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INCLUSION BODIES IN BIOTECHNOLOGY

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ABSTRACT

Protein aggregation is commonly observed phenomenon occurring during production of recombinant proteins in genetically engineered organisms over-expressing foreign genes. Aggregation of such recombinant proteins often leads to the formation of insoluble protein clusters named inclusion bodies (IBs). Although usually considered as waste by-products of protein production, over the last decade, this general misconception has been questioned by various studies. Gradual understanding of the structural, molecular and physiological features has revealed promising potential of these so far undesirable cellular products. In the present review we summarize basic characteristics of IBs, their use in antimicrobial peptides production and in various biotechnological fields, including tissue engineering, drug delivery and biocatalysis.

Keywords: inclusion bodies, protein aggregation, biotechnology, antimicrobial peptides, biomedicine

INTRODUCTION

Biotechnology is a conquering and extremely dynamically developing area, which applications are so wide that practically nowadays every industry uses biotechnology in some form. Protein production (antimicrobial peptides/proteins, enzymes, hormones, antigens, drug carriers etc.) present one of the main areas of interest in modern biotechnology. The long-term goal is to produce soluble proteins in as large quantity as possible and at the same time eliminate the formation of inclusion bodies (IBs) that have been for long time considered as waste and dysfunctional byproducts of protein production. However, production of these soluble proteins is often challenging and additional steps of inclusion bodies recovery, such as solubilization and refolding are often needed. In addition, it is usually necessary to evaluate individual expression and purification protocols for each protein and yet often a portion of proteins still stay misfolded and insoluble **(Hadj Sassi *et al.*, 2017, Upadhyay *et al.*, 2016, Mohammadian *et al.*, 2017)**. On the contrary, in recent years, many studies have been increasingly proving that IBs can retain their biological activity, which has led some scientists to concentrate their efforts on the development of methods for production of pure biologically active IBs **(Nahalka *et al.*, 2008, Huang *et al.*, 2013)**. Due to their structural and functional properties IBs can be also considered as naturally immobilized enzymes or as nanomaterials, which allows their easy recycling **(Diener *et al.*, 2016, Nahalka *et al.*, 2006)**. Although IBs also have to be isolated and purified, these protocols are much simpler than those used in the production of soluble proteins. These are just a few of the characteristics that make IBs ideal materials for many biotechnology fields.

IBS PROPERTIES

Inclusion bodies present protein aggregates accumulating during protein overexpression, or under stress conditions, such as thermal **(de Groot and Ventura, 2006)**, pH **(Castellanos-Mendoza *et al.*, 2014)** or oxidative stress **(Grune, *et al.*, 2004)**. Incorrect translation or mutations in RNA/DNA can also result in the formation of IBs **(Kopito, 2000)**. Moreover, domains and fusion tags that can drive active proteins into IBs are nowadays widely used **(Wang *et al.*, 2015, Wu *et al.*, 2011).** These protein aggregates can be formed in the cell cytoplasm and/or periplasm **(Arié *et al.*, 2006, Miot and Betton, 2004)** and consist of different conformational populations, including amyloid-like, misfolded- and native-like structures, which are present in different ratios **(Wang, 2009, Sabate *et al.*, 2010)**. In general, IBs are highly hydrated and porous proteins with a diameter in the range of 0.2 to 1.2 μm **(Margreiter *et al.*, 2008, Carrio *et al.*, 2005)**. There are several studies that describe shape of IBs usually as cylindrical, spherical or elliptical aggregates **(Bowden et al., 1991, Garcia-Fruitos *et al.*, 2010)**. The surface of IBs varies from rough, with regular folds to smooth and in transmission electron microscopy, they appear as electron-dense deposits. Size, shape and other characteristics of IBs are associated with the type of host organism and culture conditions **(Bowden *et al.*, 1991)**.
As mention above there are many factors that influence biophysical/chemical properties and the composition of IBs. However, it is generally proven that IBs consist predominantly of the recombinant product **(Neubauer *et al.*2006)**. IBs preparations usually contain some another cellular proteins (ribosomal subunit proteins, membrane proteins etc.) and small amount of other macromolecules, such as nucleic acids and lipids **(Fahnert *et al.*, 2004)**.

IBS PRODUCERS

IBs can be found in both, prokaryotic (*Escherichia coli* and lactic acid bacteria - LAB) and eukaryotic (yeast, microalgae, insect, mammalian cells etc.) cells. These aggregates are designated in eukaryotic cells as aggresomes and, unlike prokaryotic cells, also contain chaperones, chaperonins residues and proteasome subunits **(Kopito, 2000, Markossian and Kurganov, 2004)**. Bacteria, especially Gram-negative bacterium *E. coli*, has become the most popular expression systems for the production of recombinant proteins due to their rapid growth, well characterized genetics and high yield on inexpensive substrates **(Villaverde and Carrio, 2003, Rinas *et al.*, 2017, Peternel and Komel, 2011)**. LAB, particularly *Bacillus subtilis* and *Lactococcus lactis*, has been shown to be an attractive alternative for production of membrane proteins **(Boutigny *et. al.*, 2015)** and protein-based nanomaterials **(Cano-Garrido *et. al.* 2016).** The main advantage of LAB is that, unlike *E. coli*, they do not contain endotoxins in their membrane and are classified as generally recognized as safe (GRAS) organisms. Despite the very wide use of prokaryotic systems, there are a number of examples where they could not be used to produce complex functional eukaryotic proteins due to their inability to post-translational modification. Yeasts *Pichia pastoris* **(Rueda *et al.*, 2016)** and *Saccharomyces cerevisiae* **(Hou *et al.*, 2012)** present the most popular recombinant protein expression systems among eukaryotic organisms. It is because of their several advantages, including posttranslational modifications ability, rapid growth, and especially their ability to secrete proteins to the extracellular medium. Over the past few years, microalgae have received increased attention as an alternative expression system suitable for the production of valuable products, such as therapeutics proteins, biofuels and polysaccharides. The most commonly used microalgae species are *Chlorella*, *Haematococcus* and especially *Chlamidomonas* (*Ch. reinhardrii*) **(Gong *et al.*, 2011, Spolaore *et al.*, 2006).** The main advantages of using microalgae are their high productivity, cost-

effectiveness and possibility to genetically transform their nuclear and also chloroplast genomes **(Manuell *et al*., 2007, Doron *et al*., 2016).** Insect cells perform most of the posttranslational modifications present in mammalian proteins, and therefore the use of bacilovirus-insect cell expression system has become a common alternative for production of recombinant proteins **(Kost *et al*., 2005, Contreras-Gómez *et al*., 2014)**. Correct protein folding, post-translational modifications, and product assembly are main advantages of usage mammalian cells as expression systems **(Nettleship *et al*., 2010, Baldi *et al*., 2007).** CHO (Chinese hamster ovary), HEK-293 (Human embryonic kidney) and NS0 (mouse myeloma) cells have become the most commonly used mammalian cell lines and are used to produce numbers of biopharmaceutical products **(Khan, 2013)**.

Although most misfolded and aggregated proteins in the mammalian cell are usually degraded by various proteolytic systems (ubiquitin –proteasome system, chaperon mediated autophagy and macroautophagy), some of them are resistant to all known proteolytic mechanisms. The accumulation of inclusion bodies or extracellular plaques is linked to many protein misfolding disorders, including at least 30 different human diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease and transmissible spongiform encephalopathies. Most of these diseases spread rapidly from a small initial affected region to surrounding areas due to the ability of the aggregates to pass from cell to cell along connected networks. These protein aggregates usually consist of β-sheet-enriched fibrillary structures, termed amyloids, which are 0.1 – 10 μm long and about 10 nm wide. Conformation of β-sheets is stabilized by intermolecular interactions, leading to the formation of oligomers, proto-fibrils and fibrils. There is now increased understanding of the amyloid fibril formation, which may prove to be essential in the development of rational therapeutics. The use of bacteria, which express amyloid-like bacterial intracellular aggregates, as model system is one of the approaches to characterize how and when protein aggregates form inside the cell **(Ciechanover and Kwon, 2017, Moreno-Gonzalez and Soto, 2011, Ciechanover and Kwon, 2015, Ross and Poirier, 2004)**.

IBS AND THE ANTIMICROBIAL PEPTIDES PRODUCTION

Antimicrobial peptides and proteins (AMPs) are usually relatively small (10-100 amino acids), positively charged amphiphilic molecules. AMPs can be found in various organisms, including mammals, plants, invertebrates and prokaryotes. Many different kinds of AMPs have been identified in recent years, indicating their importance in the innate immune system. These antimicrobial molecules can be expressed continuously or the expression can be induced by infection, inflammation or injury **(Quinn *et al*., 2008, Steinstraesser *et al*., 2008, Zhang and Gallo, 2016)**. Despite their similar general properties, most of the folded AMPs identified to date can be classified into four groups based on their secondary structure: α-helical, β-stranded, β-hairpin (loop), and extended **(Jenssen *et al*., 2006, Bahar and Ren, 2013)**. The most common are amphiphilic peptides with two to four β–sheets (bactenecins and defensins) or peptides with amphipathic α-helices (magainin and human cathelicidin peptide LL37). According to the target organism, AMPs can be categorized into four main groups: antiviral, antibacterial, antifungal and antiparasitic peptides **(Jenssen *et al*., 2006, Bahar and Ren, 2013)**. So far the best-studied group are cationic antibacterial AMPs where their ability to kill bacteria usually depends on their interaction with bacterial membranes. Positive charge, hydrophobicity (high ratio of hydrophobic amino acids) and flexibility (the ability of protein to change conformation from soluble to membrane-interacting conformation) allows AMPs to selectively bind to negatively charged bacterial membrane. Binding of AMPs to the bacterial membrane leads to formation of pores and disruption of bacterial membrane which is followed by the collapse of the transmembrane electrochemical gradient and microbial cell death. Alternately, they may penetrate membrane without any damage, but they kill bacteria by inhibiting some important pathways inside the cell. For example, AMPs can interact with DNA, RNA or protein synthesis, protein folding, and cell wall synthesis **(Nguyen *et al*., 2011, Brogden, 2005).** It was originally thought that killing of cells was the only mechanism of action of AMPs, bud there is increasing evidence now that AMPs can also recruit and activate immune cells and even display antiviral **(Gwyer *et al*., 2013)** and anticancer **(Felício *et. al*., 2017)** activities.

In recent years, thanks to the spread of multi-drug resistant microbes ("superbugs"), the research of the AMPs attracted much more attention as promising candidates for the development of alternative antibiotics **(Aoki and Ueda, 2013)**. However, to investigate the structure and mechanism of action of specific AMP it is necessary to obtain a significant amount of this molecule. Isolation and purification from natural sources (epithelial or plant cells) is usually expensive and tedious task. Although, chemical synthesis can be used to produce certain amounts of this peptides **(Harris *et al*., 2014)**, structural determination, toxicity testing and preclinical studies usually require larger amounts of material. Furthermore, if AMPs are longer than 50 amino acids or contain more than one disulfide bond, production costs will increase substantially. In general, heterologous expression of AMPs provides a means for economical protein production. *E. coli* and yeast are the most commonly used recombinant systems. However, use of *E. coli* as an expression system to produce AMPs faces two main challenges. First, AMPs are potentially toxic to the producing host, and second, small size of these molecules makes them especially susceptible to degradation. Expression of AMPs in fusion with carrier proteins **(Li, 2011)** or use of specialized *E. coli* strains, which carry mutations making them more tolerant present two main strategies that effectively overcomes these obstacles. Although some fusion proteins provide production of soluble proteins (thioredoxin **(Bogomolovas *et al*., 2009),** glutathione transferase and small ubiquitin-related modifier **(Li *et. al*., 2011, Ma *et. al*., 2012)**), others promote the formation of inclusion bodies. Moreover, compared with soluble fusion, expression of AMPs in the form of IBs is believed to be more efficient than soluble fusion in masking the peptides' toxic effects and protecting them from proteolytic cleavage. Other advantages of protein production in the insoluble form are high rates of expression and easy collection of IBs by differential centrifugation after cell disruption. In the following section, we will focus on expressing AMPs as fusion proteins by genetically linking the AMPs to fusion protein tags with high tendency to form IBs.

There is several fusion partners that have been specially designed to pull-down peptides into inclusion bodies and selection of the appropriate one is important for effective solving of toxicity, solubility and purification challenges. Most widely used tags are PurF fragment (amidophosphoribosyltransferase), ketosteroid isomerase (KSI), autoprotease N^{pro} and baculoviral polyhedrin. These tags can also trigger an undesirable immune response so after expression of IBs they must be removed from therapeutic proteins. There are two main methods used to remove tags: use of specific endoproteases that recognize a specific linker sequence or use of intein self-cleavage systems such as tailor-made N^{pro} mutant called EDDIE **(Achmüller et. al. 2007, Kaar et. al., 2009)**.

To avoid the harmful effects on host system (*E. coli*) **Lee *et al.* (2000)** choose polypeptide F4 aggregation-promoting carrier (fragment of *purF* gene) to form inclusion bodies. They successfully used this expression system to produces seven different kinds of AMPs (MSI-344, bombinin, melittin , indolicidin , PGQ ,tachyplesin I and XPF), while the resulting expression levels were about 25-30 % of total cell proteins. KSI present another carrier protein with high inclusion body-forming tendency and is a commonly used affinity tag for expression and purification of short peptides. For example, KSI–DCD1–His6Tag protein expression system was successfully used to produce dermcidin in the form of IBs **(Čipáková et al., 2005)**. Dermcidin, anionic antimicrobial peptide, which has been discovered in human sweat, displays antimicrobial activity against pathogenic microorganisms such as *Staphylococcus aureus* and *Candida albicans.* **Amparyup *et. al* (2008)** successfully cloned and characterized the full length cDNA and genomic organization of a crustin-like antimicrobial peptide (Crus-like*Pm*). They cloned mature Crus-like*Pm* from haemocytes of *Penaeus monodon* into the hexahistidin tag and used *Vibrio harveyi* as host organism. In this expression system rCrus-like*Pm* was found in both inclusion bodies and soluble fraction, however the purity of protein was higher when purified from IBs. The purified recombinant Crus-like*Pm* protein was highly active in inhibiting the growth of Gram-positive and Gram-negative bacteria including *V. harveyi*. Baculoviral polyhedrin (Polh) is the major component of the virus occlusion body known as a polyhedral that protect the virus particle from physical and biochemical degradation. **Wei *et. al*. (2005)** investigated the use of Polh protein as a fusion partner for the expression of the model AMP - halocidin 18-amino-acid subunit (Hal18) in the *E. coli*. They successfully purified and separated recombinant Hal18 from IBs with a final yield of 30% with >90% purity and demonstrate that Polh can be used to production of AMPs. β-defensins **(Corrales-Garcia *et al*., 2011)** and cathelicidins (human LL-37) are two main families of AMPs which are express on epithelial surface and provide a first line of defense against microbial infection. These AMPs families also play important part in immunomodulatory properties, such as cell migration, proliferation and differentiation **(Niyonsaba *et al*., 2017)**. **Corrales-Garcia *et al*. (2013)** successfully expressed five variants of human β-defensins (HBD2, HBD3, HBD3-M, HBD2-KLK, HBD3-M-HBD2) in *E. coli* using two histidine contains vector systems (pET28a(+) and pQE30). While HBD2 and HBD2-KLK were expressed in soluble fractions, HBD3, HBD3-M and HBD3-M-HBD2 were located in the insoluble fraction as inclusion bodies. All variants of HBDs showed activity against pathogenic bacteria, including two strains of *Mycobacterium tuberculosis* (H37Rv and MDR). Human acidic fibroblast growth factor (haFGF) stimulates repair of delayed healing and is one of the most potential therapeutic growth factors. Delayed healings is usually associated with another serious problem – microbial infection. LL-37 is a cathelicidin-related antimicrobial peptide, which have a critical role in defense against bacterial infection. **Shen et al. (2012)** designed a novel hybrid LL-37-haFGF gene to obtained fusion protein with functional activities of both. As a host organism was used *E. coli* and protein was expressed in form of inclusion bodies then purified and refolded. LL-37-haFGF exhibit antimicrobial activity against microorganisms which are associated with wound infection (tab.1) and simultaneously stimulated proliferation of NIH 3T3 cells. Table 1 summarizes the above-mentioned antimicrobial peptides and some of their characteristics.

Table 1 Summarization of AMPs characteristics

AMPs	Size (a.a)	Host org.	Antim. Act.	Solubility	Yield (mg/L)	references
Hepcidin	25	*E.coli*	*B. subtilis*	insoluble	16-20	Zhang et. al. (2005)
MSI-344	22	*E.coli*	*E. coli, B. subtilis*	insoluble	NA*	Lee et. al. (2000)
Crus-like*Pm*	124	*V. harveyi*	*S. haemolyticus, S.aureus, , E. coli, V.harvey*i *Aerococcus viridans, Bacillus megaterium, Micrococcus luteus*	partially sol.	NA	Amparyup et. al (2008)
Dermcidin	48	*E.coli*	*E. coli, S. typhimurium L. monocytogenes, S. aureus, C. albicans*	insoluble	2.3 mg/ 10 g wet cells	Čipáková et al., 2005
HBD2	41	*E.coli*	*E. coli, P. aeruginosa, S. aureus, M. tuberculosis*	soluble	4 (95 % purity)	Corrales-Garcia et al. (2013)
HBD2-KLK	44			soluble	3 (92 % purity)	
HBD3	45			insoluble	3.4 (90 % purity)	
HBD3-M	46			insoluble	9.5 (93 % purity)	
HBD3-M-HBD2	91			insoluble	5.5 (90 % purity)	
LL-37-haFGF	156	*E.coli*	*S. aureus, S.epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter cloacae*	insoluble	5.9 (95,43 % purity)	Shen et al. (2012)
Hal18	18	*E. coli*	*E. coli S. aureus*	insoluble	0.26 (91 % purity)	Wei et. al. (2005)

*not available
a.a – number of amino acids

IBS IN BIOMEDICINE

In addition to their use in AMPs production, IBs have been recently used as biomaterials with potential biomedical applications. This so called "gold waste" can be used for example in tissue engineering, drug or gene delivery and biocatalysis.

Tissue engineering

The goal of tissue engineering is to regenerate diseased or damaged tissues. In general body cells are attached to extracellular matrix (ECM), which provides structural and biochemical support to surrounding cells. Specific composition of ECM depends on tissue type, but usually includes structural proteins (collagen, elastin), adhesive proteins (fibronectin) and proteoglycans **(Mariman and Wang, 2010, Hinds *et. al*., 2011)**. Typically, cells must be attached to ECM or other cells to function (proliferate, differentiate). Tissue engineering involves the use of tissue scaffolds (engineered ECM), cells, and biologically active molecules to create functional surrogate tissue or entire organ for a medical purposes. Scaffolds are porous materials that mimics nature ECM and act as templates for tissue regeneration **(Zhao et. al., 2013)**. The employed scaffold should be biodegradable because body cells constantly resorb and replace ECM, so over time they should replace entire implanted scaffold by natural ECM. Scaffolds must by also biocompatible and promote cell functions such as attachment, proliferation and differentiation. Another requirement for scaffold is that it must have pores, within critical range, to facilitate cell migration and transport of nutrients and regulatory factors. Nowadays, there are several materials that are used as scaffolds, including natural polymers (collagen, alginate), synthetic biopolymers (PGA, PLA) and hydrogels **(O'Brien, 2011)**.
For efficient cell attachment and colonization of scaffold surface is necessary to create correct tissue structure. In recent years, many studies have confirmed that IBs can be used as biocompatible and biodegradable materials for scaffold surface decoration. There is still more and more evidences that IB materials can be used as particulate biomaterials that dramatically support cell colonization and proliferation without any sign of cytotoxicity **(Diez-Gil *et al*., 2010, Garcia-Fruitos *et. al*., 2009)**. In one study, **Seras- Franzoso *et al.* (2012)** showed that mammalian cell expansion of IB-decorated scaffolds is a result of two independent processes, namely cell attachment and mechanical stimulation of cell division, which are triggered differently by IBs. While cell attachment is dependent on IBs variant, stimulation off proliferation, which involves the activation of the ERK pathway is based on the activity of filopodia-like sensing probes. **Seras-Franzoso *et al*. (2013)** explored how IBs decorated novel 3D porous scaffolds (constructed by polylactic acid, polycaprolactone or chitosan) promote intracellular delivery of biologically active proteins. They successfully delivered proteins up to more than 80 % of the colonizing cells, depending on the scaffold type.

Drug and gene delivery

In recent years, there has been a growing interest in the development of nano drug delivery systems (DDS) that are able to deliver embedded genes or protein drugs into mammalian cells in the form of nanopils. The purpose of these nanomedical drug delivery approaches is to provide long-term local treatment to patients. Suitable materials for nanopills construction, must meet a number of requirements, such as bioavailability, biocompatibility, biodegradability and low toxicity **(Yin *et. al*., 2017)**. Despite the fact that a number of materials are known to meet these criteria (micelles, liposomes, nanotubes), their low specificity and difficult large-scale production attracts more and more attention to the IBs as a potential therapeutic agents **(Villaverde *et. al*., 2012)**. Biological activity, nanomechanical properties, biocompatibility and slow release of protein therapeutics in aqueous conditions present the main advantages of IBs nanopills. Moreover, IBs made up of proteins of therapeutic interest are able to penetrate cells membranes and thus are capable to reach the cytoplasm as well as the nucleus. A growing number of studies confirm that IBs nanopills are suitable for protein replacement and other therapies **(Thwaite *et. al*., 2018, Talafová *et al*., 2013)**, even show potential to increase cancer treatment efficiency **(Unzueta *et. al*., 2017, Unzueta *et. al*., 2018, Céspedes *et. al*., 2016)**. **Vázquez *et.al*. (2012)** studied effects of IBs containing therapeutically potential enzymes and proteins (chaperone Hsp70, dihydrofolate reductase, catalase and the growth factor leukemia inhibitory factor) on variously injured cell lines. In all four cases these proteins had positive physiological effects on the treated cells, specifically they enhance cell survival and/or proliferation under stress conditions. Fibroblast growth factor-2 (FGF-2) is a FGF family member involved in a variety of biological processes such as embryonic development, cell proliferation, cell migration, morphogenesis, tissue repair, tumor growth and invasion **(Bikfalvi *et. al*., 1997)**. **Seras-Franzoso *et. al*. (2014)** characterize effect of temperature on the structure and biological activity (promotion of cell proliferation and the induction of cell differentiation) of FGF-2 IBs overexpressed in *E. coli*. They used two temperatures, namely 25 and 37 ºC. Protein particles produced at a lower temperature were nearly 100 nm larger than that formed at 37 ºC and the recovery of soluble proteins was higher in the IBs formed at 25 ºC. Taken together, both temperature formed IBs, but higher activity was observed for the IBs produced at 25 ºC. In another study, **Liovic *at. al*. (2012)** demonstrate that inclusion bodies can be used to deliver complex proteins into epithelial cells. As an example they choose keratin 14 (K14), polymeric cytoskeletal protein. To be function and build filaments, monomeric K14 needs first to dimerize and form heterodimers with keratin 5 (K5). They use cDNA plasmid containing EYFP (enhanced yellow fluorescent protein) labeled keratin 5 as a reporter. They perform several experiments on SW13 cells (human adrenal carcinoma cell line) that normally do not express keratin and prove that K14 IBs contain functional K14, moreover these IBs also did not appear to be cytotoxic.

Biocatalysis

The use of enzymes in biocatalysis have a broad applications in various fields of interest, including pharmaceutical and chemical industry, as well as biomedical, cosmetic and diagnostic applications **(Adrio and Demain, 2014)**. Soluble enzymes present the most commonly used form of enzymes in biocatalysis and their use is usually associated with the immobilization on the surface of an insoluble carrier **(Mateo *et. al*., 2007)**. These process on the one hand increase life time and protein stability but on the other hand decrease protein activity. IBs have been also explored as biocatalysts for many enzymatic processes. Finding that formation of bacterial IBs did not completely inactivate enzymes **(Garcia-**

Fruitos *et.al*., 2005) together with their self-immobilized character makes IBs an interesting material for efficient catalysis. The potential of IBs for biocatalytic purposes supports various studies. **Nahalka, *et. al* (2008)** used IBs of sialic acid aldolase for production of sialic acid (neuraminic acid, Neu5A). Sialic acid is a sugar with nine-carbon backbone and can be usually found on the surface of vertebrate cells, where modulate many of physiological and pathological processes **(Varki, 2008)**. IBs can be directly recovered by centrifugation, but nowadays there is several approaches that simplify and streamline separation. **Nahalka *et. al*. (2006)** immobilized catalytically active IBs of polyphosphate kinase in agar/TiO_2 beads. They used these bioenergy beads as rechargeable supply for synthesis of ATP from ADP and polyphosphates, with the yield of ATP gradually drops from initially concentration 55 % to about 30 % after 17 conversion cycles. **Köszagová *et. al*. (2018)** introduced alternative separation approach and used iron oxide particles to create magnetically modified active IBs. As example they used three magnetically modified proteins: green fluorescent protein (GFP), sialic acid aldolase (SAA), and UDP-glucose pyrophosphorylase. In all cases the magnetization of IBs enabling an easier separation process using a magnetic field and stabilize the repetitive use of the inclusion bodies in comparison with centrifugation.

CONCLUSION

In conclusion, widely accepted paradigm that IBs of recombinant protein represent a waste inactivate product, has been challenged in recent years. Instead, the newly described properties of IBs and the latest studies strongly supports the use of IBs in various fields. However, further researches are still needed to fully clarify the potential of IBs in emerging biotechnological applications.

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