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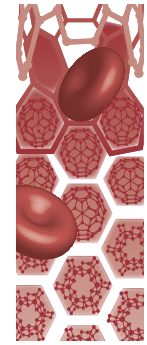

CXCR4$^+$-targeted protein nanoparticles produced in the food-grade bacterium *Lactococcus lactis*

Aim: *Lactococcus lactis* is a Gram-positive (endotoxin-free) food-grade bacteria exploited as alternative to *Escherichia coli* for recombinant protein production. We have explored here for the first time the ability of this platform as producer of complex, self-assembling protein materials. **Materials & methods:** Biophysical properties, cell penetrability and *in vivo* biodistribution upon systemic administration of tumor-targeted protein nanoparticles produced in *L. lactis* have been compared with the equivalent material produced in *E. coli*. **Results:** Protein nanoparticles have been efficiently produced in *L. lactis*, showing the desired size, internalization properties and biodistribution. **Conclusion:** *In vitro* and *in vivo* data confirm the potential and robustness of the production platform, pointing out *L. lactis* as a fascinating cell factory for the biofabrication of protein materials intended for therapeutic applications.

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Olivia Cano-Garrido[1,2,3], María Virtudes Céspedes[3,4], Ugutz Unzueta[3,4], Paolo Saccardo[1,3], Mònica Roldán[5], Alejandro Sánchez-Chardi[5], Rafael Cubarsi[6], Esther Vázquez[1,2,3], Ramon Mangues[3,4], Elena García-Fruitós*[,7] & Antonio Villaverde*[,1,2,3]

[1]Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Cerdanyola del Vallès, Spain

[2]Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Cerdanyola del Vallès, Spain

[3]CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra, 08193 Cerdanyola del Vallès, Spain

[4]Oncogenesis & Antitumor Drug Group, Biomedical Research Institute Sant Pau (IIB-Sant Pau), Hospital de la Santa Creu I Sant Pau, 08025 Barcelona, Spain

[5]Servei de Microscòpia, Universitat Autònoma de Barcelona, Bellaterra 08193 Cerdanyola del Vallès, Barcelona, Spain

[6]Departament de Matemàtica Aplicada IV. Universitat Politècnica de Catalunya. Jordi Girona 1-3. 08034 Barcelona, Spain

[7]Department of Ruminant Production, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), 08140 Caldes de Montbui, Spain

*Authors for correspondence: antoni.villaverde@uab.cat

Background

Protein materials benefit from their generic biocompatibility, full biodegradability and functional and structural convergence, which can be adjusted to particular purposes by genetic design. Also, proteins can be produced by biofabrication in cell factories by fully scalable, tuneable and cost-effective processes [1]. The optimization of methodologies for protein production has allowed the approval, by the major medicament agencies (namely US FDA and EMA), of hundreds of protein drugs for human use. In this context, the global market for recombinant protein and peptidic drugs has been growing since 2007 at an annual rate of 10%, achieving US$160.1 billion and US$50.4 billion, respectively, in 2013 [2]. Interestingly, there is a manifest trend toward the generation of protein drugs whose amino acid sequences have been modified, versus the use of natural, unmodified protein versions [3]. In this direction, and as a side aspect of protein drug development, many principles of protein engineering allow the production of self-assembling polypeptides as building blocks of complex oligomeric structures [4]. Protein self-assembling results in both amorphous and defined morphometries that include fibers, layers, ribbons, cages, particles and hydrogels. Protein materials resulting from self-assembling of building blocks are generically biocompatible and highly tuneable, exhibiting diverse applications in biomedicine (for targeted drug delivery, local drug release, protein replacement therapies and tissue engineering, among others) [5], what demand standardized and safe production methods.

Besides the *ex vivo* chemical synthesis of short peptides, recombinant protein production comprises a rich set of procedures deeply explored for biotechnological and biopharma products. Many types of cell factories are under development to expand the versatility of biological production and to adjust the final quality of products to the increas-

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ing regulatory demands [6]. In this context, the absence of endotoxins is required for *in vivo* uses because these molecules promote pyrogenicity and potent inflammatory responses [7]. This fact makes strongly advisable the use of endotoxin-free cell factories [8–12]. In this context, the Gram-positive (endotoxin-free) group of lactic acid bacteria (LAB) represents a promising platform for protein production as these species combine fast bacterial growth (vs eukaryotic platforms), easy culture with adjustable conditions and the food-grade denomination [13–15]. Among LAB, the genome of *Lactococcus lactis* was the first sequenced and the genetic tools for gene cloning and expression already developed for this species are particularly efficient. The *L. lactis* toolbox particularly favors protein secretion and high solubility levels, and the use of this platform thus addresses another of the major bottlenecks in bacterial protein production, which is protein aggregation [16]. Many proteins with therapeutic interest have been already produced in a soluble and biologically active form, for instance cytokines [17–22], hormones [23] and antigens [24–29]. Interestingly, LAB and especially *L. lactis* also offer the potential of adaptation for use in form of living vector cells for *in vivo* delivery of immunogens, therapeutic proteins and nucleic acids [30,31]. In these applications proteins are released from bacteria administered intranasal or orally, what largely expands the spectrum of therapeutic applications of this particular production system [13,32–35].

Despite its potential for the production of therapeutic polypeptides, the performance of such still emerging production system has not been explored yet regarding the biological fabrication of building blocks of complex nanostructured protein materials. As further protein assembling as functional supramolecular structures depends on protein conformation, and protein conformation is subjected to the performance of the cell's quality control system, different bacterial species might behave differently as cell factories regarding the final structure and functional quality of protein materials. To address this issue, we have produced in *L. lactis* CXCR4-targeted, smart protein nanoparticles formed by the modular protein T22-GFP-H6, previously designed for production in *Escherichia coli*. Because of its full characterization and suitability for potent recombinant gene expression, the nisin-controlled gene expression system was selected for protein production in this species [36]. Nanoparticles, as materials with regular geometry and a size below 100 nm, offer interesting advantages as drug carriers regarding distribution in organic tissues and enhanced cellular penetrability, especially regarding oncological treatments [37]. Production of T22-GFP-H6 nanoparticles in *L. lactis* has been approached for a further characterization and comparison of the properties of the same material produced in *E. coli*, at molecular, supramolecular and also systemic level. The nanoparticles produced in *L. lactis* successfully accumulated in primary tumor and metastasis in CXCR4$^+$ colorectal cancer mice models upon systemic administration, proving a specific targeting for tumoral cells that has been also confirmed *in vitro* by using CXCR4 ligands as binding inhibitors. While the data obtained here indicated robust targeting and biological behavior of the protein nanoparticles produced in *L. lactis*, confirming this system as a promising factory for complex protein materials, they also revealed detectable variability in relevant structural and functional features when comparing materials handled by alternative bacterial species with functionally different quality controls.

Methods

Strains, plasmid, culture & protein production conditions

Strains and plasmids used in this study and their most relevant features are listed in Table 1. *L. lactis* (NZ9000 strain) was transformed by electroporation with pNZ8150, while *E. coli* Origami B strain was transformed by heat shock with pET22b. Both plasmids encode, with optimized codon usage for every host (synthesized by GeneArt), the modular protein T22-GFP-H6, which when fabricated in *E. coli* (T22-GFP-H6^{coli}) self-assembles into regular toroid particles of about 14 nm. T22 is a cationic peptide that apart from promoting protein–protein contacts in nanoparticles formed by T22-GFP-H6 as building blocks [38], it binds specifically to the cell surface cytokine CXCR4, overexpressed in colorectal cancer and correlating with aggressiveness and progression of the disease [39]. T22 promotes internalization of the whole nanoparticle in CXCR4$^+$ cells [40] and its intracellular accumulation in tumor and secondary metastatic foci *in vivo* [41]. Being colorectal cancer a central health problem and metastasis the main cause or mortality in colorectal cancer, a protein nanoparticle targeted to metastatic CXCR4$^+$ colorectal cancer cells represents a promising vehicle for targeted delivery of conventional and innovative antitumoral drugs. *L. lactis* was cultured in M17 media (Sigma) enriched with 0.5% glucose and *E. coli* in lysogenic broth media (Sigma). Overnight cultures were prepared from a bacterial glycerol stock to inoculate shake flasks containing 500 ml of the appropriate media and antibiotics. *L. lactis* was cultured at 30°C without shaking and *E. coli* at 37°C and 250 rpm, growing up to 0.5 OD_{550} units. *T22-GFP-H6* gene expression in *L. lactis* was induced by 12.5 ng/ml nisin and cultures were incubated overnight at 30°C without shaking. In *E. coli*, gene expres-

Table 1. Strains and plasmids used in this study.

Bacterial strains and plasmids	Relevant genotype or phenotype	Ref.
Lactococcus lactis subsp. *cremoris* NZ9000	*pepN*::*nisRnisK*	[20]
Escherichia coli Origami B	OmpT-, Lon-, TrxB-, Gor-, Strep^R, Tet^R	[21]
pNZ8150-T22-GFP-H6	Cm^R(5 μg/ml), nisin-inducible	This work
pET22b-T22-GFP-H6	Ap^R(100 μg/ml), IPTG-inducible	[22]

sion was induced by IPTG at 1 mM and cultures were incubated overnight at 20°C and 250 rpm.

Protein purification

Both species of bacterial cells were harvested by centrifugation (5000 × *g* at 4°C, 15 min) and resuspended in wash buffer (20 mM Tris-HCl, 500 mM NaCl and 10 mM imidazole), containing an EDTA-free protease inhibitor cocktail (Roche). Afterward, *E. coli* cells were disrupted by pressuring two rounds at 1200 psi (machine pressure) and *L. lactis* cells with three rounds at 1500 psi in a French press (Thermo FA-078A). Both T22-GFP-H6^{coli} and T22-GFP-H6^{lactis} proteins were purified by His-tag affinity chromatography using 1 ml HiTrap Chelating HP column (GE Healthcare) through an ÄKTA pure FPLC (GE Healthcare). Separations were made by linear gradient of elution buffer (20 mM Tris, 500 mM NaCl and 500 mM imidazole). Purified protein fractions were dialyzed against carbonate buffer (166 mM $NaCO_3H$, pH 7.4). Protein amounts were determined by Bradford's assay [42] and analyzed by conventional denaturing SDS-polyacrylamide gel electrophoresis (15% polyacrylamide), using a commercial polyclonal antibody against green fluorescent protein (GFP; Santa Cruz Biotechnology). Protein purification was assisted by the ICTS 'NANBIOSIS', more specifically by the CIBER-BBN's Protein Production Platform [43].

Electron microscopy

T22-GFP-H6 nanoparticles were examined by transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM). For TEM, protein samples were negatively stained with uranyl acetate by conventional methods [41] and observed in a Jeol 1400 microscope operating at 80 kV and equipped with a CCD Gatan Erlangshen ES1000W camera. For FESEM, protein samples were directly deposited over silicon wafers, air dried and observed with an in-lens secondary electron detector through a Zeiss Merlin microscope operating at 2 kV.

Determination of particle size & fluorescence

Particle size was measured by two different techniques. By dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern), volume size distribution was determined at 633 nm. Using FESEM micrographs, diameters of nanoparticles were measured by ImageJ software (NIH, MD, USA). GFP fluorescence emission (510 nm) was determined on purified proteins with a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) using an excitation wavelength of 450 nm.

Cell culture, flow cytometry & confocal microscopy

Protein internalization was analyzed in CXCR4+ HeLa cell cultures in 24-well plates. Briefly, the Minimum Essential Medium medium supplemented with 10% FBS and 2 mM Glutamax (Gibco) was removed and cells were washed in PBS. Then 250 μl of 500 nM T22-GFP-H6 protein, diluted in OptiPro medium supplemented with L-glutamine, were added and incubated for 2 h, at 37°C at 5% CO_2 to allow cell binding and internalization. Then, harsh trypsin digestion (1 mg/ml for 15 min) was carried out to remove protein particles bound to the outer size of the cell membranes [44]. Intracellular green fluorescence was analyzed by flow cytometry on an FACS-Canto system (Becton Dickinson) using a 15 mW air-cooled argon ion laser at 488 nm excitation. Fluorescence emission was measured with a D detector (530/30 nm band pass filter), and manually corrected by the specific fluorescence of purified protein, to get data representative of the amount of internalized protein for comparative purposes. For competition assays, a specific CXCR4 inhibitor AMD3100 (octahydrochloride hydrate, Sigma Aldrich) was added 1 h before T22-GFP-H6 nanoparticles addition in a 1:10 (protein: AMD3100) molar ratio. For confocal analysis, cells were grown to 100,000 cells/ml on MatTek culture dishes (MatTek Corporation) for 24 h at 37°C at 5% CO_2. Then, 500 nM T22-GFP-H6 in 1 ml OptiPro medium supplemented with L-Glutamine was added, and incubated for 24 h at 37°C and 5% CO_2. Before confocal observation, nuclei were labeled with 5 μg/ml Hoechst 33342 (Life Technologies) and plasma membranes with 2.5 μg/ml CellMask™ Deep Red (Life Technologies) for 10 min in the dark. Micrographs were then taken by TCS-SP5 confocal laser scanning microscopy (Leica Microsystems) using a Plan Apo 63×/1.4

(oil HC × PL APO lambda blue) objective. In order to localize T22-GFP-H6 nanoparticles inside cells, stacks of 40–60 sections for every 0.25 µm of cell thickness were collected and 3D models were generated using Imaris software (Bitplane).

Biodistribution of T22-GFP-H6 variants in CXCR4⁺ colorectal cancer mouse models

Five-week-old female swiss nude mice, weighing 18–20 g (Charles River) and maintained in specific pathogen-free conditions were used for the *in vivo* experiments. They were performed by the ICTS 'NANBIOSIS', more specifically by the CIBER-BBN's Nanotoxicology platform of IIB Sant Pau [45], and all procedures were approved by the Hospital de Sant Pau ethical committee in accordance with Institutional guidelines.

To generate the subcutaneous (sc.) CXCR4⁺ CRC model, aliquots of 10 mg of SP5 CXCR4⁺ tumor tissue from donor animals were obtained and implanted sc. of mice by the trocher system. When tumors reached 500 mm^3 approximately, mice were randomly allocated and biodistribution was performed. T22-GFP-H6 nanoparticles in carbonate buffer (166 mM $NaCO_3H$, pH 7.4) were administered intravenously at 500 µg/mouse (n = 3). The control mice received empty buffer (n = 3). Five hours postadministration, mice were euthanized by cervical dislocation and tumors and organs were extracted for *ex vivo* recording and quantifying the fluorescence emitted by each organ. GFP fluorescence signals were detected using the IVIS spectrum equipment (Perkin Elmer). The fluorescence signal was first digitalized, displayed as a pseudocolor overlay and expressed as radiant efficiency ([p/s/cm^2/sr]µW/cm^2). Data were corrected by the specific fluorescence of purified protein, to get data representative of the amount of internalized protein, for comparative purposes. Finally, tumors and all organs were collected and fixed with 4% formaldehyde in phosphate-buffered solution for 24 h, and then embedded in paraffin for immunohistochemical evaluation.

Histology & immunohistochemistry

Four-micrometer-thick sections were stained with hematoxylin and eosin, and a complete histopathological analysis was performed by two independent observers. Immunohistochemistry (IHC) stains were performed on a DAKO Autostainer automated Link48 (DAKO) using standard procedures. The anti-GFP antibody (1:100, Santa Cruz Biotechnology) was used to detect nanoparticle accumulation and localization in tumors and normal tissue as described previously [41]. Representative images were taken using Cell B software (Olympus Soft Imaging) at 200× and 400× magnifications.

Results

The modular protein T22-GFP-H6 (Figure 1A), displaying the peptidic CXCR4 ligand T22, was produced in *L. lactis* and purified in a single peak from bacterial cell extracts. T22-GFP-$H6^{lactis}$ resulted in a polypeptide of the expected molecular mass (30.6 KDa), suffering only from mild proteolytic degradation (Figure 1B & C). The protein material occurred in form of nanoparticles that in dynamic light scattering peaked at 20.9 nm, showing also a polydisperse secondary population of around 100 nm that might correspond to soluble aggregates. Under microscopic examination, regular toroid nanoparticles were observed, similar in morphology to those formed by the same protein when produced in *E. coli* Origami B but of larger size (20 vs 14 nm).

Regarding functionality, those particles emitted fluorescent light (as expected, because of the presence of GFP), and their specific fluorescence was determined to be 3.12-fold lower than the value obtained for T22-GFP-$H6^{coli}$ nanoparticles produced in Origami B under comparable production conditions (Figure 2A). Their intrinsic fluorescence was used as reporter to check the ability of the material to penetrate CXCR4⁺ cells in a receptor-dependent way, what was comparatively done regarding the protein source. While the uptake of both nanoparticle versions was efficiently inhibited by AMD3100 (86.7% in the case of *E. coli* materials and 96.6% in the case of the food-grade nanoparticles), proving the specificity in cell entry in both cases, penetration of the material produced in *L. lactis* was surprisingly higher than that produced in *E. coli* (Figure 2A). In previous uptake analyses of GFP-based nanoparticles produced in diverse *E. coli* strains we observed a negative correlation between the capacity to internalize cells and the specific fluorescence of the material, indicative of alternative arrangements of the oligomers, affecting both the performance of the fluorophore and that of the cell active peptides (namely T22 and H6, as ligand and endosomal escape agents, respectively). Interestingly, T22-GFP-$H6^{lactis}$ materials perfectly fit in this model (red dots, Figure 2B).

The efficient and highly specific CXCR4 targeting, good penetration (Figure 2A) and the intracellular accumulation of the nanoparticles that remained fully fluorescent (Figure 2C & D), prompted us to evaluate the performance of the material *in vivo*, regarding biodistribution in CXCR4⁺ colorectal cancer mice models upon systemic administration. In animals treated with either material (Figure 3A), fluorescence largely accumulated in primary tumor as expected (Figure 3B & E), with only background signal in non-target organs (Figure 3C) that might be due to tissue self-fluorescence within the GFP emission range.

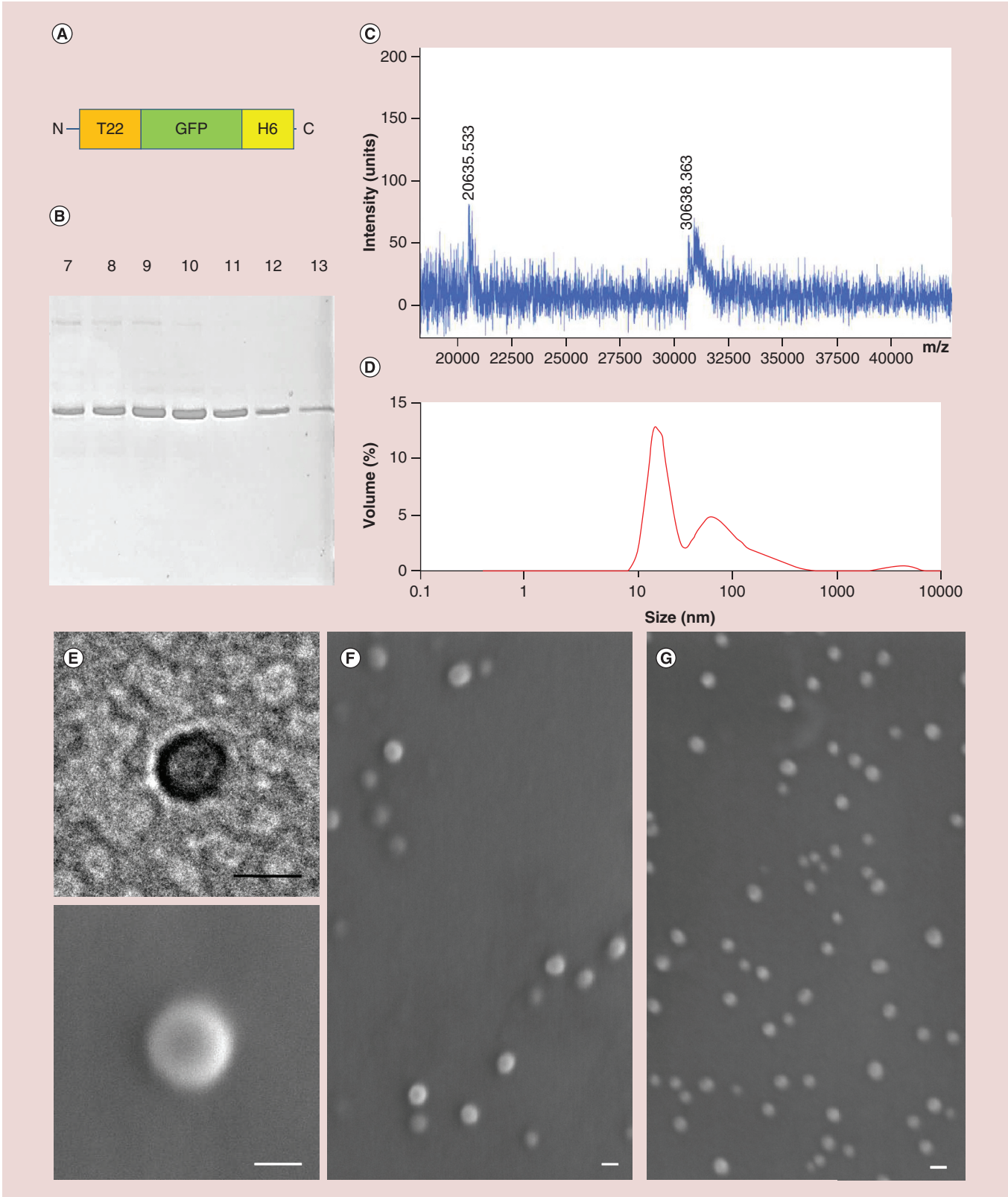

Figure 1. Nanoscale characterization of T22-GFP-H6^{lactis} nanoparticles (continued overleaf). **(A)** Modular scheme of T22-GFP-H6. Relative lengths of the modules are only approximate. **(B)** Fractions from 7 to 13 resulting from one-step protein purification of T22-GFP-H6^{lactis} in His-tag affinity chromatography that were pooled for further studies. **(C)** Mass spectrometry analysis of T22-GFP-H6^{lactis}. **(D)** Volume size distribution of nanoparticles determined by DLS.

Figure 1. Nanoscale characterization of T22-GFP-H6^{lactis} nanoparticles (cont.). **(E)** Representative micrographs of a detail of T22-GFP-H6^{lactis} nanoparticles obtained by TEM (up) and by FESEM (down). **(F)** FESEM micrograph showing a general field of T22-GFP-H6^{lactis} nanoparticles. **(G)** FESEM micrograph showing a general field of T22-GFP-H6^{coli} nanoparticles. Bars indicate 20 nm.
DLS: Dynamic light scattering; FESEM: Field emission scanning electron microscopy; TEM: Transmission electron microscopy.

While selective tumor targeting was excellent in the case of T22-GFP-H6^{coli} nanoparticles, those produced in *L. lactis* showed moderate but detectable accumulation in liver that was presumed when analyzing the whole organ but that was confirmed in liver sections (Figure 3C & F). Quantitative analysis of the signal, upon correction by the specific fluorescence of the variant nanoparticles confirmed a similar biodistribution pattern of both materials as well as the enhanced deposition in liver of T22-GFP-H6^{lactis} (Figure 3D). This might be primarily due to the slightly enhanced particle size comparing with the material produced in *E. coli* or to an expansion of the conformational spectrum of protein nanoparticles when produced in the Gram-positive cell factory.

Discussion

Among the diversity of cell factories for protein drug production [6], bacteria offer greater opportunities for cost-effective production at industrial scale and higher process versatility. Being *E. coli* the choice production system [14,47], endotoxin contamination of bacterial products is a major issue of concern [48]. Given the increasing trend in the approval of protein-based drugs [3] and in the line of the convenient biological fabrication of nanostructured protein materials [1], the production of endotoxin-free protein for biomedical uses is gaining relevance. While industrial-scale protocols for endotoxin removal have been implemented and largely tested for conventional-soluble recombinant proteins [8,11–12,48], attention has not been paid yet to self-assembling protein materials exhibiting a higher structural complexity and more prompt to retain undesired contaminants. In this context, endotoxin-free *E. coli* strains have been recently developed [10] and proved to be appropriate for the successful production of soluble protein drugs [10] as well as of more complex protein materials such as inclusion bodies [49] and tumor-targeted protein nanoparticles [50]. Interestingly, both the architecture and functionalities of smart protein materials intended for *in vivo* administration, such as LDLR-, CXCR4- or CD44-targeted nanoparticles [41,51–52], are significantly influenced by the performance of the quality control of the producing *E. coli* cells [46,53]. In turn, the quality control network might be, itself, modulated by the particular genetics that is necessary to reach the endotoxin-free status [46].

The food-grade lactic acid bacterium *L. lactis* has emerged as a promising protein production platform [16,54–55], naturally devoid of endotoxins and suitable for oral and intranasal administration as a living drug (DNA and protein) delivery system [30,35,56–59]. This microorganism has been mainly employed for the secretion of difficult-to-express proteins in soluble forms [16,60] and for the preparation of S-layers for ordered protein display purposes [61]. Although it has been also used for the controlled preparation of nanostructured biopolyester beads [62] and hybrid polyhydroxybutyrate–protein granules [63] among a few other materials, *L. lactis* has been never explored regarding the quality and activities of complex self-assembling protein materials. It is anticipated that the *L. lactis* quality control might act differently than that of *E. coli*, because of the occurrence of different regulators of stress responses [64], by the potentially divergent activities of the main cytosolic chaperone DnaK [64] and by alternative ways in which these species appear to manage protein aggregation [54,65]. Therefore, we were interested in knowing if *L. lactis* might be supportive of proper production of cell-targeted protein nanoparticles usable as *in vivo* drug vehicles for intracellular drug delivery [66]. The data obtained in the present study fully confirm the robustness of the self-assembling protein platform, since the expected toroid T22-GFP-H6 nanoparticles are produced in *L. lactis* (Figure 1E & F) specifically bind the CXCR4 cytokine receptor (Figure 2A) and efficiently internalize CXCR4$^+$ cells (Figure 2C & D), in a pathway compatible with endosome-mediated uptake. However, the slight but consistent size difference observed between T22-GFP-H6^{lactis} and T22-GFP-H6^{coli} (Figure 1), and the lowest specific fluorescence but higher penetrability of T22-GFP-H6^{lactis} (Figure 2) confirm that T22-GFP-H6 building blocks are distinctively organized in these bacterial cell factories. When analysing data from T22-GFP-H6 variants produced in several *E. coli* strains, we previously found a negative relationship between fluorescence emission and cell penetrability of the resulting nanoparticles [46]. Since T22-GFP-H6^{lactis} fits in this T22-GFP-H6^{coli} model (Figure 2B) indicates that the impact of protein conformation (or the oligomeric organization) on the material performance is irrespective of the given protein production platform used for biofabrication.

Furthermore, T22-GFP-H6^{lactis} efficiently accumulates in tumor upon systemic administration (Figure 3), escaping from renal clearance and proving the stability of the material and the good performance *in vivo* of the tumor homing peptide T22, as folded in *L. lac-*

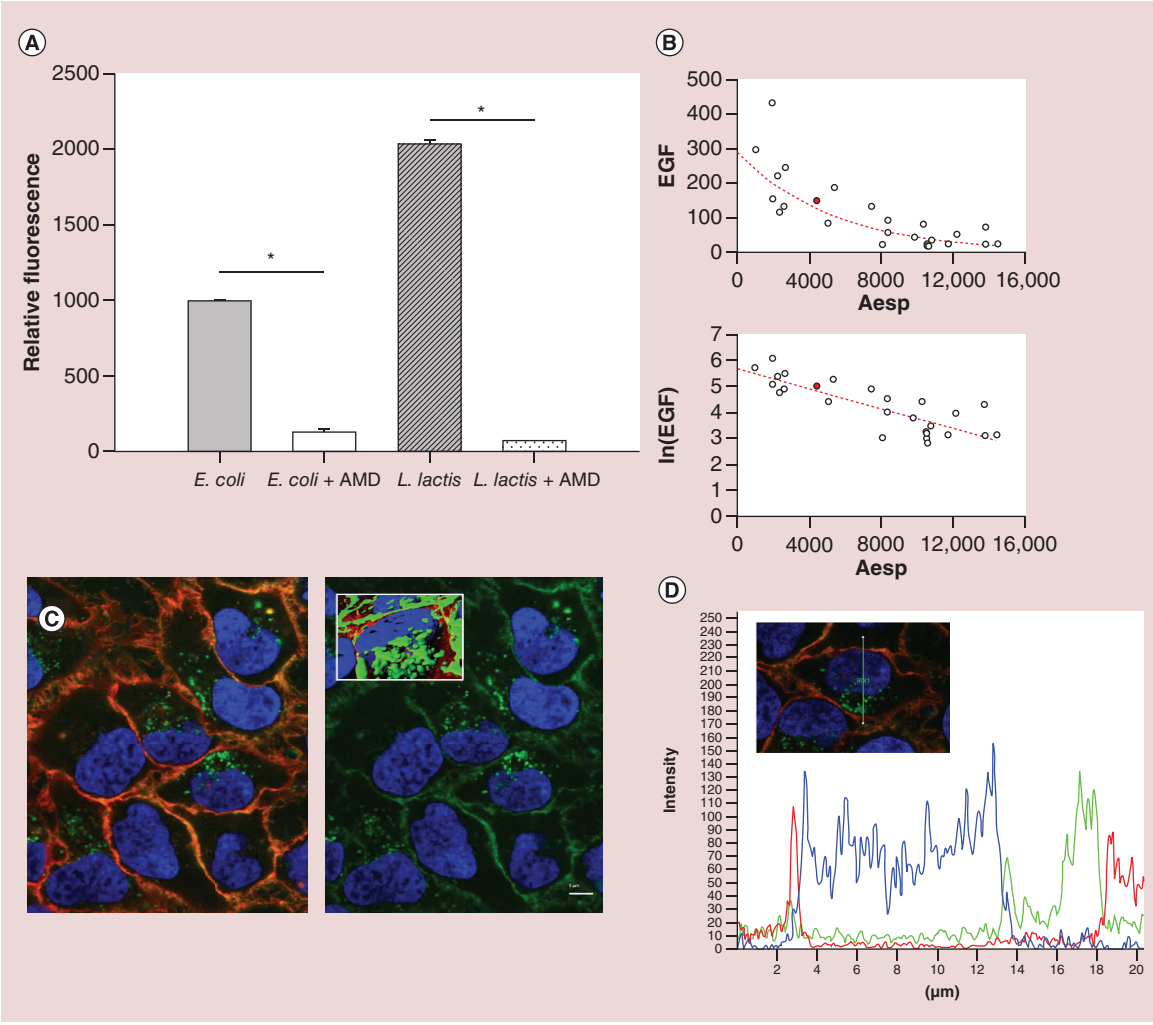

Figure 2. Cell penetrability of T22-GFP-H6^{lactis} nanoparticles into CXCR4+ HeLa cells. (A) Protein amounts internalized into cells depending on the cell factory used for production, and uptake inhibition promoted by the natural CXCR4 ligand AMD3100 (AMD). Intracellular fluorescence was corrected by specific fluorescence to render values representative of protein amounts. Asterisk indicates significant differences ($p \leq 0.05$). **(B)** Internalization efficiently represented versus specific fluorescence (as raw data, top and as logarithm, bottom) of T22-GFP-H6^{lactis} nanoparticles produced in *L. lactis* (red dots) compared with the same materials produced in several *E. coli* strains, as previously reported [46]. **(C)** Confocal images of HeLa cells exposed to 500 nM of *L. lactis* T22-GFP-H6^{lactis} nanoparticles for 24 h. Cell membranes are labeled in red and nuclei in blue. Green signals correspond to fluorescent nanoparticles. In the inset, a 3D Imaris reconstruction shows the accumulation of the material in the cell cytoplasm. This precise intracellular localization is confirmed by a fluorescence intensity profile of a representative cell exposed to nanoparticles. **(D)** The high penetrability of the material from *L. lactis* combined with its low fluorescence emission fitted very precisely in the model we had formerly generated to explain such inverse relationship (Figure 2B), supporting the concept that penetrability is linked to the precise oligomeric architecture of the materials that can impact, in its own, via conformation, on the fluorophore performance. T22-GFP-H6^{lactis} nanoparticles accumulated in the cytoplasm of target cells in absence of any detectable toxicity (Figure 2C & D).

tis. However, at difference from T22-GFP-H6^{coli} that was only observed in tumor and metastasis, T22-GFP-H6^{lactis} is also found, at minor extend, in liver (but not in other nontarget organs). Although this fact is of course undesired, the accumulation in tumor of T22-GFP-H6^{lactis} is more than two-fold than in the liver. In clinical oncology, approved and currently administered drugs such as antibody drug conjugates (e.g., trastuzumab emtansine), majorly accumulate in liver (80%), while only 1% of the injected dose reaches the tumor [67].

In this regard, the variant biodistribution map does not preclude the potential use of T22-GFP-H6^{lactis} as vehicle for drug delivery in colorectal cancer, but it indi-

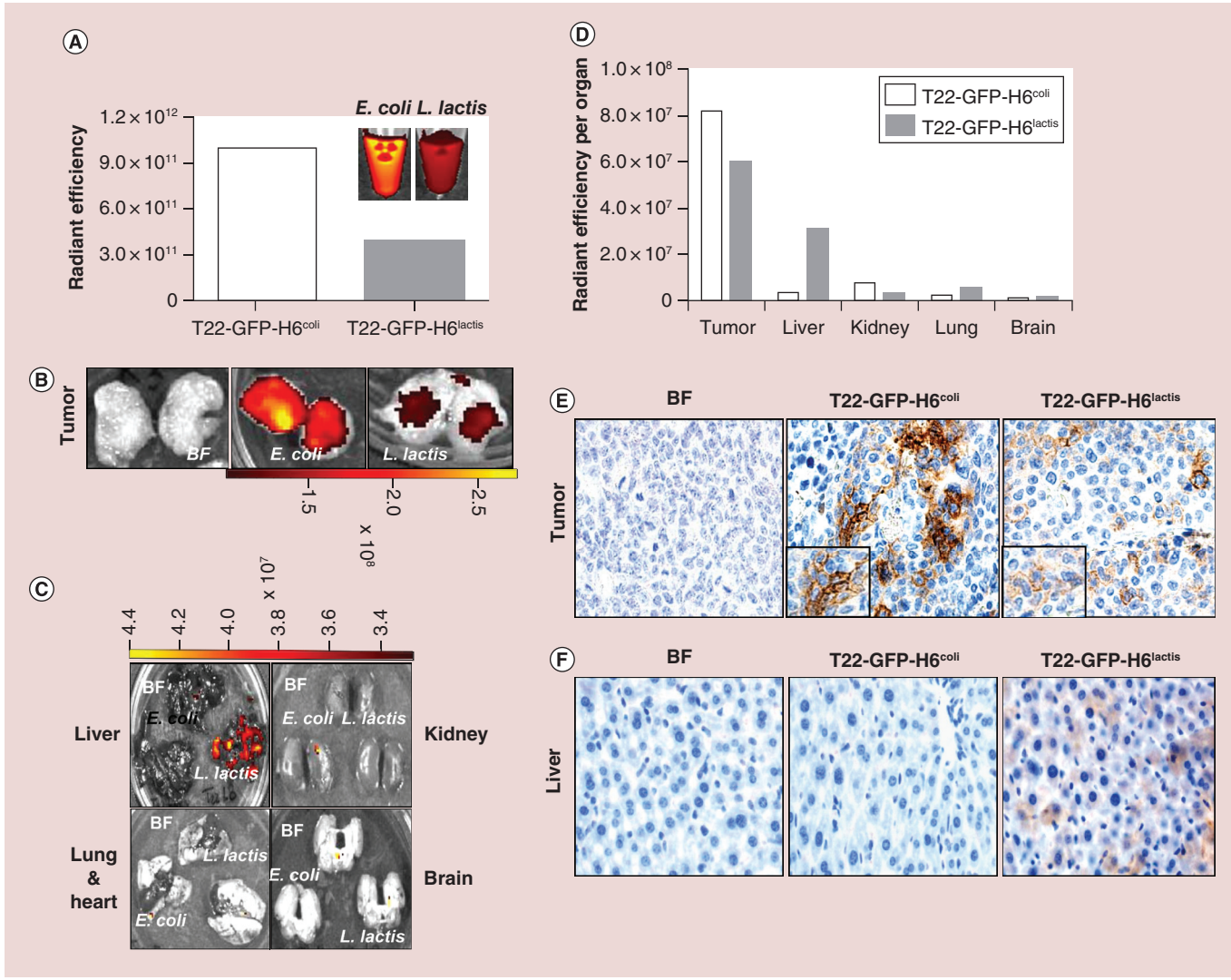

Figure 3. Comparative biodistribution of T22-GFP-H6 nanoparticles in a CXCR4⁺colorectal cancer mouse model. **(A)** Representative fluorescence (arbitrary units) recording images and quantitation of specific GFP emission signal of each nanoparticle at the same concentration by the IVIS® spectrum system. **(B)** *Ex vivo* tumor fluorescence imaging (FLI) at 5 h postadministration of 500 μg/mouse dose of each T22-GFP-H6 variants (nanoparticles produced in either *E. coli* or *L. lactis*). Note the enhanced green fluorescence associated with nanoparticle accumulation in tumors produced in *E. coli* as compared with *L. lactis* nanoparticles. BF is empty buffer. **(C)** *Ex vivo* determination of GFP fluorescence signal in relevant organs such lung, brain, kidney and liver. No fluorescence was observed in any organ except for the liver of mice administered with T22-GFP-H6^{lactis}. **(D)** Quantitation of *ex vivo* fluorescence emission (arbitrary units) in sectioned tumors and organs and expressed in radiant efficiency. Crude fluorescence values were corrected by the specific emission of each protein for comparative purposes. **(E)** Anti-GFP immunostaining showing membrane and cytosolic localization of T22-GFP-H6 variants in tumors which was absent in control animals injected with buffer (400× magnifications). **(F)** The presence and location of the T22-GFP-H6 lactic variant in liver tissue sections were demonstrated by anti-GFP immunohistochemistry (400× magnification).
GFP: Green fluorescent protein.

cates a peculiar oligomerization of the building blocks that influences targeting or promote passive accumulation, though conferring particular biomechanical properties. Interestingly, T22-GFP-H6^{coli} produced in an *E. coli* strain lacking DnaK chaperone, which have a key role in the *E. coli* quality control system, also show an aberrant distribution pattern, with a slight and unexpected accumulation in brain. In this context, the comparison of the stress responses of *L. lactis* and *E. coli* has prompted researchers suggesting different roles of chaperones among these species [64], and in fact, the quality controls of these two bacterial species show remarkable differences [68]. Then, the distinguishable biodistribution of the material observed here depending of the bacterial source suggests again a critical role of the protein quality control in defining the final fate of T22-GFP-

H6 nanoparticles upon systemic administration. This might be due to a different conformational spectrum of nanoparticle variants depending on the quality control network of the used cell factory, which can slightly influence the availability of T22 for cross-molecular contacts or some subtle architectonic properties of nanoparticles that might result in morphometric modifications leading to secondary accumulation in non-target organs such as liver. Irrespective of this fact, the biological fabrication of tumor-homing or other materials with biomedical properties in the food-grade bacterium *L. lactis* opens exciting opportunities for the delivery of these materials upon purification, as isolated protein preparations, or alternatively, through the administration of producing living cells by oral or intranasal routes already explored for this particular microbial cell factory and showing very promising potentialities in clinical trials [69]. This last approach, to be yet tested with cell-targeted protein constructs, would allow the more effective treatment of conditions such as inflammatory conditions of the digestive system in which sustained mucosal delivery is particularly appropriate.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All procedures related to animal handling were approved by the Hospital de Sant Pau ethical committee in accordance with Institutional guidelines.

Executive summary

- *Lactococcus lactis*, a food-grade lactic acid bacteria specifically exploited for the biofabrication of soluble protein species has been revealed here as a good producer of complex, self-assembling protein nanoparticles.
- Despite the robustness of the biological platform, the nanoscale architecture and biodistribution in colorectal cancer mice models reveals significant differences when compared with materials produced in *E. coli*. This fact supports a mechanistic impact of the protein quality control network on the systems level performance of the resulting product, which it is of critical relevance for the design of emerging, protein-only, nanostructured materials in biomedicine.
- Opening an exciting way for the production of high-quality, biologically safe protein nanoparticles with therapeutic interest, the present findings emphasize the suitability of *L. lactis* as a key player among the demanded, value-added cell factories for smart biomaterials.

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