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Functional protein-based nanomaterial produced in microorganisms recognized as safe: A new platform for biotechnology

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ABSTRACT

Inclusion bodies (IBs) are protein-based nanoparticles formed in *Escherichia coli* through stereospecific aggregation processes during the overexpression of recombinant proteins. In the last years, it has been shown that IBs can be used as nanostructured biomaterials to stimulate mammalian cell attachment, proliferation, and differentiation. In addition, these nanoparticles have also been explored as natural delivery systems for protein replacement therapies. Although the production of these protein-based nanomaterials in *E. coli* is economically viable, important safety concerns related to the presence of endotoxins in the products derived from this microorganism need to be addressed. Lactic acid bacteria (LAB) are a group of food-grade microorganisms that have been classified as safe by biologically regulatory agencies. In this context, we have demonstrated herein, for the first time, the production of fully functional, IB-like protein nanoparticles in LAB. These nanoparticles have been fully characterized using a wide range of techniques, including field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), dynamic light scattering (DLS), Fourier transform infrared (FTIR) spectroscopy, zymography, cytometry, confocal microscopy, and wettability and cell coverage measurements. Our results allow us to conclude that these materials share the main physico-chemical characteristics with IBs from *E. coli* and moreover are devoid of any harmful endotoxin contaminant. These findings reveal a new platform for the production of protein-based safe products with high pharmaceutical interest.

Statement of Significance

The development of both natural and synthetic biomaterials for biomedical applications is a field in constant development. In this context, *E. coli* is a bacteria that has been widely studied for its ability to naturally produce functional biomaterials with broad biomedical uses. Despite being effective, products derived from this species contain membrane residues able to trigger a non-desired immunogenic responses. Accordingly, exploring alternative bacteria able to synthesize such biomaterials in a safe molecular environment is becoming a challenge. Thus, the present study describes a new type of functional protein-based nanomaterial free of toxic contaminants with a wide range of applications in both human and veterinary medicine.

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1. Introduction

Over the last years, *Escherichia coli* has been described as a cell factory for the production of self-assembling nanostructured and functional protein materials known as inclusion bodies (IBs) [1–4], which have been studied as stimulators of cell proliferation and differentiation [5,6] and as natural protein delivery systems [2,7]. However, in terms of therapeutic applicability of this biomaterial, the presence of lipopolysaccharide (LPS) in the *E. coli* outer cell membrane becomes a major obstacle. Since LPS, also known as endotoxin, can elicit undesirable immunogenic responses [8,9], FDA regulations establish an endotoxin level limit of 5 EU/kg/h for pharmaceutical compounds and medical devices. Thus, all products from *E. coli*, as well as those from other Gram-negative microorganisms, need to be finely purified through costly processes to ensure the removal of any pyrogenic or inflammatory contaminant inherently present in the sample [8,10]. Nowadays, the development of a universal and effective method for endotoxin removal remains for the time being unresolved [10], being particularly critical for complex structures such as protein-based nanostructured materials [9,11]. This makes it necessary to seek for alternative cell factories, which offer the potential to produce protein biomaterials free from pyrogenic impurities. In this context, Gram-positive (LPS-free) lactic acid bacteria (LAB) are gaining momentum as promising microbial cell factories for both recombinant protein production purposes and as delivery live vectors [12–16]. Indeed, they have been classified by regulatory agencies as generally recognized as safe (GRAS) organisms [17]. Consequently, the development of this biologically-safe production platform based on LAB opens a new era in terms of therapeutic applicability, leaving behind all the important drawbacks associated to *E. coli*-derived recombinant products. However, thus far, the possibility to produce protein-based nanostructures in these bacterial cell factories has not been explored. Contrary to what occurs in *E. coli*, it is widely believed that recombinant proteins produced in LAB are fully soluble, rendering the production of protein-based biomaterials from these GRAS microorganisms rather impossible. Nevertheless, some recent studies, such as that published by Lu and collaborators contradict this general assumption [18]. They described that under the overexpression of a fluorescent protein, highly fluorescent protein clusters are formed in *Lactococcus lactis* cytoplasm [18]. In this line, our group confirmed the presence of protein deposits in *L. lactis* cytoplasm using a modified green fluorescent protein [19]. The consideration of all these results together, lead us to believe that *L. lactis* might potentially be used for the generation of fully safe protein-based particles that could be further explored as functional nanomaterials. Because almost nothing is known about these nanostructures, the aim of this study was the isolation and detailed characterization of the main morphometric and physico-chemical properties of such a new class of protein deposits. For that, we selected *L. lactis*, the most used LAB in the field of recombinant protein production as cell factory [20,21]. Three relevant proteins in human and veterinary medicine such as bovine metalloproteinase 9 (MMP-9) and 2 (MMP-2) and interferon gamma (IFN-γ) were used herein as model proteins. The obtained data, which revealed the possibility to produce both fully functional and safe protein-based nanoparticles in LAB, offers an attractive opportunity for the production of a new generation of biomaterials with a wide range of biotechnology applications for both human and animal medicine.

2. Materials and methods

2.1. Bacterial strains and plasmids

L. lactis subsp. cremoris NZ9000 [20] and NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ (*clpP-htrA*; Em^{R}) [22,23] (kindly provided by INRA, Jouy-en-Josas, France; patent n° EP1141337B1) strains and *E. coli* MC4100 strain (Strep^{R}) [24] were used. Three proteins from bovine (*Bos taurus*) origin were cloned in the Cm^{R} pNZ8148 plasmid (MoBiTech): the mature form of the interferon gamma (IFN-γ) (from Gln23 to Thr101 NM_173925) and the catalytic domain of metalloproteinase 9 (MMP-9) (from Phe107 to Pro449 NM_174744) and 2 (MMP-2) (from Tyr110 to Asp45 NM_174745). In addition, a fusion of MMP-9 with an aggregation-prone peptide (ELK16: $(LELELKLK)_2$) was also constructed (MMP-9ELK16). All genes were C-terminally fused to a His-tag for detection and quantification purposes in western blot analysis. Gene sequences were codon optimized (Geneart). In the sequence design we added a *NcoI* restriction site at 5′ followed by nucleotides CA to restore the reading frame and a *XbaI* restriction site at 3′. The digestion product was ligated into the expression plasmid pNZ8148 and ligation product were transformed by electroporation into *L. lactis* NZ9000 and *clpP-htrA* competent cells [25]. Electroporation was performed using Gene Pulser from Bio-rad fitted with 2500V, 200 Ω and 25 μF in a pre-cooled 2 cm electroporation cuvette. Following, samples were supplemented with 900 μL M17 broth with 0.5% glucose and incubated for 2 h at 30 °C. The electroporation mix was centrifuged for 10 min at 10,000×*g* at 4 °C and the pellet was resuspended in 100–200 μL of M17 media and plated. Besides, recombinant Green Fluorescent Protein (rGFP) previously described in [19,26] were also used.

2.2. Nanoparticle production and purification

L. lactis strains containing the previously described plasmids were grown in M17 medium enriched with 0.5% glucose at 30 °C without shaking. *E. coli* was grown in LB rich medium at 37 °C, 250 rpm. Nanoparticle production was induced by adding 12.5 ng/mL nisin (Sigma-Aldrich) in *L. lactis* or 1 mM IPTG in *E. coli* cultures at OD_{550nm} = 0.5. After induction, cultures were grown for 3 h. Antibiotics were used for plasmid maintenance at the following concentrations: chloramphenicol (5 μg/mL) and erythromycin (2.5 μg/mL) for *L. lactis* and ampicillin (100 μg/mL) and streptomycin (30 μg/mL) for *E. coli*.

Once produced, protein nanoparticles were purified using the purification protocol described by [27], including, at the beginning of the process, a mechanical disruption step by French Press. The protocol was performed under sterile conditions and all incubations were carried out under agitation.

The amount of recombinant proteins present in nanoparticles were quantified by denaturing SDS-PAGE as described in [19]. Bands were identified using a commercial polyclonal serum against histidine tag (#A00186-100 Genscript) and an anti-mouse secondary antibody (#170-6516, Bio-Rad). Recombinant protein yield were estimated with a standard curve with known amounts of a GFP-H6 protein. Quantification was performed with the Quantity One software.

2.3. Field emission scanning electron microscopy (FESEM)

For nanoparticles morphometry (size and shape), microdrops of protein aggregate suspensions were deposited during 2 min on silicon wafers (Ted Pella Inc.), air-dried and observed in a FESEM Zeiss Merlin (Zeiss) operating at 2 kV. Micrographs of nanoparticles morphology at a nearly native state were acquired with a high resolution in-*lens* secondary electron (SE) detector. A quantitative analysis of particle size was performed with a total number of 474 nanoparticles using Image J software.

2.4. Transmission electron microscopy (TEM)

For ultrastructure, samples were fixed with aldehydes, post-fixed with osmium, dehydrated in acetone, embedded in Epon