Functional protein-based nanomaterial produced in GRAS microorganism: a new platform for biotechnology

Olivia Cano-Garrido[a,b,c], Alejandro Sánchez-Chardi[d], Sílvia Parés[e], Irene Giró[f], Witold I. Tatkiewicz[c,f], Neus Ferrer-Miralles[a,b,c], Imma Ratera[c,f], Antonino Natalello[g], Rafael Cubarsi[h], Jaume Veciana[c,f], Àlex Bach[e,i], Antonio Villaverde[a,b,c], Anna Arís[e]*, and Elena Garcia-Fruitós[a,b,c&]*

[a] Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.
[b] Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.
[c] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), 08193 Cerdanyola del Vallès, Spain.
[d] Servei de Microscòpia, Universitat Autònoma de Barcelona, Bellaterra 08193 Barcelona, Spain
[e] Department of Ruminant Production, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), 08140 Caldes de Montbui, Spain.
[f] Department of Molecular Nanoscience and Organic Materials, Institut de Ciència de Materials de Barcelona (ICMAB-CSIC), Bellaterra, 08193 Barcelona (Spain)
[g] Department of Biotechnology and Biosciences, Università di Milano-Bicocca, 20126 Milano, Italy
[h] Departament de Matemàtica Aplicada IV. Universitat Politècnica de Catalunya. Jordi Girona 1-3. 08034 Barcelona, Spain
[i] Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain.
[&] Present address: Department of Ruminant Production, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), 08140 Caldes de Montbui, Spain.

* co-corresponding author: elena.garcia@irta.cat; Telephone: +34934674040; Fax: +34934674042; anna.aris@irta.cat; Telephone: +34934674040; Fax: +34934674042

Olivia Cano-Garrido: olivia.cano.garrido@gmail
Alejandro Sánchez-Chardi: Alejandro.Sanchez.Chardi@uab.cat
Sílvia Parés: silvia.pares@irta.cat
Irene Giró: irene.giroparadell@gmail.com
Witold I. Tatkiewicz: witold.tatkiewicz@gmail.com
Neus Ferrer-Miralles: Neus.Ferrer@uab.cat
Imma Ratera: iratera@icmab.es
Antonino Natalello: antonino.natalello@unimib.it
Rafael Cubarsi: rcubarsi@gmail.com
Jaume Veciana: vecianaj@icmab.es
Àlex Bach: alex.bach@icrea.cat
Antonio Villaverde: antoni.villaverde@uab.cat
Anna Arís: anna.aris@irta.cat
Elena Garcia-Fruitós: elena.garcia@irta.cat

Abstract

Inclusion Bodies (IBs) are protein-based nanoparticles formed in *Escherichia coli* through stereospecific aggregation processes, during the ~~under~~ overexpression of recombinant proteins. During 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 ~~cell~~ 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 here, 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 spectroscopy (FTIR), zymography, cytometry, confocal microscopy, and wettability and cell coverage measurements. Our results allow us to conclude that ~~they~~these materials ~~that~~ share the main physico-chemical characteristics with IBs from *E. coli* but~~,~~ ~~but~~ being devoid of any harmful endotoxin contaminant. These findings reveal a new platform ~~of~~ for the production of protein-based safe products with high pharmaceutical interest.

Keywords: endotoxin-free, nanoparticles, functional nanomaterials, GRAS, Lactic acid bacteria

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 ~~in terms of therapeutic applicability of this biomaterial~~. 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 ~~with important associated costs~~ 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 ~~is a still~~remains for the time being unresolved ~~problem~~ [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) ~~a group of Gram-positive bacteria known as~~ lactic acid bacteria (LAB) ~~has been recently~~are gaining momentum as ~~an alternative~~ promising microbial cell factor~~y~~ies for both recombinant protein production purposes and as ~~protein~~ delivery live vectors ~~purposes~~ [12-~~14~~]16]. ~~These microorganisms do not have LPS and, in consequence, their derived products do not contain pyrogenic impurities [15, 16].~~ Indeed, they have been classified by regulatory agencies as generally recognized as safe (GRAS) organisms [17]. Consequently, ~~T~~the development of this biologically-safe production platform based on LAB clearly opens a new era in terms of therapeutic applicability, leaving behind all the important drawbacks associated to *E. coli*-

derived recombinant products. However, s~~S~~o far, the possibility to produce protein-based nanostructures in these bacterial cell factories has ~~not~~ never been explored. ~~It is widely believed that LAB, C~~contrary to what occurs in *E. coli*, it is widely believed that recombinant proteins produced in LAB are ~~only able to produce~~fully soluble ~~proteins~~, rendering the production of protein-based biomaterials from these GRAS microorganisms rather impossible. ~~However~~Nevertheless, some recent studies, such as that published by Lu and collaborators ~~-~~contradict this general assumption [18]. They ~~Lu and collaborators~~ describe~~dd~~ that under the overexpression of a fluorescent protein, ~~the presence of~~ highly fluorescent protein clusters are formed ~~inside~~ in *Lactococcus lactis* cytoplasm~~, under the overexpression of a fluorescent protein~~ [18]. ~~Besides~~In this line, our group ~~also observed~~confirmed the presence of protein deposits in *L. lactis* cytoplasm using a modified green fluorescent protein [19]. Considering all these results together~~,~~ ~~it~~this ~~can be expected~~leads us to believe that *L. lactis* ~~is~~ might ~~also able~~potentially be used ~~to form~~for the generation of fully safe protein-based protein ~~clusters~~ particles that could be further explored as functional nanomaterials. Because almost nothing is known about this nanostructures,~~Thus,~~ the aim of this study is 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) have been used here as model proteins. The obtained data, which reveals 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 ~~a type of~~ biomaterials with a wide range of applications in biotechnology and human and animal medicine.

2. Materials and Methods

4

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 nº 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 ºC. The electroporation mix was centrifuged for 10 min at 10,000 x *g* at 4 ºC 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 ºC without shaking. *E. coli* was grown in LB rich medium at 37 ºC, 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 nanoparticle were purified using the purification protocol described by [27], adding at the beginning a mechanical disruption step by French Press. The protocol has been done under sterile conditions and all incubations were carried out under agitation.

The amount of recombinant proteins present in nanoparticles has been 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.

2.6. Field Emission Scanning Electron Microscopy (FESEM)

For nanoparticles morphometry (size and shape), microdrops of protein aggregates samples were deposited during 2 min in 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 size particles was performed with a total number of 474 nanoparticles using Image J software.

2.7. Transmission Electron Microscopy (TEM)

For ultrastructure, samples were fixed with aldehydes, post-fixed with osmium, dehydrated in acetone, embedded in Epon resin, and polymerized following conventional methods [28-30]. Ultrathin sections were placed on copper grids, contrasted, and observed with a TEM Jeol JEM-1400 (Jeol Ltd.) equipped with a CCD Gatan ES1000W Erlangshen camera. For MMP-

2, MMP-9 and IFN-γ immunolocalization, pellets of bacterial cells and protein nanoparticles were fixed in 4 % (w/v) paraformaldehyde and 0.1 % (v/v) glutaraldehyde in PB, cryoprotected in sucrose, cryofixed in propane, dehydrated in methanol, embedded in Lowicryl HM20 resin (Polysciences Inc.), and polymerized with UV rays. Ultrathin sections placed on carbon-coated gold grids were labeled for the 3 antigens of interest using polyclonal primary antibodies (anti-MMP-2: #AV20016, Sigma-Aldrich; anti-MMP-9: #50560-RP01, Sino Biological Inc.; and anti-IFN-γ: #ab9657, Abcam, at working dilution 1:5, 1:5 and 1:2 respectively) and protein A coupled to 10 nm-gold particles (BBI Solutions), following standard methods [30, 31]. Grids were contrasted and examined with a TEM Jeol JEM-1400 at same conditions than previously described.

2.8. Z-Potential

Z-Potential characterization of each kind of protein nanoparticles was carried out using dynamic light scattering (DLS) equipment (Malvern Nanosizer). In order to avoid the electrodes from getting burned, the samples were prepared in deionized (MilliQ) water, a low ionic strength medium. Each sample was analyzed by triplicate.

2.9. Proteinase K assay

Protein nanoparticles were resuspended in PBS ~~with protease inhibitor~~ and sonicated to obtain a homogenous sample. Kinetic analysis were performed as described in [32]. In control suspensions MilliQ water was added instead of Proteinase K. Sample was recovered after every measurement to maintain constant the reaction volume. The experiment was done by triplicate.

2.10. Mathematical modeling

The dynamics of nanoparticle disaggregation process is described through a mixture of decreasing exponential functions with up to three populations according to one of the

following two models. If the protein of nanoparticles at time t is denoted by $y_m(t)$, a mixture without a constant population is given by

$$y_m(t) = N_0 e^{-c_1 t} + \ldots + N_k e^{-c_k t}; m = 2k; k \geq 1$$

denoted with an even subscript according to the even number of terms. Otherwise, if there exist a non-disaggregated population, the nanoparticle amount is given by

$$y_m(t) = N_0 + N_1 e^{-c_1 t} + \ldots + N_k e^{-c_k t}; m = 2k+1; k \geq 1$$

with an odd subscript. In both cases the fit will depend on a set of m parameters P that is evaluated from a least squares fit. Only positive values of N_i and c_i are allowed. The parameter N_i is the initial protein of the i-th component and c_i, in the exponent, provides the expected life $T_i = 1/c_i$ (the half-life is $t_i = \ln 2 / c_i$). The number of components in the mixture model

is $C = \left[\dfrac{m+1}{2} \right]$ (integer part). From a data array $L = \{(x_i, t_i); i = 1, \ldots, n\}$, where x_i is the nanoparticle amount at time t_i, we minimize the χ^2 of the fit [32]. Among all the possible fits, as increasing m we choose the one satisfying $\ldots > \chi^2_{m-1} > \chi^2_m \leq \chi^2_{m+1}$.

In the context of the disaggregation process, not all fits are admissible. Indeed, the procedure can be applied in two ways: (a) As for each strain, the experiment is repeated q times, there is a number of q arrays by strain that we average to build a single array $\bar{L} = E(L)$ (E means the expected value). We then determine the specific parameters P' of the strain. (b) Alternatively, for each strain, each experiment may be fitted separately. This provides q families of parameter sets P that we average as $\bar{P} = E(P)$ in order to characterize the strain components. A meaningful fit must fulfill that the values $\bar{P}$ and P' match within a low error margin. This is a sufficient condition to accept the fitting, otherwise the strain average values $E(L)$ would not represent the individual experiments. In particular, this implies that the number of components of the strain obtained from the average $E(L)$ and that obtained for each individual experiment must match. The necessary condition for comparing both

procedures is to determine the same number of strain components C from the average array and the single arrays. If both approaches provide a different number of strain components, other fittings to reconcile both procedures must be chosen among those with low χ^2, preferably among the simplest models, although they not provide the lowest value.

2.11. Metalloproteinases activity assay (zymography)

MMP enzymatic activities were determined by gel 10 % SDS-PAGE + 1 % gelatin under non-denaturing conditions. IBs were loaded onto the gel diluted 1:1 with a sample loading buffer (0.125 M Tris, 0.005 % bromophenol blue, 20 % glycerol, 4 % SDS). Then, the gel was incubated with developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02 % Brij 35) for 48 h at 37 ºC. Finally, it was dyed with Coomassie and discolored until degradation bands become visible. Densitometry analyses of the bands were performed with the Image J software. Soluble MMP-9 produced in *L. lactis* has been used as a positive control.

2.12. Fourier transform infrared spectroscopy (FTIR)

The protein hydrated films were measured by the infrared microscope Varian 610-IR, coupled to the Varian 670-IR spectrometer (both from Varian Australia Pty Ltd.), as reported in [33, 34]. In particular, drops of 1-5 µL of the soluble protein and nanoparticles were deposed on a BaF_2 window. The FTIR spectra were then collected in transmission mode after the solvent evaporation in order to obtain a uniform protein film on the window. Under these conditions [33, 34] spectra with high signal-to-noise ratio without detectable scattering effects were obtained [35] (Suppl Fig. 1).

For hydrogen/deuterium (H/D) exchange experiments, the protein film on the BaF_2 window was hydrate by adding 5 µL of a solution of 1/4 of glycerol/D_2O ratio (w/w) around the dried film. The sample was then tightly closed by a second window using a flat O-ring.

2.13. Proliferation Assay

Human skin fibroblast cells (1BR3.G) were grown at 37 ºC and 10 % CO_2 on protein nanoparticles-decorated surfaces [36]. A total of 1 µg of rGFP based protein nanoparticles from *L. lactis* and *E. coli* were added to each well of an untreated Costar 3370 plate and incubated with 100 µL of Dulbecco's Modified Costar Medium (DMEM) without serum at 4 ºC overnight. After incubation, 1BR3.G cells were added (5,000 cells per well) in 100 µL DMEM medium with 2% fetal bovine serum (FBS). As controls, we plated cells on non-decorated wells and we also used wells decorated with nanoparticles but without cells. Plates were incubated at 37 ºC for 24, 48 and 72 h, and the MTT proliferation cell assay was performed as described [37][35].

2.14. Nanoparticles internalization (cytometry)

HeLa cells (60,000 cells per well) were seeded in treated 12-well plates in the presence of Minimum Essential Medium (MEM-α) medium supplemented with 10 % FBS and 2 mM Glutamax (Gibco) [30]. After incubation at 37 °C and 5 % CO_2 for 24 h, the medium was removed and the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS). Then, 5 µg of rGFP based protein nanoparticles from *L. lactis* and *E. coli* were suspended in MEM-α containing 10 % FBS and 2 mM Glutamax and added per well. After 48 h, cell samples were treated for 15 min in 1 mg/mL trypsin and samples were analysed on a FACSCanto system (Becton Dickinson) using a 15 W air-cooled argon-ion laser at 488 nm excitation for GFP. Fluorescence emission was measured with a 530/30 nm band pass filter.

2.15. Nanoparticles internalization (confocal microscopy)

Hela cells (100,000 cells/mL) were seeded on MatTek culture dishes with MEM-α medium supplemented with 10 % FBS and 2 mM Glutamax (Gibco), and incubated at 37 °C and 5% CO_2. 5 µg rGFP nanoparticles were added to cells in the presence of Optipro medium (Gibco) and incubated O/N. For confocal analysis, cell membrane and nuclei were stained and stacks were obtained as described elsewhere [38][36].

2.16. Modification of gold substrates with mixed self-assembled monolayers (SAMs)

SAMs with different proportions of 1-undecenethiol (-CH$_3$ terminated) and 11- mercapto-1-undecanol (-OH terminated) were prepared by immersion of gold substrates in an ethanolic solution of the thiols with the appropriate molar ratio for 24 h as descrived elsewhere [5].Then, the substrates were dried under a stream of N$_2$ getting surfaces with different wettability properties.

2.17. Wettability

The wettability of mixed thiols SAMs on gold surfaces before and after protein nanoparticles deposition was determined via static contact angle (CA) measurements using a DSA100 from KRÜSS.

2.18. Deposition of protein nanoparticles on SAM modified substrates and surface covering analysis

Substrates with mixed SAMs were immersed in the protein nanoparticles suspensions for 2 h, and rinsed with MilliQ water. Light microscopy images were obtained using an Olympus Bx51 microscope and surface coverage analysis using ImageJ 1.47

2.19. Statistical analysis

Data were analyzed using a general linear model (JMP, SAS Institute Inc.). For the analysis, strain, protein, and the interaction between strain and protein were used as fixed effects. When more than two means were compared, differences were established using the Tukey's multiple mean separation test. Data were previously transformed when necessary to achieve a normal distribution. Results are expressed as the means of non-transformed data ± standard error of mean (SEM), except otherwise stated.

3. Results

3.1. Ultrastructural analysis of L. lactis nanoparticles by electron microscopy

TEM micrographs obtained of *L. lactis* cells under overproduction conditions showed the formation of regular -intracytoplasmic round protein deposits in practically all cells (Fig. 1A). Furthermore, the specific immunolocalization confirmed that these deposits are formed by the recombinant protein (Fig. 1A, right images). In a second step, once the production of these protein deposits in *L. lactis* was successfully provenassessed, MMP-9, MMP-2 and IFN-γ nanoparticles were isolated and quantified by Western blot (data not shown). The ultrastructural morphology of the nanomaterial was analyzed by FESEM and TEM (Fig. 1B and C) and the obtained micrographs allowed us to conclude that these protein deposits clearly corresponded to well structured and compact-defined particles at within the nanoscale range (Fig. 1B and C).

3.2. Size determination of L. lactis nanoparticles

Using By the analysis of FESEM micrographs we determined the size of the nanostructures, obtaining values ranging between 300 and 450 nm (Table 1). Extreme mean values were found in NZ9000 *clpP⁻ htrA⁻* strain (hereafter called *clpP-htrA)*, with lowest mean values in IFN-γ and highest mean values in MMP-9 (Table 1). The statistical analysis showeds that the size of the particles is was determined by the combination of the specific protein and the strain. MMP-9 round particles (Fig. 1B) were bigger in *clpP-htrA* strain than those produced in wild type strain NZ9000 (Table 1). In marked contrast, more elongated IFN-γ nanoparticles (Fig. 1B) showed high variable size and shape and higher mean values in NZ9000 strain (Table 1).

3.3. Electrodensity and immunolocalization analysis of L. lactis nanoparticles

The internal ultrastructure reported by TEM (Fig. 1C) not only strengtheneds the analysis performed by FESEM (Fig. 1B), but also showeds that the electrodensity of protein aggregates is again protein- and strain-dependent. Interestingly, the electrodensity and compactness observed by TEM (Fig. 1C) perfectly correlateds with the solubility of each

protein (Table 1). The higher the solubility of a protein ~~is~~was, the less prone to form protein nanostructures it ~~is~~was. Among tested proteins, IFN-γ showed~~s~~ higher solubility and its nanoparticles present low electrodensity and a less compact ultrastructure (Fig. 1C). Additionally, these particles, in marked contrast to what ~~occur~~occurred~~s~~ with particles formed by prone to aggregate proteins (~~,~~ such as MMPs, that formed highly compact, smooth surface~~,~~ and round-shaped nanostructures), exhibited rough surface and more variable size and shape (Fig. 1B). To evaluate in more detail the distribution of the protein forming such nanomaterial we performed an immunolocalization of purified nanoparticles using TEM. Ultrathin section of central parts of MMPs nanoparticles showed cortical electrodense parts of the nanostructure (heavily labeled), whereas central and less electrodense areas were less marked (or not marked) (Fig. 2). This finding, observed both in nanoparticles inside cells (Fig. 1C) and in purified ~~ones~~ material (Fig. 2), ~~is~~ was indicative of high differences in protein concentrations in cortical and central parts of nanoparticles.

3.4. Study of the stability and supramolecular organization of L. lactis nanoparticles

The structure and stability of the protein forming such nanomaterials ~~are~~ were important parameters to be dissected. Z-potential measurements showed that all nanoparticles present negatively charged surfaces with large negative values ranging from -33 to -26 mV (Table 1), being an indication of stable suspensions. To get further detail of supramolecular organization of nanoparticles produced in *L. lactis*, a time-course of ~~nanoparticle~~ proteolytic stability was performed (Fig. 3). After proteinase K~~k~~ ~~incubation~~treatment, the three evaluated proteins ~~evaluated~~ showed a population susceptible to degradation (population 1), with half-life values in the same range for all proteins (Fig. 3). Besides, a fully resistant population (population 2), ranging from 33 to 40 %, was preserved. Thus, nanoparticles produced in *L. lactis* presented two differentiated populations in terms of proteolytic resistance (Fig. 3).

3.5. Determination of L. lactis nanoparticles functionality in vitro

13

At this point it was critical to determine the biological activity of the nanoparticles, produced and purified for the first time from *L. lactis*. Fig. 4A shows that nanoparticles of proteins relevant for human and veterinary medicine produced in *L. lactis* are biologically functional. All MMPs tested showed the ability to degrade the substrate in a strain- and protein-dependent manner (Fig. 4A). It is worth mentioning that it was also possible to modulate the conformational quality of the protein embedded in such nanomaterials (Fig. 4A). The selection of the appropriate strain would allow increasing the specific activity of *L. lactis* nanoparticles, as it occurs with MMP-9. Besides, MMP-9 improves significantly its specific activity, just by the presence of an aggregation tag (ELK16) fused to the protein (corresponding to the activity of 0.08 ng of soluble MMP-9 protein). Moreover, the statistical analysis indicated that the strain, combined with the protein used, are important parameters to take into account in the production of such functional protein delivery agents in safe production systems.

Interestingly, data obtained from the FTIR analysis confirmed the coexistence of intermolecular β-sheets in nanoparticles (components at around 1695 and 1627 cm^{-1}) with the presence of native-like structures (Fig. 5 and Suppl Fig 2) [33, 34]. These two populations perfectly correlated with the model mentioned above (Fig. 2), in which resistant (intermolecular β-sheets) and sensitive (native-like structures)-proteinase K populations coexist in the same nanostructure. As an example, the second derivative spectra of MMP-9 and MMP-9-ELK16 were dominated by the marker bands of intermolecular β-sheets in protein aggregates, but an important component around 1658 cm^{-1} was also present, indicating the occurrence of native-like α-helices and of random coil structures. The comparison of MMP-9 and MMP-9-ELK16 spectra allowed concluding that MMP-9-ELK16 was characterized by a lower level of aggregation, particularly in the case of *clpP-htrA*

sample. This was, ~~which is~~ in agreement with a higher specific activity observed with these nanoparticles (Fig. 4A).

The ability to stimulate cell proliferation is another interesting property of IBs from *E. coli*. Interestingly, surfaces decorated with the protein-based nanomaterial produced in *L. lactis* ~~are~~ were also able to stimulate the proliferation of mammalian cells at a level similar to that achieved by the particles produced in *E. coli* (Fig. 4B). Moreover, *L. lactis* nanoparticles ~~are~~ were able to internalize inside the cells even ~~better~~ more efficiently than those produce in *E. coli* (Fig. 4C and D).

3.5. Study of the hydrophilic/hydrophobic nature of L. lactis nanoparticles

Since it has been already described that the hydrophilicity of a surface influences its cell adhesion properties [39]~~[37]~~, we have determined the nanoparticles wettability. Thus, the wettability of mixed self-assembled monolayers (SAMs) on gold substrates before and after being functionalized with *L. lactis* nanoparticles was determined via contact angle (CA) measurements (Fig. 6). As expected, prior to nanoparticle deposition the CA decreased as the hydrophilicity of the SAM increased. An increase of 10% of the molar fraction of OH-terminated thiols implied a decrease of 6º of the contact angle of the SAM-covered substrates. However, after MMP-9 nanoparticle deposition, the reduction of the contact angle in response to a 10%-increase of the hydrophilic thiols concentration relative to the hydrophobic one was smaller (4.0º in NZ9000 and 2.1º in *clpP-htrA*). Regarding MMP-2, after deposition of both strains, the contact angle remained rather constant around 60º, which ~~is~~ was an indication of its higher hydrofilic character. The MMP-9-ELK16 presented different wettability properties~~y~~ depending on the genetic variant it ~~is~~ was obtained from. Thus, MMP-9-ELK16 from the wild type strain ~~gives~~ rendered also a constant contact angle around 60º but the one obtained from *clpP-htrA* strain ~~gives~~ showed~~place to~~ a reduction of 3º of the contact angle in response to a 10%-increase of the hydrophilic thiols concentration. After IFN-γ deposition produced in both strains, the contact angle remained rather constant at around 65°-70°. From the change of CA

15

after nanoparticle deposition, we concluded that the deposition of nanoparticles produced in *L. lactis* buffered the change of wettability of the substrates. On the other hand, IFN-γ and MMP-2 nanoparticles, kept wettability relatively constant around a certain value.

Coverages of nanoparticle surface are shown in Table 2. From these data, it was possible to affirm that the affinity of protein nanostructures toward hydrophilic and/or hydrophobic surfaces also depended on the genetic background in which they have been produced. Thus, the interactions between these protein-based biomaterials and substrates, and the density of deposited nanoparticles can be tuned to a certain extent choosing a specific *L. lactis* strain. With MMP-2 and MMP-9-ELK16 there was a higher coverage and higher affinity of the nanoparticles towards the substrate for the NZ9000 than for the genetically modified *clpP-htrA*. Regarding the MMP-9 and the IFN-γ, the potential difference between NZ9000 and the mutant strain was not clear. The surface coverage significantly varied also in response to changes of substrate wettability. For some proteins, high coverages were obtained for substrates of different wettability, which could be attributed to the presence of amphiphilic properties of the obtained nanoparticles.

4. Discussion

E. coli IBs have been shown to be a mechanically stable nanomaterial with interesting properties as a drug delivery system, but also as a promising biomaterial for tissue engineering. However, the use of *E. coli* as recombinant cell factory for their production has an important shortcoming. Because of their endotoxic nature due to the presence of LPS, even at low concentration, IBs can trigger a non-desired immunogenic response upon administration. Therefore, any *E. coli*-derived product have to be necessarily further purified through a time-consuming, costly, and, in many cases, ineffective depyrogenation process [14]. For this reason, and with the goal of producing protein-based biomaterials in an

endotoxin-free environment, we explored *L. lactis* as cell factory. Although *L. lactis* has already been proven to be an excellent choice for the production of other LPS-free biomaterials such as polyhydroxybutyrate (PHB), the main mechanical and chemical properties of protein-based nanostructures produced in this Gram-positive microorganism have never been studied. Thus, the present study show for the first time an accurate analysis of a novel protein-based nanomaterial produced in a safe cell factory, which allows us to set the basis to further explore the applicability of this platform to produce biomaterials for the pharmaceutical industry.

As starting point, we successfully produced and isolated such nanomaterial from *L. lactis*. The obtained nanoparticles were carefully assessed by high resolution microscopy techniques, noticing that proteins acting as building blocks of these nanoparticles are heterogeneously distributed within the nanomaterial (Fig. 2). In particular, immunolocalization analysis reveals high differences in protein concentrations in cortical and central parts of nanoparticles, being cortical regions much more rich in protein than particle core. This is probably a phenomenon closely related to the activity of cell mechanisms during nanostructure formation and growth, previously observed in IB formation [30]. In addition, the ultrastructure observed by FESEM and TEM (Fig. 1 and Table 1) also resembled that of IBs formed in *E. coli* [3, 30, 40][3, 30, 38]. Interestingly, our data proved that nanoscale features such as size, compactness and shape can be easily tailored by selecting the appropriate combination of protein and strain (Table 1, Fig. 1B and C). The interaction between strain and protein had also a clear effect on the biological activity of our nanoparticles (Fig. 4A). This finding opens a whole range of possibilities in the customization of this functional and safe biomaterial. This is particularly interesting considering that these protein-based nanomaterials can be also genetically engineered, being possible not only to define size, shape and electrodensity, but also the organization and the biological activity of the produced protein-based nanoparticles in this safe microorganism. Considering the enzymatic activity of MMP nanoparticles (Fig. 4A) and

their ability to stimulate cell proliferation (Fig. 4B), this biomaterial has a great potential for both drug delivery (Fig. 4A) and tissue engineering purposes (Fig. 4B).

It has also been described that *E. coli* IBs show a bimodal supramolecular organization of the embedded protein. In this case, *E. coli* IBs contain a fraction of protein ranging from 9 to 33% with an amyloid-like organization, which is fully resistant to proteinase K and degradation acting as a nanoparticle scaffold, and two other protein populations, one of them being immediately degraded and another one sensitive to proteinase K digestion being associated with the biological activity shown by IBs [32]. The food-grade nanomaterial characterized in the present study exhibited a degradable population, with a half-life comparable to the populations described in *E. coli* [32], and a resistant population (Fig. 3). The coexistence of these two distinguished populations was further confirmed by FTIR analysis (Fig. 5). Proteins adopting an intermolecular β-sheet conformation would correspond to the proteinase-K resistant population, whereas proteinase-K sensitive population exhibited a native-like structure which contributed to its remarkable biological activity.

The determination of the chemical properties of these nanoparticles also supports that such nanoclusters have many characteristics in common with those obtained when using *E. coli* as cell factory. Depending on the genetic background chosen to produce the nanoparticles, the wettability properties of the nanostructures and thus, the wettability of surfaces modified with them, can be modulated [5]. Besides, the presence of a simple aggregation tag, such as ELK16 in MMP-9, clearly modifies the behavior of the nanostructure produced. However, Z potential values of *L. lactis*-derived nanoparticles determined in this study are higher than those observed in IBs producing in LPS enriched environment [5], indicating that, although endotoxin-free nanostructures share many characteristics with those derived from *E. coli*, they are more stable in suspension.

Considering *E. coli* limitations, this study opens a range of possibilities in terms of applications. The implementation of lactic acid bacteriaLAB as a routine cell factory for the

production of functional nanomaterials would allow the development of safe production processes, avoiding at the same time complex downstream purification steps to eliminate toxic components.

4. Conclusions

Data presented in this study reveal for the first time, that it is possible to produce, tune and isolate endotoxin-free IB-like nanoparticles from LAB through a cost-effective and fully scalable process. All results evidence that under overexpression conditions *L. lactis* is able to produce protein nanoparticles with the same properties that those produced in *E. coli*. Interestingly, these functional nanostructures isolated from *L. lactis* show even higher stabilities and activity that those obtained from *E. coli*. This finding allows making a qualitative step, since it opens a field of opportunities for the production of recombinant proteins in a cost-effective format using GRAS systems as cell factories. ~~Thus~~Overall, these data~~, this makes~~ indicates that LAB ~~an~~ are ideal alternative~~s~~ for the synthesis of tuned protein-based endotoxin-free nanoparticles for a wide range of applications in both human and veterinary medicine. ~~In summary, this work indicates that LAB could become the workhorse bacteria in a near future, replacing~~ *~~E. coli~~* ~~in this role.~~

6. References

[1] Ferrer-Miralles N, Rodriguez-Carmona E, Corchero JL, Garcia-Fruitos E, Vazquez E, Villaverde A. Engineering protein self-assembling in protein-based nanomedicines for drug delivery and gene therapy. Crit Rev Biotechnol 2015;35:209-21.

[2] Garcia-Fruitos E, Vazquez E, Diez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, Veciana J, Villaverde A. Bacterial inclusion bodies: making gold from waste. Trends in Biotechnology 2012;30:65-70.

[3] Wang X, Zhou B, Hu W, Zhao Q, Lin Z. Formation of active inclusion bodies induced by hydrophobic self-assembling peptide GFIL8. Microb Cell Fact 2015;14:88.

[4] Singh A, Upadhyay V, Panda AK. Solubilization and refolding of inclusion body proteins. Methods Mol Biol 2015;1258:283-91.

[5] Diez-Gil C, Krabbenborg S, Garcia-Fruitos E, Vazquez E, Rodriguez-Carmona E, Ratera I, Ventosa N, Seras-Franzoso J, Cano-Garrido O, Ferrer-Miralles N, Villaverde A, Veciana J. The nanoscale properties of bacterial inclusion bodies and their effect on mammalian cell proliferation. Biomaterials 2010;31:5805-12.

[6] Loo Y, Goktas M, Tekinay AB, Guler MO, Hauser CA, Mitraki A. Self-Assembled Proteins and Peptides as Scaffolds for Tissue Regeneration. Adv Healthc Mater 2015;4:2557-86.

[7] Villaverde A, Garcia-Fruitos E, Rinas U, Seras-Franzoso J, Kosoy A, Corchero JL, Vazquez E. Packaging protein drugs as bacterial inclusion bodies for therapeutic applications. Microb Cell Fact 2012;11:76.

[8] Wakelin SJ, Sabroe I, Gregory CD, Poxton IR, Forsythe JL, Garden OJ, Howie SE. "Dirty little secrets"--endotoxin contamination of recombinant proteins. Immunol Lett 2006;106:1-7.

[9] Rueda F, Cano-Garrido O, Mamat U, Wilke K, Seras-Franzoso J, Garcia-Fruitos E, Villaverde A. Production of functional inclusion bodies in endotoxin-free Escherichia coli. Appl Microbiol Biotechnol 2014;98:9229-38.

[10] Petsch D, Anspach FB. Endotoxin removal from protein solutions. J Biotechnol 2000;76:97-119.

[11] Beenken-Rothkopf LN, Karfeld-Sulzer LS, Zhang X, Kissler H, Michie SA, Kaufman DB, Fontaine MJ, Barron AE. Protein polymer hydrogels: effects of endotoxin on biocompatibility. J Biomater Appl 2013;28:395-406.

[12] Taguchi S, Ooi T, Mizuno K, Matsusaki H. Advances and needs for endotoxin-free production strains. Appl Microbiol Biotechnol 2015;99:9349-60.

[13] Villaverde A, Corchero JL, Seras-Franzoso J, Garcia-Fruitos E. Functional protein aggregates: just the tip of the iceberg. Nanomedicine (Lond) 2015;10:2881-91.

[14] Parlane NA, Grage K, Lee JW, Buddle BM, Denis M, Rehm BH. Production of a particulate hepatitis C vaccine candidate by an engineered Lactococcus lactis strain. Appl Environ Microbiol 2011;77:8516-22.

[15] Cano-Garrido O, Seras-Franzoso J, Garcia-Fruitos E. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. Microb Cell Fact 2015;14:137.

[16] Garcia-Fruitos E. Lactic Acid Bacteria: a promising alternative for recombinant protein production. Microb Cell Fact 2012;11:157.

[17] Konings WN, Kok J, Kuipers OP, Poolman B. Lactic acid bacteria: the bugs of the new millennium. Current Opinion in Microbiology 2000;3:276-82.

[18] Lu WW, Kong J, Kong WT. Construction and Application of a Food-Grade Expression System for Lactococcus lactis. Molecular Biotechnology 2013;54:170-6.

[19] Cano-Garrido O, Rueda FL, Sanchez-Garcia L, Ruiz-Avila L, Bosser R, Villaverde A, Garcia-Fruitós E. Expanding the recombinant protein quality in Lactococcus lactis. Microb Cell Fact 2014;13:167.

[20] Mierau I, Kleerebezem M. 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. Appl Microbiol Biotechnol 2005;68:705-17.

[21] King MS, Boes C, Kunji ER. Membrane protein expression in Lactococcus lactis. Methods Enzymol 2015;556:77-97.

[22] Cortes-Perez NG, Poquet I, Oliveira M, Gratadoux JJ, Madsen SM, Miyoshi A, Corthier G, Azevedo V, Langella P, Bermúdez-Humaran LG. Construction and characterization of a Lactococcus lactis strain deficient in intracellular ClpP and extracellular HtrA proteases. Microbiology 2006;152:2611-8.

[23] Poquet I, Saint V, Seznec E, Simoes N, Bolotin A, Gruss A. HtrA is the unique surface housekeeping protease in Lactococcus lactis and is required for natural protein processing. Molecular Microbiology 2000;35:1042-51.

[24] Thomas JG, Baneyx F. Roles of the Escherichia coli small heat shock proteins IbpA and IbpB in thermal stress management: Comparison with ClpA, ClpB, and HtpG in vivo. Journal of Bacteriology 1998;180:5165-72.

[25] Holo H, Nes IF. High-Frequency Transformation, by Electroporation, of Lactococcus lactis subsp. cremoris Grown with Glycine in Osmotically Stabilized Media. Appl Environ Microbiol 1989;55:3119-23.

[26] Garcia-Fruitos E, Gonzalez-Montalban N, Morell M, Vera A, Ferraz RM, Aris A, Ventura S, Villaverde A. Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. **Microb Cell Fact 2005;4:27.**

[27] Seras-Franzoso J, Peternel S, Cano-Garrido O, Villaverde A, Garcia-Fruitos E. Bacterial inclusion body purification. Methods Mol Biol 2015;1258:293-305.

[28] Mussa T, Ballester M, Silva-Campa E, Baratelli M, Busquets N, Lecours MP, Dominguez J, Amadori M, Fraile L, Hernández J, Montoya M. Swine, human or avian influenza viruses differentially activates porcine dendritic cells cytokine profile. Vet Immunol Immunopathol 2013;154:25-35.

[29] Rodriguez-Carino C, Duffy C, Sanchez-Chardi A, McNeilly F, Allan GM, Segales J. Porcine circovirus type 2 morphogenesis in a clone derived from the l35 lymphoblastoid cell line. J Comp Pathol 2011;144:91-102.

[30] Seras-Franzoso J, Sanchez-Chardi A, Garcia-Fruitos E, Vazquez E, Villaverde A. Cellular uptake and intracellular fate of protein releasing bacterial amyloids in mammalian cells. Soft Matter 2016;12:3451-60.

[31] Rodriguez-Carino C, Sanchez-Chardi A, Segales J. Subcellular immunolocalization of porcine circovirus type 2 (PCV2) in lymph nodes from pigs with post-weaning multisystemic wasting syndrome (PMWS). J Comp Pathol 2010;142:291-9.

[32] Cano-Garrido O, Rodriguez-Carmona E, Diez-Gil C, Vazquez E, Elizondo E, Cubarsi R, Seras-Franzoso J, Corchero JL, Rinas U, Ratera I, Ventosa N, Veciana J, Villaverde A, Garcia-Fruitós, E. Supramolecular organization of protein-releasing functional amyloids solved in bacterial inclusion bodies. Acta Biomater 2013;9:6134-42.

[33] Ami D, Natalello A, Taylor G, Tonon G, Maria Doglia S. Structural analysis of protein inclusion bodies by Fourier transform infrared microspectroscopy. Biochim Biophys Acta 2006;1764:793-9.

[34] Natalello A, Doglia SM. Insoluble protein assemblies characterized by fourier transform infrared spectroscopy. Methods Mol Biol 2015;1258:347-69.

[35] Bassan P, Sachdeva A, Kohler A, Hughes C, Henderson A, Boyle J, Shanks JH, Brown M, Clarke NW, Gardner P. FTIR microscopy of biological cells and tissue: data analysis using resonant Mie scattering (RMieS) EMSC algorithm. Analyst 2012;137:1370-7.

[36] Seras-Franzoso J, Diez-Gil C, Vazquez E, Garcia-Fruitos E, Cubarsi R, Ratera I, Veciana J, Villaverde A. Bioadhesiveness and efficient mechanotransduction stimuli synergistically provided by bacterial inclusion bodies as scaffolds for tissue engineering. Nanomedicine 2012;7:79-93.

[37] Garcia-Fruitos E, Rodriguez-Carmona E, Diez-Gil C, Ferraz R**M, Vazquez E, Corchero JL,** Cano-Sarabia M, Ratera I, Ventosa N, Veciana J, Villaverde A. Surface Cell Growth Engineering Assisted by a Novel Bacterial Nanomaterial. Advanced Materials 2009;21:4249-+.

[38] Pesarrodona M, Ferrer-Miralles N, Unzueta U, Gener P, Tatkiewicz W, Abasolo I, Ratera I, Veciana J, Schwartz S Jr, Villaverde A, Vazquez E. Intracellular targeting of CD44+ cells with self-assembling, protein only nanoparticles. Int J Pharm 2014;473:286-95.

[39] Faucheux N, Schweiss R, Lutzow K, Werner C, Groth T. Self-assembled monolayers with different terminating groups as model substrates for cell adhesion studies. Biomaterials 2004;25:2721-30.

[40] Garcia-Fruitos E, Seras-Franzoso J, Vazquez E, Villaverde A. Tunable geometry of bacterial inclusion bodies as substrate materials for tissue engineering. Nanotechnology 2010;21.

[1] Ferrer-Miralles N, Rodriguez-Carmona E, Corchero JL, Garcia-Fruitos E, Vazquez E, Villaverde A. Engineering protein self-assembling in protein-based nanomedicines for drug delivery and gene therapy. Crit Rev Biotechnol 2015;35:209-21.

[2] Garcia-Fruitos E, Vazquez E, Diez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, et al. Bacterial inclusion bodies: making gold from waste. Trends in Biotechnology 2012;30:65-70.

[3] Wang X, Zhou B, Hu W, Zhao Q, Lin Z. Formation of active inclusion bodies induced by hydrophobic self-assembling peptide GFIL8. Microb Cell Fact 2015;14:88.

[4] Singh A, Upadhyay V, Panda AK. Solubilization and refolding of inclusion body proteins. Methods Mol Biol 2015;1258:283-91.

[5] Diez-Gil C, Krabbenborg S, Garcia-Fruitos E, Vazquez E, Rodriguez-Carmona E, Ratera I, et al. The nanoscale properties of bacterial inclusion bodies and their effect on mammalian cell proliferation. Biomaterials 2010;31:5805-12.

[6] Loo Y, Goktas M, Tekinay AB, Guler MO, Hauser CA, Mitraki A. Self-Assembled Proteins and Peptides as Scaffolds for Tissue Regeneration. Adv Healthc Mater 2015;4:2557-86.

[7] Villaverde A, Garcia-Fruitos E, Rinas U, Seras-Franzoso J, Kosoy A, Corchero JL, et al. Packaging protein drugs as bacterial inclusion bodies for therapeutic applications. Microb Cell Fact 2012;11:76.

[8] Wakelin SJ, Sabroe I, Gregory CD, Poxton IR, Forsythe JL, Garden OJ, et al. "Dirty little secrets"--endotoxin contamination of recombinant proteins. Immunol Lett 2006;106:1-7.

[9] Rueda F, Cano-Garrido O, Mamat U, Wilke K, Seras-Franzoso J, Garcia-Fruitos E, et al. Production of functional inclusion bodies in endotoxin-free Escherichia coli. Appl Microbiol Biotechnol 2014;98:9229-38.

[10] Petsch D, Anspach FB. Endotoxin removal from protein solutions. J Biotechnol 2000;76:97-119.

[11] Beenken-Rothkopf LN, Karfeld-Sulzer LS, Zhang X, Kissler H, Michie SA, Kaufman DB, et al. Protein polymer hydrogels: effects of endotoxin on biocompatibility. J Biomater Appl 2013;28:395-406.

[12] Taguchi S, Ooi T, Mizuno K, Matsusaki H. Advances and needs for endotoxin-free production strains. Appl Microbiol Biotechnol 2015;99:9349-60.

[13] Villaverde A, Corchero JL, Seras-Franzoso J, Garcia-Fruitos E. Functional protein aggregates: just the tip of the iceberg. Nanomedicine (Lond) 2015;10:2881-91.

[14] Parlane NA, Grage K, Lee JW, Buddle BM, Denis M, Rehm BH. Production of a particulate hepatitis C vaccine candidate by an engineered Lactococcus lactis strain. Appl Environ Microbiol 2011;77:8516-22.

[15] Cano-Garrido O, Seras-Franzoso J, Garcia-Fruitos E. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. Microb Cell Fact 2015;14:137.

[16] Garcia-Fruitos E. Lactic Acid Bacteria: a promising alternative for recombinant protein production. Microb Cell Fact 2012;11:157.

[17] Konings WN, Kok J, Kuipers OP, Poolman B. Lactic acid bacteria: the bugs of the new millennium. Current Opinion in Microbiology 2000;3:276-82.

[18] Lu WW, Kong J, Kong WT. Construction and Application of a Food-Grade Expression System for Lactococcus lactis. Molecular Biotechnology 2013;54:170-6.

[19] Cano-Garrido O, Rueda FL, Sanchez-Garcia L, Ruiz-Avila L, Bosser R, Villaverde A, et al. Expanding the recombinant protein quality in Lactococcus lactis. Microb Cell Fact 2014;13:167.

[20] Mierau I, Kleerebezem M. 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. Appl Microbiol Biotechnol 2005;68:705-17.

[21] King MS, Boes C, Kunji ER. Membrane protein expression in Lactococcus lactis. Methods Enzymol 2015;556:77-97.

[22] Cortes-Perez NG, Poquet I, Oliveira M, Gratadoux JJ, Madsen SM, Miyoshi A, et al. Construction and characterization of a Lactococcus lactis strain deficient in intracellular ClpP and extracellular HtrA proteases. Microbiology 2006;152:2611-8.

[23] Poquet I, Saint V, Seznec E, Simoes N, Bolotin A, Gruss A. HtrA is the unique surface housekeeping protease in Lactococcus lactis and is required for natural protein processing. Molecular Microbiology 2000;35:1042-51.

[24] Thomas JG, Baneyx F. Roles of the Escherichia coli small heat shock proteins IbpA and IbpB in thermal stress management: Comparison with ClpA, ClpB, and HtpG in vivo. Journal of Bacteriology 1998;180:5165-72.

[25] Holo H, Nes IF. High-Frequency Transformation, by Electroporation, of Lactococcus lactis subsp. cremoris Grown with Glycine in Osmotically Stabilized Media. Appl Environ Microbiol 1989;55:3119-23.

[26] Garcia-Fruitos E, Gonzalez-Montalban N, Morell M, Vera A, Ferraz RM, Aris A, et al. Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. Microb Cell Fact 2005;4:27.

[27] Seras-Franzoso J, Peternel S, Cano-Garrido O, Villaverde A, Garcia-Fruitos E. Bacterial inclusion body purification. Methods Mol Biol 2015;1258:293-305.

[28] Mussa T, Ballester M, Silva-Campa E, Baratelli M, Busquets N, Lecours MP, et al. Swine, human or avian influenza viruses differentially activates porcine dendritic cells cytokine profile. Vet Immunol Immunopathol 2013;154:25-35.

[29] Rodriguez-Carino C, Duffy C, Sanchez-Chardi A, McNeilly F, Allan GM, Segales J. Porcine circovirus type 2 morphogenesis in a clone derived from the l35 lymphoblastoid cell line. J Comp Pathol 2011;144:91-102.

[30] Seras-Franzoso J, Sanchez-Chardi A, Garcia-Fruitos E, Vazquez E, Villaverde A. Cellular uptake and intracellular fate of protein releasing bacterial amyloids in mammalian cells. Soft Matter 2016;12:3451-60.

[31] Rodriguez-Carino C, Sanchez-Chardi A, Segales J. Subcellular immunolocalization of porcine circovirus type 2 (PCV2) in lymph nodes from pigs with post-weaning multisystemic wasting syndrome (PMWS). J Comp Pathol 2010;142:291-9.

[32] Cano-Garrido O, Rodriguez-Carmona E, Diez-Gil C, Vazquez E, Elizondo E, Cubarsi R, et al. Supramolecular organization of protein-releasing functional amyloids solved in bacterial inclusion bodies. Acta Biomater 2013;9:6134-42.

[33] Ami D, Natalello A, Taylor G, Tonon G, Maria Doglia S. Structural analysis of protein inclusion bodies by Fourier transform infrared microspectroscopy. Biochim Biophys Acta 2006;1764:793-9.

[34] Natalello A, Doglia SM. Insoluble protein assemblies characterized by fourier transform infrared spectroscopy. Methods Mol Biol 2015;1258:347-69.

[35] Garcia-Fruitos E, Rodriguez-Carmona E, Diez-Gil C, Ferraz RM, Vazquez E, Corchero JL, et al. Surface Cell Growth Engineering Assisted by a Novel Bacterial Nanomaterial. Advanced Materials 2009;21:4249-+.

[36] Pesarrodona M, Ferrer-Miralles N, Unzueta U, Gener P, Tatkiewicz W, Abasolo I, et al. Intracellular targeting of CD44+ cells with self-assembling, protein only nanoparticles. Int J Pharm 2014;473:286-95.

[37] Faucheux N, Schweiss R, Lutzow K, Werner C, Groth T. Self-assembled monolayers with different terminating groups as model substrates for cell adhesion studies. Biomaterials 2004;25:2721-30.

[38] Garcia-Fruitos E, Seras-Franzoso J, Vazquez E, Villaverde A. Tunable geometry of bacterial inclusion bodies as substrate materials for tissue engineering. Nanotechnology 2010;21.

7. Figure captions

Figure 1. FESEM and TEM micrographs of *L. lactis* and purified nanoparticles. A) On the left, FESEM micrographs of *L. lactis* bacteria. In the middle, TEM sections of *L. lactis* with MMP-9 nanoparticles inside (light grey particles). On the right, TEM immunolocalization of MMP-9 nanoparticles (labeled by anti-MMP-9) in *L. lactis*. Scale bars: Upper micrographs 500 nm; lower micrographs 200 nm. **B)** FESEM micrographs of purified nanoparticles. Scale bars: 200 nm. **C)** TEM micrographs of purified nanoparticles. Scale bars: left inset 200 nm; right inset 100 nm; lower micrographs 500 nm.

Figure 2. TEM micrographs of purified nanoparticles labeled by specific antibodies. Scale bars: Left and right inset 200 nm; lower micrographs 500 nm.

Figure 3. Time-course stability of nanoparticles. MMP-9, IFN-γ and MMP-2 produced in the *clpP-htrA* during proteinase K digestion (dark grey diamonds). Controls without proteinase K (light grey squares). The standard error is represented by black lines at each time point. The values detailed in the table indicate the distribution of protein populations in the nanoparticles according to their half-life under proteinase K treatment.

Figure 4. Functionality of *L. lactis* protein-based nanoparticles. A) Specific activity of metalloproteinases. Different letters depict differences between proteins (P < 0.0001) and due to the interaction between strain and protein (P = 0.0022). B) Biological effects of GFP nanoparticles on human mammalian cell cultures. ~~i.~~ Proliferation assay with 1BR3.G cells cultured on surfaces decorated with 1 µg of GFP nanoparticles at 24, 48 and 72 h. Bars with asterisk differ (24 h, P = 0.0433; 48 h, P = 0.0008; 72 h, P = 0.0055) from negative control. **CB)** ~~ii.~~ Percentage of fluorescent HeLa cells analysed by cytometry after 24 h exposure to GFP nanoparticles. Significant differences depicted by asterisk (P = 0.0010). **DC)** ~~iii.~~

29

Confocal micrographs of HeLa cells after 24 h exposure to GFP nanoparticles and their orthogonal sections in both the x and y axis. Scale bar in micrograph represents 5 µm. In all cases, results are expressed as means of non-transformed data ± SE, which correspond to un-transformed data.

Figure 5. FTIR analysis of purified nanoparticles. Second derivatives of absorption spectra of **A)** MMP-9 and MMP-9-ELK16; the component around 1658 cm^{-1} can be assigned to α-helices and random coils and the two components at around 1633 cm^{-1} and 1692 cm^{-1} to the native intramolecular β-sheet structures of the protein. **B)** IFN-γ; the components around 1656 and 1681 cm^{-1} can be assigned to the native α-helices and to turn structures of the protein, respectively. **C)** MMP-2; the component around 1637 cm^{-1}, along with the peak at ~1692 cm^{-1}, can be assigned to native, intramolecular β-sheets. The component around 1658 cm^{-1} is due to the native α-helices and to the random-coil structures of the protein. In A), B) and C) the two components at around 1627 and 1695 cm^{-1} are the typical marker bands of intermolecular β-sheet in protein aggregates.

Figure 6. Contact angle measurements. Contact angles of 6 different functionalized gold surfaces, containing different molar ratio of −OH terminated (X_{OH}) alkanethiols. Surfaces before nanoparticle deposition (dark grey diamond) and after nanoparticle deposition (light grey square).