* 76, 5563-5569

57 Rodriguez-Carmona, E.*, et al.* (2015) A novel bio-functional material based on mammalian cell aggresomes. *Applied microbiology and biotechnology* 99, 7079-7088

58 Torrealba, D.*, et al.* (2016) Complex Particulate Biomaterials as Immunostimulant-Delivery Platforms. *PloS one* 11, e0164073

59 Corchero, J.L.*, et al.* (2014) Recombinant protein materials for bioengineering and nanomedicine. *Nanomedicine* 9, 2817-2828

60 Loo, Y.*, et al.* (2015) Self-Assembled Proteins and Peptides as Scaffolds for Tissue Regeneration. *Advanced healthcare materials* 4, 2557-2586

61 Villaverde, A.*, et al.* (2015) Functional protein aggregates: just the tip of the iceberg. *Nanomedicine* 10, 2881-2891

62 Seras-Franzoso, J.*, et al.* (2014) Topographically targeted osteogenesis of mesenchymal stem cells stimulated by inclusion bodies attached to polycaprolactone surfaces. *Nanomedicine* 9, 207-220

63 Tatkiewicz, W.I.*, et al.* (2013) Two-dimensional microscale engineering of protein-based nanoparticles for cell guidance. *ACS nano* 7, 4774-4784

64 Diez-Gil, C.*, et al.* (2010) The nanoscale properties of bacterial inclusion bodies and their effect on mammalian cell proliferation. *Biomaterials* 31, 5805-5812

65 Seras-Franzoso, J.*, et al.* (2014) Improving protein delivery of fibroblast growth factor-2 from bacterial inclusion bodies used as cell culture substrates. *Acta biomaterialia* 10, 1354-1359

66 Seras-Franzoso, J.*, et al.* (2013) A nanostructured bacterial bioscaffold for the sustained bottom-up delivery of protein drugs. *Nanomedicine* 8, 1587-1599

67 Seras-Franzoso, J.*, et al.* (2013) Functionalization of 3D scaffolds with protein-releasing biomaterials for intracellular delivery. *Journal of controlled release : official journal of the Controlled Release Society* 171, 63-72

68 Seras-Franzoso, J.*, et al.* (2016) Cellular uptake and intracellular fate of protein releasing bacterial amyloids in mammalian cells. *Soft matter* 12, 3451-3460

69 Liovic, M.*, et al.* (2012) Inclusion bodies as potential vehicles for recombinant protein delivery into epithelial cells. *Microbial cell factories* 11

70 Torrealba, D.*, et al.* (2016) Nanostructured recombinant cytokines: A highly stable alternative to short-lived prophylactics. *Biomaterials* 107, 102-114

71 Talafova, K.*, et al.* (2013) Bacterial inclusion bodies as potential synthetic devices for pathogen recognition and a therapeutic substance release. *Microbial cell factories* 12

72 Wang, X.*, et al.* (2015) Formation of active inclusion bodies induced by hydrophobic self-assembling peptide GFIL8. *Microbial cell factories* 14

73 Maji, S.K., *et al.* (2009) Functional amyloids as natural storage of peptide hormones in pituitary secretory granules. *Science* 325, 328-332

74 Corchero, J.L., *et al.* (1996) The position of the heterologous domain can influence the solubility and proteolysis of beta-galactosidase fusion proteins in E. coli. *Journal of biotechnology* 48, 191-200

75 Choi, S.L., *et al.* (2011) Generation of catalytic protein particles in Escherichia coli cells using the cellulose-binding domain from Cellulomonas fimi as a fusion partner. *Biotechnol Bioproc E* 16, 1173-1179

76 Nahalka, J. and Nidetzky, B. (2007) Fusion to a pull-down domain: A novel approach of producing Trigonopsis variabilis D-amino acid oxidase as insoluble enzyme aggregates. *Biotechnology and bioengineering* 97, 454-461

77 Hwang, P.M., *et al.* (2012) A PagP fusion protein system for the expression of intrinsically disordered proteins in Escherichia coli. *Protein Expres Purif* 85, 148-151

78 Zhou, B.H., *et al.* (2012) Small surfactant-like peptides can drive soluble proteins into active aggregates. *Microbial cell factories* 11

79 Hayashi, M., *et al.* (2013) Efficient production of recombinant cystatin C using a peptide-tag, 4AaCter, that facilitates formation of insoluble protein inclusion bodies in Escherichia coli. *Protein Expres Purif* 88, 230-234

80 Goda, N., *et al.* (2015) A Method for Systematic Assessment of Intrinsically Disordered Protein Regions by NMR. *International journal of molecular sciences* 16, 15743-15760

81 Li, Q.X., *et al.* (2012) Expression and purification of the p75 neurotrophin receptor transmembrane domain using a ketosteroid isomerase tag. *Microbial cell factories* 11

82 Diener, M., *et al.* (2016) Fusion of a Coiled-Coil Domain Facilitates the High-Level Production of Catalytically Active Enzyme Inclusion Bodies. *Chemcatchem* 8, 142-152

83 Billman-Jacobe, H., *et al.* (1995) Expression and secretion of heterologous proteases by Corynebacterium glutamicum. *Applied and environmental microbiology* 61, 1610-1613

84 Lu, W., *et al.* (2013) Construction and application of a food-grade expression system for Lactococcus lactis. *Molecular biotechnology* 54, 170-176

85 Welsch, N.*, et al.* (2015) Stepwise optimization of a low-temperature Bacillus subtilis expression system for "difficult to express" proteins. *Applied microbiology and biotechnology* 99, 6363-6376

86 Matsuyama, A.*, et al.* (2006) ORFeome cloning and global analysis of protein localization in the fission yeast Schizosaccharomyces pombe. *Nature biotechnology* 24, 841-847

87 Cohen, A.*, et al.* (2012) Aggregation of polyQ proteins is increased upon yeast aging and affected by Sir2 and Hsf1: novel quantitative biochemical and microscopic assays. *PloS one* 7, e44785

88 Rueda, F.*, et al.* (2016) Functional inclusion bodies produced in the yeast Pichia pastoris. *Microbial cell factories* 15, 166

89 Cano-Garrido, O.*, et al.* (2016) Functional protein-based nanomaterial produced in microorganisms recognized as safe: A new platform for biotechnology. *Acta biomaterialia* 43, 230-239

90 Mamat, U.*, et al.* (2015) Detoxifying Escherichia coli for endotoxin-free production of recombinant proteins. *Microbial cell factories* 14, 57

91 Rueda, F.*, et al.* (2014) Production of functional inclusion bodies in endotoxin-free Escherichia coli. *Applied microbiology and biotechnology* 98, 9229-9238

92 Villaverde, A.*, et al.* (2012) Packaging protein drugs as bacterial inclusion bodies for therapeutic applications. *Microbial cell factories* 11, 76