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Bacterial inclusion bodies are industrially exploitable amyloids

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Abstract

Understanding the structure, functionalities and biology of functional amyloids is an issue of emerging interest. Inclusion bodies, namely protein clusters formed in recombinant bacteria during protein production processes, have emerged as unanticipated, highly tunable models for the scrutiny of the physiology and architecture of functional amyloids. Based on an amyloidal skeleton combined with varying amounts of native or native-like protein forms, bacterial inclusion bodies exhibit an unusual arrangement that confers mechanical stability, biological activity and conditional protein release, being thus exploitable as versatile biomaterials. The applicability of inclusion bodies in biotechnology as enriched sources of protein and reusable catalysts, and in biomedicine as biocompatible topographies, *nanopills*, or mimetics of endocrine secretory granules has been largely validated. Beyond these uses, the dissection of how recombinant bacteria manage the aggregation of functional protein species into structures of highly variable complexity offers insights about unsuspected connections between protein quality (conformational status compatible with functionality) and cell physiology.

1. Introduction

Many bacterial species, as transformers of organic and inorganic substances, are used in food technologies and other fields with established or rising economic impact such as fabrics, bioremediation or mining, for performing controlled processes of industrial interest (Demain, 2000). This is done by exploiting the metabolic diversity and versatility of prokaryotic cells that, due to their evolutionary adaptation to different environments and physicochemical conditions, developed a vast spectrum of alternative physiologic strategies. In addition, bacteria have been also engineered as cell factories for the production of macromolecules (mainly proteins, nucleic acids and polymers), metal particles and secondary metabolites. The production of these substances is continuously optimized by process (tailored culture conditions for fast grow or high productivity) and genetic approaches (mutant strains with improved functionalities and metabolic routes). The resulting spectrum of biological products infiltrates diverse areas in biotechnology and pharmaceutical industries. Furthermore, an increasing number of applications emerges also in very diverse fields such as energy, electronics, material sciences and nanotechnologies (Rodriguez-Carmona & Villaverde, 2010, Du*, et al.*, 2011, Lee*, et al.*, 2012, Malik*, et al.*, 2018).

Among bacterial products, proteins are of special interest as structural but also functional agents, and they can be easily produced by conventional recombinant DNA strategies or by gene synthesis (Hartley, 2006). Proteins are produced not only in bacteria (Ferrer-Miralles & Villaverde, 2013, Overton, 2014) but also in other cell factories (mainly yeasts, filamentous fungi, mammalian and insect cells, and whole plant or animals) (Corchero*, et al.*, 2013). Recombinant enzymes are employed in food technologies, in chemicals, detergent and fabric manufacture, as well as biopharmaceuticals for protein replacement therapies and in life sciences research (Vellard, 2003). In this context, hormones, enzymes, antigens and antibodies are common protein drugs and a significant number of them, approved and marketed for human therapies, are produced in bacterial cells (Ferrer-Miralles*, et al.*, 2009, Overton, 2014, Sanchez-Garcia*, et al.*, 2016). Many vaccines are based on conventional recombinant proteins (Nascimento & Leite, 2012) or on a specific category of self-

assembling protein materials named virus-like particles (Pattenden*, et al.*, 2005, Lua*, et al.*, 2014), that mimic structural and antigenic features of natural viruses.

Protein production in bacterial cell factories was indeed made possible through the development of recombinant DNA technologies (Ferrer-Miralles*, et al.*, 2009), that raised in the late 70's with the discovery and application of restriction enzymes. Since then, recombinant protein production has become a routine practice worldwide. Protein production is reached by exploiting an expanding catalogue of bacterial species as biological factories. These show distinguishable properties regarding culture requirements, use of carbon sources, metabolic capabilities and protein secretability (Corchero*, et al.*, 2013, Ferrer-Miralles & Villaverde, 2013). The enterobacterium *Escherichia coli* was the initial recombinant cell factory and still the most universally preferred system for protein production, because of its easy and fast culture, deep understanding of genetics and physiology as well as for the availability of numerous tools for genetic manipulation (Rosano & Ceccarelli, 2014).

The vast majority of proteins in nature are active as soluble species. Then, protein production processes are aimed to obtain soluble proteins with native, functional structure to nicely mimic their natural activities. While the native conformation is reached through a correct folding process,-protein misfolding and consequent aggregation in the cytosol (for intracellular) or the periplasm (for secreted) is a very common event. As a consequence, in most production processes the occurrence of insoluble protein species has been reported, often accompanied by a fraction of soluble protein (de Marco, 2013). The ratio between soluble fraction and total protein (the supposed percentage of soluble and functionally folded protein) is then commonly used as an indication for a successful process.

2. Inclusion bodies in recombinant bacteria

Aggregates of recombinant proteins occur as small supramolecular entities in the soluble cell fraction or as larger protein clusters that precipitate as insoluble materials. Therefore, the recombinant protein species range along a spectrum of conformational versions that embrace soluble protein species, soluble aggregates and insoluble

aggregates (Schrodel & de Marco, 2005, Martinez-Alonso*, et al.*, 2008). Soluble aggregates or proto-aggregates have been usually considered as precursors of large protein deposits (Martinez-Alonso*, et al.*, 2009). Aggregated proteins tend to eventually accumulate in recombinant cells as refractile (under optical microscope visualization), electrodense (under transmission electron microscope visualization) submicron particles known as inclusion bodies (IBs, Figure 1A, B) (Villaverde & Carrio, 2003). IBs have been considered to be a main bottleneck in the protein production processes and their formation has been associated to improper protein folding or misfolding (Villaverde & Carrio, 2003, Baneyx & Mujacic, 2004). Despite the development of diverse approaches and algorithms to predict protein solubility based on the primary sequence (Smialowski*, et al.*, 2007, Tjong & Zhou, 2008, Magnan*, et al.*, 2009, Diaz*, et al.*, 2010, Agostini*, et al.*, 2012, de Groot*, et al.*, 2012, Huang*, et al.*, 2012, Smialowski*, et al.*, 2012, Chang*, et al.*, 2014, Yang*, et al.*, 2016, Paladin*, et al.*, 2017, Roche*, et al.*, 2017, Khurana*, et al.*, 2018, Rawi*, et al.*, 2018), the tendency of a given recombinant polypeptide to form IBs cannot be accurately anticipated. While the progress in *in silico* prediction of solubility would be highly convenient (as it might allow preventing unsuccessful efforts in the production of insoluble proteins and then save costs) it is still an unmet target. This is likely because solubility of a recombinant protein does not merely lie on the amino acid sequence, but also on multifactorial and highly dynamic physiological issues, such as cell energetics, the availability of molecular chaperones, foldases, stabilizing osmolytes, the folding and secretion kinetics, or the effect of incorrect post-translational modifications (Bandyopadhyay*, et al.*, 2012). The formation of IBs is then an important obstacle in protein production processes, as their occurrence undermine the relative accumulation of soluble protein species versus the aggregated versions, that is, it reduces the solubility of the target protein.

In addition, protein aggregation has been historically associated to lack of functionality, because reaching the native protein conformation is generally linked to solubility (Baneyx & Mujacic, 2004). The generic tendency to aggregation and insolubility of recombinant proteins has then pushed to develop strategies for protein production addressed to minimize IB formation, which has been only moderately

successful and strongly protein-dependent (Sorensen & Mortensen, 2005, Sorensen & Mortensen, 2005). Being mechanically stable (Vogel*, et al.*, 2002, Garcia-Fruitos*, et al.*, 2009), IBs can be purified from bacterial cells upon cell breaking and mild-to-harsh rinsing treatments or density gradient centrifugation (Schrodel & de Marco, 2005, Rodriguez-Carmona*, et al.*, 2010, Seras-Franzoso*, et al.*, 2015). This offers a particulate material usable as relatively pure source of recombinant protein. In this context, when attempts to prevent IB formation failed, IBs have been exploited in protein biotechnology as relatively pure source of proteins (Gatti-Lafranconi*, et al.*, 2011) to be recovered *in vitro*. This approach considers first the disintegration of IBs by the application of more or less aggressive chaotropic agents or detergents and a successive protein refolding process under controlled conditions. These approaches have been developed in parallel to the advances in recombinant protein production and are summarized elsewhere (Vallejo & Rinas, 2004, Singh & Panda, 2005, Freydell*, et al.*, 2007, Burgess, 2009, Simpson, 2010, Basu*, et al.*, 2011, Yamaguchi & Miyazaki, 2014, Singh*, et al.*, 2015).

The lack or poor solubility of recombinant proteins produced in bacteria is a multifactorial event. Which different genetic and environmental parameters (that is, bacterial culture conditions) and how they might be involved in the aggregation process have been a matter of controversial discussions, especially regarding the application of palliative measures (Sorensen & Mortensen, 2005, Sorensen & Mortensen, 2005). Recombinant protein aggregation is associated to incomplete or inadequate protein folding (Hlodan*, et al.*, 1991), the consequent occurrence of exposed hydrophobic patches (Murby*, et al.*, 1995), the inability of the cell quality control machinery (mediating both folding and proteolysis) to accurately perform under protein overproduction conditions (Tomoyasu*, et al.*, 2001, Baneyx & Mujacic, 2004), the triggering of cell responses to conformational stress (Gasser*, et al.*, 2008), an inappropriate codon usage (Parret*, et al.*, 2016) and the molecular overcrowding associated to high protein yields in recombinant bacteria (Martinez-Alonso*, et al.*, 2008). The frequent heterologous origin of the recombinant polypeptides is also favouring aggregation, often due to the absence or inadequate posttranslational

modifications executed by bacterial cells that might be critical to adopt a native conformation (Schmidt, 2004, Demain & Vaishnav, 2009).

Bacterial IBs are mainly composed of the recombinant protein that is accompanied by some/relatively few host cell proteins and traces of other macromolecular contaminants, such as lipids and nucleic acids (Rinas & Bailey, 1992, Fahnert*, et al.*, 2004). IBs are not mere clustered protein deposits, as the recombinant protein is not associated in an amorphous form, but structured as formerly unexpected amyloidal architectures (Carrio*, et al.*, 2005, Cano-Garrido*, et al.*, 2013), as discussed below. Amyloidal structures are formed through sequence-dependent, stereospecific interactions (Speed*, et al.*, 1996) that exclude protein-protein associations between different protein species in a single aggregate (Morell*, et al.*, 2008). IB formation in recombinant bacteria, based on such recruitment of homologous polypeptide chains is an extremely dynamic process (Carrio*, et al.*, 1998, Carrio*, et al.*, 1999, Carrio & Villaverde, 2001, Carrio & Villaverde, 2002, Baig*, et al.*, 2014), that results from an unbalanced equilibrium between protein association and deposition, proteolytic digestion and disaggregation (Carrio*, et al.*, 1999, Carrio*, et al.*, 2000, Carrio & Villaverde, 2001, Carrio & Villaverde, 2002). This complex process is mediated by the arms of the quality control cell machinery that handle protein aggregates (Weibezahn*, et al.*, 2004, Mogk*, et al.*, 2018). In this way, the arrest of recombinant protein synthesis in metabolically active cells promotes the physiological disintegration of IBs. This is a fast process that results in the refolding of the protein into the native conformation (Carrio & Villaverde, 2001, de Marco, 2007) but also in the proteolytic digestion of an important fraction of the IB material (Carrio & Villaverde, 2001, Carrio & Villaverde, 2002). The integration of IB protein within the cellular quality control is also demonstrated by the conformational rearrangements observed within the IB particles (Gonzalez-Montalban*, et al.*, 2008, Elia*, et al.*, 2017).

Although this is a still moderately understood issue, IB formation causes or it is linked to conformational stresses in the producing bacterial cell (Gasser*, et al.*, 2008) and aggregated species are suspected to show some extent of intrinsic toxicity that might negatively affect bacterial cell growth. However, when eukaryotic cells are exposed to IBs in suspension or these particles are administered to whole organisms through

different routes, including injection or oral administration, IBs do not appear to be harmful (Garcia-Fruitos*, et al.*, 2009, Diez-Gil*, et al.*, 2010, Garcia-Fruitos*, et al.*, 2010, Liovic*, et al.*, 2012, Seras-Franzoso*, et al.*, 2012, Vazquez*, et al.*, 2012, Seras-Franzoso*, et al.*, 2013, Seras-Franzoso*, et al.*, 2013, Tatkiewicz*, et al.*, 2013, Seras-Franzoso*, et al.*, 2014, Seras-Franzoso*, et al.*, 2014, Seras-Franzoso*, et al.*, 2015, Cespedes*, et al.*, 2016, Seras-Franzoso*, et al.*, 2016, Torrealba*, et al.*, 2016, Torrealba*, et al.*, 2016, Unzueta*, et al.*, 2017, Stamm*, et al.*, 2018, Unzueta*, et al.*, 2018). Therefore, IBs have gained interest as intriguing biocompatible protein materials (Villaverde, 2012, Villaverde*, et al.*, 2012, Loo*, et al.*, 2015, Seras-Franzoso*, et al.*, 2015, Rinas*, et al.*, 2017) that might be substantially different from protein aggregates linked to prion diseases or to degenerative disorders (Chiti & Dobson, 2017, Hartl, 2017). In this regard, IBs represent a paradigmatic example of non-toxic amyloids, associated with a diversity of functions and whose prevalence in living beings seems to be more spread than previously expected (Badtke*, et al.*, 2009, Maji*, et al.*, 2009, Romero & Kolter, 2014, Villaverde*, et al.*, 2015, Jacob*, et al.*, 2016, Molina-Garcia*, et al.*, 2016, Dragos*, et al.*, 2017, Hewetson*, et al.*, 2017, Jackson & Hewitt, 2017, Piscitelli*, et al.*, 2017, Shin & Cherry, 2017).

In 2005, it was reported that IBs are at least partially composed by functional protein (Garcia-Fruitos*, et al.*, 2005, Jevsevar*, et al.*, 2005) (Figure 1C). The fraction of functional polypeptides is not externally attached to the protein particles but it represents a structural part of the aggregates, being stable and tightly linked to IBs in aqueous solvents (Garcia-Fruitos*, et al.*, 2007). The fact that an important fraction of the IB-embedded protein retains its biological activity (Gonzalez-Montalban*, et al.*, 2007) and that solubility and functionality are not equivalent parameters (Garcia-Fruitos*, et al.*, 2007) have allowed to further explore IBs as immobilized enzymes for catalysis (Hrabarova*, et al.*, 2015), as biocompatible materials for tissue engineering (Garcia-Fruitos*, et al.*, 2009, Seras-Franzoso*, et al.*, 2012, Tatkiewicz*, et al.*, 2013, Tatkiewicz*, et al.*, 2018) and as protein delivery agents for *in vivo* applications (Garcia-Fruitos*, et al.*, 2012, Rinas*, et al.*, 2017). Despite the exponentially growing number of examples supporting that IB functionality is a generic and transversal concept (Villaverde*, et al.*, 2015), it still remains unfamiliar to an important fraction of the

scientific community that retains the obsolete concept linking protein solubility and functionality (Figure 1D). The bases of IB protein biological activities and the resulting applicability of IBs as functional materials will be discussed below.

2.1 IBs are depot-type bacterial amyloids

The combination of a significant set of amyloid-like properties (Ventura & Villaverde, 2006) and the occurrence of biological activity of IB protein (Garcia-Fruitos*, et al.*, 2005) suggests a specific type of non-toxic functional amyloids, apparently uncommon in bacteria. Non-toxic functional amyloids have been widespread found in living beings including bacteria (Seviour*, et al.*, 2015, Dragos*, et al.*, 2017, Van Gerven*, et al.*, 2018). They perform a significant set of recognized functions necessary for or favourable to cell or organic functions, though the activation/inactivation of defined cell circuits. These activities include catalysis, regulation of gene expression, control of DNA replication, signalling, mechanical structuring and complex functions at the systems level such as memory and reproduction (Hafner Bratkovic, 2017, Hewetson*, et al.*, 2017). In bacteria, amyloids have been associated to biofilm formation and quorum sensing (Schwartz*, et al.*, 2012, Seviour*, et al.*, 2015, Schwartz*, et al.*, 2016, Stenvang*, et al.*, 2016, Besingi*, et al.*, 2017, Dragos*, et al.*, 2017), virulence and toxicity (Marcoleta*, et al.*, 2013, Syed & Boles, 2014, Van Gerven*, et al.*, 2018), plasmid replication (Molina-Garcia*, et al.*, 2016), bioenergetics (Molina-Garcia*, et al.*, 2017) and transcriptional regulation (Pallares*, et al.*, 2015, Yuan & Hochschild, 2017, Kaur*, et al.*, 2018), among others. In these examples, the target protein is found in two alternative conformational versions, namely amyloid and non-amyloid, that represent the ON-OFF states of molecular switchers. The coexistence of native or native-like and amyloidal forms of recombinant proteins in bacterial IBs places these particles out of this category. However, there are significant functional and structural similarities between bacterial IBs and secretory granules in the endocrine system. These are functional amyloids that act as repositories of peptidic hormones that are released from glands upon defined stimuli (Maji*, et al.*, 2009). As far as we know, IBs are the first fully characterized depot-type amyloids found in bacteria. This amazing architecture, explained in next sections, make them particularly appealing as fully tailored

manufactured protein depositories that can be obtained by standard protein production procedures in recombinant cells.

3. Protein aggregation as soluble protein aggregates

As mentioned above, according to an assumption still largely accepted among biologists, soluble proteins correspond to monodispersed, native, and fully functional proteins. This simplification contrasts with both the evidence that soluble aggregates have a critical role *in vivo* in the progression of neurodegenerative diseases (Gerson*, et al.*, 2014, Kumar*, et al.*, 2016) and the data collected studying recombinant proteins. In two seminal papers (Nomine*, et al.*, 2001, Nomine*, et al.*, 2001) it was demonstrated that recombinant fusion constructs (composed by carriers such as GST, maltose-binding protein or thioredoxin, known for their solubility plus the target proteins) expressed in *E. coli* and designed to improve overall yield, could form soluble micellar aggregates. These were described as composed by an aggregated nucleus originated by the condensation of hydrophobic patches made available by the misfolded target protein and by an external layer made of hydrophilic, highly soluble carrier protein (Figure 2 A). The overall structure remained soluble, but the functionality of the target protein was partially or totally lost. This condition can be very frequent when a difficult-to-produce protein is fused to a "solubilizing" partner, but can arise also when multi-domain proteins with components characterized by differing aggregation propensity are produced (Souillac, 2005). It must be also stressed that protein aggregation inside bacterial cells is an extremely dynamic process regulated by both chemical (osmolytes) and molecular chaperones (Singer & Lindquist, 1998, Carrio & Villaverde, 2001, Mogk*, et al.*, 2003, Schultz*, et al.*, 2007, Natalello*, et al.*, 2008). Chaperone availability influences the process of aggregation, the structural characteristics of the aggregates, and their re-solubilisation into less complex or even native monodispersed molecules (Figure 2 B). In some cases, protein (re)folding to the native conformation is not achieved and chaperones stick to partially misfolded proteins, impairing their further condensations into larger aggregates (de Marco*, et al.*, 2000).

Nevertheless, the proteins complexed in these soluble aggregates are neither native nor fully functional. The contamination with GroEL and DnaK (easily detected by SDS-PAGE) is a strong indicator that recombinant target proteins expressed in *E. coli* are at least partially unfolded/misfolded (de Marco*, et al.*, 2000). The data collected in the last 20 years using recombinant proteins and controlled conditions show that in the same protein preparation it is possible to identify different soluble aggregates with increasing level of structural complexity, functionality, and capacity to interfere with cell metabolism (Calamai*, et al.*, 2005, Schrodel & de Marco, 2005, Ami*, et al.*, 2009, Li*, et al.*, 2009). Multi-step sucrose gradients, native-blue electrophoresis and electron microscopy analyses enabled to appreciate the large structural variety of soluble aggregates recovered from samples purified from bacteria with different genetic background, grown at different conditions, and challenged with alternative stress factors (Stegemann*, et al.*, 2005, Natalello*, et al.*, 2008, Liu*, et al.*, 2013). Part of the proteins trapped in these aggregates remains functional and these proteins are in a dynamic equilibrium with both monodispersed (native) forms and conventional (insoluble) IBs (de Marco*, et al.*, 2005, Stampolidis*, et al.*, 2009, Van der Henst*, et al.*, 2010). The kinetic of aggregate formation suggests that aggregation proceeds progressively into denser forms once the bacterial folding machinery is overwhelmed and osmolytes become limiting (de Marco*, et al.*, 2005, Stampolidis*, et al.*, 2009, Van der Henst*, et al.*, 2010). Intrinsically disordered proteins are particularly prone to be recruited during aggregate nucleation, for instance, by proteins such Huntingtin which possess poly-glutamine patches (Wear*, et al.*, 2015). The aggregation process can be minimized *in vivo* by tuning the cell culture parameters. This can be done according to the information inferred from “aggregation probes”, which quantify the relative aggregation level at different growth conditions (Schultz*, et al.*, 2006, Paul*, et al.*, 2015). It means that native monodispersed proteins will be produced when the expression rate remains below the maximal capacity of the bacterial cells to fold the specific polypeptide. Under this perspective it must be considered all those precautions that reduce the cell growth rate (low temperature). These include the controlled increase of foldases and molecular chaperones (induction of stress response by ethanol/ benzyl alcohol addition or heat shock, overexpression of recombinant molecular chaperones), or the stabilization of the folding intermediates (chemical

chaperones) (de Marco*, et al.*, 2005). Post-harvesting steps are also relevant since purification, storage, and stress conditions strongly influence the formation and the nature of soluble aggregates (Natalello*, et al.*, 2009, Lebendiker & Danieli, 2014, Guo*, et al.*, 2015, Smirnova*, et al.*, 2015), and particularly, the aggregation process of intrinsically disordered proteins and proteins with intrinsically disordered regions (Churion & Bondos, 2012, Lebendiker*, et al.*, 2015). Osmolytes of different chemical compositions have been effectively exploited to prevent the progressive formation of inactive soluble aggregates of therapeutic proteins (Liu*, et al.*, 2013). The contribution of other components of cell chemical environment, such as salts and nucleic acids, to protein stability and aggregation has been reported (Futami*, et al.*, 2014, Song, 2017), but is still largely unappreciated. What is clear but often underestimated, is that data obtained using protein preparations of soluble aggregates can be totally misleading (Nomine*, et al.*, 2001). Consequently, protein quality control guidelines have been proposed to evaluate biophysical and functional features of soluble proteins and to prevent the use of material that is not suitable for both research and biotechnological applications (Paul*, et al.*, 2015). Size exclusion chromatography (SEC) is the most accessible method to assess the presence of oligomeric forms and soluble aggregates in a sample. Large aggregates usually show a peak corresponding to the void volume and can be directly separated by native species (mono or oligomeric). Multi-angle light scattering (MALS) coupled to SEC or asymmetrical flow-field flow fractionation (AF4) results in more precise information and has been conveniently used to evaluate the effect of expression, purification, and storage protocols on the monodispersity of recombinant proteins (Zhou*, et al.*, 2006, Pavisic*, et al.*, 2010, Chen*, et al.*, 2016, Amartely*, et al.*, 2018). Dynamic light scattering (DLS) is another standard technique available for measuring the size of particles in solution and frequently used for detecting the presence of protein soluble aggregates. However, it has been criticized for its bias towards larger aggregates and alternative methods such as Taylor dispersion analysis have been suggested (Hulse*, et al.*, 2013).

4. Protein aggregation as IBs

Since their discovery as a common outcome during overexpression of heterologous proteins in *E. coli*, the perception on the composition and structure of IBs has dramatically changed. The aggregation process was not reversible by simple dilution of the polypeptide chains. Therefore, formation of protein aggregates in cell factories could not be attributed to a salting-out process as usually observed when proteins exceed their solubility limits. Moreover, it soon became obvious that active protein could be recovered following denaturation-renaturation protocols (Marston, 1986, Kane & Hartley, 1988, Schein, 1989). This suggested that the polypeptide chains within IBs were not covalently damaged and the information for reaching the native state was still present in the IB polypeptides. Due to the fact that IBs were formed starting from a vast diversity of recombinant proteins unrelated in sequence, size, structure and origin, it was initially thought that the chains engaged within the aggregates were adopting unfolded or misfolded conformations associated in a form of "statistical spaghetti", that was escaping cell quality control.

This was subsequently challenged by deep studies of the intracellular folding and aggregation of a unique model system at the time, the tailspike protein of bacteriophage P22. These revealed that aggregates originated from early, soluble partially folded intermediates in the folding pathway that could either proceed further to the native pathway or alternatively, form intermolecular aggregates (Haase-Pettingell & King, 1988). The notion of the competition between productive and aggregation pathways was subsequently introduced, with the partitioning being dependent on temperature, concentration, etc. This partitioning was also influenced by point mutations that were either favoring or disfavoring the aggregation pathway (Goldenberg & King, 1982 , Goldenberg*, et al.*, 1983 , Yu & King, 1984, Yu & King, 1988, Mitraki*, et al.*, 1991). The notion of specificity of aggregation has subsequently emerged when heterogeneous co-aggregates could not be observed in mixed renaturation experiments of entirely different proteins (Wetzel, 1994 , Wetzel, 1996, Betts*, et al.*, 1997, Fink, 1998). This also implied that IBs were not amorphous protein deposits, but they were built through specific association of structural segments that could even retain "quasi-native" features (Mitraki & King, 1989, King*, et al.*, 1996 Jan).

4.1 Amyloidal architecture of IBs

It was well known that the target recombinant proteins were the major constituent of IBs (Ventura & Villaverde, 2006). However, their structural characterization *in vivo* was very challenging, due to the complexity of the folding and aggregation processes under molecular crowding conditions, the presence of heterologous proteins, and the dynamic nature of the process. Many methods for the characterization of protein aggregates can be applied only to dilute protein solutions *in vitro* and were not suitable to evaluate the insoluble, quasi-solid nature of the IBs. The first structural insights became possible thanks to Fourier Transform Infrared Spectroscopy (FTIR) that evidenced how IB fractions displayed either increased intermolecular beta-sheet structure or, alternatively, native-like spectra. This suggested the occurrence of both amyloidal protein forms and folded polypeptides (Oberg*, et al.*, 1994, Przybycien*, et al.*, 1994, Fink, 1998). The possibility of recording FTIR spectra in intact cells enabled also following the kinetics of IB formation in real time (Ami*, et al.*, 2005, Ami*, et al.*, 2006). In a landmark 2005 study, it was reported that IBs had intermolecular beta-structure as probed by FTIR and were binding to amyloid-diagnostic dyes such as Congo Red and Thioflavin T (Carrio*, et al.*, 2005). Moreover, following extraction from cells, they were able to seed formation of amyloid fibrils from homologous soluble counterparts *in vitro,* suggesting an amyloid-like character (Carrio*, et al.*, 2005). In subsequent electron microscopy studies, freshly extracted IBs appeared as electron dense, round objects from which fibrillar structures were occasionally emanating (Figure 3A). These fibrillar structures became more evident after treatment with proteinase K (Morell*, et al.*, 2008). X-ray fiber diffraction studies showed the characteristic cross-beta pattern, confirming the amyloid structure of the material (Wang*, et al.*, 2008). When model fluorescent proteins such as GFP were fused to short aggregating peptides, such as Alzheimer's Aβ42 or the foot-and-mouth disease VP1 capsid protein, the formed IBs retained fluorescence. This fact indicated that aggregation was driven, and that it exclusively involved the aggregation-prone peptides. The fusion moieties represented by the intrinsically soluble GFP remained free to adopt native structure inside the protein clusters (Garcia-Fruitos*, et al.*, 2005). The preference for aggregation *in vivo* of molecules belonging to the same species was

addressed by Fluorescence Energy Transfer (FRET) experiments of fusion proteins expressed in *E coli* cells. FRET is a sensitive method to probe close proximity of fluorescent proteins since it occurs between two fluorophores when located in the range of 10-100 Å from each other. Three-fold higher FRET was observed when two different fluorescent proteins were fused to the same aggregation-driving domain (ie either Aβ42 or VP1) compared to FRET observed upon co-expression of fusions of the same fluorescent proteins to two different aggregation domains. Thus, the requirement for sequence similarity for aggregation reinforced the notion of the stereospecific intermolecular contacts driving IB formation (Morell*, et al.*, 2008).

Sequence-specific structural information on the aggregated material was then made possible thanks to the development of methods such as Hydrogen/Deuterium exchange coupled with Nuclear Magnetic Resonance (NMR) and solid-state NMR (Wang*, et al.*, 2008). Regardless of the initial conformation of the starting proteins, short (7-10 amino acids) stretches were found to form segments protected from hydrogen-exchange and able to mediate intermolecular beta-structure and IB formation (Wang*, et al.*, 2008). IBs formed from the prion domain of the Het-s protein from the filamentous fungus *Podospora anserina* and amyloid filaments formed by the same domain *in vitro* share the same amyloid structure as suggested by solid-state NMR studies (Wasmer*, et al.*, 2009). The conformation of selected residues within IBs formed by Influenza virus hemagglutinin expressed *in E. coli* was studied with solid-state NMR in whole cells and native-like, alpha helical conformation was reported for four selected residues (Curtis-Fisk*, et al.*, 2008). Short sequences engaged in beta-amyloid structures could apparently coexist with native – like conformation originated from the rest of the polypeptide chain, or parts of it.

The latest perception on the structure of IBs converges towards a model where beta-sheet, amyloid-structured parts and native-like, or even biologically active parts coexist (Gonzalez-Montalban*, et al.*, 2007, Villaverde*, et al.*, 2015). This conveys both mechanical stability and functional properties, allowing recovery of biological activity with a combination of mild solubilization conditions and traditional denaturation-renaturation protocols (Peternel*, et al.*, 2008, Singh*, et al.*, 2015). Finally, the application of analytical methods such as contact angle measurements, zeta potential

and nanoindentation allowed the evaluation of IB wettability, surface properties, and stiffness (Diez-Gil, *et al.*, 2010). Thus, the view of IBs as particulate, porous and highly hydrated bio-nanomaterials with interesting biological properties and a high mechanical stability has recently emerged. This awareness opened unexpected and promising avenues for IB use as immobilized biocatalysts, tissue engineering bioscaffolds or nanopills for controlled protein drug release (for reviews, see (Mitraki, 2010, Garcia-Fruitos, *et al.*, 2012, Loo, *et al.*, 2015, Rinas, *et al.*, 2017)), as discussed in following sections.

4.2 Biophysical nanoscale properties of IBs

As discussed above, the target recombinant protein is the major component of bacterial IBs (Neubauer, 2006). Its relative abundance in IBs varies according to the amino acid sequence (Peternel, *et al.*, 2008) and it also depends on the conditions of bacterial culture and gene expression (Strandberg & Enfors, 1991, Baig, *et al.*, 2014, Bakholdina, *et al.*, 2018). Bacterial strains with mutations affecting protein folding and metabolism (e.g. deficiencies in the chaperones DnaK and ClpA, or in the cytosolic protease ClpP), also influence the IBs content (Carrio & Villaverde, 2003, Martinez-Alonso, *et al.*, 2008, Garcia-Fruitos, *et al.*, 2012), that in turn impact on several biophysical and chemical properties of IBs and on their use as nanomaterials (Diez-Gil, *et al.*, 2010).

In this context, the shape and size of IBs are very much dependent on the particular target protein, the host bacterial strain as well as the production time (Garcia-Fruitos, *et al.*, 2009, Diez-Gil, *et al.*, 2010, Garcia-Fruitos, *et al.*, 2010). Different authors described slightly different IB shapes, from spherical to ellipsoidal, cylindrical and even tear-shaped (Bowden, *et al.*, 1991, Carrio, *et al.*, 2000, Garcia-Fruitos, *et al.*, 2007, Garcia-Fruitos, *et al.*, 2010, Peternel & Komel, 2010), with a diameter that is limited by the size of the bacterial cell and ranges from 50 nm (probably the detection limit under optical microscope) to about 1,000 nm (Peternel, *et al.*, 2008, Garcia-Fruitos, *et al.*, 2010). On the other hand, the size of the IBs is significantly reduced *in vitro* in low pH buffers as the result of an irreversible contraction process (Peternel, 2008). This peculiar condensation event affects the density and solubility of the IBs and it might be

of relevance for further *in vitro* and *in vivo* applications of IBs as materials. After isolation from bacterial cells, they are stable and can be stored for long periods at 4 °C, frozen at -80°C, or lyophilized, keeping the original IB architecture and the biological activity of the embedded proteins (Garcia-Fruitos*, et al.*, 2009).

IB proteins are usually not released in physiological buffers (Garcia-Fruitos & Villaverde, 2010), and such compositional stability is the basis for the exploitation of enzymatically active IBs as reusable catalysts (Krauss*, et al.*, 2017, Rinas*, et al.*, 2017). This concept of IBs as self-immobilized enzymes shows room for further improvement regarding operation reusability (Koszagova*, et al.*, 2018), scalability (Kloss*, et al.*, 2018) and functional complexity (Jager*, et al.*, 2018). However, a fraction of IB protein is released from IBs in aqueous solutions (Garcia-Fruitos*, et al.*, 2007, Unzueta*, et al.*, 2018), and the extent of such removable protein population appears to be dependent on the nature of the target protein, which influences the percentage of native-like IB protein. It is also dependent on factors such as the genetic background of the producer strain and the pH of the storage buffer (Garcia-Fruitos*, et al.*, 2012). As materials, some IBs are loose, and as indicated earlier they can be dissolved by mild detergents or high pH buffers (Jevsevar*, et al.*, 2005). This condition represents an opportunity to recover soluble IB protein *in vitro* avoiding protein denaturing agents (Singh*, et al.*, 2015, Singh*, et al.*, 2015). The particular sensitivity of IBs to such buffers and other mild-to-harsh treatments must be carefully considered to preserve the IB structure during purification (Rodriguez-Carmona*, et al.*, 2010, Rodriguez-Carmona*, et al.*, 2011, Seras-Franzoso*, et al.*, 2015), especially if intended for use as functional materials.

The modification in the host cells of the genetic background relative to components involved in the protein quality control impacts not only on the efficiency of protein removal from IBs, but also on their functionality and physicochemical parameters such as zeta potential (surface charge) (Novak, 2009, Diez-Gil*, et al.*, 2010), stiffness (elasticity) (Diez-Gil*, et al.*, 2010) and wettability (ability of an aqueous liquid to maintain contact with IBs' surface) (Diez-Gil*, et al.*, 2010). For instance, IBs recovered from wild type cells are homogenous, while in DnaK$^-$ and ClpA$^-$ cells two IB populations with distinguishable stiffness and which remained segregated were produced (Diez-Gil*, et al.*, 2010). These mechanical properties are extremely important when using IBs as

anchoring structures for the cultivation of the mammalian cells (Diez-Gil*, et al.*, 2010, Seras-Franzoso*, et al.*, 2012, Seras-Franzoso*, et al.*, 2013, Tatkiewicz*, et al.*, 2013, Tatkiewicz*, et al.*, 2018), as stiffer IBs better stimulate cell attachment and/or proliferation (Novak, 2009).

5. Physiology of IB formation

Protein aggregates formed in recombinant bacteria are not mere passive deposits of insoluble protein, but the result of complex active physiological processes that link protein folding, quality control, conformational stress and amyloid formation. The physiology of IB formation is still far to be precisely dissected but the comprehension of the process has progressively increased.

5.1 Energetics of IB formation

IBs are usually found at the polar sites of the cell and it is generally accepted that polar preference of IBs or other protein aggregates can be attributed to macromolecular crowding in the midcell nucleoid region (Lindner*, et al.*, 2008, Winkler*, et al.*, 2010, Coquel*, et al.*, 2013, Gupta*, et al.*, 2014, Neeli-Venkata*, et al.*, 2016, Oliveira*, et al.*, 2016). Occasional midcell detection of IBs can be explained by nucleoid-free regions at potential future septation sides (Winkler*, et al.*, 2010, Coquel*, et al.*, 2013). The hypothesis of crowding-provoked polar preference has never been challenged but the way protein aggregates reach the poles has been a matter of controversial debate as contradicting results were obtained suggesting an either energy-independent or energy-dependent mechanism.

For example, it was demonstrated that polar localization of aggregates occurred also in energy-deprived cells, indicating a passive mechanism for polar positioning (Winkler*, et al.*, 2010). Also, experimentally observed movements of age-related aggregates towards the poles were explainable with passive diffusion of aggregates and spatially non-homogeneous macromolecular crowding (Coquel*, et al.*, 2013). On the other hand, there was also evidence that transport of aggregates to the poles can be an energy-driven process or at least dependent on metabolic activity (Rokney*, et al.*,

2009, Govers*, et al.*, 2014, Govers*, et al.*, 2017). In this context, studies on cells with pressure dissociated IBs revealed that reassembly of small aggregates into large IBs did occur in cells exposed to fresh nutrients but not in energy and nutrient depleted cells (Govers*, et al.*, 2014).

These seemingly contradictive findings relatively to purely diffusive movement or energy-driven transport of aggregates towards the cellular poles become understandable by considering the glassy nature of the bacterial cytoplasm. Physical-chemical studies have shown that metabolic activity is required to maintain fluidity in the cytoplasm, a condition necessary for the movement of larger particles (> 30 nm) (Parry*, et al.*, 2014). Thus, it is reasonable to guess that it is not the active ATP-dependent transport of aggregates but rather the fluidizing properties of active metabolism which facilitate movements of aggregates towards less crowded regions. Passive diffusion of aggregates towards the pole, a region of less macromolecular crowding, would then depend on the fluidity of the cytoplasm as well as on the initial size of aggregation foci. Smaller aggregation foci could move to the poles by diffusion also in energy-deprived cells, but larger aggregation foci could only reach the poles in metabolically active cells (Govers*, et al.*, 2014). Polar distribution of IBs or damaged proteins can be considered as an evolutional benefit compared to unbiased dilution of misfolded and aggregated proteins as continued cell division leads to rejuvenation (Lindner*, et al.*, 2008, Rokney*, et al.*, 2009, Winkler*, et al.*, 2010).

5.2. Quality control and IB formation

The bacterial protein quality control machinery, composed by a complex catalogue of chaperones and proteases with overlapping activities, has an important role in protein folding and refolding in the bacterial cytoplasm during conformational (thermal) stresses and especially during the production of aggregation-prone recombinant proteins. DnaK/DnaJ/GrpE (the KJE set), GroELS, ClpB, and IbpAB are main chaperones acting during this process (Figure 2 B) and they are over-produced under recombinant protein production (Allen*, et al.*, 1992, Rinas, 1996, Veinger*, et al.*, 1998, Goloubinoff*, et al.*, 1999, Houry, 2001, Mogk*, et al.*, 2003, Jurgen*, et al.*, 2010, Zblewska*, et al.*, 2014). As these chaperones might be limiting for proper protein folding, many

biotechnologists have been exploring their co-production along with the recombinant protein, aiming at optimizing the production of soluble and properly folded soluble species (Sorensen & Mortensen, 2005, Sorensen & Mortensen, 2005, Kolaj*, et al.*, 2009). However, the role of chaperones and proteases goes beyond the strict control of the correct folding and refolding of misfolded soluble proteins. It has been widely proven that soluble and insoluble (aggregated) proteins are in a dynamic equilibrium between protein deposition and removal (Schrodel & de Marco, 2005) and the protein quality control machinery controls not only protein aggregation, but also protein quality in both soluble and aggregated fractions (Garcia-Fruitos*, et al.*, 2007, Rinas*, et al.*, 2007, Baig*, et al.*, 2014). Specifically, it has been described that pivotal chaperones such as DnaK, GroEL, and ClpB (heat shock proteins -Hsp-) and IbpAB (small heat shock proteins -sHsp-) are intimately associated with IBs, which confirms that they have an active role in the formation and disaggregation of these protein clusters (Allen*, et al.*, 1992, Mogk*, et al.*, 2003, Mogk*, et al.*, 2003, Mogk*, et al.*, 2003, Carrio & Villaverde, 2005, Jurgen*, et al.*, 2010, Zblewska*, et al.*, 2014).

In this context, upon the arrest of *de novo* protein synthesis, different members of the protein quality control are actively involved in the disintegration of IBs (Carrio & Villaverde, 2001). In parallel, and more specifically, the coordinated action of ClpB (disaggregase), KJE, and sHsp promotes the removal of polypeptides from protein aggregates (Mogk & Bukau, 2004, Weibezahn*, et al.*, 2004). DnaJ binds to the aggregate surface, and it recruits first DnaK and finally ClpB to form an energy-dependent chaperone complex whose coordinated action promotes protein removal (Jurgen*, et al.*, 2010, Aguado*, et al.*, 2015, Aguado*, et al.*, 2015). In absence of functional DnaK or ClpB, the disaggregation activity is minimized, while IbpAB mutants have just a mild effect on the disaggregation process (Mogk*, et al.*, 2003, Mogk & Bukau, 2004, Gonzalez-Montalban*, et al.*, 2008, Garcia-Fruitos*, et al.*, 2010). This indicates that IbpAB is not essential in the disaggregation process, although its presence can notably enhance the action of ClpB/KJE (Mogk*, et al.*, 2003, Mogk & Bukau, 2004). On the other hand, protein disaggregation is clearly an ATP-driven process (Rokney*, et al.*, 2009, Winkler*, et al.*, 2010). For example, HtpG can interact with DnaK/DnaJ/GrpE to further promote refolding of aggregated proteins in an ATP-

dependent manner (Genest*, et al.*, 2011). Alternatively, ClpB can also assist in cooperation with DnaK/DnaJ/GrpE in ATP-driven refolding of aggregated proteins (Ben-Zvi*, et al.*, 2004, Acebron*, et al.*, 2009, Winkler*, et al.*, 2010). The small heat shock proteins (sHSPs; IbpA/IbpB) stabilize and decrease the size of protein aggregates (Ratajczak*, et al.*, 2009) thereby promoting their DnaK/DnaJ/GrpE and ClpB mediated refolding (Matuszewska*, et al.*, 2005). The major disaggregating chaperones (DnaK, ClpB) (Winkler*, et al.*, 2010) but also IbpA (Lindner*, et al.*, 2008) co-localize at the poles and participate in the disintegration of polar aggregates (Rokney*, et al.*, 2009).

In absence of DnaK, IBs are unusually large (Figure 2B) and the specific activity of the forming protein is significantly higher than in the wild type *E. coli* strain (Gonzalez-Montalban*, et al.*, 2006, Garcia-Fruitos*, et al.*, 2007, Rinas*, et al.*, 2007). In contrast, GroEL and IbpAB mutant strains form IBs in which the trapped enzyme shows the same specific activity than that found in the wild type *E. coli* strain (Kuczynska-Wisnik*, et al.*, 2004, Garcia-Fruitos*, et al.*, 2007, Rinas*, et al.*, 2007). The genetic background has also been proven to determine the final morphology of IBs. While most of IBs have a spherical-like form, indicating a homogenous deposition and removal of protein on the whole IB surface, a particular tear-shape morphology has been observed in ClpP-deficient bacterial cells (Garcia-Fruitos*, et al.*, 2010). At this point it is important to emphasize that proteases such as ClpP and Lon play also a role in the IB formation and intracellular solubilization (Carrio & Villaverde, 2003, Garcia-Fruitos*, et al.*, 2010). Surprisingly, once proteins are physiologically released from IBs, they are proteolyzed irrespective of if they are misfolded or properly folded (and consequently functional) (Garcia-Fruitos*, et al.*, 2010). Such uncontrolled proteolysis is tightly regulated by DnaK, which is anchored to the IB surface (Carrio & Villaverde, 2005). In the absence of this chaperone, the proteolysis is significantly reduced (Garcia-Fruitos*, et al.*, 2010). Moreover, an excess of DnaK by controlled overexpression in IB-producing bacterial cells has been suggested to inhibit proper *in situ* folding of proteins forming such aggregates (Gonzalez-Montalban*, et al.*, 2008). It appears evident from this schematic summary that it is not sufficient inducing the overexpression of single (or combinations of) chaperones/proteases to improve automatically recombinant protein folding. Automatized approaches which enable a large array of expression combinations are

probably the most efficient approach to identify the optimal conditions for each single protein (Turchetto*, et al.*, 2017).

5.3 Controlled IB production

As discussed in previous sections, the formation of IBs is dependent on the quality control system and linked to an orchestrated cell response to stress factors. The identification of these factors opened a door to the engineering of production processes to favour the formation of IBs with defined properties, by adjusting the parameters affecting their formation. Paradoxically, many of them were recognized during the attempts to enhance the yield of soluble protein (Sorensen & Mortensen, 2005, Sorensen & Mortensen, 2005). When visualizing the protein production process as a whole, the rate at which the proteins fold in the cell can be reduced by adjusting the growth temperature of the culture, the strength of the gene promoter or even the medium composition. In the first case, the growth rate of bacteria directly affects the metabolism of the cell and specifically protein synthesis (Bosdriesz*, et al.*, 2015, Calcines-Cruz*, et al.*, 2018, Wurm*, et al.*, 2018). In the second scenario, both the transcription and translation rates for the nascent polypeptide are reduced improving the performance of the folding modulators in the same way. In addition, the same effect has been observed when medium composition reduces the cell growth (Jhamb & Sahoo, 2012) or when the inducer concentration is added in limiting amounts (Rabhi-Essafi*, et al.*, 2007). In the same line, the possibility to increase the yield of insoluble protein has been performed by the fusional addition of aggregation or pull-down tags to the recombinant protein (Nahalka & Nidetzky, 2007, Zhou*, et al.*, 2012, Costa*, et al.*, 2014, Wang*, et al.*, 2015, Jong*, et al.*, 2017).

5.4 Tailoring IBs

IBs are formed during protein production processes carried out under very diverse culture conditions, targeted to a vast diversity of proteins and supported by multiple expression systems (Taylor*, et al.*, 1986, Georgiou & Valax, 1999, Castellanos-Mendoza*, et al.*, 2014). By comparing production conditions, it has been determined that protein

aggregation and IB formation are favoured by factors affecting the transcriptional and translational regulation of the recombinant protein. These include the number of copies of the target gene (gene dosage), the promoter strength and the extent of the induction stimuli, the reducing conditions of the cytosol of the bacteria and the physicochemical characteristics of each particular protein (Mitraki*, et al.*, 1991 , Przybycien*, et al.*, 1994, Carrio & Villaverde, 2005, Ventura & Villaverde, 2006, Ignatova*, et al.*, 2007, Nahalka*, et al.*, 2008, Wang*, et al.*, 2008, Tyedmers*, et al.*, 2010, Winkler*, et al.*, 2010, Singh*, et al.*, 2015). The environment can also influence IBs formation. In fact, during recombinant protein production, temperature, culture time, nature of the inducer, growth rate, agitation and pH (Table 1, and Supplementary Table 1), have a profound impact on architectonic IB properties such as size, inner structure, content of active protein and their potential for being released or solubilized (Garcia-Fruitos*, et al.*, 2005, Garcia-Fruitos*, et al.*, 2005, Margreiter*, et al.*, 2008, Peternel*, et al.*, 2008, Upadhyay*, et al.*, 2012, Castellanos-Mendoza*, et al.*, 2014, Krauss*, et al.*, 2017, Valdez-Cruz*, et al.*, 2017, Calcines-Cruz*, et al.*, 2018).

The increase in IB size has been related with progression of the culture time, being more noticeable in the first hours after recombinant protein synthesis induction (Carrio*, et al.*, 1998, Margreiter*, et al.*, 2008, Garcia-Fruitos*, et al.*, 2009, Upadhyay*, et al.*, 2012, Castellanos-Mendoza*, et al.*, 2014). However, it has been demonstrated that IBs populations are dynamically varying respect to time, being able to increase and decrease in the same process (Castellanos-Mendoza*, et al.*, 2014). Also a rapid bacterial growth rate enhances the aggregation and the accumulation of recombinant proteins as IBs (Iafolla*, et al.*, 2008). During culture, the increase in inducer concentration (mostly exemplified by IPTG) tends to favour the growth of IBs by a hundred of nanometers (Luo*, et al.*, 2006), while at lower concentrations causes a reduction of the IB size combined with higher density of the recombinant protein within them (Margreiter*, et al.*, 2008, Jhamb & Sahoo, 2012).

On the other hand, temperature is a widely used parameter to externally manipulate protein aggregation. Growth of *E. coli* at low temperature (between 16 °C and 30 °C) minimizes the formation of IBs or enriches them with partially or fully folded proteins, enhancing their biological activity (Jevsevar*, et al.*, 2005, de Groot & Ventura, 2006, Vera*, et al.*, 2007, Peternel*, et al.*, 2008). Likewise, low temperature enlarges the extractable protein fraction from IBs, through the steps of solubilization and renaturation (Jevsevar*, et al.*, 2005, Peternel*, et al.*, 2008). On the contrary, when cultures are maintained at 37°C (or higher), IB formation is promoted as well as an increase of impurities inside them (Strandberg & Enfors, 1991, Valax & Georgiou, 1993, Villaverde*, et al.*, 1993). Even more, IBs formed at 42°C present greater resistance to solubilization and denaturation compared with those formed at 25°C, indicating that the culture temperature determines the conformational properties and stability of the proteins trapped within aggregates (Jevsevar*, et al.*, 2005, de Groot & Ventura, 2006, Vera*, et al.*, 2007, Peternel*, et al.*, 2008).

pH has also been examined regarding the formation and quality of IBs (Strandberg & Enfors, 1991, Castellanos-Mendoza*, et al.*, 2014, Calcines-Cruz*, et al.*, 2018). When the pH dropped below 5.5, an increase in the relative amount of IBs was observed compared with cultures developed at constant pH, upon temperature-mediated induction of recombinant gene expression (Strandberg & Enfors, 1991). Differences in the size and architecture of the IBs were determined when comparing bioreactor cultures under controlled pH (7.5) and uncontrolled pH. Those IBs formed under uncontrolled increasing alkaline conditions (up to 8.5) have a lower content of amyloid structures and are easier to solubilize using proteinase K or to denature by chaotropic agents if compared with those IBs recovered from bacteria cultured at controlled pH (Castellanos-Mendoza*, et al.*, 2014). Similar results were found when IBs were produced under constant basic pH of 8.5, using different model proteins and genetic backgrounds (Calcines-Cruz*, et al.*, 2018).

The architecture and size of IBs can be also affected by oxygenation conditions of bacterial culture in shake flasks. When orbital (200 rpm) and resonant acoustic mixing (RAM at 20 *g* forces) were used, a decrease in IB size combined with higher number of small aggregates was observed throughout the cytoplasm. In addition, the smaller

bodies formed under these conditions were easier to solubilize, and showed enrichment in alpha helices compared with those formed under orbital agitation or under lower *g* forces in RAM (Valdez-Cruz*, et al.*, 2017).

Culture conditions represent factors to exploit for improving the IB characteristics according to the final applications foreseen for such "raw material". For instance, the modification of process parameters allows adjusting the conformational (and functional) quality of the IB proteins and more efficient protein extraction (Peternel*, et al.*, 2008, Dong*, et al.*, 2014, Raghunathan*, et al.*, 2014, Krauss*, et al.*, 2017, Calcines-Cruz*, et al.*, 2018).

6. Applicability of IBs as non-toxic, functional amyloids

Bacterial IBs are mechanically stable protein materials (Garcia-Fruitos*, et al.*, 2009). Such structural robustness is the basis for their emerging applications as a versatile microbial biomaterial in biomedicine (Rodriguez-Carmona & Villaverde, 2010) and specially as a topographic decorator in tissue engineering (Seras-Franzoso*, et al.*, 2015). The firm attachment of functional enzymes to the amyloidal scaffold of IBs (Cano-Garrido*, et al.*, 2013) is, on the other hand, supportive of their application as self-immobilized catalysts (Rinas*, et al.*, 2017). The differential sensitivity of amyloidal and non-amyloidal forms of IB proteins to proteinase K (Cano-Garrido*, et al.*, 2013) confirms the dual structural composition of the materials, that might be linked to the occurrence of harder and softer areas in the particles (Diez-Gil*, et al.*, 2010).

6.1 Protein release from IBs

The non-amyloidal fraction of IB protein appears being in a looser conformational status supported by weaker protein-protein contacts than the cross-molecular beta-sheet architecture of the amyloidal fibril skeleton (Carrio*, et al.*, 2005, Gonzalez-Montalban*, et al.*, 2006, de Groot*, et al.*, 2009, Pesarrodona*, et al.*, 2016, Rueda*, et al.*, 2016). Upon internalization by cultured mammalian cells (Figure 3B), an important fraction of IB protein is released from the particles (Figure 3C), probably supported by the action of cell factors, presumably chaperones (Seras-Franzoso*, et al.*, 2016), in the

context of the evolutionarily conserved quality control machinery (Mogk*, et al.*, 2018). The ability of this fraction of IB proteins to be released under physiological conditions has opened a spectrum of possibilities both *in vitro* and *in vivo* to (i) recover functional protein from IBs under non-denaturing conditions (Peternel*, et al.*, 2008) and (ii) explore IBs as mimetics of secretory granules of the endocrine system for the sustained release of functional proteins in the body (Unzueta*, et al.*, 2018), respectively.

6.2 Controlled in vitro release of IB protein in biotechnology

In the context of IB protein recovery, classical denaturation and renaturation procedures are time consuming and result into a limited, product-dependent recovery of the functional protein (Vallejo & Rinas, 2004). The recognition of IBs as nanoparticles composed by properly folded proteins prompted the investigation of simpler methods to extract them from the bulk material, as the disruption of the protein structure might be not required. In fact, washing IBs with physiological buffers with mild denaturants enables the removal of functional proteins in a single step procedure (Singh*, et al.*, 2015).

Producing IBs in conditions that favour proper protein folding (for instance, low growth temperatures) enriches them with functional polypeptides at expenses of amyloidal structure. However, these highly functional IBs can be quite fragile, and their isolation from bacterial cells becomes a critical step. Lysozyme commonly used for bacterial lysis can adhere to the IB surface, representing an additional and major impurity. On the other hand, sonication can cause the loss of the target protein, and high-pressure homogenization has been recognized as the most suitable cell disruption method for looser IBs (Peternel & Komel, 2010). Upon IB isolation, mild detergents can be used for IB solubilization and high recovery rates of extremely pure and biologically active protein have been achieved in this way (Jevsevar*, et al.*, 2005). In pioneering studies, various chemicals in slightly alkaline buffer (40 mM Tris/HCl, pH 8), that do not disturb the protein structure (5% n-propanol, 2 M urea, 0.5% Triton X-100, 0.2% NDSB, 5 % DMSO and 0.2% N-lauroyl-sarcosine) were tested to extract biologically active granulocyte colony stimulating factor (G-CSF) from IBs, representing up to 50 % of the

whole protein yield (Jevsevar*, et al.*, 2005). Similar procedures for non-denaturing solubilization were later described for other proteins (Peternel*, et al.*, 2008, Upadhyay*, et al.*, 2014) (1,2,4,5). Besides low concentration of chaotropes and mild detergents, also alkaline pH in combination with low concentration of urea (Singh*, et al.*, 2015), high pressure (St John*, et al.*, 1999), and organic solvents (Singh*, et al.*, 2012) have been used for successful recovery of bioactive proteins from IBs (Singh*, et al.*, 2015). (Singh*, et al.*, 2015). On the other hand, mild extraction does not work for all IB embedded proteins; for instance, the bone morphogenetic protein 2 which still requires harsh extraction and subsequent refolding under appropriate redox conditions (Quaas, 2018).

6.3 IB protein release in vivo for biomedicine

The *nanopill* concept was around the idea that IBs, as functional protein-releasing amyloids, might be exploited for protein replacement therapies. In this context, IBs formed by functional proteins would physiologically rescue cells with deficiencies caused by either genetic (missing protein functions) or environmental (external stresses) conditions, by acting as carriers of proteins that re-establish cellular homeostasis. A deep interaction (and further embedment) between IBs and mammalian cell membranes in cultured cells was strongly suggesting that at least a significant fraction of IB material might be available intracellularly (Garcia-Fruitos*, et al.*, 2009). As a proof of concept, IBs formed by dihydrofolate reductase, catalase, leukaemia inhibiting factor and Hsp70, once added to the medium of cultured cells were able to rescue cell viability in different experimental systems (Vazquez*, et al.*, 2012). In parallel, it was also demonstrated that IBs composed by the structural cytoskeleton protein keratin 14 released enough correctly folded protein to form heterodimers together with the fluorescent keratin 5, and thus built up fluorescent precursors of the intermediate filaments in epithelial cells (Liovic*, et al.*, 2012). Other interesting applications in regenerative medicine have emerged later, in the context of wound healing (Figure 3D, (Stamm*, et al.*, 2018)). In this context, bacterial IBs appeared suitable also as structural and functional biomimetics of the secretory granules in the mammalian endocrine system by releasing protein hormones (urotensin, glucagon,

obestatin, bombesin and secretin among others, Figure 3A). Under physiological conditions these are kept in the body as amyloidal storage units to be released upon appropriate stimuli with the assistance of cell chaperones (Maji*, et al.*, 2009).

Subsequent studies have shown that nanopills are functional in delivering biologically active proteins not only upon free addition to the culture medium (top-down), but also when decorating surfaces on top of which cells grow (bottom-up) (Seras-Franzoso*, et al.*, 2013). In this second case, nanopills act rather as bioactive topographic scaffolds or *bioscaffolds* (Seras-Franzoso*, et al.*, 2013) with a dual effect. On the one side, they support cell proliferation due to physical stimuli promoted by modifications of the surface nanotopography and that favour both cell adhesion and the activation of proliferative cascades (Seras-Franzoso*, et al.*, 2012). On the other hand, growth or differentiation protein factors released by IB can induce a direct biological effect. The functionalization of 2D and 3D structures with FGF-2 bioscaffolds favoured the growth and differentiation of epithelial cells, fibroblasts and neuron-like cells (Seras-Franzoso*, et al.*, 2013, Seras-Franzoso*, et al.*, 2013, Seras-Franzoso*, et al.*, 2014). IBs releasing the lipoxygenase AmbLOXe have been recently proved to be effective in wound repair (Stamm*, et al.*, 2018), in both nanopill and bioscaffold formats.

The way through which nanopills and bioscaffolds perform their function is dual. Experimental data support protein released by IBs penetrating exposed cells and acting from within as well as accumulating in the culture medium and acting extracellularly. In this regard, free IBs interact tightly with the cell membrane through cell-emitted filopodia which promote IB internalization by micropinocytosis (Seras-Franzoso*, et al.*, 2012, Vazquez*, et al.*, 2012, Seras-Franzoso*, et al.*, 2016, U*, et al.*, 2016). Although part of the IB protein is degraded in lysosomes, significant amounts of IB protein are still detectable intracellularly by immunohistochemistry, once released from IBs into the cytoplasm after the disruption of the endosomal membrane (Figure 3C, (Seras-Franzoso*, et al.*, 2016)). A similar event, although with additional steric constraints, probably occurs with bioscaffolds (Seras-Franzoso*, et al.*, 2013). Interestingly, the biological effect of bacterial amyloids increases when IBs are produced at temperatures below 37 °C (Seras-Franzoso*, et al.*, 2014). This is because the

conformational and functional quality of IB proteins is higher under these conditions (Vera*, et al.*, 2007), as discussed above.

A step forward in the design of improved nanopills has been the generation of cell targeted-IBs that preferentially bind to a specific cell type and enable targeted protein delivery. In this context, soluble glycoproteins have been covalently conjugated to therapeutic IBs for the recognition of adhesins on the surface of pathogens. A self-cleaving peptide has been included to release the functional IB protein after pathogen recognition, creating in this way a pioneer tailored and active protein delivery platform (Talafova*, et al.*, 2013). In a further step to tailor IBs, the fusion of a ligand for a cell surface receptor to the IB-forming protein enabled receptor-dependent IB internalization and cell targeted delivery without requiring chemical modifications of IBs after their purification (U*, et al.*, 2016).

In vivo studies have proved the potential of IBs as protein releasing materials, mimicking the *in vivo* secretion of protein hormones. This effect has been simulated by intratumoral injection and also as subcutaneous nanoimplantation of functional IBs in different murine cancer models. When fluorescent (GFP- or IRFP-based) IBs are injected intratumorally, the emission remains within the tumor for at least one week, and does not induce apparent side effects. This observation indicates that IBs might represent a stable local source for releasing functional protein without toxicity effect. In addition, in this pioneering experiment, there was a certain *in situ* antitumor effect derived from the tumor receptor blockade induced by the peptide T22 contained in the IBs and used as selective binder for the tumoral marker CXCR4 (Cespedes*, et al.*, 2016). In a more challenging approach, it was possible to demonstrate that IB protein could act systematically moving through the blood stream. As an intriguing platform, IBs formed by self-assembling proteins (Figure 3E) that organize as nanoparticles (Pesarrodona*, et al.*, 2016, Unzueta*, et al.*, 2018) were used. When such IBs were applied locally as subcutaneous implants far from the primary tumor in mice, the active protein was progressively and specifically transferred over a period of more than ten days from the injection site to the tumor (Unzueta*, et al.*, 2018). This fact supports that IBs could be used *in vivo* as stable and biocompatible implantable materials for remote delivery of nanostructured protein drugs and materials (Figure

3F). The production of IBs and IB-like materials in endotoxin-free strains of *E. coli* (Rueda*, et al.*, 2014), in food-grade gram positive acid lactic bacteria (Cano-Garrido*, et al.*, 2014, Cano-Garrido*, et al.*, 2016) and in conventional yeast factories (Rueda*, et al.*, 2016) will minimize the potential regulatory constraints for further *in vivo* applications of IBs.

7. Conclusion

Bacterial IBs were observed, since early times of recombinant protein technology, as an obstacle for the production of functional proteins. A limited comprehension of the protein production machinery wrongly positioned them as waste material excluded from the cell quality control, and catalogued IBs as protein clusters composed of irreversibly misfolded or unfolded polypeptides. Gaining insights about the physiology of recombinant cell factories and also about proteomics of protein aggregates shifted this original perception to the current status in which IBs are included in a specific category of functional amyloids. A particular combination of amyloid fibrils and functional structures of the recombinant protein confers to them intriguing properties as mechanically stable and functional materials. Such unusual features point out bacterial IBs as convenient models to study how functional amyloids (especially those used in nature as protein depots) are formed and structured but also enable their exploitation in different areas of biotechnology and medicine. IBs, as protein-releasing materials, are fully biocompatible, and their properties can be modulated through the genetic engineering of the employed proteins, of the host cells, and of the bacterial culture process. In such a way, it is possible to generate nanoparticles that can be targeted *in vivo* and used as source of functional protein.

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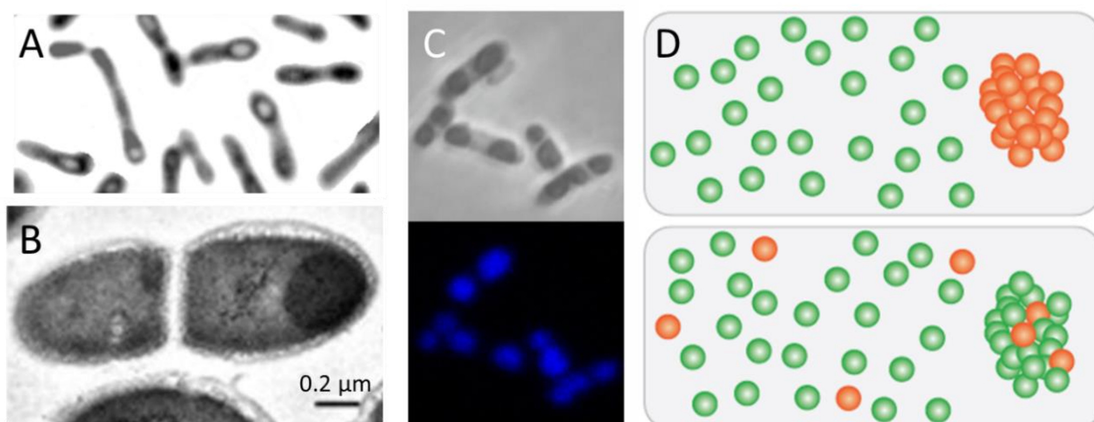

Figure 1. Properties of bacterial IBs. A. Visualization of IB-producing *E. coli* cells by optical microscopy. IBs are refractile intracellular particles. **B.** Transmission electron microscopy images of IB-producing *Lactococcus lactis* cells. IBs are observed as electrodense materials in the cell cytoplasm. **C.** Phase contrast and fluorescent microscopy of the same field of a BFP-producing *E. coli* cell culture. Blue fluorescence is apparent. **D.** Classical model (up) in which a recombinant bacterial cell can be divided into two virtual fractions, namely the soluble cell fraction containing properly folded, soluble and functional protein (green), and the insoluble fraction containing aggregated, misfolded or unfolded non-functional protein (red). Experimental data have accumulated demonstrating that both soluble and insoluble cell fractions are formed by functional (green) and non-functional (red) protein versions (bottom). Culture and genetic conditions that improve protein folding improve the conformation quality of both soluble and insoluble protein species, while those impairing folding also impact on both cell fractions. Images are modified versions from (Carrio & Villaverde, 2001) (A), (Cano-Garrido*, et al.*, 2016) (B), (Garcia-Fruitos*, et al.*, 2005) (C) and (Gonzalez-Montalban*, et al.*, 2007) (D). Images have been reproduced with permission of Elsevier and Springer Nature.

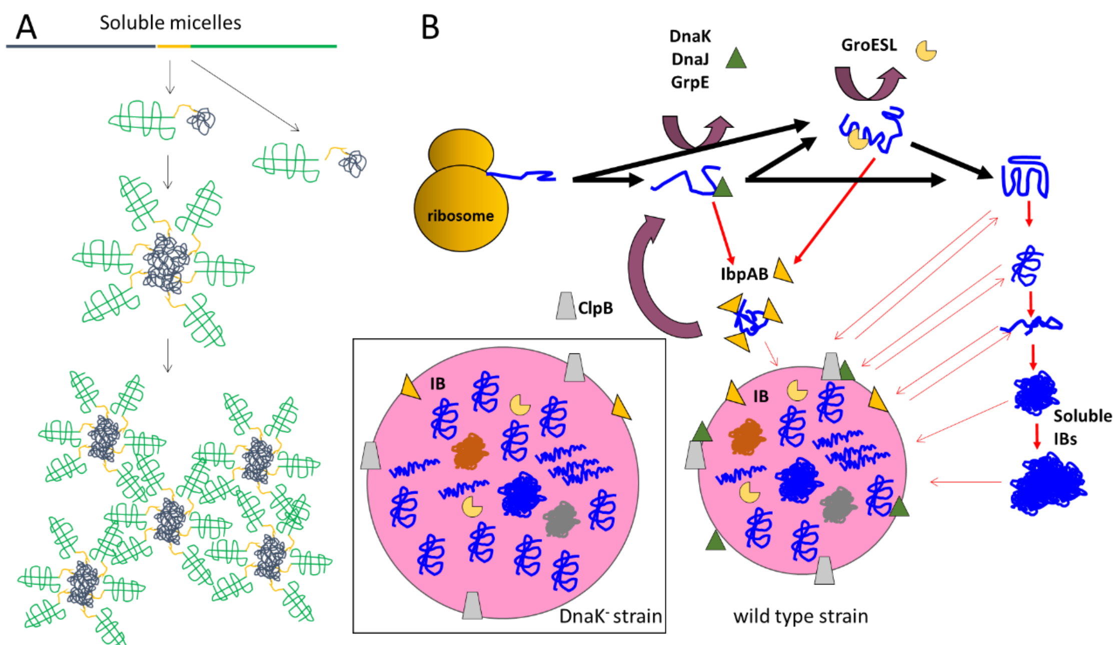

Figure 2. Protein aggregation in recombinant bacteria. A. Characteristics of soluble micelles formed by fusion proteins. Fusions between a target protein (grey) with propensity to aggregate and a passenger polypeptide with high solubility (green) are expressed to increase the chance of the target protein to remain in suspension and complete its folding. Usually a linker (yellow) is exploited to connect the sequences to avoid steric hindrance between the two moieties during folding but it has been described as a sensitive portion for proteolytic degradation. Whether it is not the case and the aggregation-prone protein does not succeed to reach a stable folding, it will interact non-specifically with similar misfolded proteins. They will form a compact hydrophobic core surrounded by the highly soluble passenger molecules. The micelle-like structures will therefore remain soluble in polar solutions. If the passenger proteins have dimerization capacity –as for instance the common passenger protein GST- they will promote the merge of single micelles to form larger aggregates which will still be able to float, at least until a critical point. **B.** Protein misfolding, progressive aggregation, precipitation and re-solubilization. Proteins might need the support of osmolytes and molecular chaperones (DnaK/DnaJ/GrpE + GroESL) to reach their native folding, otherwise remain trapped into instable folding intermediates which can aggregate. Limiting chaperone capacity as well as stress conditions which induce protein misfolding can lead to aggregation mediated by hydrophobic patches present on the molecule surface and that tend to combine with other similar regions of the same or other proteins. The consequent instability leads to form progressively larger aggregates that can finally precipitate into IBs possessing heterogeneous composition (native, quasi-native, aggregate, amyloid-like conformations; blue squiggles). At the same time, misfolded proteins and protein aggregates with variable levels of complexity can be rescued by the activity of chaperones (bidirectional arrows). Main *E. coli* cytosolic chaperones are shown as connected to the folding pathways. In absence of DnaK, IBs are significantly larger and they are formed by higher amounts of biologically active proteins.

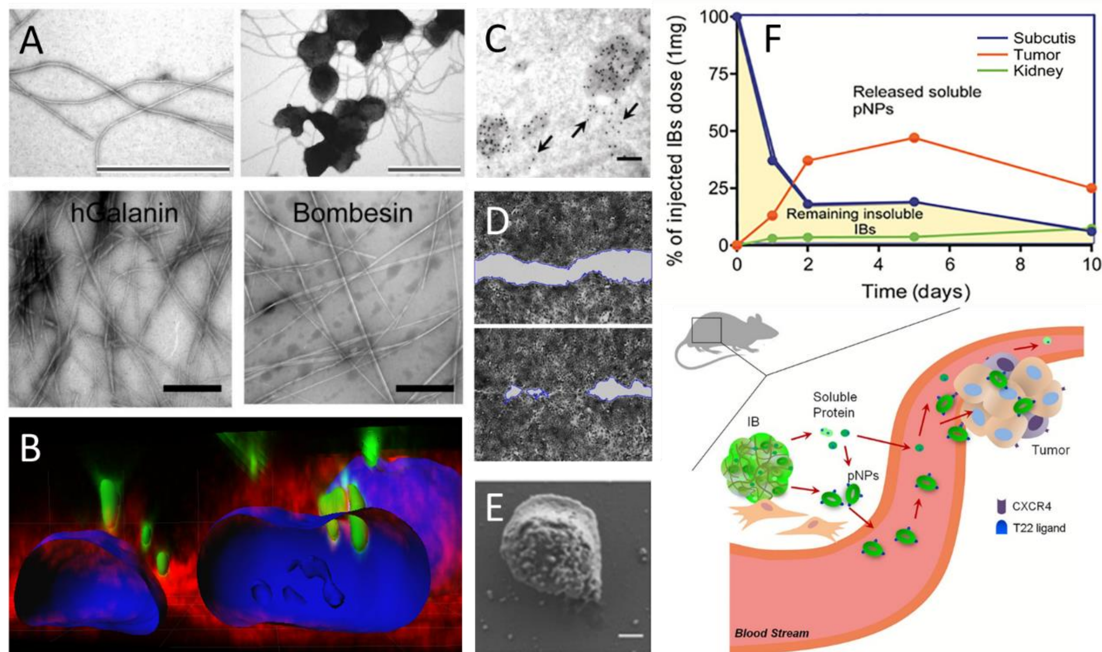

Figure 3. Amyloid structure of IBs and their uses as protein-releasing materials. A. Bacterial IBs showing detached amyloid fibrils. Bars indicate 1 µm. At the bottom, amyloidal structure of two human hormones, showing their fibrillar organization raised during *in vitro* incubation. Bars represent 0.5 µm. **B**. GFP IBs (green) penetrating cultured mammalian cells, upon addition to culture media. In red, cell membranes and in blue, the nuclear region. **C**. Immunolabelling of released IB protein upon cell internalization of IBs present in the culture media. Bar represents 100 nm. **D.** Positive effects of IBs formed by an epidermal lipoxygenase in an *in vitro* wound healing model. The bottom image reflects the IB treatment. **E**. FESEM imaging of purified IBs formed by the self-assembling protein T22-GFP-H6. This protein forms nanoparticles of 11 nm (Rueda*, et al.*, 2015), a size compatible with that of the small structures found surrounding the IB. These particles bind the cell surface tumoral marker CXCR4, though its specific ligand T22 (Unzueta*, et al.*, 2012). **F**. When IBs described in **E** are injected subcutaneously in colorectal cancer mouse models, the amount of IB protein at the injection site decreases over time, while the amount of T22-GFP-H6 protein in tumor, released from IBs, increases. At the bottom, schematic representation of how T22-GFP-H6, released from subcutaneously implanted IBs, reaches a primary $CXCR4^{+}$ tumor in colorectal cancer mouse models. The full experiment is described elsewhere (Unzueta*, et al.*, 2018). Images are reproduced or modified from (Wang*, et al.*, 2008) (A, top), (Maji*, et al.*, 2009) (A, bottom), (Villaverde*, et al.*, 2012) (B), (Seras-Franzoso*, et al.*, 2016) (C), (Stamm*, et al.*, 2018) (D), (Unzueta*, et al.*, 2018) (E, F). Images have been reproduced with permission of The American Association for the Advancement of Science, The Royal Society of Chemistry and Elsevier.

Table 1. Main properties of bacterial IBs adjustable through process conditions.

Parameter	IBs structure features	Reference
Culture time	When culture time after induction increased, IBs median diameter augmented. Also, as the culture time passed, IBs showed strong binding with Congo red and Thioflavin-T (suggesting more amyloid content), and they became more resistant to proteolysis and denaturation.	(Margreiter*, et al.*, 2008, Upadhyay*, et al.*, 2012, Castellanos-Mendoza*, et al.*, 2014)
Inductor concentration	Low concentration of inductor (IPTG) decreased the aggregation and hydrodynamic diameter. Some IBs increased in RP content under lower inductor concentration.	(Luo*, et al.*, 2006, Margreiter*, et al.*, 2008, Jhamb & Sahoo, 2012)
Growth rate	At low specific growth rates, less abundant IBs were found. However, more RP was present in IBs formed at the fastest growth rate.	(Iafolla*, et al.*, 2008)
Temperature	IBs formed at low temperature presented some properly folded and active proteins, less impurities, and were solubilized and denatured faster than those formed at high temperature. Increase in temperature promoted RP aggregation and improved the IB production rate.	(Schein & Noteborn, 1988, Strandberg & Enfors, 1991, Jevsevar*, et al.*, 2005, de Groot & Ventura, 2006, Peternel*, et al.*, 2008)
pH	Relative amount of IBs increased with the decline in pH during culture. IBs formed under basic pH, presented more α-helices, were less resistant to proteolysis and bonded less Thioflavin-T, vs. IBs produced at acidic pH.	(Strandberg & Enfors, 1991, Castellanos-Mendoza*, et al.*, 2014, Calcines-Cruz*, et al.*, 2018)
Agitation	Diffused protein clusters were seen inside cells cultured in shake flasks under acoustic resonant mixing at high energy (20 g) compared with IBs formed under orbital agitation. IBs at 20 g were less resistant to proteolysis.	(Valdez-Cruz*, et al.*, 2017)

Human interferon–α2 (IFN–α2); interferon–γ (IFN–γ); Human growth hormone (hGH); Xylanase (XynB); Alzheimer-related peptide Ab42 mutant fused to green fluorescent protein (Ab42(F19D)-GFP); green fluorescent protein (GFP); protein A from *Staphylococcus aureus* and I-galactosidase (SpA-gal); Isopropyl β-D-1-thiogalactopyranoside (IPTG); Not determined (N.D.); guanidinium chloride. (GnCl); Thioflavin-T (Th-T); Recombinant protein (RP).