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Short communication

Protein-only, antimicrobial peptide-containing recombinant nanoparticles with inherent built-in antibacterial activity

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ABSTRACT

The emergence of bacterial antibiotic resistances is a serious concern in human and animal health. In this context, naturally occurring cationic antimicrobial peptides (AMPs) might play a main role in a next generation of drugs against bacterial infections. Taking an innovative approach to design self-organizing functional proteins, we have generated here protein-only nanoparticles with intrinsic AMP microbicide activity. Using a recombinant version of the GWH1 antimicrobial peptide as building block, these materials show a wide antibacterial activity spectrum in absence of detectable toxicity on mammalian cells. The GWH1-based nanoparticles combine clinically appealing properties of nanoscale materials with full biocompatibility, structural and functional plasticity and biological efficacy exhibited by proteins. Because of the largely implemented biological fabrication of recombinant protein drugs, the protein-based platform presented here represents a novel and scalable strategy in antimicrobial drug design, that by solving some of the limitations of AMPs offers a promising alternative to conventional antibiotics.

Statement of Significance

The low molecular weight antimicrobial peptide GWH1 has been engineered to oligomerize as self-assembling protein-only nanoparticles of around 50 nm. In this form, the peptide exhibits potent and broad antibacterial activities against both Gram-positive and Gram-negative bacteria, without any harmful effect over mammalian cells. As a solid proof-of-concept, this finding strongly supports the design and biofabrication of nanoscale antimicrobial materials with in-built functionalities. The protein-based homogeneous composition offer advantages over alternative materials explored as antimicrobial agents, regarding biocompatibility, biodegradability and environmental suitability. Beyond the described prototype, this transversal engineering concept has wide applicability in the design of novel nanomedicines for advanced treatments of bacterial infections.

1. Introduction

The efficacy of antibiotics has been hampered by the increasing incidence of multi-resistant bacterial infections [1]. In this context, naturally occurring cationic antimicrobial peptides (AMPs) are of a special interest because of their potential as alternatives to conventional antibiotics [2–4]. Being an important functional arm of the innate immune system they act as a first line of mucosal defence against a broad spectrum of microorganisms [5]. AMPs are rather short, with a total molecular mass between 2 and 9 kDa. The cell lytic activity of AMPs is associated to pore formation in cell membranes [6–8]. This occurs by the selective peptide binding to negatively charged cell surfaces and the subsequent membrane permeabilisation [9]. For some AMPs, alternative internal targets have been also identified, which might show multiple modes of action apart from the main lytic activity [6,10,11]. Although AMPs have been successfully incorporated into topical

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drug formulations, their applicability in systemic therapies remains a challenge due to their low solubility, limited stability and a rapid metabolic excretion favoured by their low molecular mass.

In an attempt to increase AMP performance, protein engineering has allowed generating new or more effective AMP variants with improved biological activity and higher clinical opportuneness [12–15]. Among them, GWH1 is particularly interesting. Folding as an amphipathic helix, it shows high antimicrobial activity and low haemolytic potential [16]. In addition, GWH1 also exhibits antitumor activities both *in vitro,* over cultured cancer cells, and *in vivo,* in animal cancer models [17]. We wondered if the cationic nature of GWH1 could be exploited to prompt the formation of protein-only nanoparticles while keeping its membrane lytic activities. Cationic peptides, when fused to the amino terminus of His-tagged proteins, promote symmetric cross-protein interactions and the formation of regular oligomers ranging between 12 and 80 nm [18]. These nanoparticles are fully stable *in vivo* upon systemic administration and useful as vehicles for targeted drug delivery and imaging [18–20]. So far, GWH1 has been solely produced by chemical synthesis. However, obtaining this peptide as recombinant self-assembling proteins for nanoparticle formation might largely expand its functional versatility and biological applicability. In addition, this would represent a new biological platform for the design of antimicrobial nanomaterials, that solely based on proteins are to be environmentally friendly, and exhibit higher biocompatibility and wider structural and functional versatility than those based on metals [21], currently under intense exploration [22–24]. Investigating this possibility, protein-only nanoparticles empowered by GWH1 have been generated and successfully characterized as functional nanoscale materials with built-in antibacterial properties.

2. Materials and methods

2.1. Protein design, production and purification

Recombinant pET22b-derivatives encoding proteins GWH1-GFP-H6, T22-GWH1-GFP-H6 and their parental GFP-H6 (Fig. 1A) were designed in-house and produced by GeneArt. T22-GFP-H6, used as control, has been described in detail elsewhere [20]. *Escherichia coli Origami B* (BL21, OmpT$^-$, Lon$^-$, TrxB$^-$, Gor$^-$, Novagen) cells were transformed by heat-shock and cultured in 2 l shaker flasks with 500 ml of LB medium [25] containing 100 μg/ml ampicillin, 15 μg/m kanamycin, and 12.5 μg/ml tetracycline at 37 °C. The induction of recombinant gene expression was done at an OD between 0.5 and 0.7 by the addition of 0.1 mM isopropyl-β-thio galactopyronaside (IPTG). Then, bacterial cells were kept growing overnight at 20 °C. After sedimentation at 5000 g (4 °C, 15 min), the cell pellet was resuspended in Wash buffer (Tris 20 mM, pH 8.0, NaCl 500 mM, imidazole 10 mM) with 0.5% Triton X-100 (Roche Diagnostics GmbH) and ethylenediamine tetra-acetic acid-free protease-inhibitor (Complete EDTA-Free, Roche) for T22-GWH1-GFP-H6 and in Wash buffer with complete EDTA-Free for GWH1-GFP-H6. Cells were then disrupted by sonication (2 rounds of 10 min at 10% amplitude and 1 round of 10 min at 15% of amplitude) and soluble fractions separated by centrifugation for 45 min (15,000g at 4 °C). Protein purification was carried out with a His tag affinity chromatography using HiTrap Chelatin HP 1 ml column (GE Healthcare) in an AKTA purifier FPLC (GE Healthcare).

After filtering the soluble fraction, samples were loaded onto the column and washed to remove Triton X-100 with 60 column volumes of Wash buffer for T22-GWH1-GFP-H6 and 10 column volumes for GWH1-GFP-H6. Elution was achieved by a linear gradient of 20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0. The purified fractions were collected and analyzed by TGX gel chemistry and Western Blotting with anti-His monoclonal antibody (Santa Cruz Biotechnology Inc). Proteins were finally dialyzed against sodium bicarbonate buffer with salt (166 mM $NaHCO_3$ pH 8 + 333 mM NaCl) overnight at 4 °C. Protein purity and integrity were checked by mass spectrometry (MALDI-TOF), and protein amounts were determined by Bradford's assay [26]. Protein production has been partially performed by the ICTS "NANBIOSIS", more specifically by the Protein Production Platform of CIBER-BBN/ IBB (http://www.nanbiosis.es/unit/u1-protein-production-platform-ppp/).

2.2. Nanoparticle Characterization

Volume size distribution of unassembled proteins and resulting nanoparticles was determined by Dynamic Light Scattering (DLS) at 633 nm in a Zetasizer Nano ZS (Malvern). For that, each sample was measured three times in saline sodium bicarbonate buffer. The GFP fluorescence emission of the materials was determined in triplicate in a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at 510 nm using an excitation wavelength of 488 nm. For that, protein samples were diluted in the corresponding storage buffer to 1 mg/ml, in a final volume of 100 μl.

To characterize protein nanoparticles, a drop of 3 μl of GWH1-GFP-H6 was directly deposited on silicon wafers (Ted Pella Inc) for 5 min, excess blotted with Whatman filter paper number 1 (GE Healthcare), air dried, and observed without coating with a high resolution in-lens secondary electron detector in a field emission scanning electron microscope (FESEM) Zeiss Merlin (Zeiss) operating at 0.5 kV. A qualitative approach of size and shape at ultrastructural level of GWH1-GFP-H6 nanoparticles was evaluated at this nearly native state.

2.3. Ultrastructural analysis

To determine the ultrastructural effects of nanoparticles over bacterial cells, 1 ml of cultures of each *E. coli* and *S. aureus* growing in two media (LB and MHB) were incubated 24 h alone or with protein (2 μM) in 24-well plates with a coverglass. Coverglasses and medium were processed for scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively, by adjusting standard procedures developed for bacterial analysis [27–30]. Briefly, coverglasses with each sample were fixed in 2.5% (v/v) glutaraldehyde (Merck) in 0.1 M PB for 12 h at 4 °C, post-fixed in 1% (w/v) osmium tetraoxide (TAAB Lab) containing 0.8% (w/v) potassium hexocyanoferrate (Sigma-Aldrich) in PB, and, then, dehydrated in graded series of ethanol, dried with CO_2 in a Bal-Tec CPD030 critical-point dryer (Balzers), mounted in stubs, and observed in a FESEM Zeiss Merlin as previously described for nanoparticles. Supernatants of each sample were fixed, post-fixed, and dehydrated as for SEM, embedded in Spurr resin (Sigma-Aldrich), and polymerized at 60 °C for 48 h. Ultrathin sections (70 nm) of selected areas of semi-thin sections (1 μm) were obtained with Leica ultracut UCT microtome (Leica Microsystems, Germany), placed on 200 mesh copper grids, and contrasted with conventional uranyl acetate (30 min) and lead citrate (5 min) solutions. For the qualitative approach, 10 randomly selected areas of each grid were observed with a TEM Jeol JEM-1400 equipped with a CCD Gatan ES1000 W Erlangshen camera.

2.4. Determination of the antibacterial activity

Wild strains of two Gram-positive (*Staphylococcus aureus* ATCC-29737 and *Micrococcus luteus* ATCC-10240) and two Gram-negative bacterial species (*Escherichia coli* ATCC-25922 and *Pseu-*