

# Bacterial inclusion bodies: making gold from waste

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**Many protein species produced in recombinant bacteria aggregate as insoluble protein clusters named inclusion bodies (IBs). IBs are discarded from further processing or are eventually used as a pure protein source for *in vitro* refolding. Although usually considered as waste byproducts of protein production, recent insights into the physiology of recombinant bacteria and the molecular architecture of IBs have revealed that these protein particles are unexpected functional materials. In this Opinion article, we present the relevant mechanical properties of IBs and discuss the ways in which they can be explored as biocompatible nanostructured materials, mainly, but not exclusively, in biocatalysis and tissue engineering.**

## Biology of bacterial inclusion bodies

Bacterial inclusion bodies (IBs) are protein aggregates that have been commonly observed in recombinant *Escherichia coli* since the implementation of recombinant DNA technologies. IBs were rapidly recognized as the major bottleneck in recombinant protein production and were assumed to be formed by unfolded or highly misfolded polypeptides that failed to reach their native conformation [1].

Solubility has been considered the main macroscopic signal of successful protein conformation and functional quality at the molecular level [2]. For instance, many recombinant proteins have been produced only (or almost only) as IBs and consequently discarded before further use. Thus, in biomedical research, among the wide spectrum of proteins identified as potential drugs, only a very limited fraction are actually produced, approved for use and marketed. In this context, a significant number of the therapeutic proteins in use have been obtained in yeasts or mammalian cells instead of *E. coli*. These systems allow post-translational modifications that are absent in bacteria and overcome massive aggregation [3].

Protein aggregation in bacteria has contributed to lower than expected success for recombinant DNA technologies because the yield of functional, soluble and stable

polypeptides is often not cost-effective enough for industrial production. Even laboratory-scale production of many specific products for research purposes has a protein insolubility bottleneck. Minimization of protein aggregation through midstream approaches (by chaperone co-expression, protein engineering or by adjusting gene dosage, temperature or transcription rate) has yielded rather inconsistent and product-dependent results. Rational manipulation of foldase, protease and disaggregase activities to control protein solubility depends on metabolic engineering reaching the systems level. Unfortunately, the regulatory complexity, synergism and redundancy of the cellular quality control network make such a task currently unaffordable. At the downstream level, IBs are eventually used as a source of protein for *in vitro* refolding and recovery. This is because up to 90% of the total protein in IBs is recombinant protein and because IBs can easily be separated from cell debris by simple procedures. However, refolding strategies for proteins from isolated IBs need to be developed on a case-by-case basis and are weakly extendible to a wide spectra of products (insulin is among the exceptions). Consequently, IBs have been historically observed as undesired waste products of biotechnology processes [1].

Although recombinant protein aggregation in bacteria has been ignored as a scientific issue for decades and thus IB research has largely been neglected, a few seminal observations have revitalized both academic and industrial interest in IBs. Recent research in conformational stress of recombinant bacteria has provided insights into the physiology of IB formation and on the molecular architecture of these protein clusters. Emerging concepts regarding how bacteria survey the quality of soluble and insoluble protein species, the dynamics of protein aggregation–disaggregation, the functional characterization of IB polypeptides and the mechanical stability of IBs have unveiled unexpected potential for IBs in nanobiotechnology. In this Opinion article, we summarize these findings and support a new view on bacterial IBs. We propose reasons why IBs should not be discarded as waste byproducts of bioengineering processes. Instead, protein production processes might eventually be retargeted to obtain tailored IBs as desirable biomaterials for novel applications in industry and biomedicine.

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## Inclusion bodies are highly dynamic and contain functional protein

Interest in IBs has recently resurfaced largely because of the findings that (i) protein deposition in recombinant cells is fully reversible and (ii) a relevant fraction of IB proteins is actually functional.

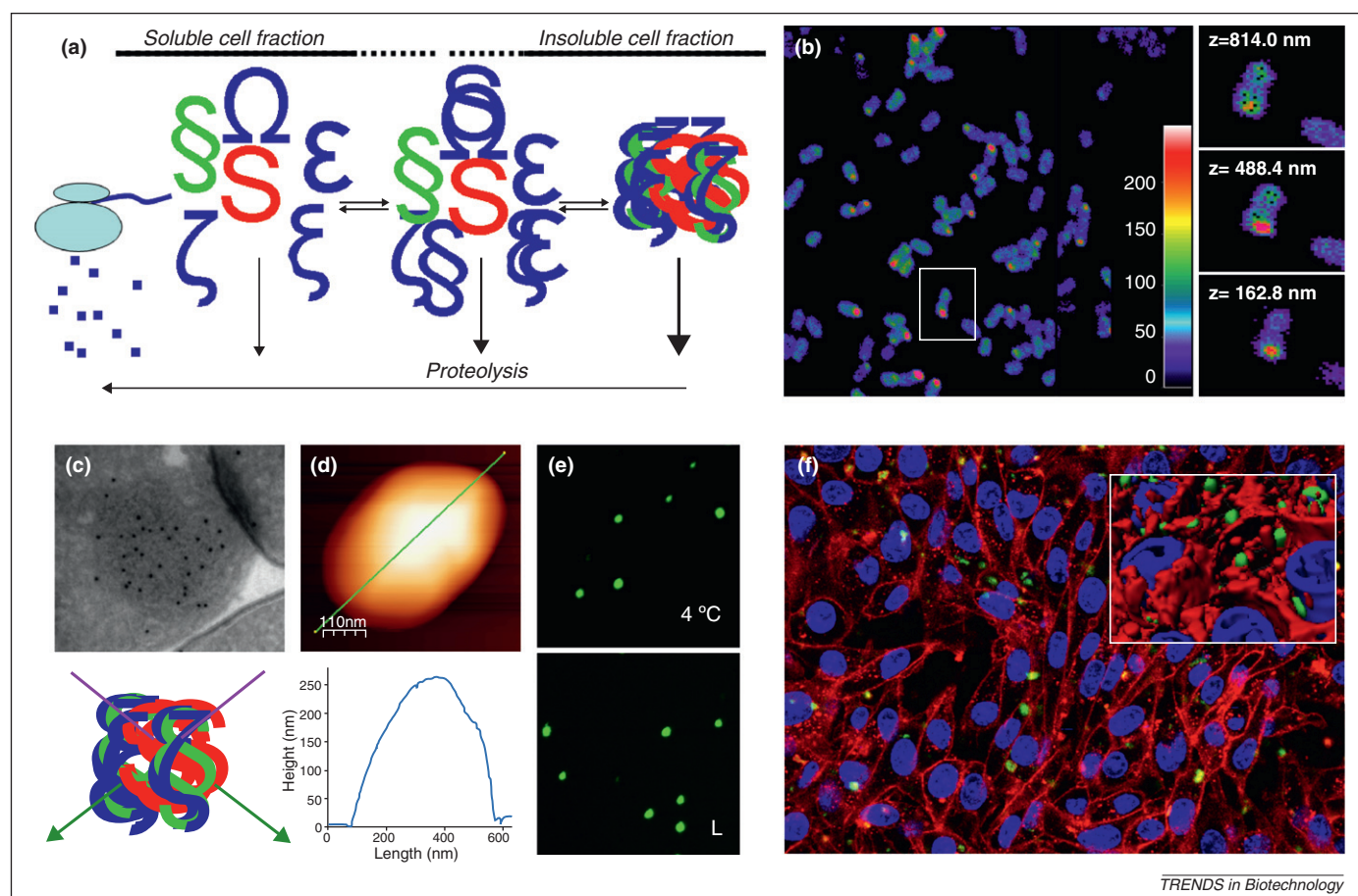
### Protein deposition in recombinant cells is fully reversible

Already formed IBs spontaneously disintegrate in the cytoplasm of *E. coli* when *de novo* protein synthesis is arrested [4]. The process is highly dependent on chaperones and proteases, which indicates that IB proteins are actively removed from the clusters by cell components. More generically, cell-controlled IB disintegration also indicates that aggregates are not excluded from the conformational surveillance of the cell. In this regard, the main chaperone DnaK is a key controller of IB protein extraction and degradation, and uncoupling of DnaK and protease activities largely stabilizes aggregation-prone recombinant proteins [5]. Observations of soluble aggregates

in recombinant bacteria largely support a continuum-of-forms model, under which bacteria contain the recombinant protein in a gradation of conformational states that steadily migrate between soluble and insoluble (virtual) cell fractions (Figure 1a) [6].

### An important fraction of the IB-forming protein is functional

Enzyme activity in IBs was first associated with  $\beta$ -galactosidase [7] and endoglucanase D [8], but it was suspected that the activity was due to contamination. In 2005, functional protein species were clearly identified in IBs of the human granulocyte-colony stimulating factor formed in *E. coli* at suboptimal growth temperatures [9]. Such IBs were termed nonclassical inclusion bodies in the belief that these protein clusters were structurally anomalous and different from conventional protein aggregates. However, a parallel study using *E. coli* cells that produced green or blue fluorescent proteins, dihydrofolate reductase or an aggregation-prone version of  $\beta$ -galactosidase (at 37 °C) showed that conventional IBs were biologically active



**Figure 1.** (a) A single recombinant protein produced in bacteria can fold into a wide spectrum of conformations, including the native conformation (red), functional isoforms (green) and inactive, largely misfolded protein forms (blue). All these species can reversibly aggregate via stereospecific crossmolecular interactions as soluble or insoluble clusters, through chaperone-modulated processes. Aggregated peptides are also substrates for proteases. (b) Confocal section of an *E. coli* culture producing GFP IBs. Magnified images of a single cell at different z values are also presented, which show the fluorescent core of the protein particles and the slightly less fluorescent surface layer. (c) Transmission electron micrograph of intracellular GFP IBs immunodetected with GFP-reactive antibodies. The porous architecture of IBs, which supports their high hydration and mechanical stability, is evident. Such a loose organization allows efficient mass transfer and permits diffusion of the substrate (purple lines) and release of the product (green lines) during enzymatic catalysis (bottom). (d) 3D AFM topographic image of a single GFP IB deposited on a mica surface. Its topographic cross-section indicated as a green line is shown at the bottom. (e) Confocal microscopy images of purified IBs formed by mGFP as in panel (d), stored for 1 month at 4 °C (top) and after lyophilization and reconstitution (L, bottom). (f) Overlay of BHK cell culture growing on polystyrene plates decorated with mGFP IBs 75 h after cell seeding. The inset shows intimate cell membrane-IB contacts visualized by confocal xyz sections stack-processed using Imaris 3D software (modified from [22]; Copyright Wiley-VCH Verlag GmbH & Co, KGaA. Reproduced with permission).

[10]. Thus, even IBs produced using standard protocols can be considered as nonclassical regarding the functional quality of the embedded proteins. The extent of biological activity exhibited by IB proteins (measured as specific activity or fluorescence) depends on the specific protein, genetic background of the host bacteria and culture temperature [11], and ranges from undetectable amounts to levels even higher than those shown by soluble counterparts [10].

The biological activity of IB proteins requires protein folding and native or native-like secondary structure of the embedded protein (Figure 1a). Protein deposition as bacterial IBs is a stereospecific event, as suggested not only by the high protein purity of the aggregates, but also by the co-aggregation *in vitro* of homologous denatured proteins [12] and the sequence-specific seeding ability of isolated IBs [13]. Furthermore, sequence-specific contacts during IB formation have been fully proved *in vivo* through the mutually exclusive formation of IBs in cells that produce two heterologous protein species [14]. Stereospecific cross-molecular contacts require native-like secondary structure, and native-like conformation of IB proteins has been widely confirmed by IR spectroscopy [15]. Moreover, fine imaging analysis of GFP IBs revealed a more intense fluorescence in the core than at the IB surface [16]. Furthermore, fluorescence confocal section analysis of individual IBs within cells supports the functional nature of the IB core and suggests a structural role of native protein in the IB scaffold rather than superficial contamination (Figure 1b) [17].

Examinations of the inner architecture of IBs have benefited from amyloidosis clinical investigation with IR, circular dichroism, X-ray diffraction and other techniques for structural analysis. As a result, both native-like secondary structures and amyloid-like cross-molecular  $\beta$ -sheet architectures have been identified in IBs [13]. Proteinase K-resistant amyloid-like fibers have been described as IB components. Although quantitative data are not available, such fibers probably represent a low proportion of the total IB mass [14]. How properly folded and amyloid-like protein stretches coexist and are organized within these protein particles remains an unsolved issue. However, structural studies on IBs have revealed that the relative abundance of cross- $\beta$  regions is much lower than in conventional amyloid fibrils [18], which indicates that important segments of IB proteins are excluded from the tightly packed  $\beta$ -sheet architecture.

### Inclusion bodies can be used as naturally immobilized enzymes in biocatalysis

The biological activity of bacterial IBs opens intriguing possibilities for their rational use in applications in which aggregation *per se* is not a major impediment [19]. Many recombinant enzymes, naturally immobilized as functional IBs, have been explored for industrial catalysis in a set of very elegant studies. So far, oxidases, reductases, phosphatases, kinases, aldolases, glucosidases, phosphorylases and others have been investigated as IB-based catalysts. *In vivo* immobilized enzymes might be advantageous because high yields of recombinant proteins can easily be driven to pure protein particles using selected peptides as pull-down

tags [20]. In addition, the porous nature of IBs is expected to permit efficient mass transfer through the IB scaffold (Figure 1c), and their surface nanorugosity (Figure 1d) would be convenient for IB immobilization in solid phase catalysis. In addition, the production cost for pure IBs is estimated to be approximately 20 times lower than for easy-to-produce soluble proteins (García-Fruitós, unpublished data).

IBs are mechanically stable enough to withstand harvest by ultrasonication, high pressure or other harsh physical procedures applied to break cells [21]. This property is very convenient for industrial-scale manipulation in enzymatic reactors. In addition, very low amounts of the IB protein are released to aqueous media from freshly isolated IBs during the first minutes of incubation [16]. Beyond this point, IBs are extremely stable in long-term storage and incubation without perceivable changes in size, geometry and biological activity [22]. In addition, IBs show good tolerance of lyophilization and freezing–thawing (Figure 1e). In an enzymatic study, 90% of the initial maltodextrin phosphorylase activity was observed in naked IBs from *Pyrococcus furiosus* after 10 reaction cycles [23]. This excellent operational stability can be further enhanced by entrapping IBs in semi-permeable gel matrices or microcapsules [based on either agar–TiO<sub>2</sub>, poly(methylene-co-guanidine), alginates and cellulose sulfate] [24,25], alone or in combination with crosslinking agents such as glutaraldehyde [26]. This approach minimizes eventual enzyme leakage and consequent product contamination. Finally, high-cell-density production of IBs is fully feasible and improved downstream protocols have been adapted to obtain highly pure protein particles [27,28].

### Inclusion bodies are mechanically stable nanoparticulate materials

IBs are harvested from bacterial cultures by harsh mechanical procedures to disrupt the cell wall. On purification, IBs are observed as pseudospherical particles (Figure 1d) with limited size dispersion, ranging from 50 to 500 nm in diameter. IB average size depends on the particular protein species, genetic background of the cell and harvest time [22]. The biological origin, mechanical stability and regulatable size of IBs, together with the increasing demand for fully biocompatible and tunable nanostructured materials, raises the question of to what extent these protein particles can function as particulate biomaterials for biomedical applications.

In recent years, numerous studies have supported functionalization and nanostructuring of surfaces in tissue engineering and regenerative medicine [29]. Among the available approaches, the generation of nanostructured and nanopatterned surfaces with either inorganic or organic materials (Table 1) is especially appealing. These coatings not only improve cell adhesion and proliferation, but also influence more complex cellular processes such as cell differentiation and motility [30]. However, the need for complicated coating techniques, low versatility, limited physicochemical characteristics and cytotoxicity have delayed further progress in this area.

In bottom-up approaches to topographic engineering, IBs formed by irrelevant proteins successfully stimulate



**Table 1. Most common materials for surface modification used in tissue engineering**

Material	Properties <sup>a</sup>	Application	Deposition or presentation method	Ref.
Inorganic coatings (DLC, apatite, TiO <sub>2</sub> , etc.)	Biocompatibility, nanophase materials, high Young's modulus (GPa)	Tissue engineering (mainly hard tissues such as bones)	Physical deposition, anodization, electrospun, chemical growth	[38]
Organic coatings (HA, FN, PL, PAA/PAM, PLGA, etc.)	Biocompatibility, versatile surface chemistry, controlled degradation	Tissue engineering (soft and hard tissues), drug or gene delivery	Layer by layer (LBL) deposition, electrospinning	[39]
Nanoparticles (metallic, magnetic, organic, ceramic, etc.)	Controlled size, versatile surface chemistry	Tissue engineering, drug or gene delivery, imaging	Chemical growth	[40]
Carbon nanotubes (CNTs)	Electroactive, high specific surface, functionalization, good mechanical properties	Tissue engineering, cell tracking and labeling, sensing of cellular behavior	Electrospinning, chemical vapor deposition (CVD)	[41]
IBs	Simple and cost-effective production, controlled size, biocompatibility, appropriate (and tunable) chemical and mechanical properties, nanoroughness	Tissue engineering	Dip coating and soft lithography techniques	[31]
IBs <sup>a</sup>	Simple and cost-effective production, controlled size, mechanical stability, porosity, reusability	Biocatalysis	Naked material, microencapsulation, gel embedment or crosslinking	[10]

<sup>a</sup>Additional properties of IBs as functional materials for catalysis are also indicated.

surface colonization by mammalian cells without any sign of cytotoxicity (Figure 1f) [22,31]. Because they are bioadhesive, IBs enhance cell retention on the decorated substrate. In addition, IBs activate filopodia-mediated cell sensing and suitable mechanotransduction circuits that stimulate proliferation via activation of the ERK pathway [32]. Acting as inert materials, their geometry, stiffness, Z-potential, wettability, size and morphology can be tuned by selective production using defined *E. coli* genetic backgrounds. In particular, deficiencies in chaperones, DnaK and ClpA, or in the protease ClpP lead to anomalous quality control and protein deposition patterns, which significantly affect the nanoscale properties of IBs produced in these mutants (Table 2) [17,22,31]. Interestingly, the impact of extracellular matrix hydrophobicity on stem cell adhesion, spread and differentiation was evaluated in a recent study by screening substrate variants with different properties [33]. The contact angle identified as optimal for surface colonization (57.99°) is within the range of angles exhibited by IBs and perfectly matches that of IB variants produced in DnaK<sup>-</sup> and ClpA<sup>-</sup> cells [31], which further supports the use of IBs as convenient substrate materials to favor mammalian cell colonization.

Surface patterning by microcontact printing with IBs influences the spatial distribution and performance of cultured cells [22]. Colloidal lithography produces a similar result. Here, dense short-range ordered arrays of circular gold holes in SiO<sub>2</sub> films are produced using dispersed colloidal monolayer masks. The hole diameter can be varied using a series of particles of sizes very similar to those of IBs. The gold holes can be modified using proteins (e.g. fibronectin or vitronectin) defined into patterns. In these studies, protein type and pattern size have an important effect on cell adhesion and spreading [34]. Compared to well-characterized hard materials such as TiO<sub>2</sub> particles, the reproducibility of cell responses to IB patterning has not been yet fully evaluated.

The density and porosity of isolated IBs can be modified by adjusting the pH (Table 2). Extreme chemical or physical changes to bacterial cultures influence the size and morphology of non-IB protein aggregates [35]. Whether these parameters could be useful in modulating IB properties at midstream or downstream levels deserves detailed investigation, as they might represent a way to minimize the heterogeneity of IB particles as natural products. Finally, the fact that polypeptides packaged as

**Table 2. Biological and physicochemical nanoscale properties of bacterial IBs**

Property <sup>a</sup>	Modulating factor	Ref.
Size	Tuneable by harvesting time and by the host genetic background	[22]
Geometry	Tuneable by the host genetic background	[17]
Stiffness	Tuneable by the host genetic background	[31]
Wettability	Tuneable by the host genetic background	[31]
Z-potential	Tuneable by the host genetic background	[31]
Bio-adhesiveness	Tuneable by the host genetic background	[32]
Proteolytic stability	Regulatable by harvesting time	[42]
Specific activity or specific fluorescence	Tuneable by production conditions (mainly by temperature) and by the host genetic background	[22]
Density/Porosity	Regulatable by pH	[43]
Release of functional proteins	Regulatable by production temperature	[36]

<sup>a</sup>All the properties listed are co-determined by the nature of the specific polypeptide forming the IBs.

non-classical IBs release high amounts of functional polypeptides under nondenaturing conditions [36] opens a possibility to use these materials as sustained protein delivery systems in cell–substrate interfaces, for instance, embedded in hydrogels.

The potential of IBs as promising advanced materials has been rapidly accepted by the scientific community [28,37]. However, full identification of the possibilities and drawbacks of these particulate materials requires more detailed investigations. Prematurely stopped, pioneering proteomic studies on IBs should be now completed. Global compositional analysis of IBs would potentially identify undesired contaminants that might compromise IB use in biological systems. This would help to improve downstream strategies to obtain cleaner materials. New-generation protocols to prepare IBs fully free from bacterial cells [27] already provide particles that do not exhibit any signs of toxicity when exposed to mammalian cell cultures [17,22].

Success of the applications of IBs as biocatalysts and as inert nanostructured materials tested so far for tissue engineering cannot be more promising. However, further multidisciplinary research is still needed to fully implement IB-based material platforms in emerging bionanotechnological applications.

#### Disclosure statement

A.V., E.G.F., E.V., J.V., I.R. and C.D.G. are co-inventors of a patent (P200900045) on the use of IBs as reagents for mammalian cell culture.

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