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BIOMEDICAL APPLICATIONS OF BACTERIAL INCLUSION BODIES

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8.1 BIOLOGY OF IB PROTEINS

The production of recombinant proteins is a major technological platform in biotechnology, pharmacology, and associated disciplines, and a convenient source of polypeptides for the biocatalysis, food, and pharmaceutical industries. Proteins of industrial and medical interest cannot be purified from natural sources at working amounts through cost-effective procedures. Prokaryotic organisms, and especially the gram-negative bacterium *Escherichia coli*, have been adapted during three decades as cellular factories for protein production (Ferrer-Miralles et al., 2009; Makino et al., 2011). Alternative bacterial species, but also yeasts, fungi, algae, insect cells, and mammalian cells, as well as plants and animals, have been engineered to express foreign genes for the controlled biological synthesis of encoded proteins (Corchero et al., 2012). Using these biological platforms, enzymes, hormones, growth factors, cytoquines, antigens, immunogens, and a diversity of supramolecular complexes (including virus-like particles and other types of protein nanoparticles) have been obtained for characterization or to be

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used as tools in catalysis or biomedicine (Rodríguez-Carmona and Villaverde, 2010). Protein production processes are aimed at the manufacture of stable and soluble protein species (Baneyx and Mujacic, 2004; González-Montalbán et al., 2007). Therefore, proteolytic instability and aggregation are primary obstacles for protein production that had severely challenged the economic viability and feasibility of many processes. This is because many protein species are highly protease-sensitive and/or tend to aggregate as insoluble deposits upon biosynthesis in producing cells (Sørensen and Mortensen, 2005a,b).

In *E. coli*, particularly, the formation of large protein aggregates termed *inclusion bodies* (IBs) is very common (Villaverde and Carrió, 2003). These are spherical or pseudospherical proteinaceous clusters resulting from the aggregation of the recombinant protein, which also contain bacterial proteins and other macromolecules as minor contaminants (Neubauer et al., 2006). Similar particulate protein aggregates are observed in other cell platforms (e.g., aggresomes in mammalian or insect cells), but they have been in general less well studied than IBs (Kopito, 2000; Markossian and Kurganov, 2004; Martínez-Alonso et al., 2009a).

Bacterial IBs are highly porous, mechanically stable proteinaceous particles in a size range between the nano and micro scales (Bowden et al. 1991; Peternel et al., 2008a; García-Fruitós et al., 2009). Being refractile under optical microscope observation and electrodense by transmission electron microscopy, they tend to appear in the cytoplasm at the bacterial poles (Rokney et al., 2009) or in the periplasmic space in the case of secretory proteins (Miot and Betton, 2004). Although it had been believed that IBs are formed uniquely by unfolded or highly misfolded protein versions, and therefore are biologically inert (Baneyx and Mujacic, 2004), it was later determined that a significant fraction of the forming protein is functional and properly folded, adopting native or native-like secondary structure and exhibiting biological activity (García-Fruitós et al., 2005; Jevsevar et al., 2005; Ventura and Villaverde, 2006; González-Montalbán et al., 2007). This observation has supported the exploration of IBs formed by industrial enzymes as naturally immobilized biocatalysts with high operational stability (Nahálka and Nidetzky, 2007; Nahálka, 2008; Nahálka et al. 2008a,b; Nahálka et al., 2009; García-Fruitós and Villaverde, 2010; Sans et al., 2012), which indicates the high mechanical stability of the nanostructured particles, further confirmed by independent reports (Margreiter et al., 2008; García-Fruitós et al., 2009). On the other hand, IBs show marked amyloid properties (Carrió et al., 2005; de Groot et al., 2009; Sabate et al., 2009; García-Fruitós et al., 2011; Espargaro et al., 2012; Villar-Piqué et al., 2012), as a fraction of the IB material is composed of proteinase K-resistant protein

clusters, at least some of them organized as fibrils (Morell et al., 2008). Such a partitioning in the conformational status of IB protein species might be responsible for the unexpected potential of IBs in nanomedicine, as discussed below.

8.2 IB PROTEIN QUALITY

IBs and their forming proteins are not excluded from a cell's quality control, which surveys the conformational status of cellular polypeptides (González-Montalbán et al., 2007). Then, deficiencies in chaperones and proteases or the overexpression of these components along with the recombinant protein have a strong impact on the amount of IB protein and on its conformational and functional quality. Although co-production of folding modulators has largely been explored as a way to minimize aggregation and IB formation (Sørensen and Mortensen, 2005a; de Marco, 2007; de Marco et al., 2007; Kolaj et al., 2009), this approach often shows paradoxical effects, and it results in undesired events or even in a negative impact on the protein yield (García-Fruitós et al., 2007; Martínez-Alonso et al., 2007, 2009b, 2010; Platas et al., 2011), usually by enhancing degradation of the target protein (García-Fruitós et al., 2007; Martínez-Alonso et al., 2009a). In fact, chaperones (such as DnaK) and proteases (such as ClpP) seem to act coordinately on the IB surface (Mogk et al., 2003; Schlieker et al., 2004; Carrió and Villaverde, 2005), as a specific machinery to promote *in vivo* disaggregation for refolding attempts or, eventually, for *in situ* proteolysis (Carrió and Villaverde, 2003).

Interestingly, the conformational quality and physical properties of bacterial protein aggregates can be modulated through parameters such as culture conditions (e.g., growth temperature, harvesting time, dose of isopropyl β -D-1-thiogalactopyranoside or other chemical inducers of gene expression) and by selecting the genetic background of the producing strain (Table 8.1). In this context, the use of *E. coli* strains deficient in chaperones or proteases cannot only result into differently sized and shaped IBs, but it also serves to tune the conformational quality of the proteins forming the aggregates (Jevsevar et al., 2005; Vera et al., 2007; Peternel et al., 2008a) and the physicochemical properties of the whole particles (de Groot and Ventura, 2006; García-Fruitós et al., 2007, 2009). How functional proteins can occur together with amyloid organization is not yet clear. However, this coexistence suggests the occurrence of heterogeneously folded protein species within IBs, a possibility that is fully supported by several observations, as discussed below.

TABLE 8.1 Main Parameters that Determine Final Size, Conformational Quality, and Physicochemical Properties of IBs^a

	Size	Conformational Quality	Physicochemical Properties
Growth temperature	García-Fruitós et al., 2009	Jevsevar et al., 2005; Vera et al., 2007; de Groot and Ventura, 2006; Peternel, 2008b	ND
Harvesting time	García-Fruitós et al., 2007, 2009; Petersson et al., 2004	García-Fruitós et al., 2007	Carrió et al., 2000
Inducer concentration	Petersson et al., 2004	ND	ND
Genetic background	García-Fruitós et al., 2007, 2009	García-Fruitós et al., 2007, 2009	García-Fruitós et al., 2009, 2010; Díez-Gil et al., 2010

^aND, not described.

8.3 IB ARCHITECTURE

Scanning electron microscopy has provided some clues about the structure of IBs, revealing a wrinkled, porous structure (Figure 8.1a). In addition, independent studies indicated that IB proteins are released from IBs under nondenaturing conditions and that IBs are soluble in mild detergents at neutral pH (Jevsevar et al., 2005), especially when they are formed below 37°C and enriched with properly folded proteins. When IBs are suspended in mild detergent (e.g., 0.2% *N*-lauroylsarcosine or 0.1% deoxycholate), a fraction of properly folded proteins is washed away, and the inner structure, composed of small protoaggregates embedded in a cotton-like matrix, is exposed (Figure 8.1b) (Peternel et al., 2008a). However, when IBs are transferred to low-pH buffer, they irreversibly contract and became insoluble in mild detergents (Peternel et al., 2008a). Based on these results, a model for IB formation as well as their architecture structure was proposed, considering the distinct conformational status of IB protein subpopulations (Peternel and Komel, 2011). Such a heterogeneous composition is supported by the different sensitivity of IB protein populations to *in vitro* digestion with trypsin and proteinase K (Cano-Garrido et al., 2013). Protein species fully resistant to proteinase K seem to correspond to amyloidal

FIGURE 8.1 Scanning electron microscope images of (a) freshly purified IBs and (b) after detergent-mediated extraction of functional proteins.

structures within IBs, and these fibers might be the basis of the scaffolding matrix of IBs.

As a bacterial cell is not compartmentalized, recombinant proteins are synthesized simultaneously on multiple locations in the cytoplasm, and many different transitional folding states of the protein are present simultaneously. In recombinant bacteria, some recombinant polypeptide chains remain unfolded or misfolded and start to aggregate into small and still soluble proto-aggregates (Schroedel and de Marco, 2005), acting as nucleation seeds. Folded or partially folded protein species might interact with these nuclei through cross-molecular stereospecific interactions, while cellular proteins are mostly excluded from these sequence-specific events (Speed et al., 1996; Carrió et al., 2005). Contaminant cell proteins present in IBs, such as folding modulators, could become entrapped because of their interaction with nascent polypeptides. At neutral pH, this network is loose, and folded proteins can easily be extracted (Makino et al., 2011). However, at an acidic pH the network of misfolded proteins irreversibly contracts and properly folded proteins remain trapped inside (Peternel et al., 2008a).

8.4 IBs AS BIOMATERIALS

Interestingly, IBs have recently been observed as emerging biomaterials with potential biomedical applications. This has been empowered by the fact that these protein clusters are biologically active and also mechanically stable.

TABLE 8.2 IB Features That Have an Impact in Their Use as Biomaterials

Determinant Parameters	IB Properties	Application
Genetic background	Geometry, stiffness, wettability zeta-potential, bioadhesiveness	
Genetic background/ process variables	Size, specific activity, specific fluorescence	Biocatalysys
Process variables	Proteolytic stability	Cell therapy
Postproduction treatment	Density/porosity	Tissue engineering

Once purified, IBs are regular particles with low polydispersity, usually spherical or ovoid, ranging from 50 to 1000 nm in diameter. IB size can be tuned according to the particular protein species, genetic background of the bacteria, and production time (García-Fruitós et al., 2009), while other physicochemical properties of relevance in material sciences, such as stiffness, zeta potential, wettability, and morphology, can also be modulated by selective production in defined *E. coli* mutants (Table 8.2). In particular, deficiencies in the chaperones DnaK and ClpA or in the protease ClpP lead to anomalous quality control and altered protein deposition patterns, which significantly affect the nanoscale properties of IBs (García-Fruitós et al., 2012). To determine how the different genetics of IB bioproduction affects their elasticity, force spectroscopic atomic force microscopy (AFM) was used to measure Young's modulus of IBs in a statistical way. Young's modulus, a measure of the stiffness of elastic materials used to characterize elasticity, is defined as the ratio of the stress along an axis, which has units of pressure, over the strain along that axis, which is dimensionless, in the range of stress in which Hooke's law can be applied. Therefore, Young's modulus has units of pressure [usually megapascal (MPa)]. While wild-type IBs showed a monomodal elasticity distribution, IBs produced in specific phenotypes (DnaK⁻ and ClpA⁻) split into two elasticity populations, in agreement with wettability data. Wettability is the ability of a liquid, usually water, to maintain contact with a solid surface and it is important in the adherence of two materials. Adhesive forces between a liquid and a solid cause a liquid drop to spread across the surface. The same technique equipped with a closed-loop tracking system made it possible to obtain spatial distribution of the elasticity regimes over the various IBs and to develop stiffness maps in which each force versus indentation measurement was mapped over the target IB. Young's modulus was spread homogeneously over wild-type IBs, with mean values of 3.6 ± 0.56 MPa. On the contrary, for DnaK⁻ and ClpA⁻ IBs, two

elasticity populations were observed, with the harder areas segregated from the softer areas. The data above indicated that the genetics of IB fabrication determines the coexistence of more rigid structures and softer ones, and that they seem to be localized on predetermined areas of individual IB particles. This fact confirmed the structural diversity inside the protein aggregates, as discussed above.

8.5 PURIFICATION OF IBs

As biomaterials, IBs could eventually be used in biological interfaces, and purification protocols have been adapted to obtain these particles free from bacterial contaminants (Rodriguez-Carmona et al., 2011; Peternel, 2013). In general, IBs show mechanical and long-term stability and good tolerance to mechanical, chemical, and enzymatic cell disruption methods such as ultrasonication and high-pressure homogenization or physical procedures such as freeze/thaw cycles (Georgiou and Valax, 1999). However, IBs tend to co-sediment with bacterial cells and debris during downstream transport, and undesired contaminants such as lipids, lipopolysaccharides, nucleic acids, and remaining whole cells must be fully removed to assure the absence of cytotoxic effects upon exposure to mammalian cells. Moreover, downstream strategies must preserve conformational stability, mechanical properties, and the biological activity of the protein aggregates. In this context, Peternel and Komel compared (2010) three methods (enzymatic lysis and two mechanical methods, high-pressure homogenization and sonication) for the isolation of biologically active IBs. In this study, homogenization was most appropriate in terms of biological activity preservation of the proteins. On the other hand, a convenient combination of mechanical (sonication) and enzymatic (lysozyme) cell disruption methods followed by DNase treatment and detergent washing steps has been adapted and optimized to provide preparations of cell-free IBs with viable cell contamination below 10^{-1} cfu/mL, while maintaining the particle size and the conformational status of the protein (Rodriguez-Carmona et al., 2010), summarized in Figure 8.2. This procedure seems to be suitable for most applications of IBs involving intimate contact with mammalian cells and organs, as discussed below.

8.6 IBs IN TISSUE ENGINEERING

Recently, numerous studies have supported functionalization and nanostructuring of surfaces for tissue engineering and regenerative medicine (Dalby, 2009). Among the approaches available, the generation of nanostructured and

FIGURE 8.2 Schematic representation of IB purification to obtain bacteria-free IB preparations for biomedical applications. This process combines enzymatic digestion of the cell wall with soft mechanical disruption.

nanopatterned surfaces with either inorganic or organic materials is especially appealing (Harrison and Atala, 2007; Chien et al., 2009; Hu et al., 2010; Whited et al., 2011). As a contribution to this area and because of the tunable properties of IBs as described above, IBs formed by the green fluorescent protein (GFP) have been used successfully for a bottom-up approach to engineer surface topographies. These reports prove that IBs can be used as particulate biomaterials to stimulate surface colonization by mammalian cells without any sign of cytotoxicity (García-Fruitós et al., 2009, 2012; Diez-Gil et al., 2010). The fast colonization of IB-modified surfaces is supported by mechanical stimulation of mammalian cell proliferation on IB-decorated surfaces, as observed by enhanced phosphorylation of signal-regulated protein kinase and by the dramatic emission of filopodia-lamellipodia in the presence of IBs (Figure 8.3) (Seras-Franzoso et al., 2012a). Interestingly, it has also been shown that IBs are highly bioadhesive

FIGURE 8.3 Scanning electron microscope images of PC12 cells cultured for 48 h on IB-based topographies, showing different individual cells (a and b). Inset frames provide magnifications with further detail of IB-induced filopodia.

materials and that mammalian cell expansion on IBs is supported synergistically by both enhanced adhesion and mechanical stimulation of cell division. The extent in which these events influence cell growth depends on the particular cell line, but it is also related to the genetic background of IB-producing bacteria, thus opening exciting possibilities for fine tailoring of the material (Seras-Franzoso et al., 2012a). In this regard, since IB properties can be tuned

during their fabrication process (Peternel et al., 2008b; Martinez-Alonso et al., 2009b), these protein clusters can represent an alternative to the use of such adhesive proteins as fibronectin and vitronectin.

It is known that not only cell adhesion but also cell migration and differentiation can be controlled by engineering the cell–material interface (Ayala et al., 2011). Interestingly, the contact angle that is identified as optimal for surface colonization is within the range of angles exhibited by IBs and perfectly matches that of IB variants produced in DnaK[−] and ClpA[−] bacterial strains (Diez-Gil et al., 2010). This further supports the use of IBs as convenient substrate materials to favor mammalian cell colonization. Moreover, these biologically inert IBs have also been observed successfully as able to induce differentiation in hMSC cultures (Seras-Franzoso et al., unpublished data).

On the other hand, cell responses, such as positioning, morphological changes, and proliferation, are the result of complex chemical, topographical, and biological stimuli. In this context, cells respond positively to two-dimensional engineering of biological interfaces at the microscale using IBs. Data obtained with fibroblasts cultivated on IB-patterned supports demonstrate that these cells preferentially adhere to IB-covered areas and align and elongate according to IB patterns (Tatkiewicz et al., unpublished data). This result fills the gap between existing techniques which are based on the local modification of surface chemistry and techniques of modification of the topography at the nanoscale level (Malmstrom et al., 2011), and it also proves that IBs are interesting and useful biocompatible nanomaterials for biomedical applications.

8.7 SLOW DRUG RELEASE FROM BACTERIAL IBs USED AS NANOPILLS

IBs formed by proteins of therapeutic interest are able to deliver functional polypeptides to the cell cytoplasm and nucleus for a biological effect. Adsorbed to two-dimensional surfaces as functional materials (bioscaffolds), IBs formed by the antiapoptotic chaperone Hsp70 or the basic fibroblast growth factor (FGF-2) get embedded in top-growing cells, make their forming proteins available to them, and rescue or enhance cell viability, respectively (Seras-Franzoso et al., 2012b). Used in suspension as nanopills, IBs formed by these two particular polypeptides (and also by other enzymes or structural proteins) can penetrate the cellular and nuclear membranes in the absence of cell toxicity (Figure 8.4), promoting cell viability through a true protein replacement therapeutic effect at the cell level, for which protein release from IBs is suspected (Liovic et al., 2012; Vazquez et al., 2012).

FIGURE 8.4 Confocal xyz stack of several sections generated with the Imaris 3D software (using the Isosurface module), showing penetration of GFP-based IBs into cultured HeLa cells. IBs are shown in green, nuclear DNA in blue, and the cell membranes in red. Experimental details can be found elsewhere [76]. (*See insert for color representation of the figure.*)

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Moreover, IBs are well tolerated in oral administration (Vazquez et al., 2012), which opens exciting opportunities for therapeutic uses of these protein particles (Villaverde, 2012; Villaverde et al., 2012). Again, functional protein release from therapeutic IBs seems to involve only a fraction of IB protein, as a mechanically stable scaffold is still observed in cell-internalized IBs. The cotton-like model of IBs seem's to account functionally and architectonically for the potential of these clusters as either bioscaffolds or nanopills, which might again be based on the heterogeneous conformational status of the IB protein. While amyloidal fibers and similar structures might confer mechanic stability, properly folded protein species are the functional part of the material that is released under convenient conditions. Interestingly, IBs seem to be natural mimetics of secretory granules of higher organisms, in which protein hormones are stored for secretion as repository amyloidal scaffolds (Maji et al., 2008, 2009; Mankar et al., 2011). The potential of bacterial IBs as functional materials in biomedicine is wide and highly promising, but it remains to be fully explored.

8.8 CONCLUSIONS

Bacterial IBs are naturally occurring proteinaceous particles that contain significant fractions of properly folded versions of recombinant proteins, conferring on them biological activity. Their mechanical stability could be

based on amyloid protein versions, which create a matrix-embedding functional species. The combination of mechanical stability and functionality, together with the potential of functional proteins to be released from IBs under nondenaturing conditions, has recently prompted researchers to explore them as functional materials for biomedical applications. Being fully biocompatible, they appear as interesting slow-delivery agents for protein replacement therapies at the organic level but also in regenerative medicine. Since essentially any polypeptide suitable to be produced in *E. coli* can be obtained as IBs, the potential of bacterial cells to generate therapeutic nanopills should be explored fully in the context of innovative medicines.

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