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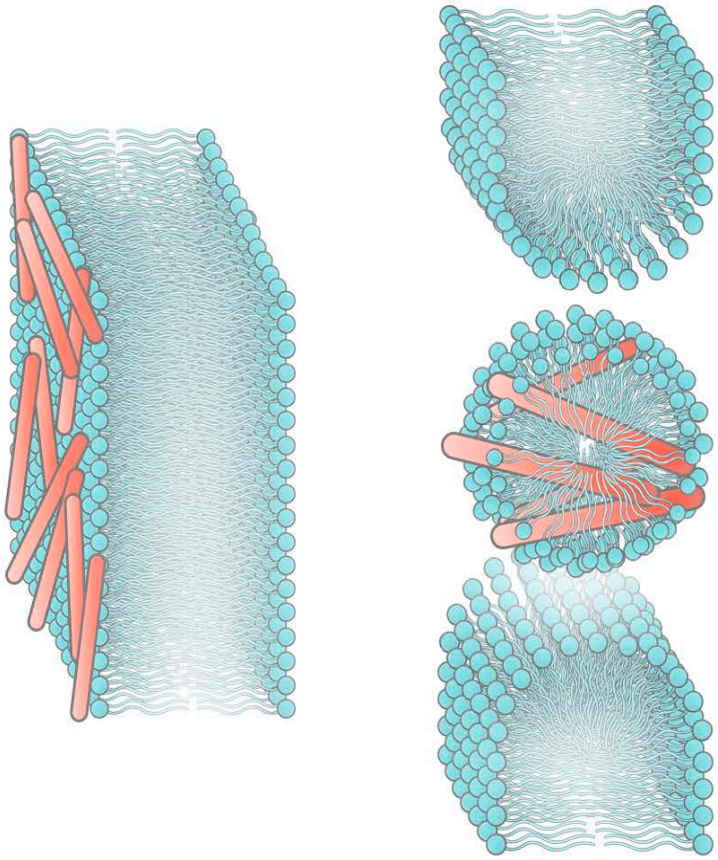

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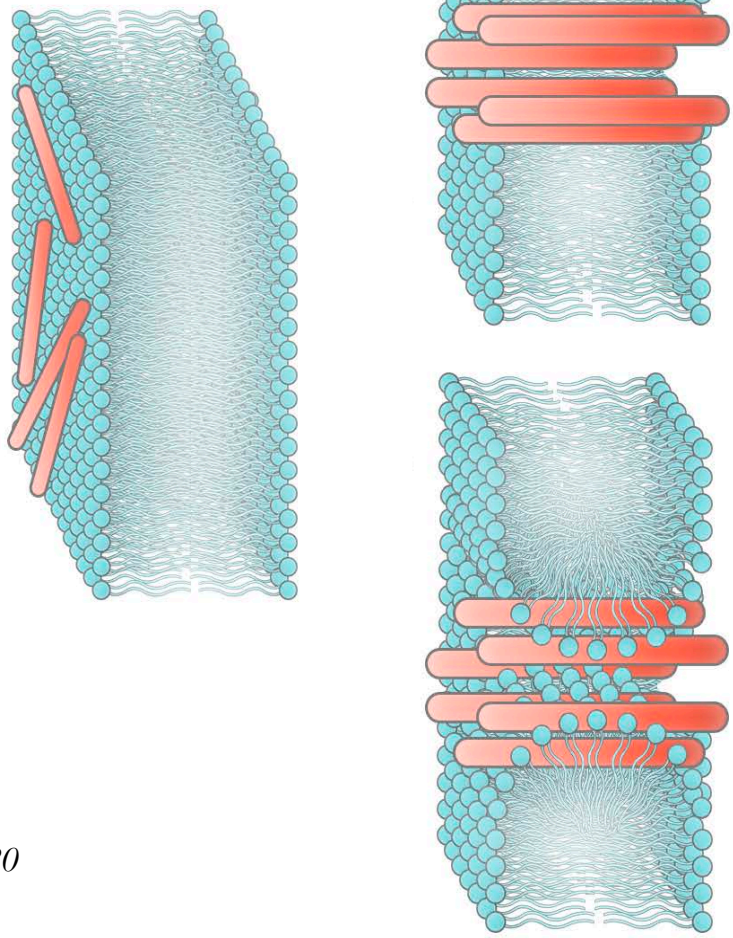

Ramon Roca Pinilla
PhD Dissertation

Directors:
Anna Arís Giralt
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PhD Dissertation

DEVELOPMENT OF A NEW GENERATION OF ANTIMICROBIAL PROTEINS BASED ON A VERSATILE NANOPARTICULATED FORMAT AND MULTIDOMAIN STRUCTURE

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PhD Dissertation, 2020

Department of Biochemistry and Molecular Biology

Dissertation submitted by Ramon Roca Pinilla as partial fulfilment of the requirements for the PhD Degree in Biochemistry, Molecular Biology and Biomedicine by the Autonomous University of Barcelona

Approval of the PhD directors:

Anna Arís Giralt

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This work has been mainly conducted in the Institute of Agriculture and Food Research and Technology, under the supervision of the doctors Anna Arís Giralt and Elena Garcia Fruitós. Part of the research has been performed at the University of Calgary, Alberta, Canada.

PhD advisor at UAB: David Reverter Cendrós

Al meu germà.

“The power of imagination makes us infinite.”

— John Muir

Contents

Summary

For most of human history, pathogens have been a leading cause of death and illness. Although we have attained the ability to treat them easily, thanks to the discovery of antibiotics, the widespread overuse and misuse of antimicrobial drugs have accelerated the appearance of antimicrobial resistances (AMRs). Because AMRs have rendered most antimicrobial drugs ineffective, the development of alternative approaches is more necessary than ever before. Host defense peptides (HDPs) have been proposed as blueprints for the generation of new antimicrobials to fight AMR infections. Despite this, most HDPs are produced by chemical synthesis, which is expensive, unsustainable, and difficult to scale-up. Alternatively, their recombinant production is very appealing but still challenging. HDPs are highly susceptible to degradation and are generally toxic to the recombinant host. However, inclusion bodies (IBs), which are protein aggregates that usually happen during recombinant production, can be used to allow HDP formation inside the host without being harmful. Also, the construction of chimeric proteins could be a strategy for successful recombinant expression of small peptides. In this context, this dissertation explores several new strategies for the recombinant production of HDPs. We tried leucine zippers as potential domains to drive the recombinant production of HDPs to the insoluble fraction and improve IBs protein quality. After that, we developed several antimicrobial multidomain proteins based on the fusion of different peptides and proteins from innate immunity. Because we also used leucine zippers with these constructs, they could be produced effectively – without toxicity to the microbial cell factory. Moreover, when needed, we were able to recover soluble antimicrobials from IBs using a mild, non-denaturing protocol. Overall, we demonstrated that these constructs have a broad-spectrum antimicrobial action against multi-drug resistant (MDR) bacteria, in both the soluble and IB format, and that they could trigger the release of IL-8 within a range of potential immunomodulatory properties. These outcomes invited us to use our constructs in the biofunctionalization of self-assembled monolayers to avoid biofilm formation. We observed that the chimeric proteins could be anchored to these materials and avoid biofilm growth. In sum, these results reinforce multidomain antimicrobial proteins as potential antimicrobial alternatives with immunomodulatory properties and open up the possibility for many applications of this new generation of antimicrobial protein nanoparticles as well as their soluble analogs.

Resum [Catalan translation]

Durant la major part de la historia humana, els patògens han estat una de les principals causes de morts i malalties. Gràcies al descobriment dels antibiòtics hem aconseguit tractar aquestes malalties amb facilitat, però el seu mal ús ha accelerat l'aparició de resistències als antimicrobians (AMRs). Atès que les AMRs han provocat que la majoria de fàrmacs antimicrobians siguin ineficaços, el desenvolupament de tractaments alternatius és mes necessari que mai. Els pèptids de la defensa del hoste (HDPs) han estat proposats com a models per la generació de nous antimicrobians per lluitar contra les infeccions AMR. Tot i així, la majoria d'HDPs és produeixen mitjançant la síntesi química, un procés que és car, insostenible i difícil d'escalar. Alternativament, la producció recombinant d'HDPs és molt atractiva però complicada, ja que són pèptids altament susceptibles de ser degradats i són tòxics per l'hoste recombinant. Malgrat això, els cossos d'inclusió (IBs), que són agregats de proteïna formats durant els processos de producció recombinant, es poden utilitzar com a format alternatiu al de la proteïna soluble per permetre la producció d'HDPs dins l'hoste sense efectes tòxics. D'altra banda, la construcció de proteïnes quimèriques podria ser una estratègia per expressar pèptids petits amb èxit. En aquest context, aquesta tesi explora diverses estratègies per la producció recombinant d'HDPs. Per una banda, hem explorat l'ús de les cremalleres de leucina com a dominis potencials per fomentar la producció recombinant d'HDPs en la fracció insoluble i per augmentar la qualitat de la proteïna recombinant dels IBs. A més a més, hem desenvolupat diverses proteïnes antimicrobianes multidomini basades en la fusió de diferents pèptids HDP i proteïnes de la immunitat innata. Com que també hem utilitzat cremalleres de leucina en aquests constructes, es poden expressar de manera efectiva – sense toxicitat per la cèl·lula productora. A més, en cas de necessitat, podem recuperar antimicrobians solubles a partir dels IBs gràcies a un protocol de solubilització suau i no desnaturalitzant. En conjunt, hem demostrat que aquests constructes tenen un ampli espectre d'acció antimicrobiana contra bacteris multi resistents (MDR), tant en el format soluble com en el format d´IB. És més, els constructes també són capaços d'estimular l'alliberament de IL-8 dins d'un potencial rang de propietats immunomoduladores. Aquests resultats ens han convidat a utilitzar les nostres proteïnes en la biofuncionalització de monocapes autoacoblants per evitar la formació de biofilms, i hem observat que aquestes proteïnes poden ancorar-se a aquests materials i evitar el creixement de biofilms. En resum, aquests resultats reforcen les proteïnes antimicrobianes multidomini com a potencials alternatives antimicrobianes amb propietat immunomoduladores.

Introduction

Antimicrobial resistance

Antimicrobial resistance (AMR) is a global public health crisis.[1] Our ability to treat common infectious diseases is challenged by the overuse and misuse of antibiotics and the lack of new alternatives to common antibiotics. Every year across Europe and the US, antibiotic resistant bacteria (ARB) are the cause of 50,000 deaths and a total of 700,000 globally.[2] A situation that will be even worse in the future. The consumption of antibiotics will rise globally by 67 % in 2030 and nearly double in Brazil, Russia, India, South Africa and China – among the most populated countries in the world.[3] Yet the appearance of new resistances is accelerating. In 2050, antibiotic resistances are estimated to cause around 10 million deaths, making them the leading cause of death in the world (Figure 1).[2,4]

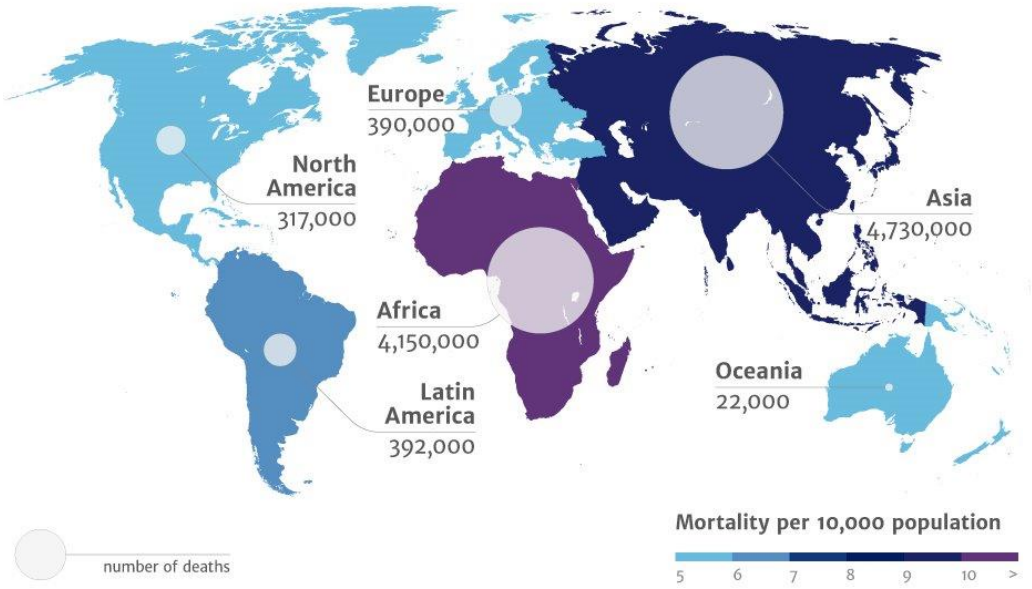

Figure 1. An estimate of deaths caused by AMR in the world in 2050. Source: *Review on Antimicrobial Resistance*.[2]

The development of resistance to new antimicrobials by microorganisms is inevitable due to selective pressure, a natural evolutionary process.[5] However, both human and veterinary medicine are accelerating the selection of resistant bacteria.[6] Yet in most countries in the world, over 50 percent of antibiotics are used not in humans but in livestock to prevent infection and to slightly increase growth. This widespread use of non-therapeutic antibiotics promotes antibiotic resistances that also affect humans.[6,7] Without any firm action, the

uncontrolled spread of AMR will also have a significant economic impact on a global scale.[8] So measures to curb the spread of existing AMRs and novel therapeutics are on demand.[1]

The burden of AMR

Awareness of antibiotic resistance took place soon after the discovery of these compounds. Sir Alexander Fleming – the discoverer of the first antibiotic substance – already noticed the danger from underdosing. In his words, "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them".[9] However, these early warnings were largely neglected. Antibiotic use increased, and so did AMRs.[10] But the realization of the problem became evident when many different multidrug-resistant (MDR) infectious organisms appeared about 50 years ago.[10] Many infectious agents that could be successfully treated with several drugs have acquired resistance to most, and in some cases, nearly all of the available compounds.[2,11]

But how does antimicrobial resistance happen? The origins of AMRs can be found in the environment. We now know that resistance genes were already present in samples from ancient permafrost, isolated caves, long preceding the mass production of antibiotics.[5] Most of the antibiotics used clinically or in agriculture derive from metabolites produced by a group of bacteria called Actinomycetes.[12] Streptomycin, tetracycline, chloramphenicol, erythromycin or the last-resort medication vancomycin are all "natural products" made by these microorganisms. Logically, these organisms must also be resistant to their antibiotics, or their toxic compounds would otherwise kill them.[5] Antibiotic producers, therefore, may have been the source for many AMR genes.[12,13]

Bacteria can acquire genes from the parent microorganism during division, which is called vertical gene transfer. Modern-day overuse puts microbes under increased selection pressure. That means that through the process of Darwinian selection, bacteria that already have resistance genes have increased survival fitness, passing these genes to their offspring. What makes bacteria unique, though, is that they can also pick up genes from their community, also known as horizontal gene transfer (Figure 2).[12] Thus, the environment is a vast reservoir of AMR genes, accessible by members of the microbial community. As an example, we know that resistance gene transfer in the human intestinal microbiome is pervasive.[14] Therefore, if AMR bacteria that come from the food supply enter the intestinal tract of consumers, they will have the opportunity to share the genes with the human microbiota.

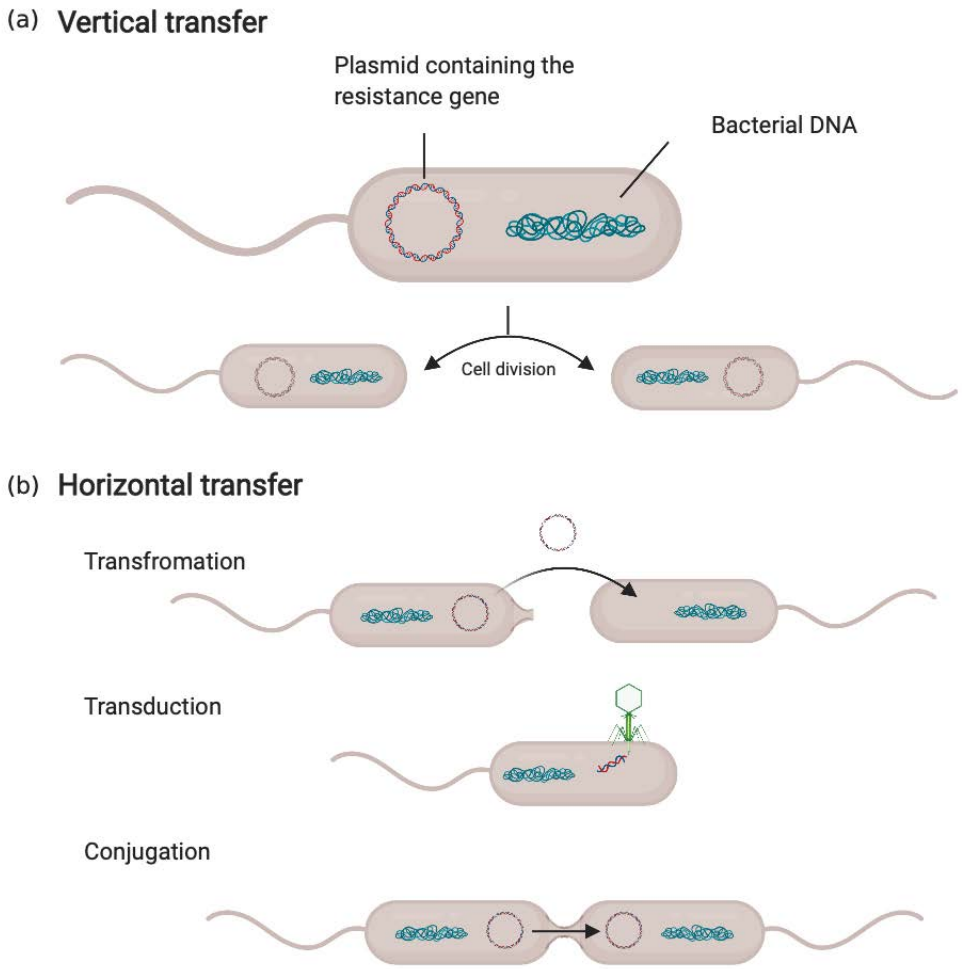

Figure 2. Mechanisms of gene acquisition by bacteria. (a) During replication, bacteria can transfer resistance genes from a parent cell to the next generation (vertical gene transfer). (b) There are three major mechanisms of horizontal gene transfer: Bacteria can uptake free DNA from the environment (transformation), a bacteriophage can transfer resistance genes from one bacterium to another (transduction), or two bacteria can share AMRs through cell-to-cell contact (conjugation).

As a consequence, hospitals are not anymore the only source of drug resistance.[10] Wherever antimicrobials are used, there are pools of resistance, including farms, the food supply chain, and the environment in general. And this is all the aftermath of sewage pollution, pharmaceutical industry waste, and manure drainage from farms. Because bacteria move their genes relatively effortlessly within and between humans, animals, and the environment, AMR appearance in one sector is reflected in the others as well.[6,15] What follows is that antimicrobial resistance is an ecological problem, defined by complex interactions between microbial populations, disturbing human, animal, and environmental health (Figure 3). Because of its complexity and multi-sectorial nature, a coordinated effort of multiple health science approaches is needed to tackle AMR, such as the One Health approach.[1,16]

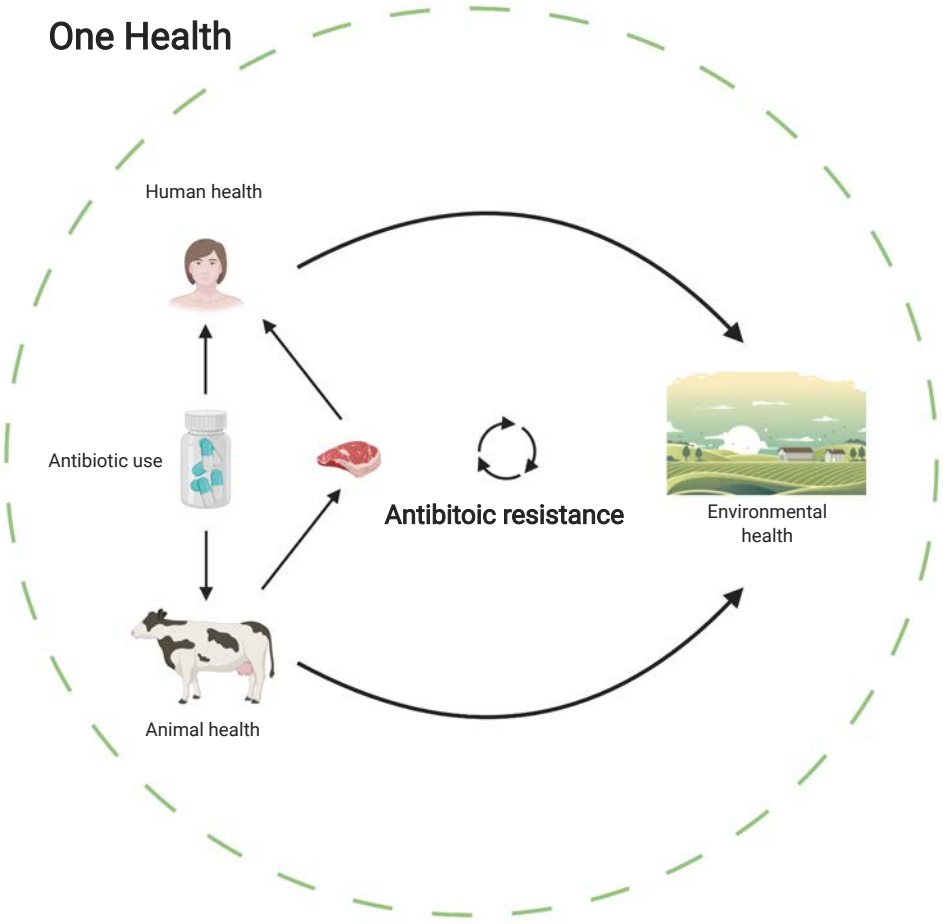

Figure 3. The ecological nature of AMRs. Transmission of antimicrobial resistance between humans, livestock and the environment. AMR is not only a human health problem, but also an environment and veterinary health issue. Adapted from ref.[16]

The health burden of infections due to bacteria with AMR can be challenging to estimate, especially in low- and middle-income countries, where there is limited access to essential antimicrobials and laboratory equipment, often with inadequate surveillance.[17] Although more work is needed to evaluate the exact impact of AMR, we have some estimates. In Europe, there are 131 infections per year with AMR bacteria per every 100,000 inhabitants, with around six of these cases being mortal. This is comparable to the burden of HIV, influenza, and tuberculosis in the same year together.[18] A burden that has doubled since 2007 and is highest in infants (less than one year) and older people (above 65 years old).[18] Although perhaps limited, these estimates make the strain of high AMR loads more apparent than ever and have forced many European countries to develop national plans to reduce AMR.[19–22]

In addition to the health costs, there are economic costs of AMRs, defined as the productivity losses due to the health problems and increased mortality of resistant infections and the

associated treatment costs.[23] Again, we rely on predictions to estimate the impact. One of these models, made by the World Bank, looked at the global economic impact of AMR in two scenarios, corresponding to a low AMR impact and high AMR impact. In both cases, a conservative approach based only on the effects on labor supply and livestock productivity was used, which probably miscalculated AMR's full economic effects. These simulations found that by 2050, annual gross domestic product (GDP) would likely fall by 1.1 %, compared to a "control" scenario with no AMR effects. This deficit would exceed $1 trillion annually after 2030. Instead, in the high AMR-scenario, the GDP decrease would be of 3.8 % by 2050, or $3.4 trillion by 2030.[8]

The costs of AMR on GDP would be worse than the 2008-2009 global financial crisis, at least in two ways. They would be felt during the period through 2050 from now, not just a couple of years. Furthermore, low-income countries would experience a significant dip in economic growth than wealthy countries, increasing economic inequality, since they have higher infectious disease prevalence and greater dependence on labor. Because of the ecological nature of the AMR problem, and because the majority of antibiotics are used on animals, livestock products are especially vulnerable. In low-income countries, livestock production would decrease 11 percent by 2050 in a high AMR-impact scenario.[8]

To date, action on AMR has been dangerously lacking.[8] Without proper AMR containment, we have already seen the health, well-being, economic, and environmental costs of AMR globally. Indeed, the burden of AMR is heavy, but it can be tackled.

New strategies to fight back antimicrobial resistances

The first logical step in reducing the burden of infection is to have good infection prevention strategies, avoiding antibiotic treatment and, thus, further spread of AMR.[2] This includes better hygiene and vaccines, which have a high potential to reduce the need for antibiotics. There is also room for improvement by optimizing the use of antimicrobials. Because food-producing animals consume most of the antibiotics, reducing antibiotic use in the livestock sector is vital.[2] Additionally, early and fast diagnosis can prevent or stop an outbreak and support better antibiotic prescriptions.[24] Finally, there is a need to incentivize research and development of new antimicrobials, which is within the scope of this thesis.[2,8,25] No major new class of antibiotic has been discovered since the 1980s, and too few antibacterial agents

are being developed to face the challenge of AMR.[25] But there are some new sources of antimicrobial therapies. Some alternatives that can be marketed within the next ten years are antibodies, probiotics, bacteriophages, lysins, peptides, proteins and enzymes, and immune stimulators.[26–28] Based on ongoing early and late-stage clinical trials, between 2020 and 2025 many of these alternatives are expected to be registered if they are successful: probiotics and lysins (2022), immunostimulants (2021&2022), bacteriophages (2022&2023), antibodies (2021-2025) and antimicrobial peptides and proteins (2022-onwards)(Figure 4).[29]

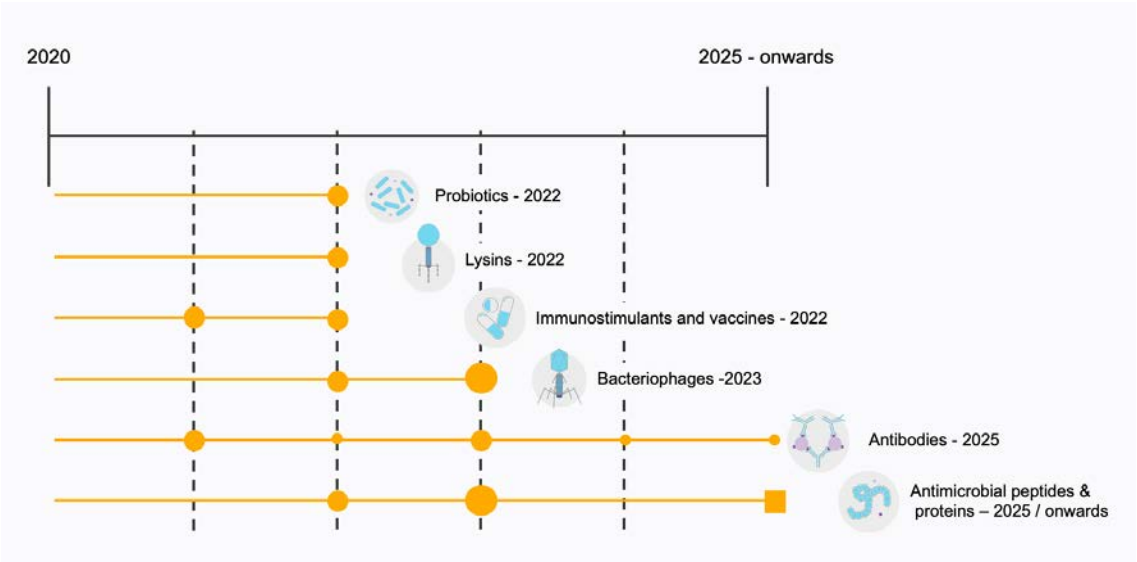

Figure 4. Timeline of the expected registration of alternatives to conventional antibiotics in the next years.[29] Dashed lines represent each year between the 2020 and 2025 period. Dots are timestamps of the earliest anticipated registration for various products under clinical trials, and a larger size symbolizes a higher number of registered products. The square illustrates different products against Gram-negative and Gram-positive bacteria that are still in preclinical phases. Icons for each alternative are from the *Review on Antimicrobial Resistance*.[2]

Antibodies

Antibodies are considered to have a strong scientific background and history of safe use, which makes them one of the alternatives most likely to have a significant clinical impact.[29] Antibodies could have the ability to bind to and inactivate a pathogen, its virulence factors, or its toxins (Figure 5).[29] They can be used as prophylactics or as treatments, holding particular promise in farm animals.[30] As an example, crops could be modified to produce therapeutic antibodies and administered through diet. Moreover, potent antibiotics can be covalently linked to antibodies and directed to a particular pathogen, increasing the effectiveness compared to giving the antibiotic alone.[31] The main limitations of antibodies are their production and delivery costs along with their narrow specificity, which limits them to a smaller market compared to broad-spectrum antimicrobials.[32] Among the possible

alternatives, antibodies are probably the first line of antimicrobials that will be first registered, since there are several phase 2 and 3 clinical trials ongoing.[29] And some have already been approved for their medical use, such as Bezlotoxumab, to prevent recurrent *Clostridium difficile* infections.[33]

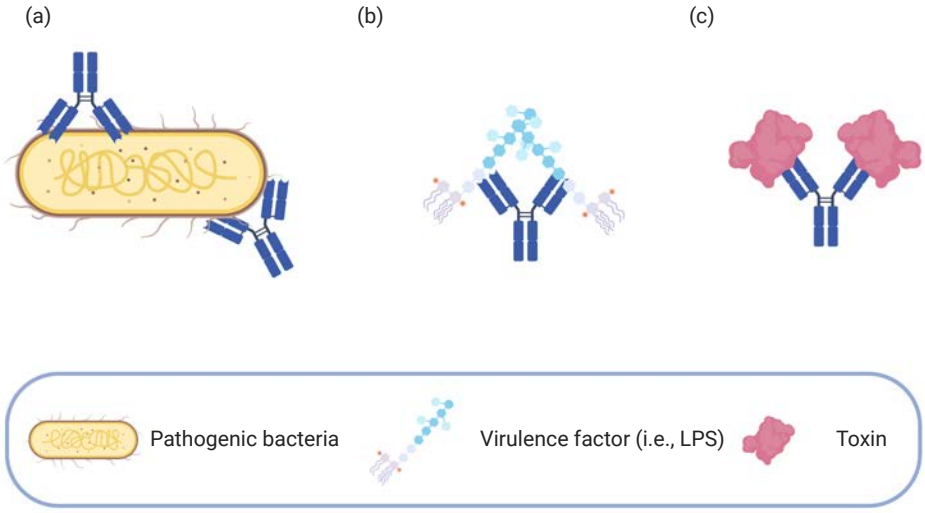

Figure 5. Antibody therapy for infectious diseases. (a) Antibodies can bind to pathogenic bacteria, (b) their virulence factors or (c) toxins, to inactivate them.

Probiotics

Probiotics are live microbial feed supplements, which can be used for the prevention and treatment of infections. How probiotics work depends on many factors, such as the bacterial strain used and dose. They can compete with pathogens either directly or indirectly.[34] For the direct competition, some probiotics secrete antimicrobial molecules or fight for similar nutrients. For the indirect competition, these beneficial microbes can lower pathogen colonization by stimulating the innate and adaptive immune responses as well as the function of the mucosal barrier. Their effect as treatments is quite limited and modest, though.[35–37] Probably, probiotics are better suited to prevent disease,[38,39] but are also in a good place, as there are several phase 2 and 3 clinical trials.[40,41] Still, more research is needed to understand their mechanism of action and how they might be used in combination with other therapies, such as bacteriophages or other antibiotics.[26,42]

Bacteriophages and lysins

Bacteriophages, as a type of virus able to infect and destroy bacteria, have the potential to replace antibiotics for some indications (Figure 6).[43,44] One advantage of bacteriophages is that they can be given in small doses since they replicate if their host bacterium is present. Moreover, they multiply and evolve during the treatment of an infection, with the potential to be deadlier to the pathogen and more specific, making them unique as a pharmaceutical product.[26] Moreover, bacteriophages can be used as wild-type or can be genetically engineered, adding new properties that could be advantageous.[45] Bioengineering can be used to overcome many of the challenges of wild-type phages, such as strain spectrum coverage, development of resistance by the bacteria, and rapid elimination by the patient.

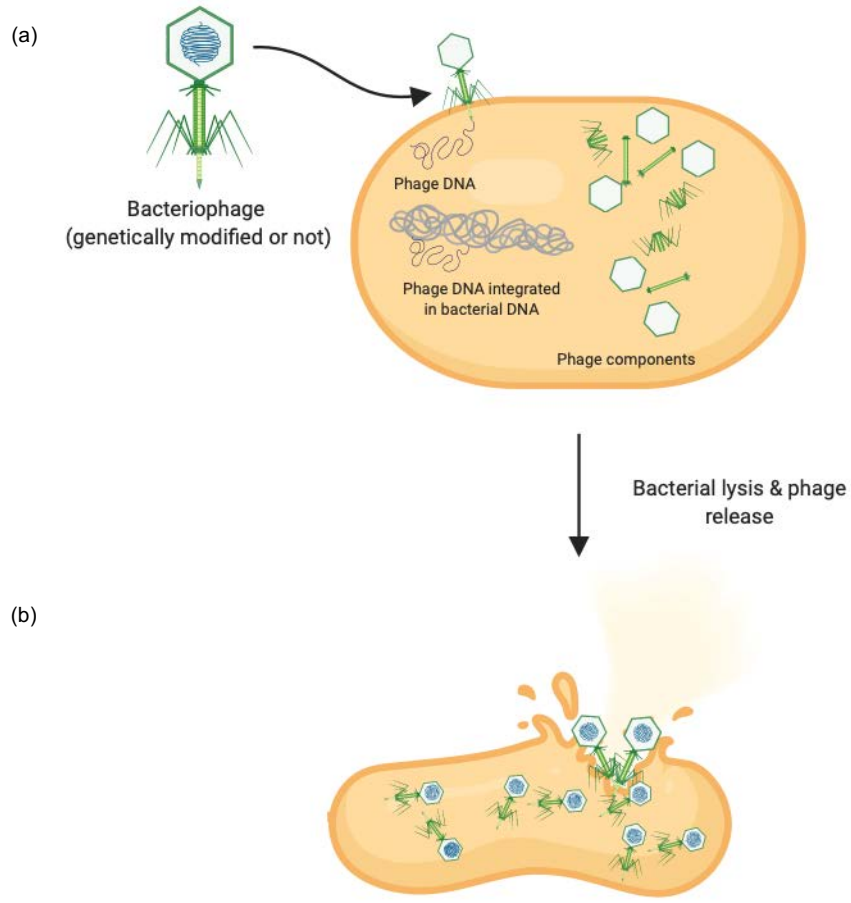

Figure 6. Bacteriophages as an alternative to conventional antibiotic therapy. (a) Wild-type or genetically modified bacteriophages target a specific bacterial strain. After that, the phage integrates its genetic material within the host genome, and then, for replication, starts using the host machinery. (b) Phage particles assemble, encapsidating their DNA, and eventually bursting the bacterial cell – killing it.

One of the advantages of bacteriophages can also be a substantial drawback. Phages are extremely specific, which might protect the host microbiome. But the narrow specificity also requires exact diagnostics to treat infections effectively. Therapeutic phages need to be precisely characterized, otherwise, the use of not well biologically and genetically defined lytic phages may lead to the expression of undesirable virulence factors as an adaptive response from the pathogenic bacteria to the phage infection.[46] Finally, since they are an "evolving" drug that self-replicates, bacteriophages are very hard to regulate.[47]

Lysins are enzymes naturally used by bacteriophages to destroy the cell wall of their target bacterium. These hydrolytic enzymes can cleave the peptidoglycan, the main component of bacterial cell walls.[48] Because of their direct antibacterial action, lysins are potential substitutes for antibiotics. They can also be used in combination with other medicines to reduce the bacterial load or weaken biofilms.[29] However, although they are very specific and resistance mechanisms are hard to develop, they have a short plasma life, are immunogenic, and potentially toxic.[49] Only a limited number of bacteriophages and lysins have entered clinical trials, and both require strict regulatory measures.[49,50]

Peptides

Antimicrobial peptides (AMPs) are small, amphipathic, and cationic peptides that are part of the innate immune defense.[51] Their modes of action by which they directly kill microbes are diverse. They may interfere with DNA and protein synthesis, protein folding, or disrupt bacterial membranes.[52] Moreover, they might also be able to modulate the immune response, helping to kill bacteria indirectly. To capture their multifunctional nature and their additional roles that go beyond antimicrobial activity, some researchers use the broader definition of host defense peptides (HDPs).[53] Although not as thoroughly tested as antibodies and bacteriophages, which have very strain-specific actions, they have the potential for broad-spectrum activity.[26] It is worth mentioning that not all peptides come from the innate immune defense, as is the case of bacteriocins. Bacteriocins are made by bacteria to inhibit the growth of closely related bacterial strains, and thus, are being studied as potential narrow-spectrum antimicrobials.[54] Yet AMPs, in general, are probably the last alternative to have a real impact since most clinical trials are still in early phases.[55,56]

Proteins and enzymes

The majority of antimicrobial proteins and enzymes with antimicrobial potential come from the immune system.[57] They usually contain more than 100 amino acids, being larger than their smaller counterparts, the AMPs.[27,28] Their modes of action are diverse. Some are lytic enzymes, and others bind to essential nutrients for survival or bind to specific microbial macromolecules. Furthermore, some might also have immunomodulatory activities.[28] Perhaps, the most notable member of this class of alternatives is lysozyme, an enzyme found in tears, saliva, and milk. Lysozyme is able to cleave the peptidoglycan from bacterial cell walls, which results in cell death, and it has been used to promote growth in livestock.[58] Another example is lactoferrin found in neutrophil granules and in epithelial cells. Lactoferrin hydrolyzes RNA, inhibiting retrovirus reverse transcription, and is also able to sequester free iron, which is an essential substrate required for bacterial growth. Moreover, lactoferrin binds to the bacterial cell wall, forming peroxides that ultimately lead to bacterial lysis. Its multiple mechanisms of action make lactoferrin not only antibacterial and antiviral but also an antiparasitic, anti-cancer, and an anti-allergic protein.[59]

Two more important examples of this diverse family of proteins would be ribonucleases (RNases) and secreted phospholipases A2 ($sPLA_2$). RNases are produced by circulating immune cells and by epithelial surfaces that have been challenged by microbes.[60] Like other antimicrobial peptides and proteins, RNases also have a net positive charge and can interact with bacterial membranes, which leads to membrane permeabilization and lysis.[61] Another exciting feature of RNases is that they are able to tie Gram-negative bacteria together, which might keep pathogens away at the infection site.[61] Instead, the antibacterial properties of $sPLA_2$ seem to be more effective against Gram-positive bacteria, because they lack an outer membrane.[62] They have a similar size as RNases – around 14 kDa – and are present in various body fluids from mammals, as long as snake, arachnid and insect venoms.[63,64] Nonetheless, their bactericidal mechanism of action is very different from that of RNases, and it depends on the effective hydrolysis of the phospholipid components of the cell membrane.[62]

We have discussed antibacterial proteins from the immune system of animals.[65,66] But the sources are varied and apparently unrelated, such as yeasts, marine bacteria, or plants.[67] What is possibly more important is that many of them show potent antibacterial activities against

different pathogens.[65,67] Still, more studies are needed to understand their safety, biocompatibility, and other bioactivities – such as immunomodulatory effects.

Immune stimulation

Immune stimulation can be used with antibiotic therapy since successful antimicrobial treatments rely on an adequate immune response. A strong immune response can substantially reduce treatment times, and therefore, the overuse of antibiotics – and the resulting selection of AMR bacteria.[68] There are two main categories of immunostimulants, specific and non-specific. Perhaps, the best-known class of specific immunomodulators are vaccines. Non-specific immunostimulants, unlike vaccines, stimulate the immune system components without any antigenic specificity and include a wide array of molecules, such as minerals, hormones, vitamins, or cytokines.[69–71] In general, immune stimulation refers to the non-specific type. However, many modulators of the innate immunity are probably unable to cure disease by themselves. In addition, immune modulation also has to be finely balanced so that it does not trigger an excessive immune response that ends in damage to the host or an immune response that cannot be turned off.[72]

Concluding remarks about the new strategies to fight back antimicrobial resistances

Alternatives to antibiotics have the potential to be used soon – before 2030 – as new therapies, perhaps as adjunctive or preventive therapies in the initial development stages.[26] Yet some technical problems must be solved. Most of these alternatives are narrow-spectrum agents, limiting their use unless doctors can make an accurate and fast diagnosis of the specific pathogen.[2] On the positive side, their narrow spectrum may reduce collateral damage to the host microbiome, which we already know is linked to chronic health problems such as asthma and obesity.[2]

Some of the approaches might not be superior to existing traditional treatments, but as resistance to conventional treatment rises, they still might be valuable alternatives regardless. Alternative methods can also be challenging to regulate since the end product might be very variable (i.e., phage therapy). Finally, their development might not be commercially attractive, making it difficult to get treatments to the market.[2,26]

Host-defense peptides

Among the possible alternatives from the previous section, HDPs are quite unique. They exhibit broad-range antimicrobial activities, not only against Gram-negative and Gram-positive bacteria, including MDR strains, but also against fungi, viruses and parasites.[73] Further, their ability to modulate immunity, recruiting immune cells to the infection site, makes them a very interesting alternative to conventional antibiotics.

HDPs are small peptides – around 10 – 50 amino acid residues in length – and are generally cationic due to an abundance of the aminoacidic residues lysine and arginine.[74] In addition, they contain several hydrophobic amino acids, which promote the formation of secondary structures. These amphipathic structures allow the peptides to interact with membrane-associate lipids and at the same time, the surrounding aqueous environment.[75,76] Usually, they are called AMPs when they have direct antimicrobial killing activites.[77]

Natural HDPs are an essential component of both the adaptive and innate immune systems. The discovery of cecropin in 1980 from the hemolymph of the moth *Hyalophora cecropia* was the first study to put the focus on HDPs.[78] From then, other landmark studies showed the existence of α-defensins in human neutrophils,[79] and magainins in the skin of frogs (*Xenopus laevis*).[80] These studies were already pointing on the ubiquity of such broad-spectrum antimicrobial molecules. And now more than 2,000 AMPs are known, coming from all three domains of life.[81,82] Given the variety of sources, it comes as no surprise that they have diverse conformations.[82] They are classified according to the secondary structure they adopt in the presence of membranes, belonging to four main classes (Figure 7):

1. α-Helical
2. β-Sheet
3. Looped peptides
4. Extended structures

The most common secondary structures found in HDPs are α-Helix (LL-37 and magainin are classic examples) and β-Sheet (i.e., defensins). Extended peptides lack marked folding patterns and are often rich in specific amino acids. An example is the peptide PR-39, found

in pigs, with around 30 % of its amino acidic composition made up of proline and arginine residues.[83] Lastly, antimicrobial looped peptides have their characteristic structure due to the formation of a disulfide bond. For instance, thanatin is an insect HDP with a looped frame with potent bactericidal and fungicidal activities.[84] Although HDPs are classified according to their structure, it does not always correlate with their function.[53] A given HDP may act against multiple targets and have various effects on both the pathogen and the host cells.

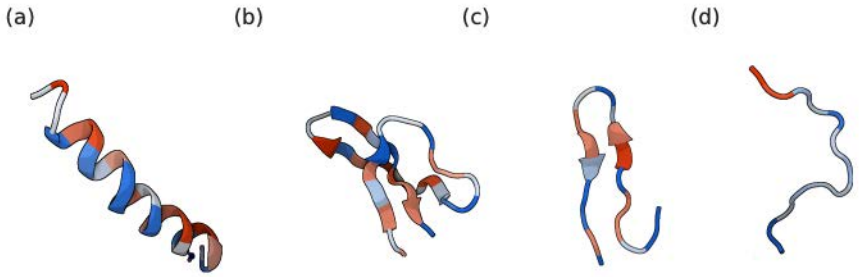

Figure 7. Different antimicrobial peptides and their secondary structures. (a) α-helical structure of magainin 2 (PDB : 2MAG). (b) β-sheeted structure of human α-defensin-5 (PDB: 1ZMP). (c) looped β-sheet structure of polyphemusin-1 from the atlantic horshoe crab (PDB: 1RKK). (d) extended structure of the bovine AMP indolicidin (PDB: 1G89). Red and blue colors symbolize hydrophilic and hydrophobic residues, respectively, showing the amphipathic nature of these molecules.

There are other ways of classifying HDPs and the classification in cathelicidins, defensins, and histatins is one of the most widely used.[85] Cathelicidins are activated through proteolysis and have varying structures and aminoacidic compositions.[86] Some have α-helical structures, whereas others have β- hairpins, and some are proline/arginine-rich peptides.[86] This is very different from defensins, which have two or more β-sheets stabilized by disulfide bonds. Lastly, histatins from humans do not have a specific structural motif but are rather defined by their high content in histidine residues.[87]

Cathelicidins

Cathelicidins, together with defensins, belong to the large group of HDPs that represent the main part of HDPs from the immune system in many vertebrates.[86] They can be found stored in neutrophil and macrophage granules in humans and other species, such as farm animals. They all share a conserved cathelin domain, and the first cathelicidin that was identified was cecropin, in 1980.[86] There are about 30 known cathelicidin family members in mammal

species. Curiously, there is only a single cathelicidin that has been found in humans, although other mammal species have more. And that is LL-37, which is codified by the *CAMP* gene and expressed after activation by pathogens or the active form of vitamin D.[88]

The LL-37 peptide has 37 amino acid residues and begins with two leucines in the N-terminus, therefore its name.[89] This peptide comes from the proteolysis of the C-terminal end of the human CAP18 protein (hCAP18), and is expressed in neutrophils, monocytes, natural killer cells, T and B cells, and epithelial cells. Besides its antimicrobial activity, LL-37 also binds and neutralizes LPS,[90] protecting against anaphylaxis. In addition, it is a chemotactic peptide for neutrophils, monocytes, and mast and T cells.[91] Finally, LL-37 can also induce degranulation of mast cells, transcriptional modifications in macrophages, vascularization and wound healing.[92]

Defensins

While cathelicidins are highly heterogeneous and found in vertebrates, defensins, instead, share structural features and are also found in invertebrates.[93,94] Defensins are rich in cysteines, forming disulfide bonds with different patterns, and depending on their bridging arrangement, they are classified into α-defensins and β-defensins. In humans, there are tens of defensins described, and all of them fall into these two categories. There is, however, a third category known as θ-defensins, the only cyclic peptides known of animal origin. θ-defensins were discovered in primates but have not yet been identified in humans.

Alpha defensins are small peptides of 2 to 6 kDa – 29 to 35 amino acids – and are abundant in neutrophils, some types of macrophages, and Paneth cells of the small intestine.[95] In their latter location, they are also called crypticidins and help reduce the number of bacteria in the intestinal lumen. In this work, we have used the human alpha defensin 5 (HD5), which is a highly expressed crypticidin in secretory granules of the ileum, and the most abundant enteric antimicrobial peptide in humans.[96] HD5 can be cleaved by proteolysis in the duodenum into many fragments, which are also antimicrobial, increasing antimicrobial diversity.[97] Its bactericidal activity is quite powerful, against hypervirulent pathogens such as *C. difficile*.[96] HD5 also probably plays an essential role in maintaining urinary tract sterility, since it is produced in the kidney and can be detected in the urine.[98] Last, HD5 dysregulated expression is likely implied in disease outcomes, such as colitis. Some researchers argue that aberrant

expression of HD5 transcripts can be a useful biomarker that can distinguish between ulcerative colitis – low levels of HD5 – and Crohn's disease – high levels of HD5.[99]

Beta defensins as a group are the most extensively studied class to date.[94] In mammals, there is a diverse and large family of β-defensin peptides with very different amino acid sequences but nearly identical tertiary structures, based on their unique disulfide bonds and beta sheets.[94] They maintain microbial homeostasis in epithelial surfaces, including the bronchial tree, oral mucosa, and genitourinary and digestive tract.[94] And as α-defensins, they also can enhance the innate and adaptive immune system, by triggering degranulation, phagocytosis enhancement, and chemotaxis of various leukocytes.[94,100]

Histatins

Histatins are a family of cationic, histidine-rich peptides found in eye and salivary secretions of higher primates.[101,102] They have a unique structure depending on the histatin sub-class, but in all sub-classes, the amino acid histidine appears to be critical to their proper antimicrobial function.[103] Most histatins are proteolytic products of histatin 3, except histatin 2, which comes from histatin 1.[104] They are especially active against fungi (i.e., *Candida* spp.). In addition, aside from their antimicrobial properties, histatins also have other critical roles, such as wound healing or the ability to bind metal ions to generate reactive oxygen species (ROS).[105]

The reported mechanism by which histatins act as microbicidals is through membrane disruption.[104] We also know that histatins effectively kill yeasts by binding to potassium transporters and eliciting a potassium release that ends in membrane damage.[104] Finally, these HDPs mediate DNA damage due to the generation of ROS,[104] having several mechanisms to achieve their homeostatic functions.

Antimicrobial mechanism of action of HDPs

As previously explained, HDPs can have direct antimicrobial activities against all categories of microorganisms, including archaea, bacteria, fungi, viruses, and multicellular parasites. But how does their antimicrobial activity work? Most HDPs show the ability to directly disrupt the microbial membrane, which results in cell death. Because of their cationic and

amphipathic nature, AMPs are attracted by electrostatic forces to the lipid bilayer. These forces come from the negative phospholipid headgroups found in bacterial membranes, including lipopolysaccharides (LPS) in Gram-negative bacteria and teichoic acids (TA), lipoteichoic acids (LTA) and lysylphosphatidylglycerol in Gram-positive. Once the HDP interacts with the cytoplasmatic membrane, they change the membrane structure in such a way that a physical hole is created, causing the leakage of cellular components.

There are several complex models by which the pore formation can be explained. These include the toroidal-pore, the barrel-stave, or the carpet model. In the toroidal-hole and barrel-stave models, a pore in the membrane is created. The main difference is that in the toroidal model, the peptides are always associated with the lipid head groups, even when inserted perpendicularly in the lipid bilayer. In the carpet model, the peptides act in a detergent-like manner, accumulating parallelly to the membrane and at high peptide concentrations, creating micelles.[106] Although most HDPs induce microbial killing by disrupting cell membranes, a few HDPs kill bacteria without lysis, binding to different intracellular targets such as DNA and proteins, suppressing effective bacterial growth (Figure 8).[107]

Whereas defensins and cathelicidins kill cells by creating a pore that leads to lysis, histatins can work very differently. As the first line of defense inside the oral cavity, histatins are a clear example of non-lytic antimicrobial killing.[87] As we have seen, histidine is crucial for its function as antimicrobial, wound-healing, antifungal, and antiviral activities.[87,104] In *Candida albicans*, histatins enter the cytoplasm and induce cell cycle arrest, efflux of ATP or potassium ions out of the cell, and reactive oxygen species. They can also target intracellular structures – such as mitochondria. Histatins do not appear to lyse lipid membranes, but instead, make cells lose integrity as a secondary effect of all these mechanisms. Yet in bacterial pathogens, histatins use an uncommon blend of energy-independent and energy-dependent lysis, making their mechanisms ambiguous.[108]

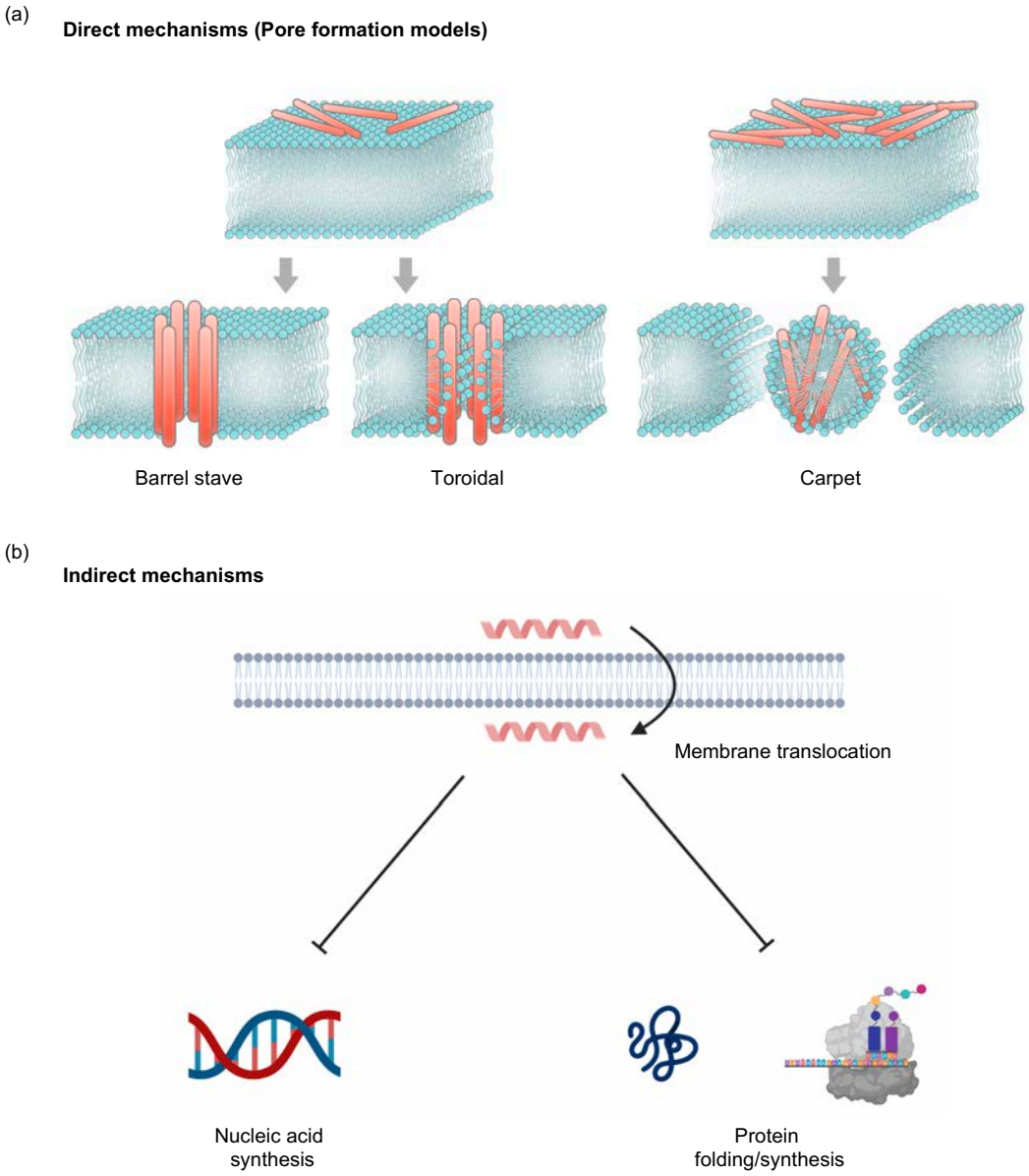

Figure 8. Direct and indirect antimicrobial mechanisms of action of HDPs. (a) The primary mode of antimicrobial activity of HDPs is through membrane perturbation and pore formation. There are three proposed pore formation models: the barrel stave, the toroidal, and the carpet model. (b) Indirect mechanisms include membrane translocation of the HDP followed by binding to intracellular targets such as DNA or proteins to finally impair critical cellular processes such as replication, transcription, translation, and protein folding.

Another example of this mode of action is indolicidin, a tryptophan-rich cathelicidin found in cytoplasmatic granules of bovine neutrophils. Indolicidin induces membrane permeation at high concentrations, but it does not lead to cell lysis. Rather, the increased permeabilization allows for continuous entry of the peptide into the cytosol to inhibit DNA biosynthesis – but not RNA. In contrast, PR-39 is internalized through a membrane receptor and then proteolyzes many DNA replication-associated proteins, leading to secondary inhibition of DNA synthesis. Finally, HNP-1, a human α-defensin, has additional activities besides its lytic properties, promoting DNA, RNA, and protein synthesis failures in *E. coli*.

The rapid membrane-lytic mechanism of HDPs makes them less susceptible to resistance development. Microorganisms would need to redesign its membrane, which is probably very complicated for most species.[109] Moreover, destruction of HDPs is problematic because they usually lack unique recognition sites for proteases.[109] Their additional non-lytic complementary activities, perhaps, also make resistance harder to occur, as there are many different mechanisms simultaneously leading to cell death. And a large number of HDPs have potent bactericidal effects against drug-resistant bacteria.[110] Some examples of susceptible MDR bacteria are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis*, MDR *Pseudomonas aeruginosa*, colistin-resistant *Klebsiella pneumoniae*, and AMR *Escherichia coli* strains.[111–113] Even more impressive is that some bacteria that acquire AMRs also alter their membrane composition, which makes them more susceptible to HDPs.[113] The most widely tested peptides against MDR bacteria are the cathelicidin LL-37 and defensins (i.e., human-β defensin 3).[94,114] But there is also plenty of research on peptides from non-mammal origin such as colistin from bacteria, thanatin from insects or several HDPs from amphibians and reptiles, to name a few.[114]

The diverse mechanisms of action and the vast repertoire of peptides from all different domains of life make HDPs an excellent potential strategy for combating MDR bacteria. And if we take into account their ability to synergize with conventional antibiotics, HDPs can be used either alone or as adjuvants in combinatorial drug therapies to control infection, making their case even more robust.[115]

Immune modulatory effects

To add a further layer of complexity, rather than directly inhibiting bacterial growth, most HDPs regulate the immune system of the host. They do so in a multitude of ways. For instance, they are able to neutralize LPS, upregulate the production of chemokines and chemokine receptors, induce cytokine production, promote angiogenesis, wound healing, and they are also able to modulate dendritic and T cell immune response (Figure 9).[116,117] All these immunomodulatory effects have only recently begun to receive attention, with some authors arguing that the main role of HDPs under physiological conditions is to modulate the immune system rather than direct bacterial killing.[116,118]

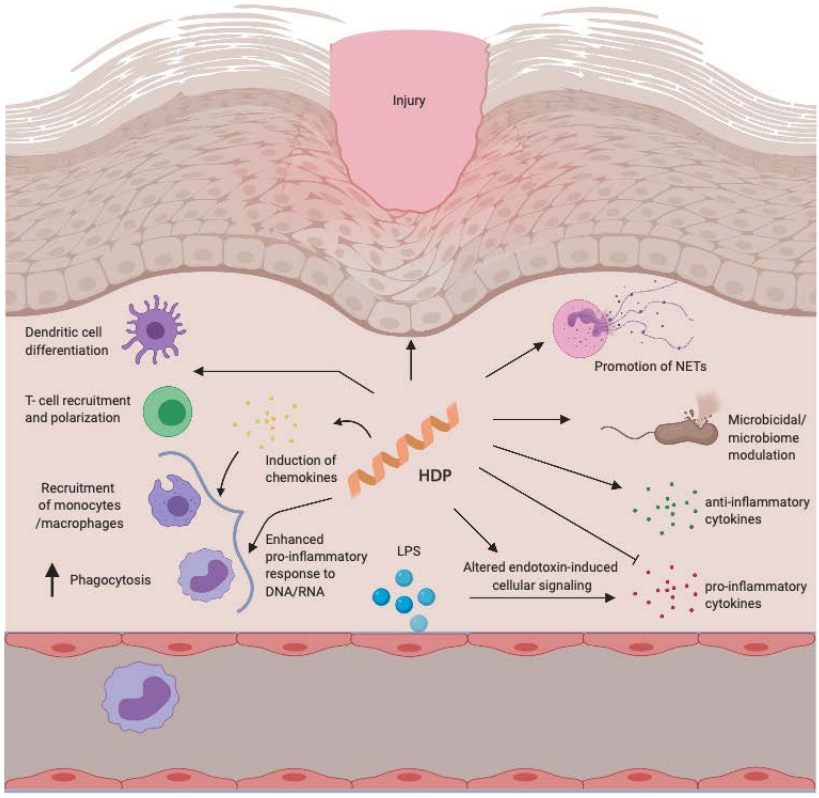

Figure 9 Immunomodulatory activities of HDPs. HDPs can be involved in the upregulation of chemokines, induction of cytokine production, and modulation of dendritic and T-cell responses. Moreover, HDP activities also include the activation of neutrophil extracellular traps (NETs). Finally, HDPs alter pathways that are activated by bacterial endotoxin (LPS), increasing phagocytosis and pro-inflammatory responses to nucleic acids. Adapted from ref.[119]

Cathelicidins trigger degranulation and release multiple antimicrobials and pro-inflammatory substances, including more cathelicidins.[120] LL-37 and PR-39 stimulate nitric oxide (NO) generation in macrophages, endowing them with cytostatic and cytotoxic activities. LL-37 also enhances the generation of reactive oxygen species. On the contrary, PR-39 inhibits it, which could be a negative feedback loop to prevent tissue damage. We know that LL-37 has

both pro- and anti-inflammatory roles depending on the microenvironment and disease background, as is the case for other HDPs. TNF-α and IL-1 are upregulated by LL-37, which are potent pro-inflammatory cytokines. Yet LL-37 also dampens endotoxin-mediated immune reactions by binding to LPS and preventing toll-like receptor (TLR) signaling. It could be that this dual immune-modulating role of LL-37 is crucial in gut microbiome homeostasis, allowing to promote inflammation under an infection, but avoiding an immune reaction to beneficial bacteria. Finally, human LL-37 can opsonize bacteria, enhancing their phagocytosis when LL-37 is bound to them and the complement receptor MAC-1.[120,121]

Defensins, too, have both inflammatory and anti-inflammatory activities.[122] At low or steady-state concentrations, HD5 has pro-inflammatory properties, and this is probably beneficial to keep epithelial integrity and to regulate the infiltration of immune cells in healthy individuals.[123] Researchers have found that HD5 synergizes with TNF-α to increase IL-8, therefore stimulating phagocytosis and chemotaxis. Or it can act on immune cells directly, being a potent chemoattractant for macrophages, mast cells, naïve and memory T lymphocytes, but not dendritic cells.[124] We also know that to mediate these effects, α-defensins use an evolutionarily conserved receptor, which suggests that immunomodulation could represent one of their primary roles, if not the principal.[124]

Antitumor properties of HDPs

While HDPs may be harmful to mammalian cells, toxic concentrations are commonly ten times higher compared to the MIC needed against bacteria.[125] Still, some HDPs specifically target cancer cells. The reason behind it is that transformed, cancerous cells generally incorporate phosphatidylserine (PS) in the outer leaflet of the plasma membrane – up to 9 % of the total phospholipids. PS is negatively charged and is usually found in the inner leaflet of the plasma membrane in healthy mammalian cells. This change in asymmetry and charge, typical of cancer cells, gives cancerous mammalian cells a chance to interact with HDPs.[126] Besides, other changes may increase the negative charges on cancer cells, including heparin sulfates and O-glycosylated mucins on the exterior of cancer cells.[126]

The density of negative charges on bacterial cells is relatively higher in comparison to cancer cells, due to the phosphate groups of LPS, LTA, and PS. As a consequence, HDP affinity for tumor cells is naturally weaker than the affinity for bacteria. Still, some cancer cells might

display other features such as lower cholesterol, having an increased membrane fluidity, which might potentiate the lytic mechanisms of HDPs, as seen in cecropins.[126] Or they can enter the cytoplasm more easily and bind to mitochondria, leading to cell death by similar means to those that histatins use.[126] In addition, we know that human α-defensins 1-3 can also inhibit neovascularization, required for tumor growth, adding to their lytic and apoptotic mechanisms (Figure 10).[127] Since the discovery of HDPs, no other class of compounds has matched their versatility.

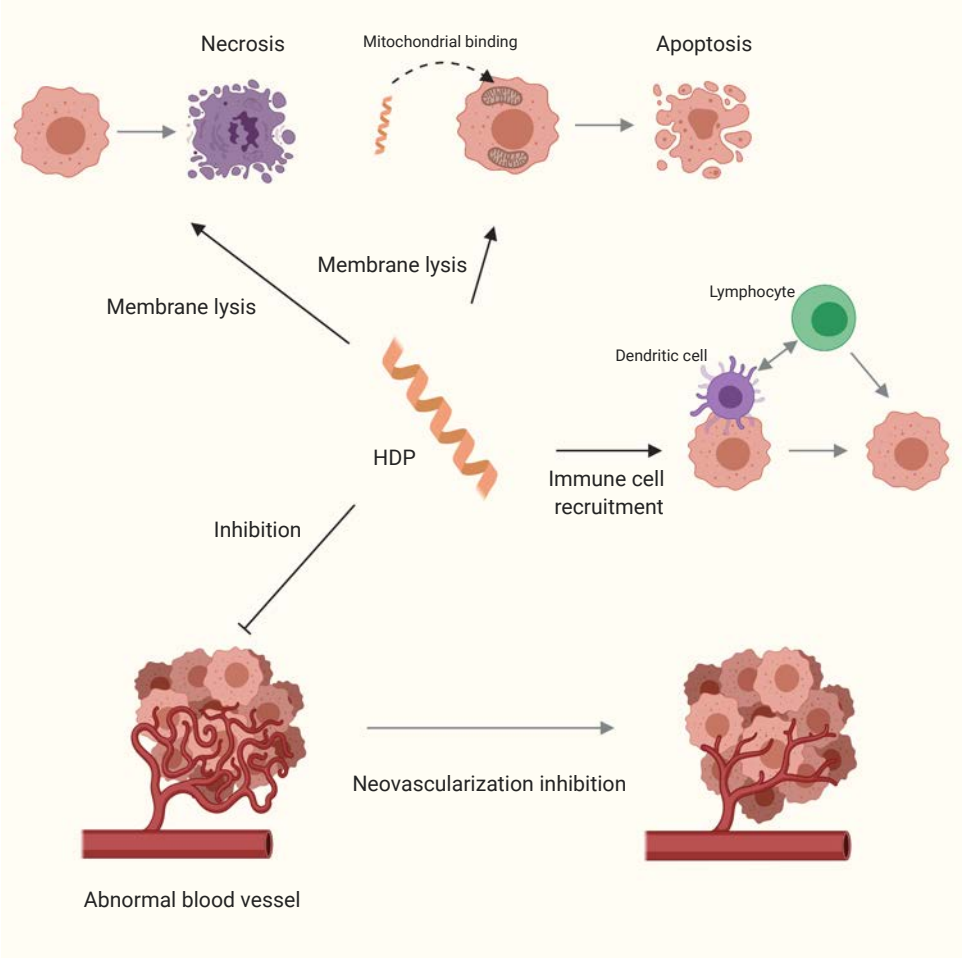

Figure 10. Anticancer properties of HDPs. HDPs can have anticancer properties through different mechanisms. They can destabilize the membrane of cancerous cell, target mitochondria intracellularly, recruit immune cells so that tumor cells are recognized and killed, or inhibit angiogenesis, effectively avoiding nutrient delivery to tumors for their proper growth. Adapted from ref.[128]

Limitations

Although HDPs have been known for some time now, therapeutic breakthroughs for systemic treatment have not happened for unknown reasons.[129] Their development has been limited to topical treatments such as in oral candidiasis, rosacea and diabetic foot ulcers.[116,130]

Critical concerns are toxicity and stability, which need to be resolved before they can be translated to therapeutics. HDPs are too early in development, so further research is needed to validate them and convince pharmaceutical companies, investors and clinicians.

A major barrier is their high manufacturing costs, as most of HDPs are produced synthetically. Solid-phase chemical synthesis can cost between $100 to $600 per gram.[29] Some estimates speculate that at least 604 million pounds will be needed to add them as alternatives in a timely manner in the future.[29] Furthermore, natural HDPs tend to have longer sequences– more than 20 amino acids – than those produced synthetically and complex secondary structures. The longer sequences increase synthesis costs – if feasible at all- and the secondary structures might make synthesis not an optimal approach, since structure might be necessary for function.

Clinical applications

The clinical application of HDP-based therapies has been evaluated over the past few decades, without any fruitful results.[116] For instance, the development of pexiganan for the treatment of diabetic foot ulcers was not approved by the US Food and Drug Administration (FDA) because of ethical concerns over the trial design and lack of advantage over conventional therapies.[131] Notably, though, pexiganan reached phase III clinical trials. Many other promising drug candidates such as iseganan (for oral mucositis), and omiganan (for rosacea) also reached this late stage. Finally, LL-37 has also been explored in a Phase IIa clinical trial, where patients showed high tolerability and almost a 6-fold increase in leg ulcer healing, compared to the placebo group, which is quite impressive.[132]

Three significant factors forestall their clinical development and widespread use: stability, toxicity, and cost. Since some HDPs are salt-sensitive, their systemic use is limited. And they show poor pharmacokinetic profiles because they are relatively susceptible to proteolysis upon systemic administration.[133] Nonetheless, there are more than 10 phase II and III clinical trials using HDPs, in progress or already completed.[134] This is why there is hope on HDP therapeutics as a new class of antibiotics, since all these problems could be overcome through different strategies, which is what the next section is all about.

Design strategies for new AMPs

Most of the currently used AMPs are produced by chemical synthesis.[135] One of the main problems of chemical synthesis is its environmental impact, due to the use of organic solvents during the process.[135] Moreover, as previously mentioned, synthetic peptides have high production costs and cannot be produced in large-scale. Thus, researchers have used several approaches to develop HDPs while addressing their inherent drawbacks.[136] Biological synthesis – also known as recombinant protein production – is being explored as an alternative approach that allows to produce HDPs through a more cost-effective process that can be easily scaled-up.[137]

The following section examines the different approaches using both synthetic and recombinant that have been used in a bid to improve HDP toxicity, stability and potency.

Peptide-drug conjugates

Peptide-drug conjugates consist of the coupling of a specific ligand or drug to a synthetic HDP with the aim to enhance antibiotic activity or add modes of actions (Figure 11a). A very common method to do so is to fuse the drug compound at the α amino group of the N-terminal end of the HDP with a carboxyl group.[138] Usually, this is done while the peptide is still attached to a solid support – in the case of synthetic HDPs. The first application that comes to mind is to modulate an HDP by covalently binding a traditional antibiotic, to induce synergistic antibacterial effects. A group from Utrecht University used several HDPs based on the magainin 2 sequence and coupled vancomycin to them.[139] They found an increase in antimicrobial activity for this vancomycin-magainin conjugates against vancomycin-resistant *Enterococci*. Moreover, they found that the minimal inhibitory concentration (MIC) values were in the range or lower than for vancomycin alone. This has been done for other HDPs and antibiotic combinations, sometimes improving their effects, but not always.[140,141] Other drugs that have anti-inflammatory effects or anti-cancer effects have been used, expanding the therapeutic properties of HDP-drug combinations.[142,143]

The activity of HDPs can also be enhanced by improving their interaction with the bacterial membrane. One way of doing so is by lipidating HDPs with fatty acids of different lengths.[144] There is a limit in the length that can be introduced, because acyl chains that are too long

increase aggregation and self-assembly of conjugates. Yet the MIC values can be dramatically lowered, suggesting a better interaction with bacterial membranes, which leads to increased antimicrobial killing.[144]

The conjugation of a drug to an HDP improves its activity and adds novel therapeutic properties to the final molecule. This approach can also be used to add drugs to HDP mimetics, which we will see in the next sub-section.[145] Still, sizeable challenges need to be tackled. The problems range from low aqueous solubility during peptide synthesis, to the formation of undesired side products, which can be toxic.[146]

Peptidomimetics

To improve the stability, price, and pharmacokinetic shortcomings of synthetic HDPs, investigators have designed non-peptide oligomers and polymers (Figure 11b). They take inspiration from the structural features of natural AMPs to mimic their biological functions. Thus, the peptidomimetics have an amphipathic backbone and a net positive charge. Yet peptidomimetics still rely on chemical synthesis in standard solid-phase procedures.[147]

Magainin was used to design the peptide mimetic *meta*-phenylene ethynylene (mPE). mPE shows antimicrobial activity at low concentrations – in the nanomolar range – against a variety of pathogenic bacterial and yeast strains found in oral infections.[148] It inhibits the growth of *S. aureus*, *Porphyromonas gingivalis*, and *Streptococcus mutans* and also prevents *S. mutans* biofilm formation. Moreover, mPE also binds DNA intracellularly, potentially interfering with DNA replication, mediating non-lytic bacterial cell killing. There is also good synergy of mPE with common oral antiseptics, which might support its use in conjunction. Finally, the immune-modulatory potential of this peptide mimetic was also assessed. mPE was able to reduce TNF-α secretions of macrophages that had been stimulated with LPS. Moreover, low concentrations adequately suppressed IL-1β or induced IL-8 release in myeloid and epithelial cells, restating its potential utility as an anti-inflammatory.[149]

Another class of peptidomimetics that mirror HDPs are cationic steroid compounds (CSA), with hydrophobic residues and positive charges on opposing sides. They show far superior antimicrobial activities than the human cathelicidin LL-37 against a wide array of bacteria from oral and upper respiratory tract infections.[150] In addition, they are able to induce IL-8

release in keratinocytes, again suggesting their potential role not only as AMP mimetics but also as HDP mimetics.[150]

Many other designs have also improved antimicrobial activity, promising synergies with other antibiotics, such as enhancing their uptake, antibiofilm[151] and anticancer activities,[152] and immune-modulatory effects.[153] However, they have critical hurdles that need to be solved if they want to be used as therapeutics. Their availability, bioavailability, toxicity, and regulation should be addressed. Their synthesis needs to be optimized and simplified to produce them in large scales. Their rapid antimicrobial killing *in vitro* also comes with immunogenic side effects when tested *in vivo*.[154] To mimic HDPs, a minimal amount of hydrophobicity is required, but this also leads to toxicity at the same time. All these characteristics may explain why HDP mimetics still have not reached the market.[154]

Genomic mining strategies

We already know that bacteria themselves produce most antibiotic compounds.[12] Competition between members of the same ecological niche drove the process. Similarly, bacteria also developed bacteriocins, which are AMPs produced by bacterial species to kill other bacteria. Sometimes, unless bacteria are under stress – i.e. competition with another bacterial strain for survival– these AMPs are not expressed. These bacterial defense mechanisms – and from other organisms too – might be more prevalent and diverse than what we know.[155] One way to discover them is to analyze sequenced genomes for their diverse range of AMPs and HDPs (Figure 11c).

It is possible to prospect for genes that encode for bacteriocins. It has been done in cyanobacteria,[156] thermophilic bacteria,[157] or bacteria from the gastrointestinal tract.[158] But we can also look beyond, and indeed researchers have found phage-derived AMPs with potent antibacterial and immunoregulative characteristics.[159] Moreover, novel AMPs can be found in within the sequence of apparently unrelated proteins. For instance, pepsin A, the main human stomach proteolytic enzyme, contains AMPs. Through a computational approach, investigators found these AMP candidates *in silico*, displaying a wide-spectrum activity in the range between 2-50 μM.[160] Given the abundance of genomic information at our disposal, these innovative approaches are more than welcome in our endeavors to expand our antibiotic arsenal.

De novo designs

Similar to what researchers do in the case of peptidomimetics, *de novo* design strategies are based on the characteristic features of HDPs. In peptidomimetics, non-aminoacidic organic structures are used whereas the *de novo* approach considers charge, hydrophobicity, and amphiphilicity to create new peptide sequences (Figure 11d). Besides, the complexity of protein folding and the resulting secondary structures are considered to make a generic sequence for short synthetic peptides, usually of less than 20 amino acids.[161] For instance, the broad spectrum Trp-rich peptide indolicidin is used as inspiration to design short Trp-rich sequences with enhanced antibacterial and anti-endotoxin activities.[162] Several of these synthetic analogues possessed superior killing properties against MRSA compared to indolicidin.

De novo designs can go as low as the smallest unit possible – dipeptides. Diphenylalanine (FF), naturally isolated from kefir, can be used to make supramolecular polymer assemblies that inhibit bacterial growth entirely, trigger the upregulation of the stress response, and destabilize the bacterial membrane.[163] Another case of supramolecular architectures is viral capsid-inspired designs. Capsids do not disrupt microbial membranes and generally do not show any antimicrobial activity. Yet their make-up is ideal for designing artificial capsids assembled from AMP sequences, now with the ability to destroy bacterial membranes.[164] Any AMP sequence can be used to construct a self-assembling capsid, either naturally occurring or designed.

Some researchers argue that the lack of innovation in antibiotic discovery is because we have reached a limit in antibiotic discovery in the natural world.[165,166] Since there is an urgent need to come up with out-of-the-box solutions, they teach computers the basic principles of HDP design. Computers have already been used to produce HDPs *in silico* that are effective *in vitro* and *in vivo*.[167,168] Perhaps, pattern recognition may play an important role in the discovery of potent computer-made HDPs or maybe go a step further and make new molecular structures not found in nature. Nonetheless, a suitable approach must be chosen to materialize the results and test them empirically – either by synthesis or recombinant production.

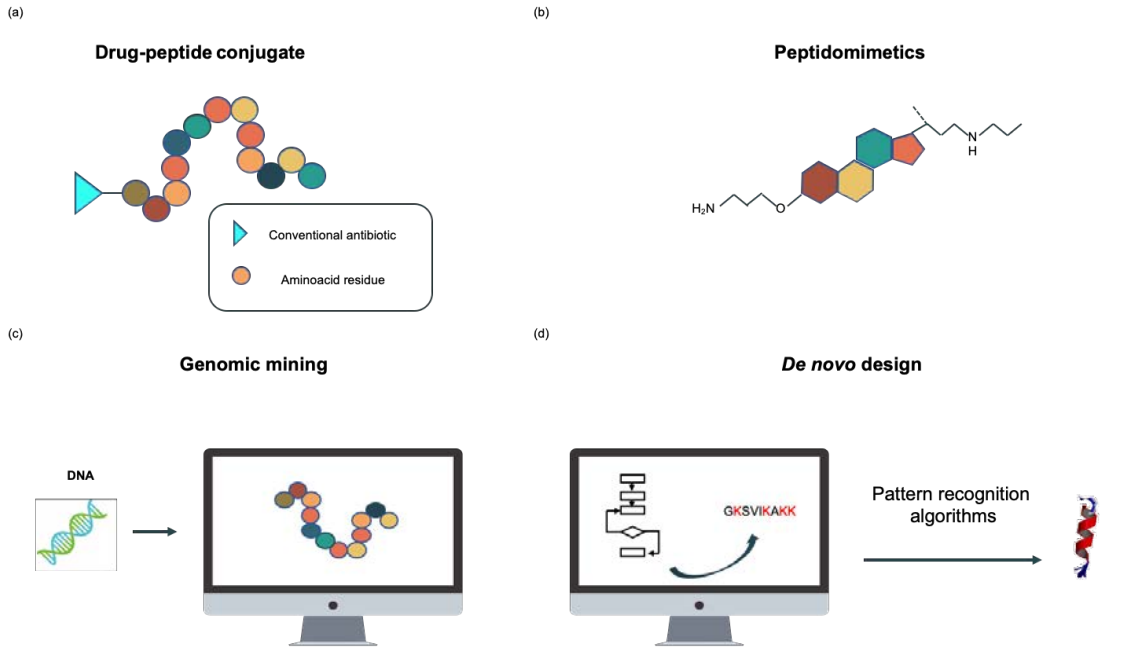

Figure 11. Different strategies to obtain new new HDPs. (a) Drug-peptide conjugates combine a mix of AMP with another molecule, to enhance the HDP activity or bacteria targeting. (b) Peptidomimetics use non aminoacidic structures that have similar activities to HDPs, along with improved stability, pharmacokinetics, or price. (c) Genomic mining strategies use bioinformatics to search for DNA sequences that code for potential, potent HDPs. (d) *De novo design* takes into account HDP general properties to make artificial molecules from scratch, with the help of algorithms.

Recombinant protein production

Many organisms can be used as hosts for recombinant protein production, including bacteria, yeasts, insect and mammalian cells. Recombinant protein production in microbial systems revolutionized biochemistry. Before it, large volumes of animal and plant tissues or biological fluids were needed for the purification of minimal amounts of proteins. The development of recombinant technology allowed to express and purify in large quantities any protein of interest, which, in turn, allows for their biological characterization and industrial and commercial exploitation.[169,170]

Choosing the optimal expression organism is critical to ensure proper protein yields and biological function. However, the most popular recombinant protein expression system is *E. coli*, by and large.[170,171] Because it is the most used and well-studied host, there are many genetic engineering tools adapted to it. Although *E. coli* has many drawbacks for protein production and downstream purification,[171] it is still a fine choice, particularly for proof-of-concept explorations, such as the multidomain antimicrobial polypeptides produced for the studies of this dissertation.[172]

The process for recombinant protein expression is the following: the sequence of the gene of interest is selected – which codes for your protein of interest – and cloned to an expression vector of choice. After that, the cloned genetic construct is transformed into the chosen host, and the protein production is induced. Then, if the protein is expressed intracellularly, cells are disrupted, and the protein is purified and characterized (Figure 12). If the protein is secreted, the growth media is recovered, and the protein is purified from it. These steps are pretty straightforward, in theory. In practice, however, many things can go unexpectedly. Proteins produced in recombinant systems are heterologous. In some cases, proteins are proteolyzed by host proteases and in other cases, they are produced at very low yields, which could be due to their toxic effect on the producer cell. It is also frequently observed that proteins aggregate forming what is known as inclusion bodies (IBs).[169]

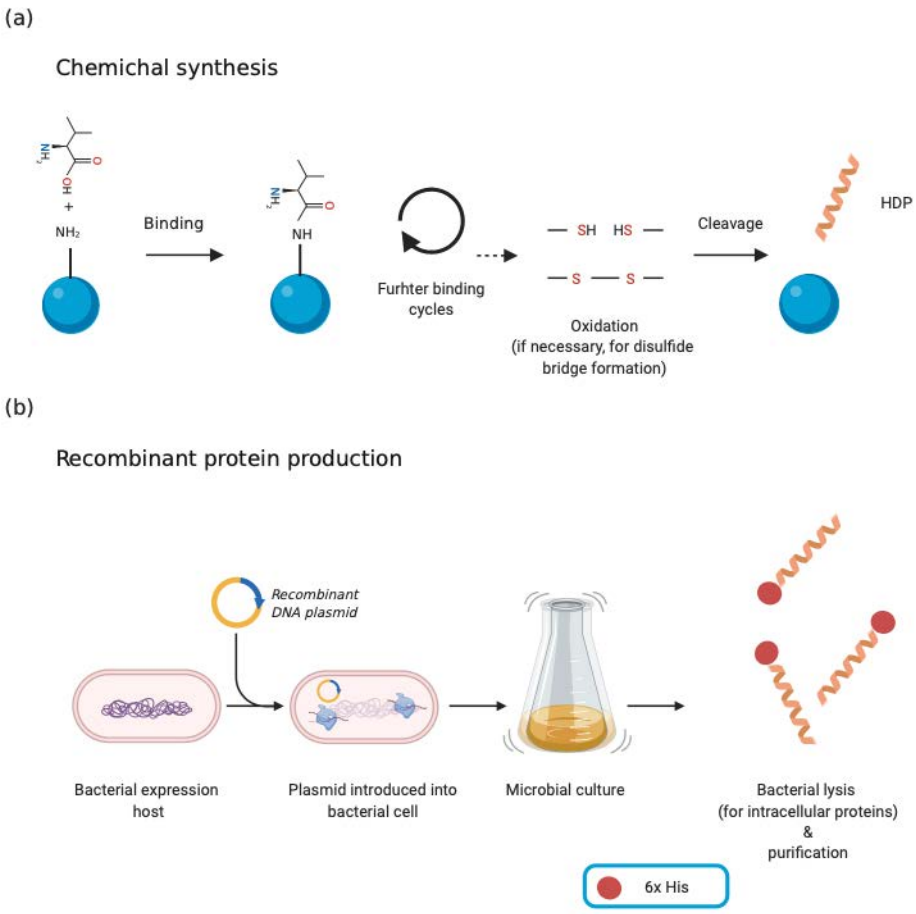

Figure 12. HDP production methods. (a) Solid-phase synthesis of an HDP: At every binding cycle, the desired amino acid is added until the sequence is obtained, and then cleaved from the solid phase. If there is a disulfide bridge, an oxidation step can be done before cleaving. (b) Recombinant production of an HDP: A plasmid that contains the gene for the desired peptide or protein is introduced into a microbial cell factory. Protein production is induced under suitable growth conditions, and then the protein can be recovered (i.e., a six histidine-tag that allows for selective downstream purification).

Inclusion bodies

During recombinant protein expression, we usually take genes from one organism – i.e., the gene coding for a human HDP – and express it in another organism – i.e., *E. coli*. This heterologous expression often risks the formation of IBs, which are protein particles with sizes that range from 50 to 800 nm that almost exclusively contain the over expressed protein (Figure 13a). They are generally found in the cytoplasm, often polarly distributed and denser than many of the cellular components (Figure 13b). Yet at the same time are highly hydrated and have a porous, mechanically stable architecture.[173] The protein in IBs is classically thought to be in a misfolded state.[173,174] However, some researchers have contested this view, upon the observation of green-fluorescent protein IBs that fluoresce.[174–176] The generally accepted idea that low recombinant protein solubility means no biological activity is not always necessarily accurate.[177–179] Besides, IBs are stable and keep functionality under harsh storage conditions, which soluble protein might not withstand easily.[180]

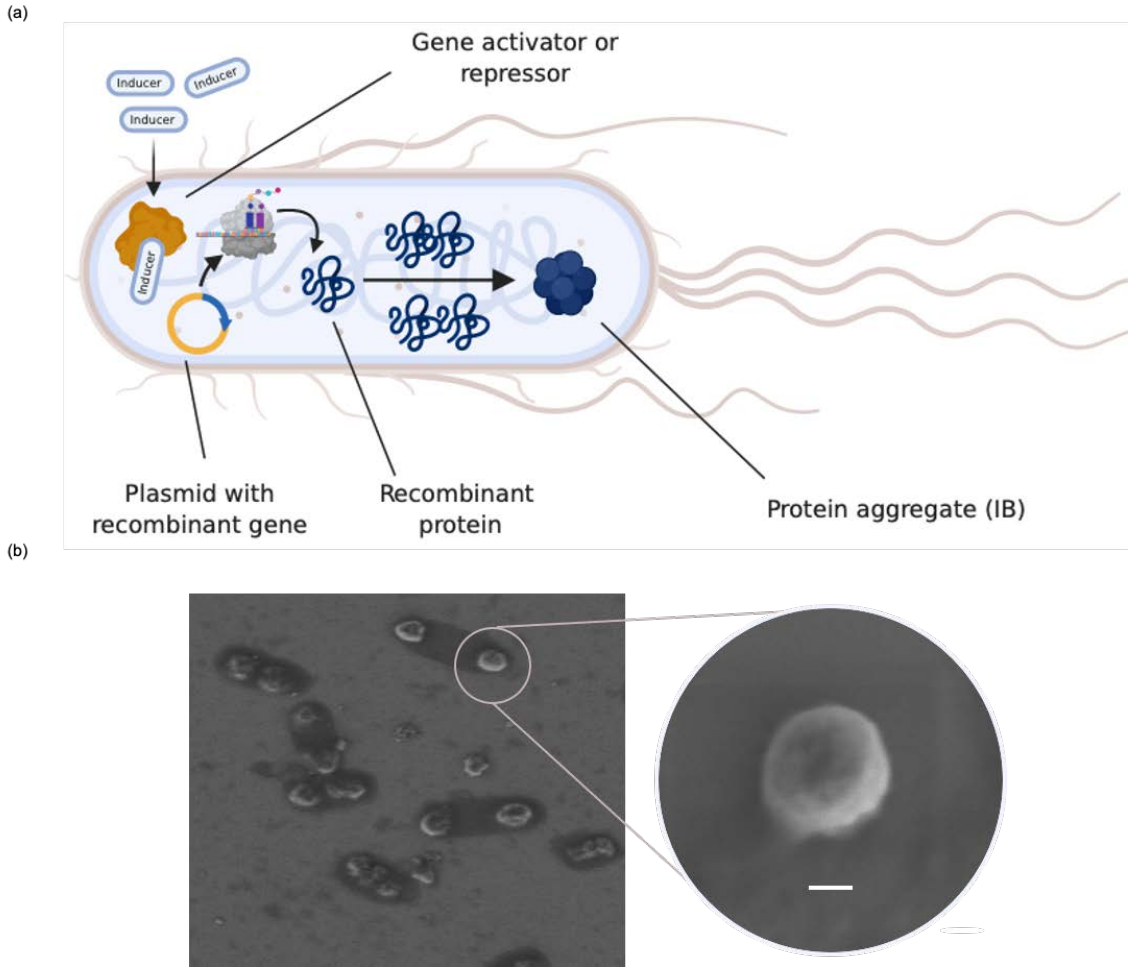

Figure 13. Recombinant protein expression. (a) After adding an inducer, the expression of the recombinant gene begins. The recombinant gene is transcribed to mRNA and the host ribosome will translate it to protein. Often, protein will be overexpressed, leading to the formation of IBs. (b) Scanning electron microscopy of IBs, where we observe their polar distribution inside the bacteria. Bar size = 200 nm.

Because of their properties, IBs have been tested in many applications. For instance, IBs keep almost all of the protein embedded in them in physiological buffers, with only a small part released into the surrounding environment. Thus, IBs formed by enzymes can be used as self-immobilized biocatalysts.[181,182] Many enzymes, such as reductases, oxidases, kinases, synthases, and lipases, have been used as self-immobilized biocatalysts in an IB format.[182,183] IBs also have appealing drug delivery qualities, such as high cell penetrability and slow release of functional IB protein, allowing for the non-toxic administration of higher doses.[184] IB size, geometry, and stiffness can also be adjusted to make biomaterials with useful topographies for tissue regeneration.[185,186] Moreover, they have been used against tumors to penetrate and break tumoral tissue selectively.[187,188] Finally, IBs are obtained in one single step, without the need to encapsulate them in a separate process.[189] Yet one application that remains to be explored is the use of HDPs in an IB format.

The intracellular accumulation of partially folded heterologous proteins results in IB formation, as outlined. Some groups have tried to control and increase the aggregation tendency of proteins as a result of the increasing interest in these nanomaterials. To do so, different aggregation tags used as fusion partners to the protein of interest, such as VP1,[174] GFIL8[190] and ELK16.[191] However, recombinant proteins forming this biomaterials coexist with other proteins such as chaperones, and also with lipids,[192,193] but the presence of these impurities has been poorly studied. This is an important challenge that needs to be solved in terms of IB translatability, as their composition needs to be controlled precisely for some applications. Consequently, there is room to optimize IB composition and one possible strategy is by using an adequate aggregation tag.

Nonetheless, in some applications, the soluble format may still be necessary. But it has been demonstrated that IBs can be used as a source of soluble protein, after a mild extraction protocol in non-denaturing conditions.[194,195] The active protein can be retrieved from IBs without any renaturation procedures. And after that, purified using Immobilized Metal Affinity Chromatography (IMAC)[194] or any other desired purification method, making IBs an attractive source for tricky to produce proteins in a soluble format.

Recombinant HDPs

Large quantities of HDP are required to test them, and recombinant production shows many advantages over the strategies used for their extraction from their natural source or when chemically synthesized.[171] Chemical synthesis, for instance, is limited when the peptide length increases and when secondary structures, such as β-sheets and α -helix, are necessary for protein function. The chemical reagents for synthesis also have a very high cost hampering the scale-up, and proteins sometimes need post-translational and conformation modifications to show biological activity, which cannot be achieved easily or at all through chemical synthesis.[196]

Recently, some AMPs have already been successfully produced using recombinant expression systems.[197–199] One example is LL-37, made at a small scale utilizing *E. coli* with yields of 2.4 mg/L. Another example is Plectasin, a defensin from the mushroom *Pseudoplectania nigrella*, with a similar output of 5.5 mg/L. And at least 20 more other HDPs have been produced with yields that range from 0.6 to 36 mg/L.[200] Still, their small size makes them easily degradable and their recombinant production is limited because they are toxic for the produced bacterial cell due to their antimicrobial nature.[201,202]

To address these shortcomings, different approaches to make the production of recombinant HDPs linked to a carrier that stabilizes the peptide have been described.[203] Some examples of carriers for these fusion proteins are small ubiquitin-like modifier (SUMO), thioredoxin, glutathione S-transferase (GST), biotin carboxyl carrier protein (BCCP), green fluorescent protein (GFP), calmodulin and human serum albumin.[204–208] These carriers help to overcome the toxicity of the HDP and at the same time increase their protein expression yields.[209,210] Alternatively, researchers from Korea have patented another strategy for the recombinant production of HDPs that is also based on fusion carriers.[211] They use acidic peptides as fusion partners, which have a charge-charge interaction that neutralizes the potential bactericidal effect of the overexpressed HDP. By adding these acidic peptides, they are able to prevent the antimicrobial peptide-mediated killing of the host microorganism.

However, there is one main disadvantage when using fusion partners. These fusion proteins contain the AMP of interest but also a carrier without antimicrobial activity. The removal of the fusion partner requires expensive enzymatic cleavage or toxic reagents to retrieve the

peptide of interest from the original fusion protein.[212,213] This can be solved by including self-cleaving tags that enable purification and cleavage in a single step, saving time, labor and cost but with the risk that cleavage might be incomplete or uncontrolled.[214]

Multidomain proteins as new AMPs/HDPs

Most proteins consist of at least two domains, which are the functional parts of any protein that could exist independently of the rest.[215] By using recombinant DNA technologies, we could engineer artificial multidomain proteins by coupling functional domains that are not naturally together. Indeed, the design of multidomain proteins has been tested for some applications. Already in 1998, some researchers made flavodoxin and cytochrome chimeras, to improve electron transfer proteins and create a bio-transformer.[216] And a very common application of this "molecular lego" approach is to tag a protein with a fluorescent protein – usually green fluorescent protein (GFP).[217]

But can we enhance killing activities or create new properties not found in natural HDPs with protein engineering?[218] We already know that nearly all HDPs that are produced recombinantly use fusion partners with no additional activities, just as carriers.[219] Yet some researchers have explored what happens when the fusion partner adds other functions.[220]
In particular, they tried to improve the broad-spectrum AMP C6 – a plant defensin – specificity. They did so by fusing rationally improved versions of the C6 AMP to the peptide pheromone (cCF10) that is species-specific and binds to bacterial membrane receptors with high affinities. That might be interesting if the killing of pathogenic bacteria is achieved without killing beneficial microorganisms found in the host microbiome, which is what most antibiotics in clinical use do.[220]

Indeed, other research groups have tried a similar approaches,[221–224] and even define these narrow-spectrum hybrid AMPs as specifically targeted antimicrobial peptides (STAMPs).[225] In general, they join two independent peptides components with a linker, having a targeting and a killing region.[225] Many combinations can be tried, so the killing and targeting domain can be natural, artificial, or even rationally enhanced designs.[221–223] All these hybrid antimicrobials show improved antimicrobial activity, selectivity, and kinetics against their specific targets. Nonetheless, inevitable bactericidal effects on other bacteria are still observed, and very few are being evaluated for their clinical use.[220,226]

Multidomain polypeptides are probably not suitable for synthetic approaches due to their amino acid length. Because of that, recombinant methods are the go-to method. An advantage of the recombinant approach is that the structural folding, although limited by the chosen expression system, will probably be better than in synthetic methods. Also, the recombinant approach yields two protein formats: soluble and insoluble, and each might be useful in different contexts.

It is true, however, that for multidomain AMPs might result in unpredictable folding outcomes. To prevent this, short-linker sequences between each domain can be very effective, and certainly, the choice of amino acids in these linkers is essential for the construction of functional hybrids.[227] Several properties have to be taken into account, such as linker length, hydrophobicity, and potential secondary structures.[228] An example is the amino acid glycine, often present in linkers since it has no side chain – which could interfere with folding and bioactivity. Proline and serine are also very common. In the case of proline, its unique – and rigid - cyclic side chain restricts the linker conformation and prevents the formation of hydrogen bonds with other amino acids, reducing possible undesired interactions. Instead, the small, polar amino acids serine, like glycine, provides excellent flexibility due to its small size and stability in aqueous solvents through the formation of hydrogen bonds with water.[228,229]

In any case, a primary concern for all the explained approaches – synthetic or recombinant - is the development of AMRs against these compounds. HDPs obtained through any of these methods resemble their natural counterparts. If bacteria evolve resistance mechanisms against synthetic or recombinant HDPs, the innate immune responses during infections could be jeopardized.[230] Using multiple bacterial killing mechanisms in one molecule – i.e., each domain of a recombinant multidomain polypeptide – might difficult the appearance of AMRs. Therefore, multidomain antimicrobial proteins might provide a versatile and tunable approach to construct broad-spectrum HDPs that do not generate AMRs.

Summary

The growing threat of AMRs has increased the pressure to develop new therapeutic strategies to fight drug-resistant pathogens. HDPs have drawn considerable attention as a source of antibiotics, due to their broad-spectrum and fast bacterial killing - either alone or synergistically with traditional antibiotics. And even after 40 years after their discovery, we still get inspiration from natural peptides to develop innovative therapeutics. Nevertheless, natural HDP sequences and structures are often not enough, being toxic in systemic administrations and expensive to produce, limiting their clinical applications.

Recombinant multidomain polypeptides designs emerge as a unique solution to overcome these problems. They offer the potential for large-scale production at an acceptable cost.[231] Fusion partners can make these hybrid HDPs more resilient to degradation and confer additional mechanisms to enhance antimicrobial activity. Since each domain contributes to the overall molecule functionality, there is no need to use toxic chemicals or expensive enzymes to cleave irrelevant fusion partners.[212] Besides, they are easily purifiable either in a soluble, IB, or solubilized format.[232,233] As a result, this dissertation first explores the feasibility of rationally designed recombinant multidomain antimicrobial peptides.

Objectives

This work aims to develop and explore the potential of a new generation of antimicrobial proteins based on the combination of different modules forming a multidomain polypeptide, to achieve an easy recombinant production, avoid toxicity problems associated to the production of HDPs in recombinant systems, and merging different functionalities in an optimal final antimicrobial effect. These proteins are produced using recombinant DNA technologies in either a soluble or nanoparticulated format (IBs), being this last format especially relevant for its high stability and slow-release behavior.

To achieve our general objective, we took the following steps:

1. To study the use of alternative aggregation tags, such as Jun and Fos leucine zippers, and in this way, increase protein aggregation and improve the IBs composition and purity using *E. coli* as a recombinant host. (**Study 1**)

2. To design, produce and test the first antimicrobial multidomain protein, which combines different antimicrobial domains and Jun and Fos leucine zippers (JAMF1) to be produced as IBs. (**Study 2**).

3. To produce different variants of the JAMF1 multidomain protein named JAMF1.2, JAMF2 and AM2, and compare their antimicrobial and immunomodulatory activities in their soluble form. (**Study 3**)

4. To explore a potential suitability of these multidomain constructs to decorate surfaces, and thus, create materials with antibiofilm properties. (**Annex 1**).

Results

Study 1

Exploring the use of leucine zippers for the generation of a new class of inclusion bodies for pharma and biotechnological applications

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Preface

IBs are functional protein aggregates with many appealing properties, such as a slow release of protein, high stability, and low production costs. These features might make IBs particularly useful as biocatalysts, in tissue engineering, and for some human and animal therapies. To our knowledge, however, these protein clusters were not explored in conjunction with antimicrobial proteins and peptides when we began this work.

Although usually highly pure, IBs still have many host-derived impurities, such as lipids and carbohydrates. Besides, the purity pattern is very protein specific. So before trying to produce antimicrobial proteins in a nanocluster format, we wanted to seek means to control the composition and standardize the IB formation process among the different antimicrobial constructs that we wanted to design. This is why we examined if the addition of leucine zippers could be used as an aggregation promoting domain and also as a mechanism to improve the IB physicochemical properties. To do so, we used GFP as a model protein in this first set of experiments. What we found is that we could increase the aggregation ratio, protein purity, and the specific activity of some of these leucine zipper-GFP constructs, compared to GFP alone. Thus, this study opened the opportunity to use leucine zippers as aggregation tags during the recombinant production of our antimicrobial multidomain proteins.

Exploring the use of leucine zippers for the generation of a new class of inclusion bodies for pharma and biotechnological applications

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Abstract

Background: Inclusion bodies (IBs) are biologically active protein aggregates forming natural nanoparticles with a high stability and a slow-release behavior. Because of their nature, IBs have been explored to be used as biocatalysts, in tissue engineering, and also for human and animal therapy. To improve the production and biological efficiency of this nanomaterial, a wide range of aggregations tags have been evaluated. However, so far, the presence of bacterial impurities such as lipids and other proteins coexisting with the recombinant product forming the IBs has been poorly studied. These impurities could strongly limit the potential of IB applications, being necessary to control the composition of

these bacterial nanoparticles. Thus, we have explored the use of leucine zippers as alternative tags to promote not only aggregation but also the generation of a new type of IB-like protein nanoparticles with improved physicochemical properties.

Results: Three different protein constructs, named GFP, J-GFP-F and J/F-GFP, have been engineered. J-GFP-F is a GFP flanked by two leucine zippers (Jun and Fos); J/F-GFP was formed coexpressing a GFP fused to Jun leucine zipper (J-GFP) and a GFP fused to a Fos leucine zipper (F-GFP); and, finally, GFP was used as a control without any tag. All of them were expressed in *E. coli* and formed IBs, where the aggregation tendency was especially high for J/F-GFP. Moreover, those IBs formed by J-GFP-F and J/F-GFP constructs were smaller, rougher, and more amorphous than GFP ones, increasing surface/mass ratio and, therefore, surface for release. Although the lipid and carbohydrate content were not reduced with the addition of leucine zippers, interesting differences were observed in the protein specific activity and conformation with the addition of Jun and Fos. Moreover, J-GFP-F and J/F-GFP nanoparticles were purer than GFP IBs in terms of protein content.

Conclusions: This study proved that the use of leucine zippers strategy allows the formation of IBs with an increased aggregation ratio and protein purity, as we observed with J/F-GFP approach, and the formation of IBs with a higher specific activity, in the case of J-GFP-F IBs. Thus, overall, the use of leucine zippers seems to be a good strategy for the production of IBs with more promising characteristics useful for pharma or biotech applications.

Keywords: inclusion bodies, aggregation, recombinant protein, leucine zippers, Jun, Fos, purity

Background

Inclusion bodies (IBs) are protein nanoparticles ranging from 50 to 800 nm formed during the production of recombinant proteins and often localized in the cytoplasmic space of bacterial cells,[1] although their formation in the periplasm has also been described.[2] In the past, IBs have been regarded as an undesired byproduct of recombinant protein production processes.[3] However, over the last 15 years it has been broadly proven that proteins forming IBs are biologically active.[4-7] The presence of native proteins forming such aggregates has prompted to assess their potential as biomaterials for a wide range of applications, including biocatalysis, tissue engineering, antimicrobial and cancer therapies.[7-10] They offer important advantages over their soluble counterparts such as high stability,[11] slow-release behavior,[12,13] and production through cost-effective processes.[14] Aiming to improve the production and biological efficiency of this nanomaterial, the increase of the aggregation tendency of proteins of interest is a key aspect and has been evaluated through the use of different aggregation tags, being VP1,[6] GFIL8[15] and ELK16[16] three representative examples. Besides, the scale-up of IB production and purification protocols have also been improved during the last decade.[14,17] It has been described that the recombinant protein forming this biomaterial coexists with other proteins such as chaperones, and also with lipids.[18,19] However, the presence of these impurities has been poorly studied, and consequently there is room to optimize IB composition. This is an important challenge to be solved in terms of IB applicability, since for specific applications it is important to control the exact composition of these bacterial nanoparticles. Thus, exploring ways to control the IB formation process emerges as a central strategy to improve IB purity and better control their physicochemical properties. In the present study, we have used an alternative approach for the generation of a new type of IB-like protein nanoparticles using leucine zippers (LZ) as aggregation-seeding domains with the aim to drive protein aggregation and improve IB properties for industrial

applications. In marked contrast to aggregation tags used till present to increase protein aggregation, LZ are protein–protein interaction domains consisting of amphipathic α helices that dimerize in parallel, either as homodimers or heterodimers, to form a coiled-coil.[20-22] LZ dimerization motifs have already been explored as protein-protein interactions drivers both in recombinant mammalian[23] and bacterial[24,25] cells. Thus, LZ specific properties make these peptides promising players to improve, control, and stabilize IB quality, thus boosting their potential for pharma and biotech industrial applications. In this study, we have explored the aggregation profile of a GFP reporter protein fused to Jun and Fos LZ[23] at different positions. The purity and formation of GFP IBs have been studied to determine LZ possible role in obtaining purer and better controlled IBs.

Results

Construct design and modelling

In this study three different protein constructs, named GFP, J-GFP-F and J/F-GFP, have been engineered to evaluate the effect of Jun and Fos LZ on the protein aggregation process (Figure 1a). J-GFP-F is a single fusion protein consisting on a GFP flanked by Jun and Fos at N- and C-terminal, respectively, whereas J/F-GFP is constituted by two proteins (a GFP with Jun LZ at N-terminal (J-GFP) and a GFP with Fos at N-terminal (F-GFP)), simultaneously coexpressed (Figure 1). GFP protein has been used as a control without any LZ tag. The three-dimensional structural arrangements of the constructs were guessed by iterative treading, taking advantage of the I-TASSER webserver.[26] The generated models were visually inspected and possible three-dimensional arrangements of the J/F units with respect to the GFP were selected as representative conformational arrangements (Figure 1b). In all constructs, the J/F subunits tended to assume a helical structure, as expected, but it was only possible to generate highly ordered starting domains for J/F-GFP. All models

underwent 250 ns of atomistic molecular dynamics simulations in full water solvent (Additional file: Supplementary Figure S1) showing the J/F elongated domains to be structurally unstable leading to partially disordered arrangements similar to those initially modelled for J-GFP-F. Among the generated models for J-GFP-F, no highly ordered structure was present, and the pool differed by the local arrangement of the J/F subdomains (as exemplified by two structures in Figure 1b).

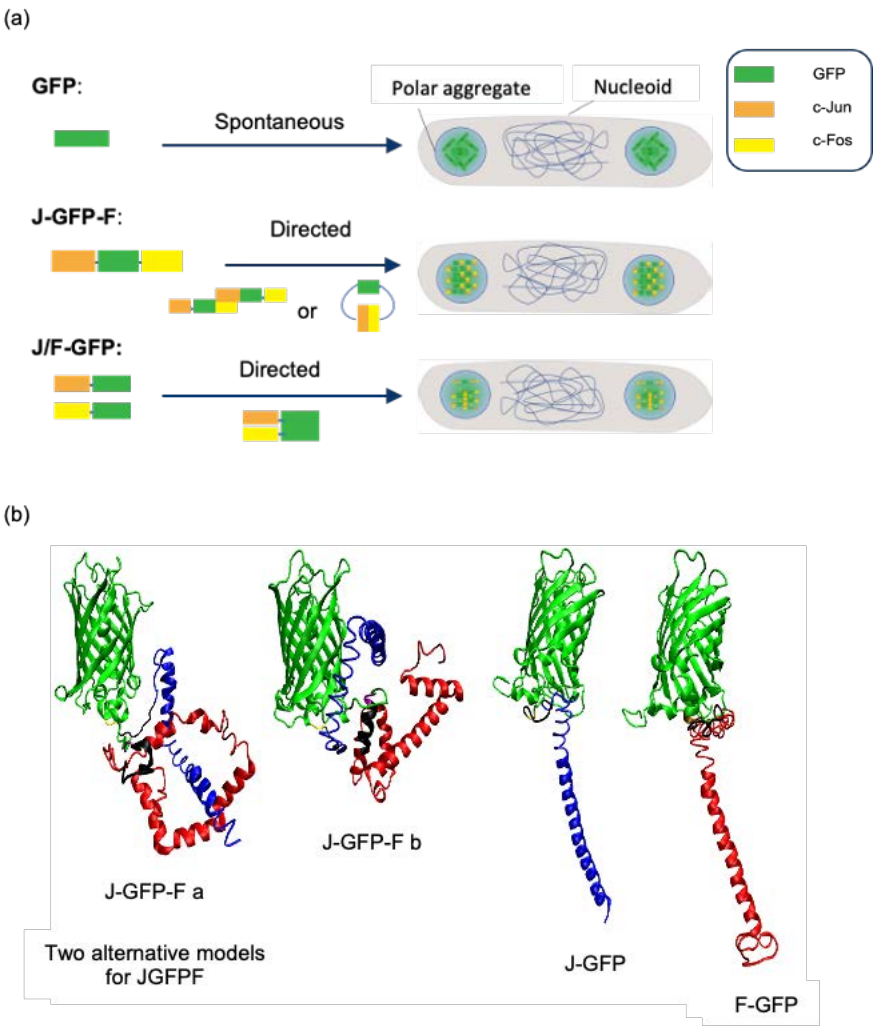

Figure 1. Recombinant construct. a) Diagram of the protein-based constructs and their possible aggregation process as IBs depending on the presence or not of LZ. b) . Representative three-dimensional models of the J-GFP-F (two models: a and b) and J/F-GFP constructs (one model each), as generated by iterative threading. Construct domains are color coded as follow: GFP (green), Jun (blue), Fos (red).

Protein production and aggregation

The cell growth was determined under the overexpression of the three different constructs used in this study (Figure 2). Interestingly, a reduction in the bacterial growth was observed when J-GFP-F and J/F-GFP were produced, especially at 5 h (Figure 2). Moreover, those proteins that impaired the growth (J-GFP-F and J/F-GFP) were produced at lower levels than their GFP counterpart (Table 1). Analyzing the protein yields at different production time points, it can be observed that there is a time-dependent production of both GFP and J/F-GFP, while for J-GFP-F production values kept similar at different time post-induction (Table 1).

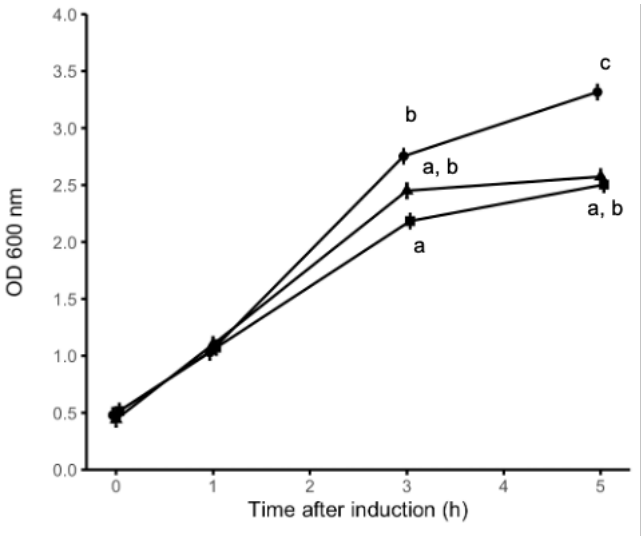

Figure 2. Optical Density values of recombinant bacteria cultures in LB medium after protein expression induction. Circles represent E. coli pET22b/GFP (GFP), triangles E. coli pET22b/Jun-GFP-Fos (J-GFP-F), and squares represent E. coli pETDuet-1-Jun-GFP/Fos-GFP (J/F-GFP). Different letters depict significant differences between the growth curves ($p \leq 0.0001$).

Table 1. Total protein yield (μg) of each recombinant construct at different times post-induction (1, 3, and 5 h).

	GFP			J-GFP-F			J/F-GFP			p value		
Time (h)	**1**	**3**	**5**	**1**	**3**	**5**	**1**	**3**	**5**	**Treat**	**Time**	**Treat x time**
Protein yield (μg)	52.39 ±0.79	99.43 ±1.03	223.82 ±1.48	1.44 ±0.12	1.23 ±0.08	0.93 ±0.07	5.12 ±0.23	9.98 ±0.2	20.66 ±1.96	<0.0001	0.029	0.014

Despite of the reduction in protein levels and in cell viability for those proteins carrying Jun and Fos (Figure 2 and Table 1), all constructs aggregated (Figure 3). Specifically, the analysis of the aggregation ratio showed differences among the constructs used, being the aggregation ratio higher for J/F-GFP than for GFP and J-GFP-F (Figure 3 and Additional file: Supplementary Table 1).

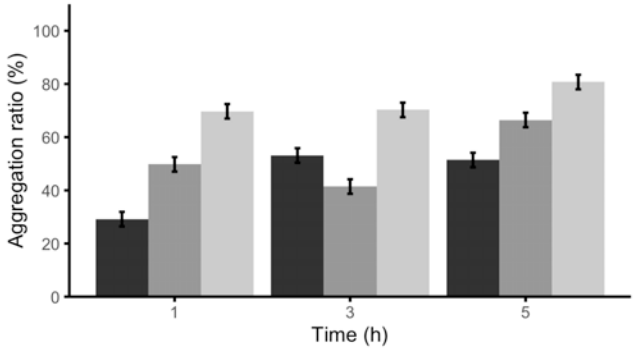

Figure 3. Protein aggregation ratio (%) for each construct over time. Black, dark grey, and light grey bars represent GFP, J-GFP-F, and J/F-GFP, respectively. Significant differences for construct ($p \leq 0.05$) and for time ($p \leq 0.1$; Additional file: Supplementary Table 1).

Characteristics of purified IBs

Aiming to explore the specific nanoarchitectural characteristics of the protein nanoparticles formed using the three different tag combinations, IBs of each construct were purified. In all samples, qualitative and quantitative approaches with high resolution electron microscopy imaging (FESEM) of the ultrastructural morphometric of IBs showed a high number of well-formed nanoparticles with round shape and nanoscale size in the three constructs (Figure 4). However, slight and important shape and size differences between GFP IBs and those nanoparticles formed with Jun or Fos LZ were detected. First of all, control GFP IBs showed a very homogeneous round shape and smooth surface, suggesting a highly compact structure

(Figure 4). In contrast, Jun-Fos based IBs showed a high variability in shape, significantly more amorphous than GFP ones and with a high percentage of IBs showing rough and porous surface. About the size, GFP nanoparticles showed the highest size with a diameter of 400-500 nm and a large mean area (Figure 4), whereas Jun-GFP-Fos and Jun-GFP/Fos-GFP IBs were significantly smaller with a lower area and a diameter around 250 nm in both cases (Figure 4).

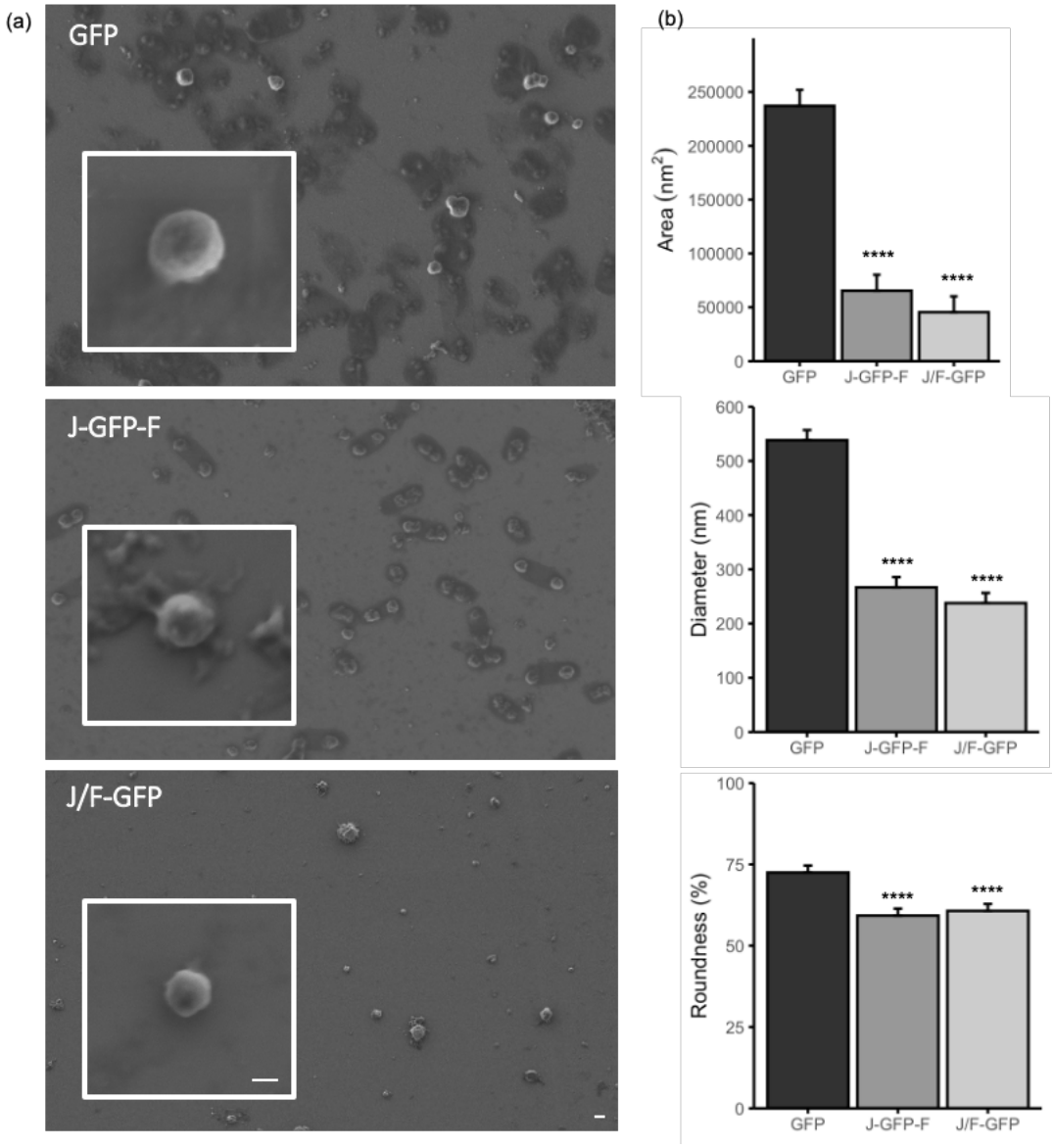

Figure 4. Representative FESEM images of the isolated IBs for each construct: GFP IBs, J-GFP-F IBs and J/F-GFP IBs. Bars size: 200 nm. IB mean area (nm^2), mean diameter (nm) and roundness (%) was calculated for each construct IB (**** $p \leq 0.0001$).

The analysis of the protein quality in terms of protein activity of GFP, J-GFP-F, and J/F-GFP IBs indicated that the addition of Jun and Fos improved the functional protein content when flanking the GFP, while no difference were observed for J/F-GFP compared to GFP (Figure 5a). Interestingly, the comparison of the specific fluorescence of IBs with the soluble version indicated that although the activity of the soluble form of all the proteins was higher than when forming IBs, the difference between soluble and IBs was especially greater in the case of GFP (Figure 5b).

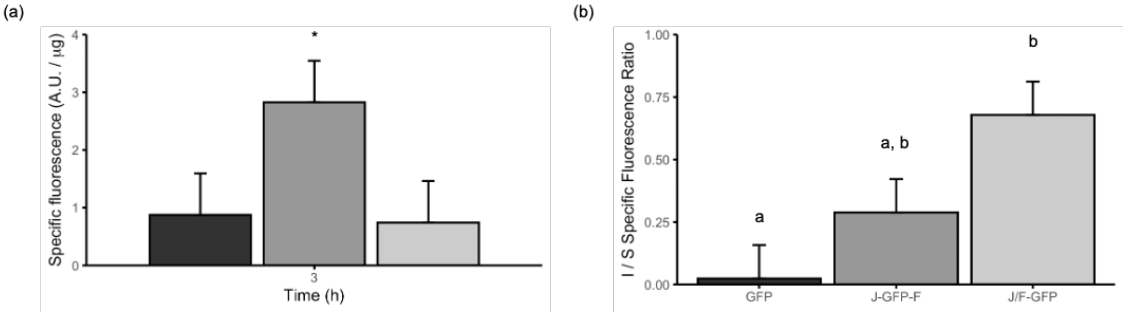

Figure 5. Specific fluorescence of the protein constructs. IB specific fluorescence for the three constructs (a). Specific fluorescence ratio of the insoluble fraction compared to the soluble fraction specific fluorescence (b). Black, dark grey, and light grey bars represent GFP, J-GFP-F, and J/F-GFP, respectively. * and different letters show statistically significant differences ($p \leq 0.05$).

The conformational properties of the proteins embedded in the IBs were investigated by Fourier transform infrared (FTIR) spectroscopy.[27] The spectra were collected before and after hydrogen/deuterium (H/D) exchange to allow a better assignment of the Amide I band components to the protein secondary structures.[28,29] GFP and J/F-GFP IBs displayed comparable IR response, while J-GFP-F IBs showed distinct peak positions and relative intensities of the spectral components assigned to β-sheets (Figure 6 and Figure S3). In particular, the absorption spectra (both before and after H/D exchange) of J-GFP-F IBs were more similar to those of the soluble GFP (Figure S3) and the main β-sheet peak showed a higher downshift after H/D exchange compared to GFP and J/F-GFP IBs (Figure 6). In the deuterated J-GFP-F, this component occurred at around 1620 cm^{-1}, a peak position near to that observed for the main β-sheet band of soluble GFP, which indeed was observed

around 1622 cm^{-1} (Figure 6).[30,31] Overall, these data suggested that the Jun and Fos motifs modulated the conformational features of the formed IBs.

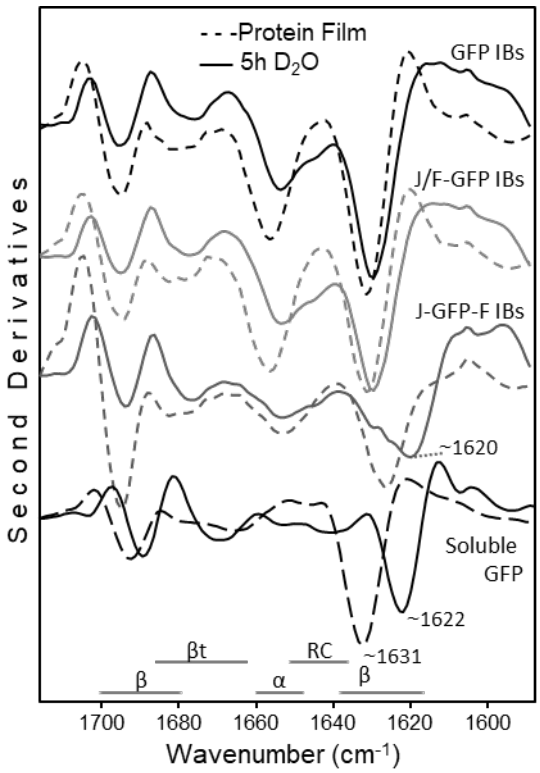

Figure 6. Second derivatives of the FTIR absorption spectra of GFP, J-GFP-F and J/F-GFP IBs and of soluble GFP. Samples were measured in form of protein films, obtained by solvent evaporation, and after re-hydration by D_2O in order to allow H/D exchange. Selected peaks and the typical spectral region of the different protein secondary structures after H/D exchange are indicated. α, α-helices; β, β-sheets; βt, β-turns; RC, random coils.

Purity of IBs

Interestingly, J-GFP-F and J/F-GFP nanoparticles have a degree of protein purity more than twice than that observed for parental GFP nanoparticles (Figure 7). Besides, the analysis of lipid and carbohydrate content in IBs indicated that the presence of Jun and Fos in J-GFP-F increased the presence of these co-contaminants while the strategy for the production of IBs using J/F-GFP showed no significant differences with GFP in lipid and carbohydrate content (Figure 8).

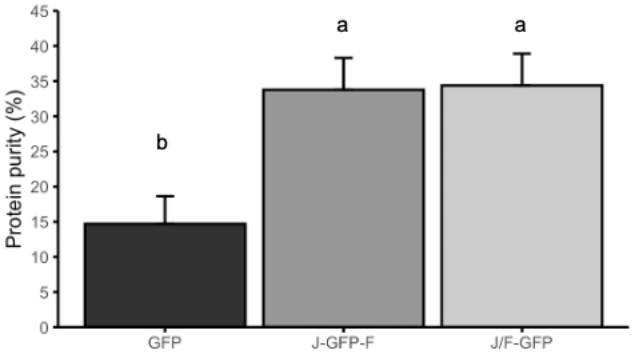

Figure 7. **Protein purity of IBs**. For each construct, protein purity was determined by densiometric image-analysis, comparing the amount of the specific recombinant construct to the whole protein amount of the IB. Different letters depict statistically significant differences ($p \leq 0.05$).

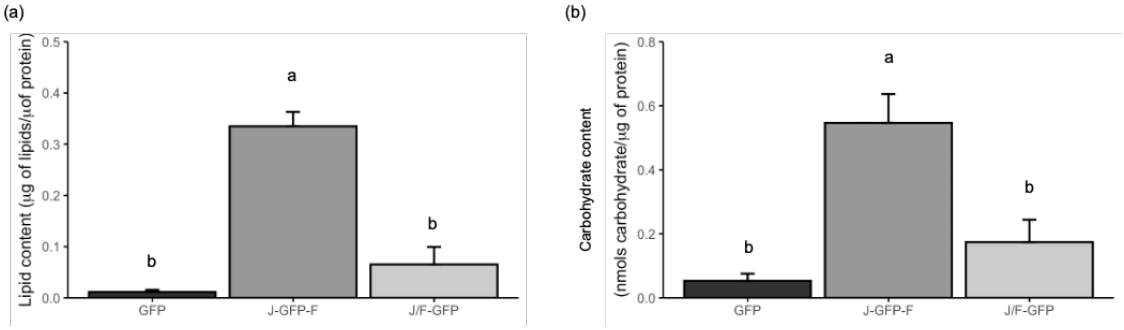

Figure 8. Lipid (a) and carbohydrate (b) content of for each IB construct. Different letters depict statistically significant differences. Different letters indicate statistically significant differences ($p \leq 0.01$ (a) and $p \leq 0.05$, (b)).

Discussion

IBs are protein aggregates ranging at nanoscale that have been widely studied from different perspectives. Since most of the recombinant proteins form IBs when overexpressed in bacteria,[3] and some of them are only produced in this insoluble format, these nanoparticles have been broadly used as a source to obtain the soluble form of a wide range of protein of interest.[32] On the other hand, by being protein nanoparticles rich in functional recombinant protein, IBs have also been explored as a new class of biomaterial with promising applications in biocatalysis, tissue engineering, and human and animal therapies.[7-9,11] However, although different aggregation tags have been used to promote their formation, the impact of these aggregation domains in the quality of protein aggregates has not been addressed so far. For that, in this work, we have explored if the use of Jun and Fos LZ

dimerization motifs could drive the formation of IBs in a more controlled way in terms of quality.

The results obtained proved that the constructs containing Jun and Fos (Figure 1a) were produced as IBs with aggregation ratios higher than in the GFP (Figure 3), proving that the aggregation propensity can be improved by using this strategy. The enhancement of the aggregation propensity can be attributed to the formation of Jun and Fos interactions among constructs, confirming that while the Jun and Fos domains seem to explore a number of partially disordered conformations when free in solution, this does not hinder the formation of stable aggregates. Interestingly, the aggregation ratio was observed to be higher for J/F-GFP than for J-GFP-F (Figure 3). This can be rationalized in terms of competition between intramolecular and intermolecular Jun/Fos interactions. In J-GFP-F the Jun and Fos domains are entangled due to intramolecular interactions between the two (Additional file: Supplementary Figure S1).

Their entanglement in the same construct competes with the formation of dimers, or multimers, where Jun and Fos fragments belonging to different molecules interact and bond with each other. In both cases the two interacting fragments are the same, thus their energetic is expected to be close. At room temperature we might expect the competition and coexistence of monomers, dimers, and multimers in solution. However, it is also worth noting that in J-GFP-F each Jun unit will be close to a Fos domain, this corresponds to a high local concentration of the partner, thus favoring the intramolecular entangled J-GFP-F. On the other hand, when the Jun/Fos domains are uncoupled, independently bound to different GFPs (as in J/F-GFP) there is obviously no competition between intermolecular and intramolecular interactions.

The study of the specific physicochemical characteristics of purified GFP, J-GFP-F and J/F-GFP IBs showed that, depending on the LZ strategy used, it's possible to modulate IB features such as specific activity, size, protein purity and presence of contaminants such as lipids and carbohydrates. The presence of LZ has a significant impact on both the size and shape in the aggregations, with IBs diameters around 250 nm and more amorphous forms, while GFP IBs have a size of 400-500 nm and a higher average surface and higher roughness (Figure 4). However, despite the differences in size, surface rugosity and roundness, ultrastructural morphometry of J-GFP-F and J-/F-GFP IBs (Figure 4) agrees with other conventional IBs produced in *E. coli*[33] and other cell factories such as *Lactococcus lactis*[34] or *Pichia pastoris*.[35] Interestingly, more amorphous shape, rough surface, and lower size of J-GFP-F and J/F-GFP IBs could be indicative of significant differences in protein production and aggregation and/or nanoparticle formation. In fact, nanoarchitectural aspect of GFP IBs shows nanostructures with higher size, compactness and smooth surface than Jun and Fos IBs that appear as more soft particles with rough surface more amorphous shape, and lower mean size.

These nanoscale differences can play a great role in differential functionalities of proteins forming IBs in potential therapeutical applications. Then, low size, amorphous shape, and rough surfaces increase ratio surface/mass and, therefore, potential protein release at *in vitro* and *in vivo* conditions, becoming suitable and desired morphometric characteristics for more efficiently releasing nano or micro-platforms of drug delivery systems. Moreover, although smaller, J-GFP-F had a specific activity significantly higher than the other constructs tested (Figure 5a), which is in accordance with FTIR spectra (Figures S3 and 6). In particular, after H/D exchange the main β-sheet peak of J-GFP-F IBs was observed to be very close to that observed for the soluble GFP, indicating the maintenance of native-like conformational features. Although J/F-GFP IBs showed no differences in the quality of the protein forming

the IBs when compared to GFP (Figure 5a), both J-GFP-F and J/F-GFP aggregates presented activities closer to the soluble form than GFP produced without tags (Figure 5b), which indicated that the presence of Jun and Fos had a positive impact on the IB protein quality.

On the other hand, the analysis of the elements forming such aggregates showed that J-GFP-F and J/F-GFP IBs had less protein impurities (Figure 7), which indicates that Jun and Fos sequences drive a more controlled formation of the protein nanoparticles in terms of protein composition. The presence of lipids and carbohydrates also was affected by the presence of LZ, but unfortunately not improved, being the values detected in J/F-GFP IBs comparable to the levels in GFP IBs and even higher in the case of J-GFP-F (Figure 8).

Overall, these results demonstrate that aggregation-seeding domains based on LZ peptide-peptide interaction can drive the formation of a specific type of IBs, improving their quality in terms of protein content (Figure 7) and in one of the approaches increasing specific activity (Figures 5a and 6). However, those IBs with higher specific activity (J-GFP-F) are produced through a strategy that has a negative impact in the presence of contaminants such as lipids and carbohydrates (Figure 8). On the contrary, the strategy based on the coexpression of Jun-GFP and Fos-GFP (J/F-GFP) to form IBs, even though did not display any increase in the protein quality (Figure 5a), had no negative impact in the content of lipids and carbohydrates (Figure 8), showing that could be a promising approach for the production of IBs with higher recombinant protein content and less protein impurities.

Conclusions

Altogether this study proved that the use of Jun and Fos LZ is a good strategy for the production of IBs with more promising characteristics that might be useful for pharma or biotech applications. This is especially relevant for J/F-GFP approach which allowed producing hybrid IBs with increased aggregation ratio and protein purity without affecting negatively their activity and lipid and carbohydrate content.

Methods

Construction of expression plasmids

The sequence encoding amino acid residues 2-238 of the enhanced GFP (EGFP) was fused downstream of sequence encoding Fos 118-210 (bFos) or Jun 257-318 (bJun) using the linker sequence encoding SGGGSGGS to construct Fos -GFP and Jun -GFP, respectively. For the Jun -GFP-Fos construct, the sequence encoding Jun 257-318 (bJun) was fused at the N-terminal, while the sequence encoding Fos 118-210 (bFos) was fused at the C-terminal, using in both cases the linker sequence encoding SGGGSGGS. The Jun -GFP-Fos construct was cloned into pET22b (Amp^R) vector (pET22b-Jun -GFP-Fos), while Jun -GFP and Fos -GFP constructs were cloned in pETDuet-1 (Amp^R) to co-express them (pETDuet- 1- Jun -GFP/ Fos -GFP). As a control, residues 2-238 of EGFP were cloned into pET22b. The DNA sequences corresponding to each gene sequence were codon optimized for its expression in *Escherichia coli* (GeneArt, Germany).

E. coli competent cells preparation

E. coli BL21 (DE3) cultures was grown overnight (ON) in LB medium at 37°C with shaking at 250 rpm. A 1/100 inoculum was done in 50 ml of LB and the culture was grown until the optical density (OD_{600nm}) reached a value between 0.2 and 0.4. After that, cultures were

centrifuged (4,000 × *g*) at 4 °C for 15 min. Pellets were resuspended in 12.5 ml of cold and sterile 50 mM $CaCl_2$ and incubated for 45 min in an ice bath. Cells were centrifuged again as described above and resuspended in 1.25 ml of cold and sterile 50 mM $CaCl_2$ in glycerol (15 % v/v) to prepare aliquots of 200 µl, which were stored at −80°C. To transform the cells, 40 ng of plasmid DNA were added to 200 µl of competent cells. The mixtures were incubated on ice for 30–60 min, warmed up to 42 °C for 45 s and placed on ice for 30 s. After incubation, 800 µl of LB media were added, and transformed cells were incubated at 37 °C for 1 h. Finally, the cells were plated on LB-agar plates containing the corresponding antibiotic.

Protein production

E. coli BL21 (DE3)/pET22b-GFP, *E. coli* BL21(DE3)/pET22b-Jun -GFP-Fos (J-GFP-F) and *E. coli* BL21(DE3)/pETDuet-1- Jun -GFP/ Fos -GFP (J/F-GFP) ON cultures were inoculated in 50 ml of LB media with 100 µg/ml ampicillin in 200-ml flasks at an initial OD_{600nm} = 0.05. Each culture was grown at 37 °C and 250 rpm until the OD_{600nm} was 0.5 and 1 mM isopropyl-β-d-thiogalactoside (IPTG) was added to induce recombinant protein expression. At times 0, 1, 3, and 5 h after IPTG induction, 1 ml samples were collected for protein fractioning. These cultures were performed by triplicate.

Protein fractioning

Samples of 1 ml were harvested by centrifugation at 6,000 × *g* at 4°C for 15 min and the pellet was resuspended in 0.5 ml phosphate buffered saline (PBS) supplemented with protease inhibitor (Complete EDTA-free, Roche, Switzerland) to prevent protein proteolysis. Then, ice-jacketed samples were disrupted by sonication (2 cycles of 1.5 min at 10% amplitude under 0.5 s cycles) (Branson Ultrasonic SA, Switzerland). These samples were centrifuged at 15,000 × *g* and 4°C for 15 min to separate soluble and insoluble fractions protein fractions. Samples were stored at -80ºC.

Protein determination

Soluble and insoluble protein fractions were analyzed by denaturing SDS-PAGE (15 % acrylamide). Denaturing buffer (Laemli 4x: Tris base 1.28 g, glycerol 8 ml, SDS 1.6 g, β-mercaptoethanol 4 ml, urea 9.6 g in 20 ml) was added to the insoluble and soluble fractions to a final concentration of 1x (see protein fractioning). Soluble and insoluble protein fractions were boiled for 10 and 45 min, respectively. At that time, samples were loaded onto the gel. SDS-PAGE protein bands were transferred onto PVDF membranes and identified using a commercial anti-GFP antibody (1:1,000, sc-9996, Santa Cruz Biotechnology, USA), followed by an incubation with a secondary ALP-conjugated anti-mouse IgG (whole molecule) antibody (1:20,000, A4313, Sigma-Aldrich, USA). The amounts of recombinant protein were estimated by comparison with known amounts (usually ranging from 125 to 1000 ng) of T22-GFP protein.[36] Protein bands were visualized with a solution of NBT/BCIP (B6404, Sigma-Aldrich, USA), and images were obtained using a Color Image Scanner. ImageJ software was used to perform densitometric analyses of the bands.

Fluorescence intensity

Fluorescence intensity of the three constructs was determined in a Varian Cary Eclipse fluorescence spectrometer (Agilent Technologies, Australia) at excitation and emission wavelengths of 480 and 510 nm, respectively.

Protein purity assessment

After separation, the gels were carefully transferred to a plastic tray filled with 200 ml of distilled water and agitated at 50 rpm to remove SDS traces. The staining solution (Coomassie Brilliant BlueR-250 Staining Solution, Bio-Rad, USA) was added into the gels (and incubated for 1 h at room temperature (RT) and revealed with destaining solution (50 % H_2O, 40% methanol, 10% acetic acid (v/v)) until bands were clearly visible. The gel images were acquired by a Color Image Scanner and analyzed with the ImageJ software.

Protein aggregation ratio

The amount of recombinant protein of the soluble and insoluble fraction for each construct was determined as explained above. After that, the aggregation ratio was calculated by dividing the quantity of the insoluble fraction for each time, replicate and construct by the quantity of protein in the respective soluble fraction.

Purification of protein nanoparticles

Bacterial cultures were processed 3 h post-induction through a combination of mechanical and enzymatic disruption methods. Protease inhibitors (Complete EDTA-free, Roche, Switzerland), and phenylmethanesulphonylfluoride (PMSF) and lysozyme were added to the culture at a final concentration of 0.4 mM (Sigma-Aldrich, USA) and 1 μg/ml (Sigma-Aldrich, USA), respectively. After 2 h of incubation at 37 °C and 250 rpms the culture was centrifuged at 6,000 x *g* and resuspended in 30 ml of PBS supplemented with protease inhibitors (Complete EDTA-free, Roche, Switzerland). Then, the mixture was ice-jacketed and sonicated for 4 cycles of 1.5 minutes at 10 % amplitude under 0.5 s cycles (Branson Ultrasonic SA, Switzerland). After sonication, the mixture was frozen ON at -80 °C. The mixture was thawed and Triton X-100 was added (0.4% (v/v)) and incubated for 1 h at RT. After this treatment, the mixture was frozen at -80 °C for 2 h and then thawed between for several cycles until no viable bacterial growth was detected. After that, 125 μl of Nonidet P40 (NP-40) was added and incubated for 1 h at 4 °C. Then, DNA was removed with DNAse at a final concentration of 0.6 μg/ml and 0.6 mM $MgSO_4$ for 1 h at 37 °C. Samples were centrifuged at 15,000 × *g* for 15 min at 4 °C. Pellets containing IBs were washed with 25 ml lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA and Triton X-100 0.5 % (v/v)). Finally, pellets were centrifuged at 4 °C for 15 min and 15,000 × *g* and stored at -80 °C until analysis. The IBs were quantified by western blot using a monoclonal anti-GFP antibody (1:1000, sc-9996; Santa Cruz Biotechnology, USA). All incubations were done under agitation.

Electron microscopy

Field Emission Scanning Electron Microscope (FESEM) was used to visualize the ultrastructural morphology (size and shape) of protein nanoparticles in a nearly native state. For that, protein samples were directly deposited over silicon wafers (Ted Pella, USA), air dried, and observed with a high resolution standard secondary electron detector through a FESEM Merlin (Zeiss, Germany) operating at 2 kV. As a quantitative morphometric measurement, the mean area of IBs for each construct was analyzed as estimator of size with the Image J software. The number of particles was 30, 9, 11, for GFP, J-GFP-F and J/F-GFP, respectively. Using mean area values, the mean diameter of each particles was calculated, were the diameter was also used an estimator of size. The roundness of IB particles for each construct was also evaluated (n = 50/construct), as an assessment of particle shape.

Total carbohydrate and lipid analysis

The total lipid amount in IBs was determined following a sulfo-phospho-vanillin colorimetric assay. Briefly, 500-1,000 µl of each sample was centrifuged at 15,000 × *g* and 4 °C for 15 min. The supernatants were removed, and each pellet was dried by means of a vacuum lyophilizer (SpeedVac™, ThermoFisher) and dissolved in 200 µl of chloroform in a capped glass tube. The chloroform was evaporated at 63 °C in a fume hood. To each tube, 2 ml of 18 M sulfuric acid was added. The samples where then incubated for 10 min in a boiling water bath. After that, each tube was cooled down in ice for 5 min. Five milliliters of phosphoric acid-vanillin reagent (100 ml of 85 % (v/v) phosphoric acid and 0.12 g of vanillin, (Sigma-Aldrich, Germany)) were added to the tubes and incubated for 15 min at 37 °C. The tubes were cooled for 15 min in ice and absorbance was measured at 530 nm. For the standard curve, a range of 10-100 µg triolein (dissolved in chloroform) was used. To determine the total content of carbohydrates present in the IBs samples, a phenol-sulfuric assay was performed. A glucose standard was prepared with a range from 0 to 150 nanomols

and 150-225 µl of IBs were centrifuged at 15,000 × *g* and 4°C for 15 min. The supernatant was removed, and each pellet was dried by with a vacuum lyophilizer. A total of 150 µl of 18 M sulfuric acid was added, followed immediately by 30 µl of 5 % phenol. Samples were incubated at 90 °C for 5 min and then cooled to RT, and absorbance was measured at 490 nm. All assays were performed in triplicate.

Structure Modelling

J-GFP-F and J/F-GFP were modelled by iterative threading, as implemented the I-TASSER server[26] without applying any additional restrains. Given the primary sequence of the three constructs the server allows to generate possible three-dimensional models by multiple threading alignement. The quality of the models was assesed both by their C-score, which was negative in all cases, and visually. For GFP-F, both the first (C-score = -1.86) and the second (C-score = -2.36) modes were chosen as these models showed different arrangements of the Jun /Fos domains. For both J-GFP (C-score = -1.68) and F-GFP (C-score = -2.87) the first model was chosen.

Molecular dynamics protocol

For all the systems, the free construct was minimized, placed in a cubic box with a water layer of 0.7 nm and Na^+ Cl^- ions to neutralize the system, and a second minimization was performed. We used AMBER99SB-ILDN[37] force field and Simple Point Charge water. NVT and NPT equilibrations were run for 100 ps, followed by 250 ns NPT production run at 300 °K. The temperature was controlled with a modified Berendsen thermostat, the pressure with an isotropic Parrinello-Rahman at 1 bar. The iteration time step was set to 2 fs with the Verlet integrator and LINCS constraint. Periodic boundary conditions were used. All simulations and their analysis were run as implemented in the GROMACS package.[38]

FTIR analysis

Protein samples were resuspended in sodium phosphate buffer pH 7.4 and a few µl were deposited on a BaF_2 infrared window and dried at RT in order to obtain a protein film.[28] The

transmission FTIR absorption spectra were then acquired by the Varian 670-IR FTIR spectrometer coupled to the Varian 610-IR infrared microscope (both from Varian, Australia) equipped with a mercury cadmium telluride nitrogen-cooled detector. The following conditions were employed: 2 cm^{-1} spectral resolution, 25 kHz scan speed, 512 scan co-additions, and triangular apodization. Several areas for each sample were measured to verify the reproducibility of the spectral results. Only absorption spectra with the Amide I band intensity below 0.8 were considered reliable. For H/D exchange, the protein film on the BaF_2 window was rehydrated by the deposition of 8 µL of D_2O around the dried film. The chamber was then tightly closed by a second window using a flat O-ring and incubated for 5 h at RT to allow H/D exchange.[28,34] The FTIR spectra of the D_2O-rehydrated samples were collected as described above.

Protein spectra were obtained after subtraction of the proper reference spectra strictly collected under the same conditions. The second derivatives[39] were calculated after spectral smoothing by the Savitsky-Golay method. Data collection and analysis were performed using the Resolutions-Pro software (Varian, Australia).

Statistical analysis

All quantitative data are presented as mean values ± standard error of the mean ($\bar{x}$ ± SEM). Normality of the data was determined by a Shapiro-Wilk test. For IB roundness, the data were normalized with the following formula: sqrt(max(x+1) - x), where x is IB roundness and max the maximal roundness value in the data. A one-way ANOVA was conducted for all quantitative results except for IB surface and specific fluorescence measures. For the latter, values were compared with an independent sample *t*-test. Finally, we did Post hoc comparisons using the Tukey HSD test for all data analyzed by the ANOVA method. The level of significance was set at $p < 0.05$. Measures were done in triplicate, except for IB mean area, diameter and roundness, as indicated above. All statistical analyses were performed using the RStudio Statistical Software (RStudio, Inc., USA).

Abbreviations

LZ: leucine zipper; IB: Inclusion body; GFP: green fluorescent protein; IPTG: isopropyl-β-d-thiogalactoside; FESEM: High resolution electron microscopy; H/D: Hydrogen/Deuterium

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RRP carried out most of the experiments, performed the data analysis and prepared a draft of the manuscript. SF performed the structural modeling and computational study. AN and DA carried out the FTIR analysis and ASC was in charge of electron microscopy analysis. EGF and AA directed the study and prepared the final manuscript. All authors read and approved the final manuscript.

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Study 2

A new generation of recombinant polypeptides combines multiple protein domains for effective antimicrobial activity

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Preface

HDPs are tricky to produce recombinantly, mainly for two reasons: i) HDPs are antimicrobial in nature, and therefore, toxic for the recombinant bacterial cell factory and ii) HDPs are usually short, which makes them prone to degradation. To overcome these problems, we envisioned a strategy that combines different antimicrobial peptides and aggregation tags in a single multidomain protein produced as IBs. Specifically, we have combined an HDP domain with a bacteria binding gelsolin domain and the antimicrobial enzyme $sPLA_2$. Besides, since we have already seen that Jun and Fos leucine zippers promote IB formation, improving also some of their properties such as activity and purity (Study 1), we included these tags in the multidomain protein. Introducing all these domains not only might make our final construct less prone to degradation, but also add additional mechanisms to achieve the desired antimicrobial activity. In this way, we designed a single antimicrobial polypeptide named JAMF1, which was produced with no toxic effects and primarily in a nanocluster (IB) format. In addition, we could also retrieve soluble protein from the IBs, which might be useful in applications where the non-soluble form is not acceptable. Finally, the JAMF1 construct showed an unequivocal antimicrobial effect against different Gram-negative and Gram-positive bacteria. The findings of this study paved the way for the design of new and improved antimicrobial constructs based on recombinant multidomain proteins.

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A new generation of recombinant polypeptides combines multiple protein domains for effective antimicrobial activity

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Abstract

Background: Although most of antimicrobial peptides (AMPs), being relatively short, are produced by chemical synthesis, several AMPs have been produced using recombinant technology. However, AMPs could be cytotoxic to the producer cell, and if small they can be easily degraded. The objective of this study was to produce a multidomain antimicrobial protein based on recombinant protein nanoclusters to increase the yield, stability and effectivity.

Results: A single antimicrobial polypeptide JAMF1 that combines three functional domains based on human α-defensin-5, human XII-A secreted phospholipase A2 (sPLA$_2$), and a gelsolin-based bacterial-binding domain along with two aggregation-seeding domains based on leucine zippers was successfully produced with no toxic effects for the producer cell and mainly in a nanocluster structure. Both, the nanocluster and solubilized format of the protein showed a clear antimicrobial effect against a broad spectrum of Gram-negative and Gram-positive bacteria, including multi-resistant strains, with an optimal concentration between 1 and 10 μM.

Conclusions: Our findings demonstrated that multidomain antimicrobial proteins forming nanoclusters can be efficiently produced in recombinant bacteria, being a novel and valuable strategy to create a versatile, highly stable and easily editable multidomain constructs with a broad-spectrum antimicrobial activity in both soluble and nanostructured format.

Keywords: Antimicrobial peptides, Antimicrobial resistance, Inclusion bodies, Multidomain protein, Solubilization, Recombinant production, Protein nanoclusters

Background

The growing number of antibiotic-resistant pathogens is a pressing healthcare challenge. As a consequence, the development of novel antimicrobial drugs is more necessary than ever, especially against multidrug-resistant (MDR) microorganisms. One source of potential broad-spectrum antibacterials with increasing promise are antimicrobial peptides (AMPs), which are peptides from the innate immune system of nearly all multicellular organisms [1, 2]. AMPs also known as host defense peptides (HDPs), are cationic amphiphilic peptides [3]. These positively charged peptides are classified into defensins (alpha-defensins and beta-defensins), cathelicidins, and histatins [1]. Because they interact and disrupt the negatively charged bacterial cell envelope, they have broad-spectrum antibacterial activity [4, 5]. Although AMPs hold therapeutic potential, even against MDR bacteria [4–6], important drawbacks largely hinder final in vivo applications [3]. Most of the currently used AMP are produced by chemical synthesis, being molecules highly susceptibility to proteolytic degradation by microbial and host enzymes (short half-life) [7]. Besides, due to the high doses needed they are frequently toxic and high production costs are still a problem for large-scale development [8]. Alternatively, AMPs can

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be recombinantly produced [9–11]. Still, their small size makes them easily degradable, and their recombinant production is limited because they are toxic for the producer bacterial cell due to their antimicrobial nature. To address these shortcomings, different approaches to make the production of recombinant AMPs linked to a carrier that stabilizes the peptide have been described. Some examples of carriers for these fusion proteins are small ubiquitin-like modifier (SUMO), thioredoxin (Trx), glutathione *S*-transferase (GST), biotin carboxyl carrier protein (BCCP), green fluorescent protein (GFP), calmodulin and human serum albumin [12–15]. Another strategy to efficiently produce these peptides is the use of acidic partners [16]. In all the cases, these carriers or partners help to overcome the toxicity of the AMP and at the same time increase their protein expression yields [16–18]. However, to retrieve the AMP of interest, it is often necessary to remove these carriers, which requires expensive enzymatic cleavage or toxic reagents [19].

To address this gap, here, we have explored a new strategy for the production of a recombinant antimicrobial protein based on a multidomain polypeptide that combines different functional domains in a single molecule but without a carrier protein. The combination of several domains has been previously reported for other proteins [20–23], but none of them for antimicrobial treatment purposes. Additionally, taking into consideration the specific requirements of price, stability, toxicity, effectiveness, and delivery that appear to be key parameters in the development of a new generation of antimicrobials [3], we have added aggregation-seeding domains based on leucine zippers that increase the recombinant production of the antimicrobial molecules as protein nanoclusters (also known as inclusion bodies (IBs)) [24]. IBs are non-enveloped, porous and mechanically stable protein nanoparticles, mainly formed by the polypeptide of interest and generated during recombinant protein production process, having the potential to be a protein-slow release form when administered [25]. Another advantage of this protein format is its production through a one step-process. This means that, in contrast to most of encapsulation strategies, which involve two separate processes (the production of the carriers and the biomolecule separately), IB production is achieved in one single step. Finally, it has also been previously proven that IBs can be used as a source of soluble protein after a mild extraction protocol in non-denaturing conditions [26, 27].

Results and discussion

Construct design and protein production of JAMF1 as protein nanocluster

Our construct, named JAMF1 and formed by the combination of an HDP (HD5) [28], a bacterial binding domain (gelsolin) [29] and an enzymatic antimicrobial peptide ($sPLA_2$) [30], flanked by two aggregation-seeding domains (c-Jun and c-Fos leucine zippers at N- and C-terminal, respectively), has been designed (Fig. 1 and Additional file 1: Figure S1). During their production in a recombinant bacterial system, no toxicity effects were observed in the producer cell (data not shown) and, as desired, the multidomain JAMF1 protein (54 kDa) was mainly produced as protein IBs (Additional file 1: Figure S2). The percentage of JAMF1 aggregation as IBs was $74 \pm 3.1\%$ of the total multidomain protein overproduced, reaching a yield of 96.5 mg/l and a purity of 95% once purified. FESEM micrographs of the purified JAMF1 IBs showed a porous morphology with a round shape and a particle size of around 500 nm (Fig. 1).

Antibacterial activity of JAMF1 nanoclusters

To determine the antimicrobial activity of JAMF1 IBs, we evaluated the survival of both Gram-positive and Gram-negative pathogenic bacteria treated with JAMF1 IBs, following the procedure described in Fig. 2a. First, the survival of an *E. coli* DH5α model strain in presence of increasing concentrations of JAMF1 IBs was determined and a dose dependent effect was observed ($p \leq 0.01$) (Fig. 2b). Using the concentration of JAMF1 IBs giving the lowest values of *E. coli* survival (10 μM), we tested the antibacterial effect of these nanoparticles with different Gram-positive strains, including extended-spectrum beta-lactam-resistant *Enterococcus* spp. (SHV-12), extended-spectrum beta-lactam-resistant *Enterococcus* spp. (CTX-M-14), and *E. faecalis* (ECF), and Gram-negative strains, including Carbapenem-resistant *Klebsiella pneumoniae* (KPC), quinolone-resistant *K. pneumoniae* (qnrA), and extended-spectrum beta-lactam-resistant *E. coli* (CMY2) (Fig. 2c). In all strains tested we observed a clear decrease in the survival ($p \leq 0.001$), reaching viability reduction values of $96.3 \pm 0.2\%$ for KPC, $91 \pm 0.2\%$ for qnrA, 85.3 ± 0.6 for CMY2, $82.8 \pm 2\%$ for SHV-12, $89.8 \pm 0.9\%$ for ECF, and $94.4 \pm 0.7\%$ for CTX-M-14 (Fig. 2c).

Anti-biofilm activity of JAMF1 nanoclusters

To further evaluate the potential of this new class of antimicrobial proteins we have assessed the capacity of JAMF1 nanoclusters to inhibit biofilm formation. For that, KPC was grown in multiwell plates in which JAMF1 IBs were previously immobilized, as detailed in Fig. 3a. The results obtained showed a decrease of $81.4 \pm 2.3\%$ in biofilm formation ($p \leq 0.0001$) when surfaces were decorated with JAMF1 IBs (Fig. 3b), which confirms that antimicrobial nanoclusters are also active when deposited on plastic surfaces to inhibit biofilm formation.

Several works have studied the IBs appealing features in contexts such as cancer [31], tissue regeneration [32],

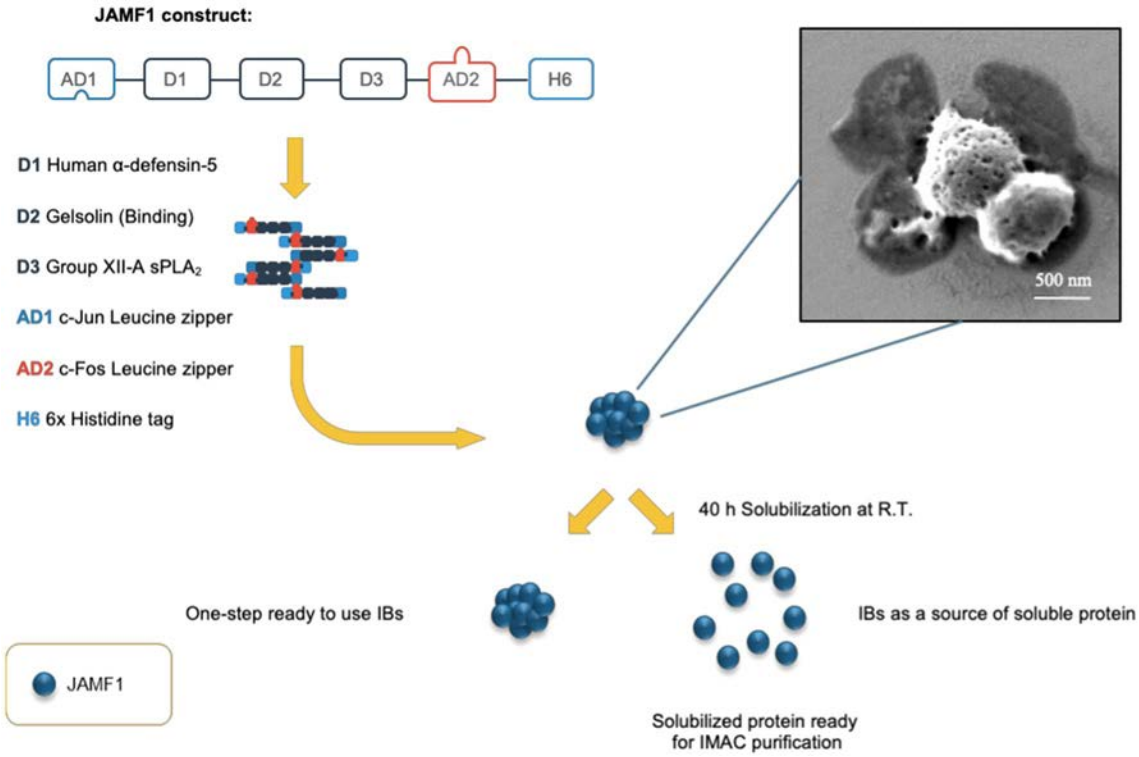

Fig. 1 Construct design. Schematic representation of JAMF1 protein construct and protein production format. Inset image: FESEM micrography of purified JAMF1 nanoparticles

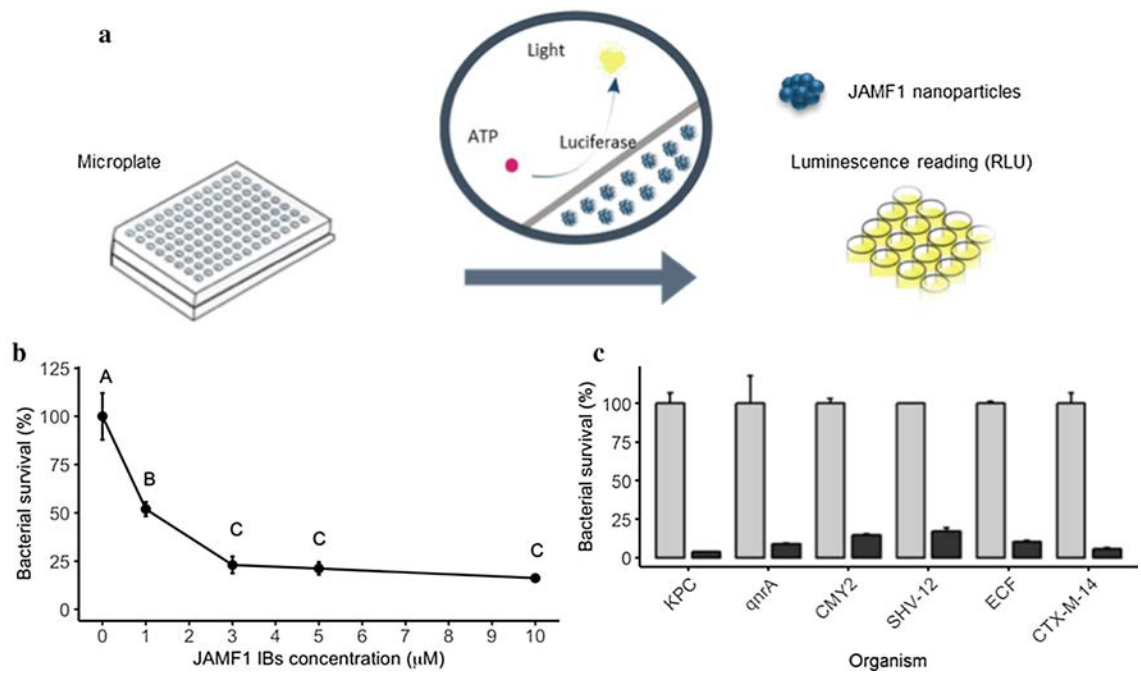

Fig. 2 Antibacterial activity of JAMF1 nanoclusters. **a** Graphic representation of the BacTiter-Glo™ Microbial Cell viability assay. **b** Bacterial survival (%) of *E. coli* DH5α in the presence of JAMF1 IBs at a range of 0-10 μM. Different letters describe significant differences ($p \leq 0.01$). **c** Bacterial survival of KPC, qnrA, CMY2, SHV-12, ECF and CTX-M-14 bacterial strains in the presence of 10 μM of JAMF1 IBs. Survival of JAMF1 treated bacterial cells (black bars) is significantly different from the negative control (grey bars) ($p \leq 0.001$)

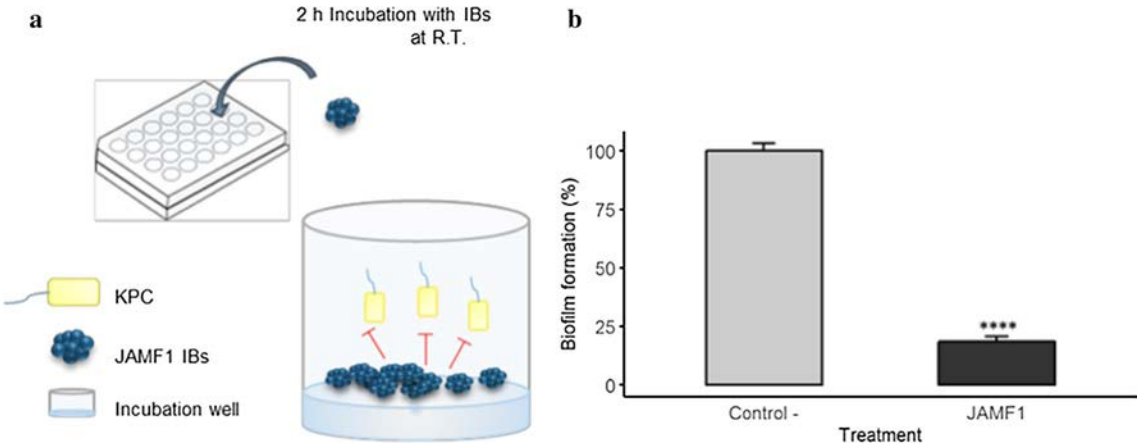

Fig. 3 Anti-biofilm activity of JAMF1 nanoclusters. **a** Biofilm inhibition assay. Plate wells were incubated for 2 h with JAMF1 IBs and then a diluted (1:200) KPC cell culture with 0.2% glucose was added and incubated for 24 h to allow biofilm formation. **b** Biofilm formation ability (%) of KPC after treating plastic wells with JAMF1 IBs (black bar) vs non-treated wells (grey bar). ****Indicates significant differences ($p \leq 0.0001$)

and immunostimulation [33], demonstrating its great potential as a new biomaterial. However, to the best of our knowledge, this is the first study exploring the antimicrobial effect of a multidomain protein embedded in IBs. Whereas previous studies have used fusion partners such as SUMO [12], Trx, GST [13], and human serum albumin [14] to overcome the difficulties to express relatively short peptides, the current work shows that it is also possible to produce a non-toxic and stable AMP-based molecule as a combination of several AMPs. This offers versatility in the construction of molecules and the possibility to explore several combinations to merge complementary antimicrobial activities without the need to use biologically irrelevant carrier proteins. The production of this new generation of antimicrobial multidomain polypeptides as nanoclusters seems to be a good strategy to escape proteolytic and host-toxicity pathways in the recombinant bacterial host.

Solubilized JAMF1 antibacterial activities

For some specific applications a soluble version of the multidomain antimicrobial polypeptide would be more appropriate than the nanostructured or IBs version, for example those applications in which an intravenous administration of the protein would be necessary. Interestingly, the IB format has also been previously described as a reservoir of functional protein [25] and, in consequence, an appealing source of the soluble version of proteins tricky to produce, available after using a mild-extraction protocol [26]. Considering that JAMF1 is mainly produced as IBs (74% of the total protein overproduced), we also explored if JAMF1 IBs could be used as a source to obtain pure antimicrobial multidomain polypeptide in its soluble form using a non-denaturing protocol and evaluate its antimicrobial potential (Fig. 4a). The results proved that the purified soluble version could not only be isolated from IBs (Additional file 1: Figure S2) but also showed antimicrobial activity against either *E. coli* ($p \leq 0.0001$) and KPC ($p \leq 0.001$) in a dose-dependent manner, where the growth inhibition reached values of $78.7 \pm 2\%$ and $91.3 \pm 8.5\%$ for *E. coli* and KPC at 3 and 2 μM, respectively ($p < 0.0001$) (Fig. 4b). Interestingly, the antimicrobial efficiency of this new multidomain antimicrobial molecule is pretty similar at 3 μM, achieving efficiencies of around 78% in both cases (Figs. 2b and 4b). This contrasts with the majority of examples comparing the activity of soluble and IB formats, in which the soluble version is usually more biologically active that its nanoclustered counterpart [34].

Conclusions

Our findings demonstrated that multidomain antimicrobial proteins produced as nanoclusters can be efficiently obtained in recombinant bacteria, being a valuable strategy to create a versatile, highly stable and easily editable constructs with antimicrobial activity against both Gram-positive and Gram-negative bacteria. Moreover, we have proven that the antimicrobial protein forming the IBs can be easily solubilized to obtain also active proteins in its soluble form.

Methods

Bacterial strains and medium

Escherichia coli BL21 (DE3) was used for heterologous protein expression. Strains used for antibacterial and antibiofilm activity assays were *E. coli* DH5α, Carbapenem-resistant *Klebsiella pneumoniae* (KPC), quinolone-resistant *K. pneumoniae* (qnrA), extended-spectrum

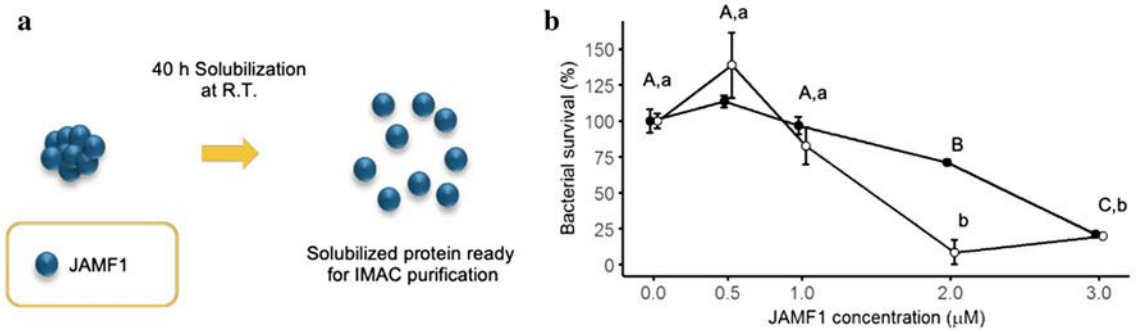

Fig. 4 Solubilized JAMF1 antibacterial activities. **a** Schematic representation of JAMF1 IB solubilization at RT. **b** Bacterial survival (%) of *E. coli* DH5α and KPC at different concentrations (0, 0.5, 1, 2 and 3 μM) of solubilized JAMF1. Capital letters depict significant differences for *E. coli* DH5α ($p \leq 0.0001$) and lower case for KPC ($p \leq 0.001$). Filled circles correspond to *E. coli* DH5α and empty circles correspond to KPC

beta-lactam-resistant *E. coli* (CMY2), extended-spectrum beta-lactam-resistant *Enterococcus* spp. (SHV-12), extended-spectrum beta-lactam-resistant *Enterococcus* spp. (CTX-M-14) and *E. faecalis* (ECF). All strains were grown in Brain–Heart Infusion (BHI) broth (Scharlau, Barcelona, Spain), except for *E. coli* strains, which were grown in Luria–Bertani (LB) medium.

Genetic construct design

From N-terminal to C-terminal, the gene for the JAMF1 (Additional files 2, 3, 4) construct consisted of the sequences encoding Jun257-318 (Uniprot entry P05412), human α-defensin-5 (HD5) precursor (Uniprot entry Q01523), gelsolin188-196 (Uniprot entry P06396), human XII-A secreted phospholipase A_2 ($sPLA_2$) precursor (Uniprot entry Q9BZM1) and Fos118-210 (Uniprot entry P01100). A linker sequence (SGGGSGGS) was used between each of the domains and a C-terminal H6-Tag for protein purification. The fusion construct was codon optimized by GeneArt (Lifetechnologies, Regensburg, Germany) and cloned into pET22b (Amp^R) (Novagene, Darmstadt, Germany) vector.

Inclusion body production and purification

Escherichia coli BL21 (DE3)/pET22b-JAMF1 (Additional file 3) culture (0.5 l) was grown at 37 °C and 250 rpm in LB broth with ampicillin at 100 μg/mL. Protein expression was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG) at an $OD_{600} = 0.4–0.6$. Cultures were grown 3 h post-induction and after that processed as previously described [35]. Briefly, protease inhibitors (Complete EDTA-free, Roche), and phenylmethanesulphonyl-fluoride (PMSF) and lysozyme were added to the culture at a final concentration of 0.4 mM (Sigma-Aldrich) and 1 μg/ml (Sigma-Aldrich), respectively. After 2 h of incubation at 37 °C and 250 rpm the culture was centrifuged and resuspended in 50 ml of PBS supplemented with protease inhibitors. Then, the mixture was ice-jacketed and sonicated for 4 cycles of 1.5 min at 10% amplitude under 0.5 s cycles. After sonication, the mixture was frozen overnight (ON) at − 80 °C. The mixture was thawed and Triton X-100 was added (0.4% (v/v)) and incubated for 1 h at room temperature (RT). After this treatment, the mixture was frozen at − 80 °C for 2 h and then thawed between for several cycles until no viable bacterial growth was detected. After that, 125 μl of Nonidet P40 (NP-40) was added and incubated for 1 h at 4 °C. Then, DNA was removed with DNAse at a final concentration of 0.6 μg/ml and $MgSO_4$ 0.6 mM for 1 h at 37 °C and 250 rpm. Samples were centrifuged at 15,000×*g* for 15 min at 4 °C. The pellet containing IBs was washed with 25 ml lysis buffer (50 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM EDTA and Triton X-100 0.5% (v/v)). Finally, a centrifugation at 15,000×*g* and 4 °C for 15 min was carried out obtaining pellets that were stored at − 80 °C until analysis. Purified IBs were quantified by western blot using a monoclonal anti-His antibody (His-probe, Santa Cruz).

IB solubilization and purification of the solubilized JAMF1

Escherichia coli BL21 (DE3)/pET22b-JAMF1 culture (2 l) was grown as previously described. The whole volume was centrifuged at 6000×*g* and the pellet was resuspended in 120 ml of PBS 1x in presence of protease inhibitors. Samples of 30 ml were subjected to 4 rounds of sonication for 5 min at 10% amplitude under 0.5 s cycle, intercalated by a minimum of 5 min repose in ice. Protein pellets were recovered and washed twice with distilled water. Pellets were weighted and solubilized in 0.2% *N*-lauroyl sarcosine, 40 mM Tris and protease inhibitors at a ratio of 40 ml/g of wet pellet as previously described [27]. The mixture was incubated 40 h ON at RT under agitation and the supernatant was recovered through centrifugation at 15,000×*g* for 45 min at 4 °C

for further purification. NaCl and imidazole were added to the solubilized protein to equilibrate the samples with the binding buffer composition, and Immobilized Metal Affinity Chromatography (IMAC) purification was carried in an ÄKTA purifier FPLC (GE Healthcare, Chicago, IL, USA) using 1 ml HisTrap HP columns (GE Healthcare). Both the binding and the elution buffer contained 0.2% *N*-lauroyl sarcosine, and the final imidazole concentration in the elution buffer was 0.5 M. The buffer of the selected fractions was changed to 10 mM KPi (K/PO_4 buffer pH 7.4) using a desalting column (GE Healthcare). The amount of purified protein was determined by Bradford's assay, and the integrity of the protein analyzed by SDS-PAGE [26].

Antibacterial activity assay

The bacterial cell viability was assessed with the BacTiter-Glo™ Microbial Cell Viability assay (Promega, Mannheim, Germany), according to the manufacturer's protocol. Shortly, bacterial cells were grown O/N at 37 °C and 250 rpm and then diluted 1:100 in KPi buffer. Then, 150 µl from the KPi diluted cells were centrifuged in 1 ml tubes at 6200×*g* at 4 °C for 15 min. Subsequently, the supernatant was removed, and the pelleted cells were then resuspended with 150 µl of either KPi buffer (negative control) or 150 µl of JAMF1 IBs at 1, 3, 5 and 10 µM. After 5 h incubation at 37 °C in a 96-well plate, 100 µl were taken and mixed with 100 µl of the BacTiter-Glo™ reagent. Finally, luminescence was measured in a microplate luminometer (LUMIstar®, BMG LABTECH. Ortenberg, Germany). The measured arbitrary luminescence values were normalized against the control (KPi treatment).

Biofilm formation assay

KPC was used as a model strain. Briefly, an O/N was grown at 37 °C and 250 rpm. Before adding bacteria to a 24-well sterile plate for biofilm formation, IB-treated wells were incubated 2 h at RT with 80 µl/well of 500 µM of JAMF1 IBs. After that, bacteria from the O/N culture were diluted 1:200 in BHI supplemented with 0.2% (w/v) glucose and grown in 24-well sterile plate (400 µl final volume) and incubated at 37 °C for 24 h. After the incubation, the supernatant was removed and wells were washed three times with 500 µl NaCl 0.9%, then fixated with 500 µl methanol for 10 min at RT. Methanol was removed and the plate was dried at 37 °C for 15 min. Finally, the remaining cells in the well were stained with 1% (v/v) crystal violet for 15 min at RT, washed three times with sterile MQ-H_2O. Finally, stained cells were diluted in 33% (v/v) acetic acid and the absorbance was measured at 595 nm [36]. All measurements were done by triplicate and in sterile conditions.

Electron microscopy

Microdrops of JAMF1 IB suspensions were air-dried on silicon wafers and micrographed in a FESEM Zeiss Merlin (Zeiss) running at 1 kV

Statistical analysis

For all assays, each condition was performed in triplicate and represented as the mean ± standard error of the mean. All data were checked for normality. All *p*-values correspond to ANOVA analyses, except for the biofilm formation assay where a *t*-test was performed. Letters correspond to Tukey test analyses.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-020-01380-7.

Additional file 1: Figure S1. Aminoacidic sequence of JAMF1 for each of the construct domains. **Figure S2.** Western Blots of JAMF1. (a) Expression time course of JAMF1 at 0, 1, 3 and 5 h post-induction with IPTG in the insoluble and soluble fractions. MWM = Molecular Weight Marker (kDa), IF = Insoluble fraction, SF = Soluble fraction. (b) Western blot of the purified JAMF1 by IMAC (PP) and the purified IBs (PIBs).

Additional file 2. DNA coding sequence of JAMF1.

Additional file 3. DNA coding sequence of pET22b-JAMF1.

Additional file 4. JAMF1 aminoacid sequence.

Abbreviations

AMP: Antimicrobial peptide; $sPLA_2$: Human XII-A secreted phospholipase A2; MDR: Multidrug resistant; IB: Inclusion body; KPC: Carbapenem-resistant *Klebsiella pneumoniae*; qnrA: Quinolone-resistant *K. pneumoniae*; CMY2: Extended-spectrum beta-lactam-resistant *Escherichia coli*; SHV-12: Extended-spectrum beta-lactam-resistant *Enterococcus* spp; CTX-M-1: Extended-spectrum beta-lactam-resistant *Enterococcus* spp; ECF: *Enterococcus faecalis*; BHI: Brain–Heart Infusion; LB: Luria–Bertani broth; HD5: Human α-defensin-5; IPTG: Isopropyl-β-D-thiogalactoside; SUMO: Small ubiquitin-like modifier; Trx: thioredoxin; GST: Glutathione *S*-transferase; BCCP: Biotin carboxyl carrier protein; GFP: Green fluorescent protein.

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Authors' contributions

RRP carried out most of the experiments, performed the data analysis and prepared a draft of the manuscript. ALC and CS performed experiments of antimicrobial activity and protein production, respectively. EGF and AA directed the study and prepared the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Study 3

Sequence edition of different domains modulates the final immune and antimicrobial potential of a new generation of multidomain recombinant proteins

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Preface

After the promising findings regarding the JAMF1 construct (Study 2), we developed a set of new constructs based on this parental peptide, to improve their antimicrobial potential and explore the flexibility of the multidomain structure. Moreover, since we also knew that the HD5 domain in our proteins is implied in immune regulatory processes, we also wanted to test the immunomodulatory activities of all these constructs, if any. In particular, the HD5 and $sPLA_2$ domains were edited in each of the different designs, to see if we could enhance their immunomodulatory and antimicrobial activity, respectively. We sought to understand the role that each antimicrobial domain played, which at the same time mirrors the versatility of the multidomain approach. Part of this work was done in a research stay in the *Faculty of Veterinary Medicine* of the *University of Calgary* (Dr. Eduardo R. Cobo).

In the previous study, we mainly used the IB format. Here, we focused on the soluble form, obtained after the solubilization of IBs with a mild non-denaturing protocol. First, we found evidence of the importance of adding Jun and Fos leucine zippers as additional domains, since not having them made the construct toxic to the producer cells. We also discovered that the direct antimicrobial activity of these constructs is the result of the different combinations of domains – enzymatic and non-enzymatic – and that these constructs had the specific ability to increase the secretion of the chemokine IL-8 in colonic epithelial cells.

Sequence edition of different domains modulates the final immune and antimicrobial potential of a new generation of multidomain recombinant proteins

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Abstract: (1) Background: Combining several innate immune peptides into a single recombinant antimicrobial polypeptide has been recently demonstrated. However, the versatility of the multidomain design, the role that each domain plays and how the sequence edition of the different domains affects their final protein activity is unknown. (2) Methods: Parental multidomain antimicrobial protein JAMF1 and several protein variants (JAMF1.2, JAMF2 and AM2) have been designed and recombinantly produced to explore how the tuning of domain sequences affect their immunomodulatory potential in colonic epithelial cells and their antimicrobial capacity against Gram-positive and Gram-negative bacteria. (3) Results: The replacement of the sequence of defensin HD5 and phospholipase sPLA2 by other active fragments of both peptides improves the final immunomodulatory (IL-8 secretion) and antimicrobial function of the multidomain protein against antimicrobial resistant Klebsiella pneumoniae and Enterococcus spp. Further, the presence of Jun and Fos leucine zippers in multi-domain proteins is crucial in preventing toxic effects on producer cells. (4) Conclusions: Generation of antimicrobial proteins based on multidomain polypeptides allows specific immunomodulatory and antimicrobial functions, which could be easily tuned by the modification of each domain sequence.

Keywords: Host Defense Peptides; Multidomain protein; Immunomodulation; Antimicrobial; Recombinant Production

1. Introduction

The continuous rise in drug-resistant microbes is already challenging the treatment of infections and has put the health authorities on alert. Antimicrobial resistances (AMRs) are one of the health threats included within the One Health approach, which aims to find solutions to the appearance and prevalence of AMRs by the cooperation across all sectors, including human, animal and environmental health.[1] In the search for new antimicrobials, several strategies have been studied including the development of anti-infectious compounds based on natural compounds, such as essential oils or flavonoids, probiotics and prebiotics, and bacteriophages, among others.[2] However, most of them present important drawbacks such as low antimicrobial efficiencies, tough and time-consuming procedures for their isolation and/or narrowed antimicrobial spectra. Taking a new perspective in the development of antimicrobial molecules, the use of active peptides or proteins naturally present in the innate and adaptive immunity offer a powerful strategy to develop new antimicrobial molecules.[3,4] Among them, there are larger antimicrobial proteins formed by more than 100 amino acids which are often lytic enzymes, nutrient-binding proteins or proteins that contain sites that target specific microbial macromolecules.[5] The smaller antimicrobial peptides, also known as host defense peptides (HDPs), are cationic, amphiphilic and short peptides synthesized by nearly all multicellular organisms with a broad-spectrum antimicrobial activity against bacteria, viruses and fungi.[6] So far HDPs have been intensively explored, proving that they offer a great potential to treat a broad spectrum of microorganisms, including multidrug resistant strains.[7] Most of this research has been done using chemically synthesized peptides. The production of such peptides, however, is intrinsically ineffective at large scale and expensive, thus limiting the clinical application of these molecules.[8] The recombinant production of antimicrobial peptides and proteins from the immune system, whereas promising, is still challenging. The small size of HDPs impairs their stability in front of proteases, and its antimicrobial nature triggers toxic effects on the recombinant producer bacteria, usually blocking an efficient recombinant production. Different groups have addressed the fusion of a carrier protein to the antimicrobial peptide of interest to minimize the toxicity and increase their stability.[9–12] This option, however, comprises a tedious and cost-ineffective work of carrier protein removal. With the objective of overcoming these limitations, our group has recently published a new strategy based on the synthesis of several antimicrobial proteins and peptides in a single polypeptide that codifies a multidomain antimicrobial protein.[13] This approach allows to recombinantly produce antimicrobial peptides of interest at good yields, with no toxicity for the producer

bacteria, and without the need of adding non-functional carrier proteins.[13] In previous work, we combined the HDP human α-defensin-5 (HD5), a bacterial binding domain (gelsolin), and an enzymatic antimicrobial peptide ($sPLA_2$), flanked by two aggregation-seeding domains (a fragment of c-Jun and c-Fos leucine zippers at N- and C-terminal in a single polypeptide named JAMF1). The resulting antimicrobial protein showed activity against both Gram-positive and Gram-negative drug-resistant bacteria. Herein we explored whether the specific sequences of HD5 and $sPLA_2$ can be modulated to improve their immunostimulating and antimicrobial role, respectively. And to do so, we tested variations in the sequences of the antimicrobial domains and substitutions for mature forms or specific peptide domains.

2. Materials and Methods

2.1. Bacteria Strains and growth mediums

Escherichia coli BL21 (DE3) was used for recombinant protein expression. Strains used for antimicrobial activity assays were carbapenem-resistant *Klebsiella pneumoniae* (KPC) and extended spectrum beta-lactamase producing *Enterococcus* spp. CTX-M-14 (EC) (kindly provided by Dr. Lourdes Migura-Garcia, IRTA). *E. coli* strains were grown in Luria-Bertani (LB) medium and KPC and EC were grown in Brain-Heart Infusion (BHI) broth (Scharlau).

2.2. Genetic construct design

From N-terminal to C-terminal, the gene for the JAMF1 construct consisted of the sequences encoding Jun257-318 (Uniprot entry P05412), Human α-defensin-5 (HD5) (Uniprot entry Q01523), Gelsolin188-196 (Uniprot entry P06396), group-XIIA secretory phospholipase A2 ($sPLA_2$) (Uniprot entry Q9BZM1) and Fos118-210 (Uniprot entry P01100).[13] The gene encoding for the JAMF1.2 construct is identical to JAMF1 except for the Human α-defensin-5, where we used HD563-94 instead. The sequence encoding for the JAMF2 construct is identical to JAMF1.2 but the $sPLA_2$ domain was changed to $sPLA_2$23-189. A linker sequence (SGGGSGGS) was used between each of the domains. For HD5-GFP, HD563-94 was fused to GFP[14] using a linker sequence (GGSSRSS). In all constructs a H6-Tag was placed at the C-terminal for protein purification. The fusion constructs were codon optimized by GeneArt (Lifetechnologies) and cloned into pET22b (Amp^R) vector (Novagene).

2.3. Antimicrobial protein production

All constructs were produced recombinantly in *E. coli* BL21 (DE3) using the expression vector pET22b. Cultures (1-2L) were grown at 37 ºC and 250 rpm in LB broth with ampicillin at 100 μg/ml. Protein expression was induced by 1 mM isopropyl-β-d-thiogalactoside (IPTG) at an OD600=0.4-0.6. Cultures were grown 3 h post-induction. The whole volume of each protein production was centrifuged at 6,000 x *g* and the pellet was resuspended in 120 ml of PBS 1x with protease inhibitors (cOmplete EDTA-free, Roche). Samples of 30 ml were sonicated for 5 min at 10 % amplitude (0.5 s ON/OFF cycles) for 4 rounds, resting for 5 min in ice between rounds.

2.4. Protein solubilization and purification

Only HD5-GFP was obtained directly from the soluble fraction. After sonication, HD5-GFP was centrifuged 45 min at 15,000 x *g* at 4 ºC and the supernatant was recovered for Immobilized Metal Affinity Chromatography (IMAC) purification, as explained below. For all other constructs, protein pellets were recovered after sonication and washed twice with distilled water and then weighted. After that, pellets were solubilized under non-denaturing conditions for 40 h at room temperature (RT) under agitation with 40 ml/g of pellet of solubilization buffer (0.2% w/v N-lauroylsarcosine, 40 mM Tris and protease inhibitors), as previously described 15. After the incubation, the supernatant was recovered through centrifugation at 15,000 x *g* for 45 min at 4 ºC for purification. Imidazole (20 mM) and NaCl (500 mM) were added to equilibrate the solubilized samples with the binding buffer composition to prepare the sample for IMAC purification. IMAC purification was performed in an ÄKTA start protein purification system (GE Healthcare) using 1 ml HisTrap HP columns (GE Healthcare). Both the binding and the elution buffer contained 0.2 % N-lauroyl sarcosine, and the imidazole concentration in the elution buffer was 500 mM. The buffer of the selected fraction was changed to 10 mM KPi (K/PO_4 buffer, pH 7.4) with a HiTrap desalting column (GE Healthcare). Protein integrity and quantity were analyzed by SDS electrophoresis (TGX™ FastCast™, Bio-Rad), followed by western blotting with a monoclonal anti-His antibody (1:1000, His-probe, Santa Cruz). Quantification was performed by interpolation to a standard curve of soluble T22-GFP.[16]

2.5. Immunomodulatory activity in colonic epithelial cell

Human adenocarcinoma colonic epithelial (HT29) cells were maintained in Dulbecco's Modified Eagle's media (DMEM; Gibson, Life Technologies) containing 4.5 g/L glucose with 10% (v/v) fetal bovine serum (FBS; Benchmark Gemini Bio-Products), 1 mM sodium pyruvate (Gibco, Life Technologies), and 1% penicillin (100 U/ml)/streptomycin (100

μg/ml; HyClone Thermo, Fischer Scientific) in a humidified environment with 5% CO2 and at 37 °C. Cells were seeded in 24-well plates (Greiner, Bio-One, Monroe), cultured to 80-90% confluency and stimulated (or not) with recombinant HD5-GFP, JAMF1 or JAMF1.2 or HD5 (PeptaNova) (commercial synthetic peptide, positive control) (0.1 and 1 μM) or (± LPS, 1 μg/ml) for up to 24 h in culture medium without FBS or antibiotics. The dose of LPS was determined in a preliminary study using different concentrations (0.1 - 2 μg/ml), and in agreement with the literature.[17] Supernatants were collected from cells and levels of secreted IL-8 determined using a DuoSet ELISA (DY208, R&D Systems). Secretion of human IL-8 was quantified as absorbance units (AU) at 490-540 nm.

2.6. Antibacterial activity

Bacterial cell viability was determined with a BacTiter-Glo™ Microbial Cell Viability assay (Promega). Briefly, bacterial cells were grown O/N at 37 ºC and 250 rpm and then diluted 1:100 in 10 mM KPi buffer. After that, 150 μL from the KPi diluted cells were centrifuged in 1 ml tubes at 6,200 x *g* at 4 ºC for 15 min. The supernatant was removed, and the pelleted cells were resuspended with 150 μL of either KPi buffer (negative control) or 150 μL of 1 and 3 μM of the synthetic HD5 peptide or the protein constructs (HD5-GFP, JAMF1, JAMF1.2 and JAMF2). After 5 h incubation at 37 ºC in a 96-well plate, 100 μL were taken and mixed with 100 μL of the BacTiter-Glo™ reagent for 5 min. Luminescence was measured in a microplate luminometer (LUMIstar®, BMG LABTECH). The measured luminescence values (arbitrary units) were normalized to the control (KPi buffer treatment; equivalent to 100% bacterial survival). HD5 (PeptaNova) serves as a control.

2.7. Enzymatic Assay

A fluorometric assay kit was used to measure $sPLA_2$ activity (Cayman), using a PLA_2 substrate consisting of 1,2-dithio analog of diheptanoyl phosphatidylcholine. Upon enzymatic hydrolysis of the thio ester bond at the sn-2 position, free thiols were detected using 5,5'-dithio-bis-(2-nitrobenzonic acid) (DTNB) at 412 nm. Absorbance measurements were taken each minute during 15 min. The reaction rate (μmol/min/ml) was determined for constructs JAMF1, JAMF1.2 and JAMF2 from the absorbance change per minute of the linear portion of the curve, using the DTNB extinction coefficient. At least 8 time points were used for the calculations.

2.9. Statistical Analysis

Results are expressed as means with error bars representing standard errors of the mean (SEM). Data were obtained in triplicates and normality was assessed using a Shapiro-Wilk test. All comparisons were performed using either a two-sided unpaired Student's *t*-test

(asterisks) or one-way analysis of variance (ANOVA) with a *post hoc* Tukey HSD test for multiple group comparisons (letters). A P value <0.05 was considered statistically significant. All statistical analyses were performed with RStudio software (RStudio, Inc.).

3. Results

3.1. Constructs design and protein production

Several JAMF1 protein derivatives (Figure 1) were designed and produced to evaluate how changes in sequence domains influenced the antimicrobial and immunomodulatory activity of this recombinant multidomain protein. While JAMF1 construct contained the complete sequences of HD5 and $sPLA_2$ including the signal peptide, JAMF1.2, JAMF2, AM2 and HD5-GFP constructs contained a fragment of HD5 (HD563-94). JAMF2 and AM2 also presented the mature form of $sPLA_2$ domain. All multidomain proteins containing Jun and Fos tags were produced in *E. coli* mainly as inclusion bodies (IBs), which after mild solubilization and purification, yields of soluble proteins were at a range of 0.3- 2.7 mg/L culture. AM2 showed toxic effects for the producer *E. coli* strain (Figure 2), achieving just low protein yields (0.001 mg/L).

3.2. Immunomodulatory activity in colonic epithelial cells

To determine immunomodulatory effects of HD5 domain, HT29 cells were challenged (or not) with LPS and stimulated with our constructs. A different performance of the proteins was observed depending on LPS stimuli and protein concentrations (Figure 3). Under LPS challenge and at low protein concentration (Figure 3a), multidomain proteins (JAMF1 and JAMF1.2) similarly induced greater IL-8 secretion than proteins with a single active domain HD5 (HD5-GFP and synthetic HD5), which did not show any increase on IL-8 secretion compared to cells only incubated with LPS. Without LPS stimulus and at low protein concentration (Figure 3c), the recombinant proteins containing the A62-R94 fragment of HD5 (HD5-GFP and JAMF1.2) induced the greatest level of IL-8 followed by JAMF1. Synthetic HD5 peptide did not increase IL-8 secretion compared to the control (Figure 3c). At a higher concentration (Figure 3b, d), all the proteins performed in a similar way as observed at lower protein concentration (Figure 3a, c), except for JAMF1 that inhibited IL-8 secretion independently of LPS challenge (Figure 3b, d).

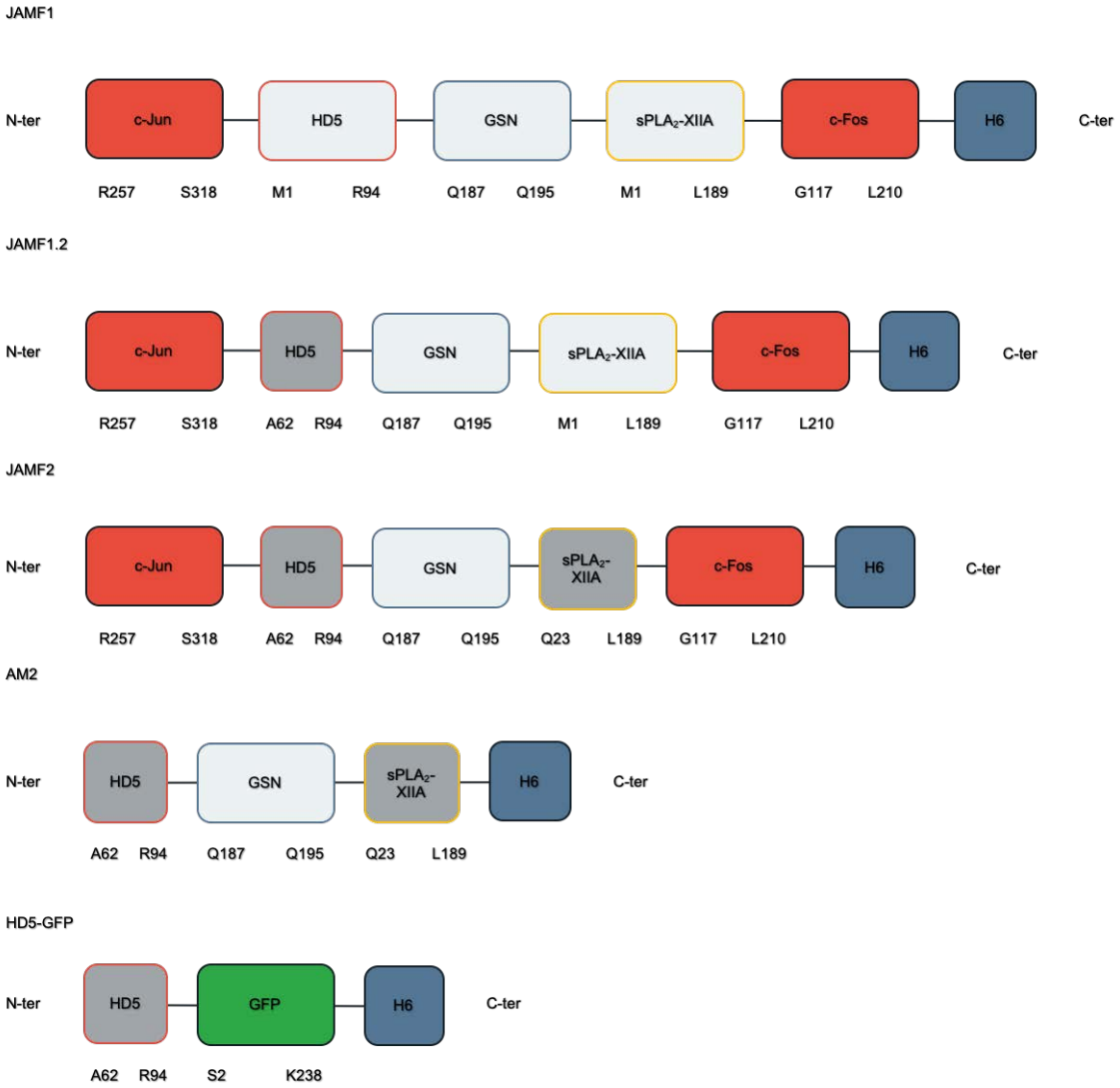

Figure 1. Schematic representation of the different antimicrobial multidomain proteins. The construct JAMF1 contains the whole amino acid sequences of the HD5 and sPLA$_2$-XIIA domains, whereas JAMF1.2 contains the whole amino acid sequence of sPLA$_2$-XIIA but a fragment (A62-R94) of the HD5. Instead, JAMF2 has the A62-R94 fragment of HD5 and the mature form of sPLA$_2$-XIIA domains, and the AM2 construct is identical to JAMF2 without the leucine zipper domains (c-Jun and c-Fos). Finally, the HD5 construct contains the A62-R94 fragment of HD5 coupled to a GFP to allow its recombinant production. All constructs have 6 histidine residues at the C-terminal for protein purification.

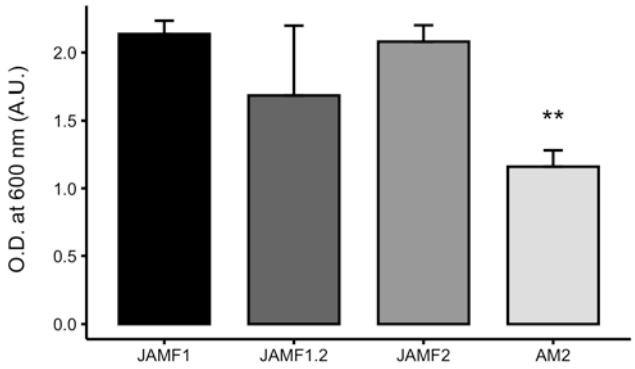

Figure 2. Biomass measure (OD$_{600}$) of *E. coli* BL21 at 3 h of production of the proteins JAMF1, JAMF1.2, JAMF2, which contain the aggregation tags Jun and Fos, and the protein AM2, without the aggregation tags. ** Shows a statistically significant difference ($p < 0.01$).

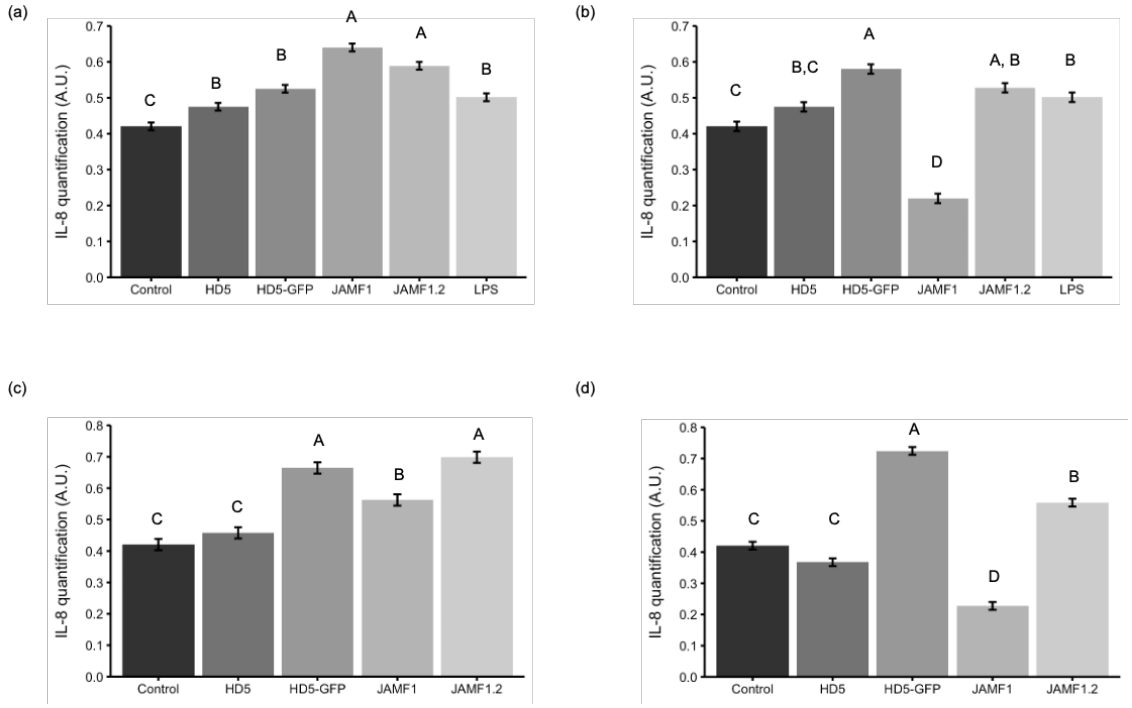

Figure 3. IL-8 secretion by colonic epithelial HT-29 cells treated during 24h with 0.1 (a, c) or 1 µM (b, d) of the different proteins in the presence (a, b) or absence (c, d) of LPS (1 µg/mL). Capital letters show statistically significant differences ($p < 0.0001$).

3.3. Antimicrobial and enzymatic activity

The antimicrobial activity of all the recombinant constructs and synthetic HD5 peptide was evaluated against Gram-negative (*Klebsiella pneumoniae* (KPC)) and Gram-positive bacteria (*Enterococcus* spp. CTX-M-14 (EC)) at two different concentrations (1 and 3 µM) (Figure 4). The antimicrobial activity was greater against Gram-negative KPC reducing the bacterial survival up to 70% (Figure 4 a, b). The most active antimicrobial protein was JAMF2 (at 1 or 3 µM) followed by JAMF1.2 and JAMF1. By contrast, the EC was slightly inhibited (40%) only at 3 µM by JAMF1 and JAMF2 (Figure 4 c, d). HD5 synthetic peptide was more bactericidal against KPC compared with recombinant HD5 peptide fused to GFP.

The analysis of the enzymatic activity of $sPLA_2$ domain showed greater values for JAMF2 protein than for JAMF1.2 and JAMF1 (Figure 5), which is in agreement with the presence of the $sPLA_2$ mature sequence.

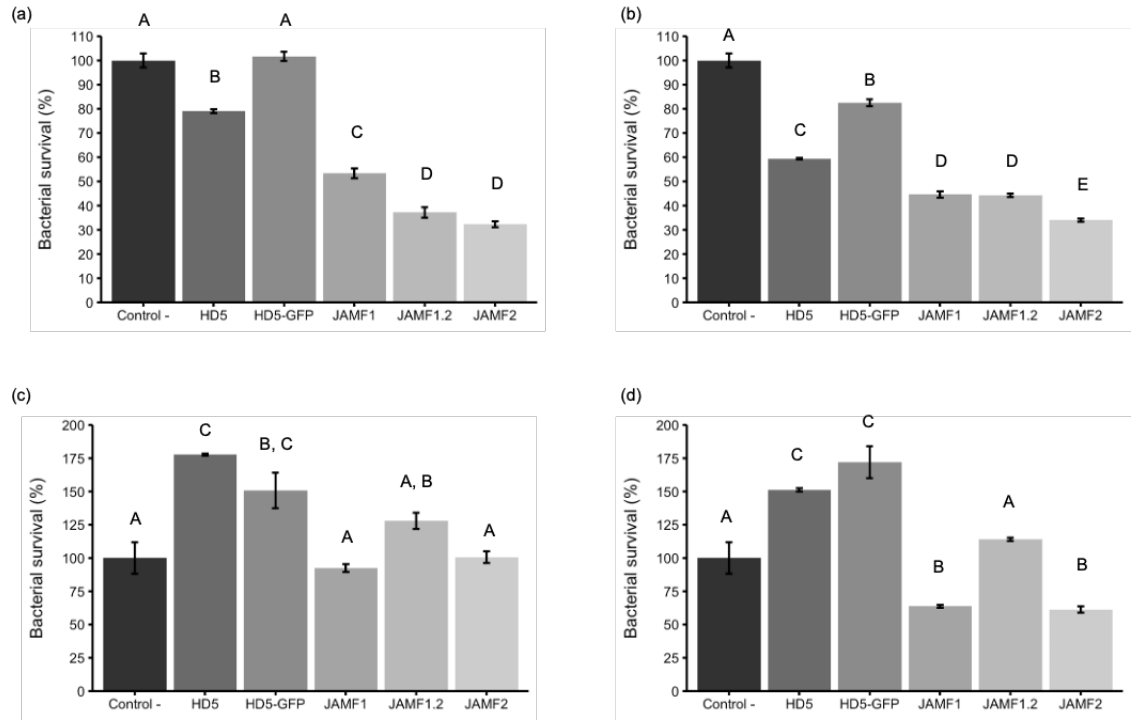

Figure 4. Antimicrobial activity of the different antimicrobial constructs at 1 (a, c) and 3 μM (b, d) against carbapenem-resistant *Klebsiella pneumoniae* (a, b) and *Enterococcus* spp. CTX-M-14 (c, d). Capital letters display statistically significant differences ($p < 0.0001$).

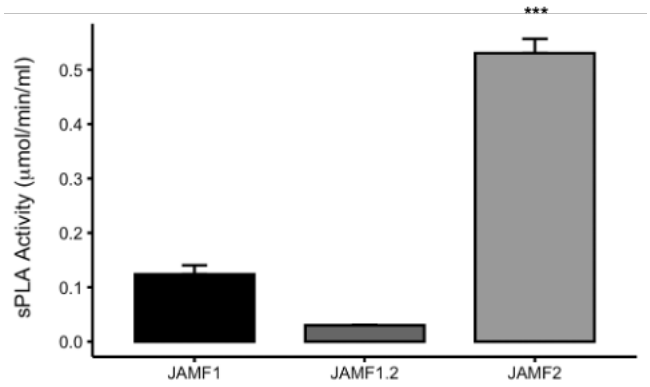

Figure 5. Enzymatic activity of the secreted phospholipase ($sPLA_2$) domain for each antimicrobial construct. Asterisks show a statistically significant difference ($p < 0.001$).

4. Discussion

We have recently described a novel strategy to create a versatile, easily editable multidomain protein.[13] The parental construct, named JAMF1, linked up the HDP human alfa defensin 5 (HD5), a bacterial binding domain (gelsolin) and an enzymatic antimicrobial peptide ($sPLA_2$), flanked by two aggregation-seeding domains (c-Jun and c-Fos leucine zippers fragments at N- and C-terminal, respectively).[13] HD5 is the major antimicrobial defensin peptide of ileal Paneth cells whose mechanism of action is based on pore-forming activity in the cell wall of

the bacterial cell. Besides, $sPLA_2$ is an enzyme involved in the defense of the host via phospholipid degradation of bacteria membranes.[18] Thus, both HD5 and $sPLA_2$ are peptides able to disrupt bacterial cells, but HD5, like other HDPs, also has an important role contributing to the regulation of host immunity. HD5 is responsible to up-regulate the expression of genes such as IL-8, IL-2 and IFN-γ involved in cell survival and inflammation in a NF-kB-dependent fashion in epithelial cells.[19] Likewise, gelsolin is involved in the host immune recognition of lipoteichoic acid (LTA) and LPS of Gram-positive and Gram-negative bacteria, respectively, and its presence in the multidomain polypeptides aims to improve the targeting of JAMF1 to pathogens.[20] The incorporation of two seeding domains such as Jun and Fos leucine zippers fosters protein aggregation inside the recombinant producer bacteria, but do not exert any activity against pathogens.

In our previous study,[13] we have demonstrated that the combination of several peptides from the innate immunity in a single recombinant polypeptide is possible. In this study, we showed that the multidomain design is versatile and the activity of the construct can be tuned by slight changes in the sequences. Several JAMF1 protein variants based on the tuning of antimicrobial sequences of HD5 and $sPLA_2$ domains have been designed and produced herein, leading to JAMF1.2, JAMF2 and AM2 proteins (Figure 1). The rest of domains, which are not antimicrobial per se have been maintained intact in all the constructs, except for AM2 where the Jun and Fos domains were removed. Accordingly, AM2 was toxic to the producer cell, reducing the final biomass of the culture (Figure 2). The seeding domains lead to the formation of protein aggregates known as bacterial IBs. IBs can be directly used as an antimicrobial material,[13] but they can also be used as a source of soluble antimicrobial proteins using a mild non-denaturing solubilization protocol previously stablished in our group.[21] Using the soluble version of JAMF1 and JAMF1.2, we compared their ability to induce chemokine IL-8 secretion on intestinal epithelial cells in comparison with HD5 peptide (Figure 3). This IL-8 chemokine (CXCL-1 in mice) is key when secreted by colonic epithelia in the recruitment of neutrophils into the colon during infectious colitis.[22] The results demonstrated that the whole sequence of HD5, present in JAMF1, behaved differently from the peptide sequence present in JAMF1.2. Specifically, the complete HD5 sequence led to bimodal behavior depending on the concentration used (Figure 3). At low concentration (0.1 μM), JAMF1 increased the secretion of the IL-8 cytokine, but at a higher concentration (1 μM) it reduced the secretion of IL-8. In contrast, recombinant proteins containing a fragment of HD5 presented the same immunostimulatory effect at both 0.1 μM

and 1 μM, even when accompanied with other concomitant host defense domains, as it occurred in JAMF1.2, or just fused to GFP carrier protein (HD5-GFP). Note that this experiment also pointed out the better function of HD5 when expressed recombinantly compared to synthetic form of HD5 (Figure 3). It is likely that when HD5 is recombinantly expressed, its folding is probably closer to its native structure, which has a clear effect in the final immunomodulatory activity of this peptide. Moreover, as expected, the capacity of the multidomain proteins to immunomodulate intestinal epithelial relied on the HD5 domain, as the inclusion of additional domains (JAMF1 and JAM1.2 compared to HD5-GFP) did not influence on its activity (Figure 3).

We showed that the direct antimicrobial activity is a result of the combination of several domains. The performance of HD5-GFP was lower than that of multidomain proteins (JAMF1 and JAMF1.2, and JAMF2), which substantially inhibited bacterial pathogens (Figure 4). Interestingly, the antimicrobial activity of synthetic HD5 peptide (Figure 4) was not impaired as it occurred with the immunomodulatory activity (Figure 3), indicating that the folding is not determinant for the pore-forming activity. The fact that multidomain proteins presented greater antimicrobial activity than the HD5 peptide and HD5-GFP is likely due to the presence of $sPLA_2$. Indeed, among multidomain proteins containing either the premature (JAMF1 and JAMF1.2) or mature forms (JAMF2) of $sPLA_2$, JAMF2 showed the highest enzymatic activity (Figure 5). The proteins JAMF1.2 and JAMF2 sequences are equal except for the $sPLA_2$ domain, that in one case is the non-mature form (JAMF1.2) while the other is the mature form (JAMF2). According to that, JAMF2 not only presented greater enzymatic activity (Figure 5) but also better ability to kill bacteria compared to JAMF1.2 and JAMF1 (Figure 4).

5.Conclusions

This study demonstrates the versatility of multidomain antimicrobial proteins and the effect of sequence tuning on the final function. The combination of a specific fragment of HD5 and the mature form of $sPLA_2$ improves both the immunomodulatory and antimicrobial function. And the use of Jun and Fos sequences flanking the multidomain protein allows its recombinant production, avoiding toxic effects to the producer cells.

Author Contributions: Conceptualization, R.R.P., A.A., and E.G.F.; methodology, R.R.P., R.H., A.L., E.R.C.; formal analysis, R.R.P, A.A., E.G.F.; investigation, R.R.P, R.H., E.R.C..;

resources, E.R.C., A.A., and E.G.F.; writing—original draft preparation, R.R.P., A.A., E.G.F.; writing—review and editing, all authors contributed; visualization, R.R.P., A.A, and E.G.F.; supervision, E.R.C., A.A., E.G.F.; project administration, E.R.C., A.A., E.G.F.; funding acquisition, E.R.C., A.A., E.G.F. All authors have read and agreed to the published version of the manuscript.

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General discussion

A new generation of antimicrobial proteins: potential and versatility

Throughout this work, we have seen how big the AMR problem is and will be in the future. The overall aim of this thesis was to develop a possible solution to help tackle AMRs. We built a group of new antimicrobials by fusing HDPs and antimicrobial proteins, which to our knowledge, were nonexistent and offer a large matrix of possible combinations. Moreover, their versatility does not only rely on their domain components but also on their possible delivery formats, including nanoparticles, still unexplored to date with antimicrobial proteins and peptides.

Although there are other structural units, such as coiled coils, domains can be viewed as the building blocks of proteins, since most proteins are made of a limited number of domains.[234] In fact, multidomain proteins are already found in nature as a result of evolutionary processes.[234] There are many natural domains, but the set of possible domain combinations massively exceeds the set of actual proteins allowed by wild type genes. Because most domains are often functional, independently stable units, we can swap them with genetic engineering to make a chimeric protein, which might have functional properties derived from each of the original peptides. If one assumes there are more than 2,000 HDP sequences alone[81,82] – not taking into account other domains that we might want to add, such as targeting domains – and we wanted to build a four domain protein, which is not particularly large,[235] we have $2{,}000^4$ possible combinations, or around $2 \cdot 10^{13}$ sequences. In other words, 20 million million new potential alternatives, a hundred times the number of galaxies in the universe (Figure 14).[236] Of course, not all permutations might be anti-infective, practical or interesting, but the vastness of possibilities makes the multidomain approach an almost infinite source of antimicrobial alternatives. To add a further layer of complexity, we can include only portions of sequences, as we have seen in Study 3. Or add additional activities, such as immunomodulatory domains, targeting domains or pull-down tags.

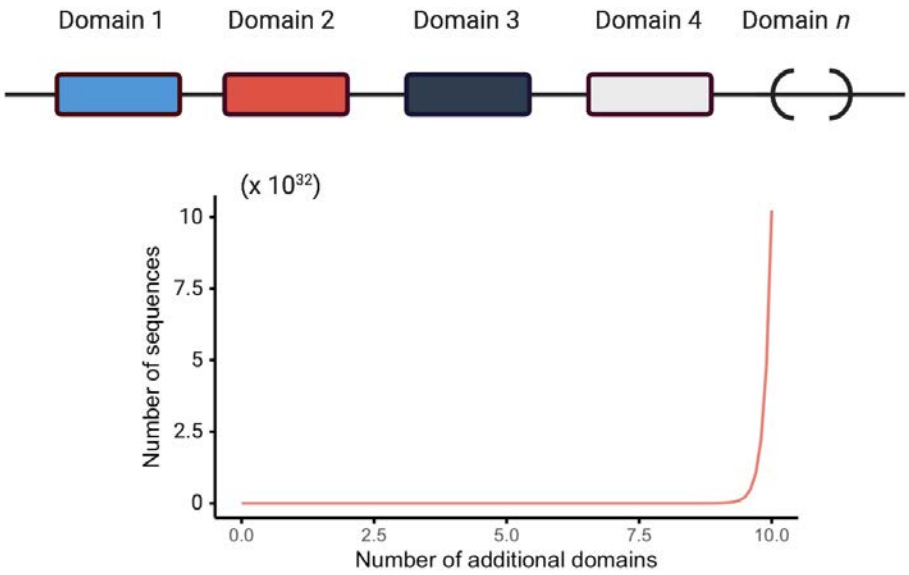

Figure 14. The boundless possibilities of the multidomain approach. For each domain that we might want to add to our chimeric constructs, there is a vast range of options to choose from. The number of sequences increases exponentially for any additional domains.

Presumably, adding domains is, in fact, a net sum of independent activities. Not so, as fusion proteins can surpass the sum of their parts,[237] although not all multidomain proteins behave in the same way.[238,239] Pinheiro and co-workers, for instance, fused two antimicrobial peptides (SP10-5 and Sub5) to the polypeptide Blad.[238] In the case of SP10-5/Blad, the final result was an improved antibacterial and anti-fungal activity compared to either of the peptides alone. Extraordinarily, these peptides that were inactive against *P. aeruginosa* or *E. coli*, after their fusion they could inhibit bacterial growth of these strains. Nonetheless, the Sub5/Blad chimera antimicrobial activity was markedly diminished after the fusion.[238] Fusion proteins with the desired domains might, sometimes, work in ways that are not foreseeable, and thus, their design is still a significant challenge.

However difficult the design is, another exciting feature of fusion proteins is that they can be constructed to interact simultaneously on two or more different pathways (Study 2 and 3), which is a significant focus in therapeutic fusion protein research and development.[240] Our antimicrobial constructs attempt to take advantage of this in multiple ways. First, because we combine two antimicrobial domains (HD5 and $sPLA_2$) that have completely different mechanisms of action (pore-formation and membrane lysis, respectively), we try to fight bacteria in various frontlines. And for this same reason, maybe making resistance to our multidomain proteins harder to occur. Secondly, besides the antimicrobial activities, our

designs also have potential immunostimulatory properties (IL-8 secretion) (Study 3). IL-8 is a potent neutrophil chemotactic factor and is crucial for their degranulation,[241] which releases more antimicrobial compounds, such as defensins or lactoferrin.[242]Moreover, it is described that HD5 domain up-regulates the genes of IL-2 and IFN-γ, involved in cell survival and inflammation.[123] By changing the expression of different immunoregulatory molecules, our proteins might help potential hosts to fight infections better.

Peptide tinkering

We could think of protein domains like car components that can be exchanged for better – or worse – ones and see how the overall performance is affected. That is what we did in Study 3, to understand how editing some domains affected the final protein activity. First, we discovered that adding additional antimicrobial domains to HD5 improved bacterial killing, hinting that the $sPLA_2$ domain was probably working well. Second, we identified differences between the parental peptide JAMF1 and its variations. For instance, we saw increased enzymatic activity in JAMF2, which has a mature form of $sPLA_2$. Accordingly, JAMF2 was also better at killing bacteria than JAMF1 or JAMF1.2, which have the full $sPLA_2$ sequence. Moreover, the HD563-94 fragment conferred higher activity to proteins JAMF1.2 and JAMF2 than the complete HD5 sequence included in JAMF1 protein (Study 3). Thus, each of the domains in the fusion antimicrobial proteins can be easily edited to tune the final performance of multidomain protein. In addition, fine-tuning sequences at the amino acid level – not only whole domains – with the help of *in silico* methods and mutagenesis strategies could open up a broad array of possibilities.

These results point, one more time, to the flexibility of multidomain proteins and how we can play with the components (domains) to refine the desired activity –in this case, antimicrobial. From the antimicrobial point of view, triggering several modes of action, bactericidal and immunomodulatory, makes the fight against infections even more effective. In this line, the series of multidomain proteins constructed in this thesis are able to kill bacteria (Studies 2 and 3) and also to stimulate IL-8 secretion to attract immune cell effectors such as neutrophils (Study 3).

Among the different domains to use, not all have to be related to the final function of the multidomain protein. It has been widely described that there are fusion partners that can be

used just to allow recombinant expression of any protein or peptide of interest. In the case of HDPs, the purpose is twofold, as these tags allow HDP expression and avoid toxicity to the host, as they are antimicrobial. From the different available carrier proteins used to improve recombinant protein expression, it appears that thioredoxin and SUMO are the most used in the case of HDP production.[243] Both have a small size (11-12 kDa), leading to high peptide-to-carrier ratios that ultimately increase HDP recovery yields.[244] Both promote soluble protein expression without toxic effects to the host. For carrier removal, however, SUMO could be superior as its enzymatic cleavage is very specific.[244] Nonetheless, in many cases choosing an adequate fusion tag can be burdensome. For example, Gomes *et al.* fused three different human HDPs to spider silk to combine antimicrobial activities with silk properties, such as robustness and polymerization ability.[245] The silk-HDP hybrids had a decrease in antimicrobial activities if their concentration was increased, due to silk self-assembly, which reduced the availability of the HDPs to which they were fused.[245] In addition, the most active peptide between the three constructs varied depending on the concentration,[245] showing how the same fusion tag might work differently on a case by case basis.

As we have seen in Study 3, another strategy to improve recombinant expression and support the host survival during recombinant production of HDPs is to add aggregation seeding domains that promote IB formation. We have demonstrated that the presence of Jun and Fos leucine zippers reduce the toxicity of antimicrobial multidomain variants to the recombinant host. These leucine zippers increase the protein potential to aggregate (Study 1) probably reducing the exposition of antimicrobial domains and its effects on the host (Study 2 and 3). Other pull-down tags have been used together with an HDP to promote IB formation, and then IBs have been resolubilized – usually under harsh conditions.[246,247] However, no one has used antimicrobial IBs as such, which brings us to the next point.

Antimicrobial inclusion bodies

Inclusion bodies are an active material that has already been used in other applications such as cancer,[187] immunostimulation[248] and tissue regeneration.[186] Because they are highly stable and have slow-release properties, they might also be a very convenient tool to fight AMRs. For example, to maintain antimicrobial activity, constant administration of antimicrobials that have a short half-life is required, or otherwise, concentrations under MIC will probably

happen during a treatment, which further increases AMRs.[249] A slow-release profile seems to be vital to maintain constant antimicrobial levels for long periods to get optimal therapeutic benefits while minimizing AMRs. Another certain benefit is that one, long-lasting administration is more convenient than multiple, short-lived ones. Better yet, IBs have these properties without the need to manipulate them further, such as encapsulation processes or embedding the proteins in a matrix, because they are naturally produced in a one-step process being carrier and active protein at the same time.

But how do IBs compare to other formats (Figure 17)? A study that compared matrix metalloproteinase-9 (MMP-9) IBs to soluble and encapsulated soluble MMP-9 showed that IBs had lower biological activities *in vitro* than the other two formats, up to 250 times less active. In contrast,, IBs were the most active protein format *in vivo*.[177] If we seek highly active proteins, it appears that the soluble format is the best alternative, which is in agreement with other studies.[174,250] In contrast, IBs were considerably more stable than soluble protein that had been encapsulated.[177] IBs were the most active format *in vivo* because they kept moderate activities even after 14 days of incubation in 50 % (v/v) serum, whereas soluble and encapsulated formats lost most of the activity after a few hours.

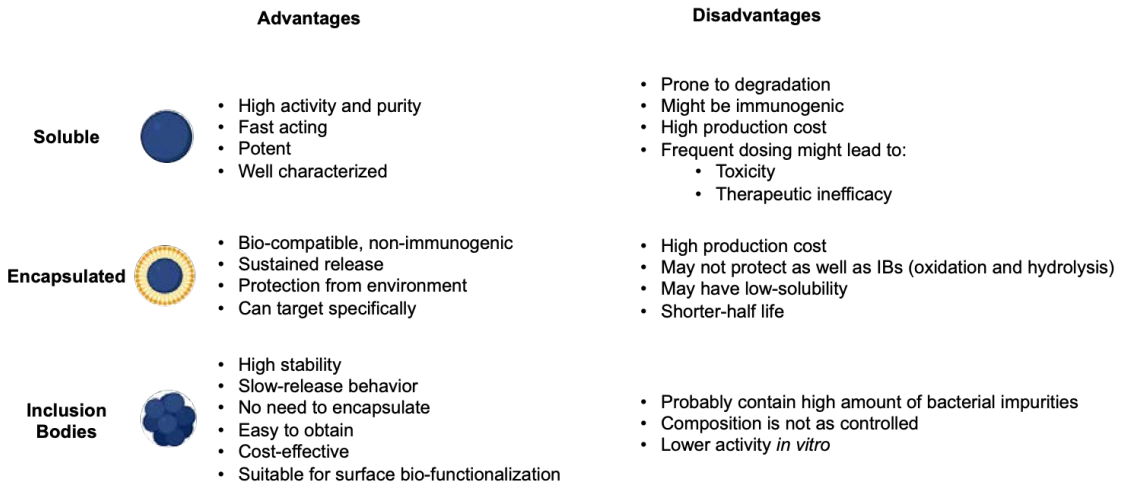

Figure 15. Different protein formats and their advantages and disadvantages. There are a variety of format options to choose from, depending on the final application and budget restrictions.

In Studies 2 and 3, we demonstrated the broad spectrum of the JAMF1 construct and its variants in the soluble and IB format. In contrast to the previous example with MMP-9[177] and other studies,[174] we demonstrated how antimicrobial IBs were as effective as the soluble format and worked at low concentrations, starting at 1-2 μM. In addition, our constructs

worked against several AMR strains. Our designs were made with the intention to be broad-spectrum, but again, the multidomain approach can be used in many ways. We can add targeting domains for particular strains, and thus, avoid the killing of non-pathogenic bacteria.[220,226] Therefore, we can make a new generation of nanoparticles with a wide or narrow strain coverage, with immunomodulatory activities and the potential to be solubilized.

IBs have another important characteristic to fight against intracellular pathogens. Although most pathogens act extracellularly,[251] a fraction of them are intracellular. Even worse, some pathogens, such as *S. aureus*, *E. coli*, *and P. aeruginosa,* traditionally thought to be extracellular, can also localize inside host cells.[252] Intracellular infections are usually persistent as they are hard to reach by the immune system[252] and the majority conventional antibiotics,[253]which show no or poor intracellular activities. Since IBs have a tendency to bind and penetrate mammalian cells, at least *in vitro*,[184,188,254,255] without being toxic, it may be that antimicrobial IBs can reach -and effectively treat- these previously inaccessible pathogens (Figure 16). We could also take advantage of the multidomain approach and include one or more domains that confer the ability to target or penetrate the infected cells more effectively.[256]

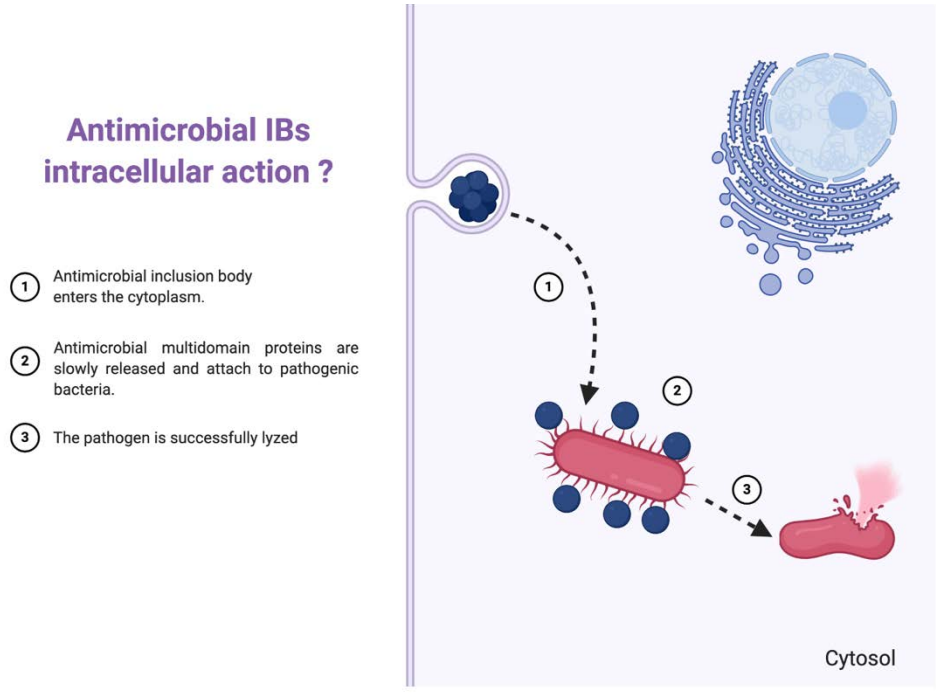

Figure 16. Can antimicrobial IBs act against intracellular pathogens? Antimicrobial IBs may have the potential to reach different intracellular compartments and exhibit a slow-release pattern that could kill intracellular pathogens.

Besides being active, solutions to the AMR crisis also need to be cheap to be accessible, as resistances to antibiotics will probably hit harder in countries that are still developing.[3] In addition, most of the current antibiotics are used for livestock farming, where price requirements are even tighter.[6,257] A further asset of IBs is that they can be produced and purified at a low cost.[179] Still, there is a long way to refine the composition and purity of IBs. For instance, IBs may contain bacterial cell wall debris as a result of cell disruption, such as LPS. But solutions are starting to emerge, such as the use of endotoxin-free engineered strains[258] or generally recognized as safe (GRAS) microorganisms such as *Lactococcus lactis* as recombinant hosts.[194,259]

Another solution to control the IB make-up is the use of aggregation seeding domains. Other researchers have previously used aggregation tags such as VP1,[174] GFIL8,[190] and ELK16[206] to drive heterologous proteins into the insoluble fraction. Yet we wanted to go beyond that in with c-Jun and c-Fos LZ (Study 1). As these LZ are able to interact between themselves as homo- and hetero-dimers,[260–262] we hypothesized they would increase protein aggregation, quality and composition. Although we found an increase in cellular contaminants in one of the constructs, LZ raised the purity of heterologous protein in IBs, adding to the arsenal of strategies to enhance IB composition.

IBs, the Klondike of active proteins

Extracting proteins from their natural sources is often not very practical. Fortunately, synthetic approaches and recombinant DNA technologies have come to the rescue. The choice between heterologous expression versus chemical synthesis depends on the cost, length of the peptide and sustainability.[135] Chemical synthesis works well for short sequences and if there is no need of secondary or tertiary – not even considering quaternary – structures.[263] However, there is a better chance to achieve a proper protein conformation with recombinant strategies, and some expressions systems offer many post-translational modifications that may be needed for the protein to work well, too.[264] In Study 3, we have observed that the immunomodulatory activity of the HD5 peptide produced as a recombinant protein is higher than that shown by the chemically synthesized peptide, indicating that probably the conformation was better achieved by recombinant strategies. Moreover, protein synthesis cannot be scaled-up as well as recombinant protein production.[200,265] As an example, in the past five years, different recombinant HDPs such as plantaricin E,[266] apidaecin[267] or Pa-MAP 2[268] have been produced in fermenters. In fact, the

number of recombinant protein drugs in the market for human medicine[269] and animal health is growing.[270]

But recombinant protein production and purification from the soluble fraction can be difficult and a unique process for each protein, so we may have to look elsewhere. If IBs are unavoidable, you might as well welcome them, as they are a pre-purified source of the protein of interest and avoid problems with the production of soluble antimicrobials, which are lethal to the producer. Because of this, we have fostered the formation of IBs by LZ domain and retrieved soluble protein from IBs at satisfactory yields (Studies 1, 2 and 3) with a modified version of the protocol described in the work by Gifre-Renom and co-workers.[194] All without extreme conditions or denaturing agents and the proteins we obtained through this universal protocol were highly pure and active. Therefore, IBs are an excellent source of soluble protein, if we know how to exploit them.

The possibility of using either the nanoparticulated (IB) or soluble version allows us to match the right antimicrobial to each application depending on the administration route, the stability needed, and action time - among others. For instance, if we need to administrate the antimicrobial parenterally, it is essential to use a well-characterized composition, so it is preferable to use a soluble or an encapsulated-soluble version, but not IBs. On the contrary, IBs might have a greater potential in topical applications or in surface decoration to avoid biofilm formation.

Potential applications

There are many possible uses of antimicrobial IBs. As an example, the main reason that dairy cows are given antibiotics is to treat intramammary infection (mastitis).[271,272] To reduce their overuse and the potential rise in AMRs – if not already present – we could try intramammary injections of IBs due to their properties, taking into account that each mammary gland has an independent performance regarding the rest of mammary glands and cow organs. IB nanoclusters steadily leak active protein because they are simultaneously nanomaterial and active compound (Figure 17). Furthermore, the addition of immune-modulatory domains such as HD5 might help resolve mastitis quicker. Indeed, some HDPs have been tried against *S. aureus* in a mouse model of mastitis, and the results are very encouraging.[273]

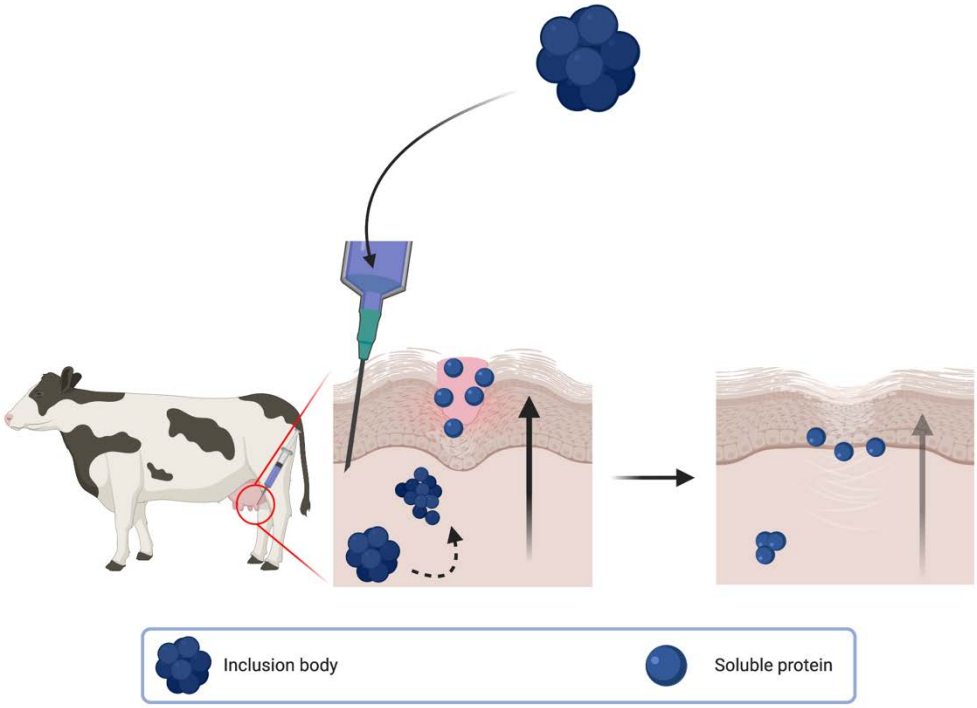

Figure 17. HDP based IBs for mastitis treatment. IB antimicrobial nanoclusters could be used in intramammary injections to treat mastitis and reduce the use of conventional antibiotics. Their properties – slow-release, stability – appear to be the perfect match for such applications.

In the work done in Annex 1 we explored if the multidomain antimicrobial proteins could be successfully anchored to gold surfaces and kept their activities. In the future, these materials could be used to protect medical equipment and devices from bacterial colonization, avoid biofilm formation, or treat it (Figure 18). Interestingly, both the soluble protein and IBs could be anchored to these materials, which adds further versatility to the approach. And we are not limited to gold materials, as we could also bind our proteins to collagen matrices, gels, films or particles.[274]

The anti-biofilm assays in Study 2 and Annex 1 are different. In Study 2, IBs were bound to 24 well plastic dishes by non-specific electrostatic interaction, whereas in Annex 1, they were explicitly bound to Ni^{2+}, similar to in IMAC purification. The non-selective binding from Study 2 proved that antimicrobial IBs were active and could avoid biofilm formation. However, selectively binding our proteins in combination to surface patterning methods (Annex 1) goes a step further and allows us to confer anti-biofilm properties in a more precise and controlled way. Moreover, the proteins might be more stably anchored, but future studies are needed to understand binding kinetics and strength in proteins that are attached

to self-assembled monolayers. It would also be interesting to compare non-covalent binding versus covalent binding of the proteins.

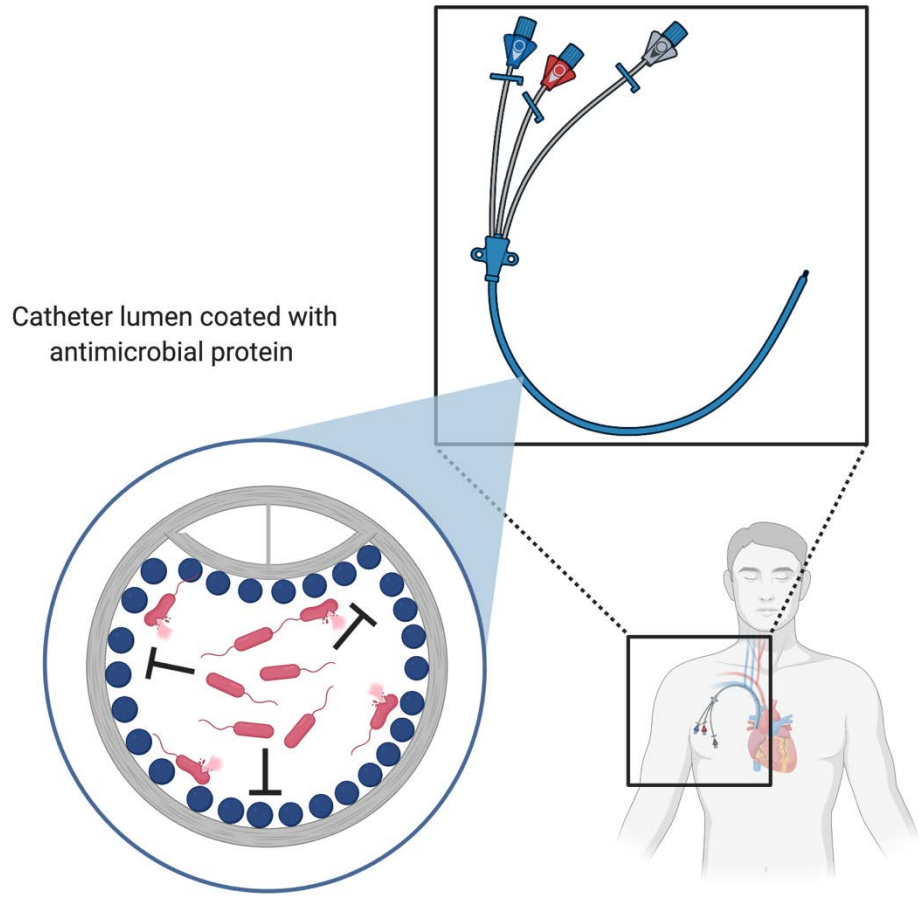

Figure 18. Surface immobilization of recombinant multidomain antimicrobial proteins. The constructs that we have developed could be used to cover surfaces of biomedical devices (i.e., the lumen of catheters) to avoid the growth of pathogens.

In sum, all of these properties make multidomain antimicrobial peptides very appealing. They can be tailored to our specific needs by the addition and edition of domains. They can be produced in a soluble or nanoparticulated format. And the combination of these two properties could make them useful in a wide range of applications – from surface biofunctionalization to slow-release parenteral injections. Best of all, we have millions of options to choose from, literally.

Conclusions

We aimed to develop an antimicrobial alternative based on multidomain recombinant proteins, inspired on peptides from the innate immunity. In addition, we explored two delivery formats: Soluble and nanoparticles (IBs). In sum, the results of this work can be found in the following statements:

1. Jun and Fos leucine zippers can be used as seeding domains to promote protein aggregation during recombinant production in *E. coli*, increasing the formation of inclusion bodies and diminishing the toxicity to the host during the production of recombinant antimicrobial proteins.

2. Jun and Fos leucine zippers position in the aminoacidic sequence might be used to adjust size, shape, surface morphology, and protein, lipid, and carbohydrate content of inclusion bodies. This might result in differential features among inclusion bodies such as the purity or specific activity of the recombinant protein embedded in these aggregates.

3. The multidomain approach can be used to construct broad-spectrum antimicrobial constructs that utilize peptidic domains from the innate immunity combined in a single recombinant polypeptide using a small linker sequence between the domains.

4. The nanoclusters or inclusion bodies of multidomain antimicrobial proteins can be easily purified and used as such. Alternatively, it can be further processed to obtain functional soluble protein after incubation with a mild detergent.

5. In addition to having antimicrobial activity, the multidomain proteins JAMF1, JAMF1.2, and JAMF2 can induce the secretion of IL-8 in colonic epithelial cells, suggesting that they are also immunomodulatory proteins, at lower concentrations (0.1 μM) than those needed for antimicrobial activity (1-10 μM).

6. The immunomodulatory activity of HD5 peptide produced as a recombinant protein is greater than that shown by the chemically synthesized peptide.

7. Each of the domains in the fusion antimicrobial proteins can be easily edited to tune the final antimicrobial and immunomodulatory performance of multidomain protein.

8. HD563-94 fragment and the $sPLA_2$23-189 domain show higher activity than the complete HD5 and $sPLA_2$ sequences, respectively.

9. The sum of HD5 and $sPLA_2$ antimicrobial domains lead to superior antimicrobial activities than HD5 alone.

10. The six-histidine tag at the C-terminal of the antimicrobial constructs allows us to immobilize them in Ni-NTA SAMs, which can be patterned through microcontact printing, displaying biofilm inhibitory properties.

Annexes

Annex 1

Anti-biofilm surfaces based on the immobilization of a novel recombinant antimicrobial protein using SAMs

Adriana Kyvik, Ramon Roca-Pinilla, Xavier Rodríguez-Rodríguez, Marc Martínez, Marta Martos, Jaume Veciana, Judith Guasch, Elena Garcia-Fruitós, Anna Arís and Imma Ratera

(In preparation)

Preface

For the most part, we have previously seen the potential of our multidomain proteins to combat AMR bacteria in a planktonic state (Studies 2 and 3). Yet the problem of AMRs gets even worse when bacteria are in biofilms. Biofilms, however, are quite pervasive in nature, as they can bind to virtually any surface, including medical devices or prosthetics. Furthermore, because the biofilm scaffold protects bacteria, they can cause chronic infections that are hard to deal with.[275] The treatment of biofilms is usually limited to either high doses of antibiotics, further contributing to the AMR problem, or directly removing the device, which can be very invasive and costly.

To find potential solutions to the formation of biofilms in surfaces, we created self-assembled monolayers (SAMs) made of gold, that can immobilize our recombinant antimicrobial constructs in either the soluble or the nanocluster format, thanks to a collaboration with the Institute of Ciències dels Materials de Barcelona (Dr. Imma Ratera). As a result of this research, we found that the SAMs with immobilized protein reduced biofilm formation of *E. coli* and carbapenem-resistant *K. pneumoniae*. An outcome that might unlock future materials that have intrinsic anti-biofilm properties, which could be used in prosthetics, catheters, and other medical gadgets prone to biofilm formation.

Anti-biofilm surfaces based on the immobilization of a novel recombinant antimicrobial protein using SAMs

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Abstract

The increasing appearance of (multi)resistant bacteria to antibiotics is a global health emergency, which is especially challenging when biofilms are formed. Here, novel biofunctionalized surfaces with the antimicrobial protein JAMF1, both in soluble form and nanostructured as inclusion bodies (IB), are developed and characterized using a multi-technique approach. A biofilm assay against *Escherichia coli* and carbapenem-resistant *Klebsiella pneumoniae* showed that the immobilized antimicrobial proteins in both forms are able to significantly reduce biofilm formation. This strategy opens up new possibilities for controlled biomolecule immobilization for fundamental biological studies and biotechnology applications, at the interface of materials science and molecular biology.

Introduction

The increasing appearance of bacteria resistant (and in many cases multiresistant) to antibiotics has become a global health emergency.[1] Far from being a phenomenon that will decrease in the coming years, it is estimated that the emergence of new resistances and the number of pan-resistant microorganisms will continue to grow reaching an increase of 67%

in 2030,[2] passing to become a leading cause of mortality, outpacing cancer, cholera, and diabetes.[3] Antibiotic resistance is even more complicated when bacteria form biofilms. Biofilms are surface-attached microbial groups embedded in an extracellular matrix that become less susceptible to antimicrobial treatments than non-adherent or planktonic cells.[4,5] Biofilms may form on a wide variety of surfaces, including living tissues, medical devices,[6,7] industrial or potable water piping systems, or natural aquatic systems.[8] Biofilms cause a significant amount of all human microbial infections.[9] In this context it is imperative to find new surface coating approaches to avoid biofilm formation. The antimicrobial activity of antimicrobial peptides (AMPs) from the innate immunity of a variety of species is gaining interest as a possible alternative to antibiotics, also for biofilm-forming bacteria.[10] Recently, we have described a new generation of antimicrobial multidomain proteins that combines several AMPs and complementary functional domains in a single polypeptide recombinantly produced as protein nanoclusters named IBs.[11] However, the anti-biofilm activity of such multidomain protein has not been explored so far.

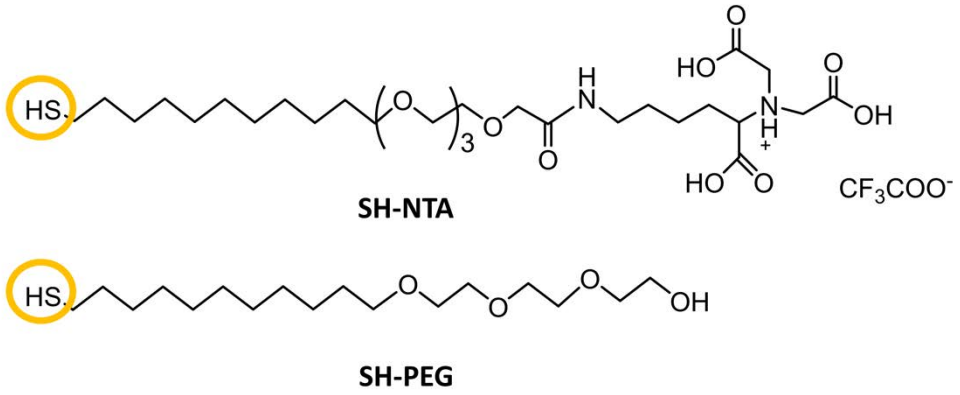
One of the strategies recently used to provide antimicrobial properties to medical devices, in order to avoid the need of systematic treatments, is based on the immobilization of AMPs through bonds on the different surfaces. Moreover, it has also been shown that the immobilized AMPs are able of keeping their antimicrobial activity under specific conditions.[12-15] This strategy allows higher availability of AMPs on the surface and more uniform distribution in comparison with the incorporation of AMPs through other adsorption methods that give place to non-homogeneous peptide distributions. In addition, the anchoring of AMPs on the surfaces has been reported to be a good strategy in order to avoid enzymatic degradation. Such advantages, allow to increase stability and completely avoid toxicity due to the use of high AMP dosages.[16,17]

One of the strategies recently used to anchor AMPs on surfaces and study the effect of its immobilization is based on the use of self-assembled monolayers (SAMs). SAMs are based on well-organized molecules on surfaces which are easy to be prepared and functionalized and allow a fine control at the molecular level.[18,19] Thus, the use of SAM strategy to anchor AMPs on surfaces has been shown to be one of the best strategies for a controlled design of antibiofilm surfaces to coat medical devices.[20]

Thus, herein we report on the use of a new antimicrobial multidomain protein (JAMF1) formed by several AMPs by means of DNA recombinant technology in a soluble and a nanocluster (IB) format. Then, we report on the formation of an antimicrobial surface through the immobilization of this novel generation of antimicrobial proteins using a mixed SAM strategy, as a proof of concept for coating medical devices. JAMF1 has been successfully anchored on a model gold surface using a mixed SAM strategy. Such mixed SAM is based on ((1-mercapto-11-undecyl)-(tetra(ethylene glycol)) terminated SAM (PEG-SH), and nitriloacetic acid (NTA) terminated EG4-SAM (NTA-PEG-SH) (Scheme 1). Specifically we have used the interaction of the JAMF1 protein terminal His-tag with the Ni-NTA complex found at the surface of the SAM. (Figure 1).

Then, the immobilized novel antimicrobial protein in its soluble and IB forms on S-NTA-Ni samples were characterized using a multi-technique approach in order to (i) verify the successful realization of each SAM formation step, (ii) the specific immobilization of the protein and (iii) compare the differences when immobilizing the soluble versus the IB form. Cyclic voltammetry, X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and fluorescence microscopy measurements have been performed. Moreover, for a deep characterization of the functionalized surfaces, prefunctionalized patterned and non-patterned mixed PEG and Ni-NTA SAMs (S-NTA-Ni) will be prepared. In order to achieve spatial control of the distribution of the molecules, optimization of the μCP procedure of the SH-NTA and SH-PEG molecules will be performed and a His-tagged green fluorescent protein (His6-GFP) will be used as a reporter to easily visualize the pattern.

Finally, a biofilm assay has been done to evaluate the actual antimicrobial effect of the surfaces modified with the novel antimicrobial protein in its soluble and IB form. The antimicrobial activity was tested against *Escherichia coli* and *Klebsiella pneumoniae* bacterial strains, being this last one an example of an antibiotic-resistant strain.



Scheme 1. Molecular structure of the molecules used for NTA SAM formation. The NTA terminated thiol (**SH-NTA**) and (b) the commercial pegylated alkanethiol (**SH-PEG**).

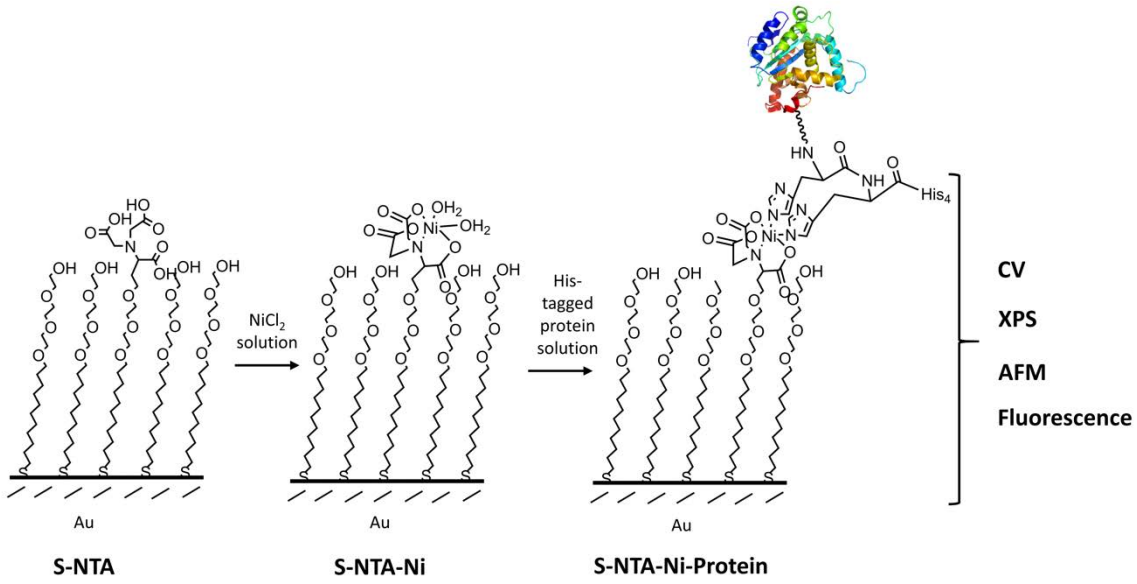


Figure 1. Strategy used for the immobilization of His-tagged JAMF1 protein using the Ni-NTA strategy: NTA SAM formation (**S-NTA**), immersion in a $NiCl_2$ solution to obtain the Ni-NTA SAM ((**S-NTA-Ni**), followed by the incubation with the His-tagged protein, obtaining **S-NTA-Ni-Protein** (Protein: GFP, Soluble or IB protein). Finally, a multi-technique approach is used for its characterization.

Materials and Methods

Bacterial strains and medium. We used *Escherichia coli* BL21 (DE3) and *E. coli* Origami B for heterologous protein expression of antimicrobial proteins and a modified version of green fluorescent protein (mGFP), respectively. *E. coli* DH5α and carbapenem-resistant *Klebsiella pneumoniae* (KPC) have been used for antibiofilm activities. *E. coli* was grown in Luria-Bertani (LB) medium and KPC in Brain Heart-Infusion (BHI) broth (Scharlau, Barcelona, Spain).

Antimicrobial protein genetic construct design. The gene encoding for the JAMF1 construct consisted of the sequences encoding Jun257-318 (Uniprot entry P05412), human α-defensin-5 (HD5) precursor (Uniprot entry Q01523), gelsolin188-196 (Uniprot entry P06396), group-XIIA secretory phospholipase A2 ($sPLA_2$) precursor (Uniprot entry Q9BZM1) and Fos118-210(Uniprot entry P01100), from N-terminal to C-terminal. A linker sequence consisting of serine and glycine residues (SGGGSGGS) was used between each of the domains and a six-histidine tag at the C-terminal for protein purification. The fusion construct was codon optimized by GeneArt (Life technologies, Regensburg, Germany) and then cloned into pET22b (Amp^R) (Novagene, Darmstadt, Germany) backbone for further *E. coli* transformation and recombinant production.

Production of Recombinant Antimicrobial Protein JAMF1. *E. coli* BL21 (DE3)/pET22b-JAMF1[11] culture (0.5 L) was grown at 37 ºC and 250 rpm in LB broth with ampicillin at 100 μg/mL. Protein expression was induced by 1 mM isopropyl-β-d-thiogalactoside (IPTG) at an OD600=0.4-0.6. Cultures were grown 3 h post-induction. To obtain JAMF1 (IBs), the bacterial culture was processed with a mechanical disruption and washing steps, as described elsewhere.[21] For soluble protein production, a 2 L culture was grown and centrifuged at 6,000 x *g* for and the pellets were recovered and resuspended in 120 ml of PBS with protease inhibitors (Complete EDTA-free, Roche). Samples were sonicated for 4 rounds of 5 min at 10 % amplitude under 0.5 s cycles, intercalated by a minimum of 5 min of rest in ice. Protein pellets were again recovered and washed twice with distilled water. The pellets were weighted and solubilized in 0.2 % N-lauroyl sarcosine, 40 mM Tris and protease inhibitors at a ratio of 40 ml/g of wet pellet as previously described.[22] The mixture was incubated 40 h overnight (O/N) at room temperature (RT) under agitation and the supernatant was recovered through centrifugation (15,000 x *g* for 45 min at 4 ºC), for purification. NaCl and imidazole were added to the solubilized protein to equilibrate the samples, and Immobilized Metal Affinity Chromatography (IMAC) purification was carried in an ÄKTA purifier FPLC (GE Healthcare, Chicago, IL, USA) using 1 ml HisTrap HP column (GE Healthcare). Both the binding and the elution buffer contained 0.2 % N-lauroyl sarcosine. The final imidazole concentration in the elution buffer was 0.5 mM. Finally, the buffer of the selected purified protein fractions was changed to KPi (potassium phosphate buffer: 80.2 % v/v 10 mM K_2HPO_4 and 19.8 % v/v 10 mM KH_2PO_4) using a desalting column (GE Healthcare). Purified IBs and soluble protein were quantified by western blot using a monoclonal anti-His antibody (1:1,000) (His-probe, Santa Cruz). As secondary anti-

body we used an anti-mouse IgG – Alkaline Phosphatase (1:20,000) produced in goat (Sigma Aldrich).

Production of mGFP-H6. *E. coli* Origami B/pET22b-T22GFPH6 (mGFPH6)[23] culture (2 L) was grown at 37 ºC and 250 rpm in LB broth with ampicillin at 100 μg/mL, kanamycin at 15 μg/ml and tetracycline at 12.5 μg/ml to an OD600 of about 0.5 before protein induction with IPTG 1 mM- T22-GFP-H6 was produced overnight at 20 ºC and 250 rpm. After overnight production, cultures were centrifuged at 6,000 x *g* and 4 ºC for 45 min. Then, the pellets were resuspended in 20 mM Tris – HCL, pH 8.0, with 500 mM NaCl, 20 mM imidazole and protease inhibitors (Complete EDTA-free, Roche, Indianapolis, USA). Intracellular soluble protein was extracted by sonication with 4 x 5 min rounds (0.5 on, 0.5 off cycles) at 10 % amplitude, with a minimum 10 min rest in ice between rounds. Finally, protein was purified by affinity chromatography using a 1-ml HisTrap HP column (GE Healthcare) in an ÄKTA start purifier (GE Healthcare). Protein separation was achieved with a linear imidazole gradient from 20 mM to 500 mM. Fractions were collected and dialyzed against 166 mM $NaHCO_3$ at pH 7.4 The amount of protein was determined with a Nanodrop 1000 spectrophotometer (Thermo Scientifc) and protein integrity was evaluated by Western blot analysis as described above.

Preparation of patterned and non-patterned His-tagged protein Ni-NTA SAMs. Substrates used were either glass slides with a 2 nm titanium adhesion layer and a 10 nm gold layer or silicon wafers with a 50 nm titanium adhesion layer and 100 nm gold layer. Substrates were cut to have an area of 1.5×1 cm and cleaned in HPLC gradient solvents for 5 minutes; first isopropanol, then acetone, and lastly ethanol. After drying them with the nitrogen gun very carefully, they were exposed to ozone (UVO CLEANER, Model No. 42-220) for 20 minutes and immersed in ethanol for 30 minutes.

For the μCP procedure, the PDMS stamp of interest was cut with a scalpel, rinsed with ethanol and dried off with a stream of nitrogen. Then, a solution of **SH-PEG** (ProChimia Surfaces, Scheme 1) 1 mM in ethanol was dropped (40 μL) on top of the PDMS stamp. The stamp was then dried off with the nitrogen gun. Substrates were then printed with the inked stamp by carefully placing the stamp on the substrate and leaving them in contact for 2 minutes. An empty and flat petri dish was placed on top of the stamp to increase and homogenize the pressure. The stamp was removed carefully with tweezers and substrates were incubated with 80 μL of **SH-NTA** (ProChimia Surfaces, Scheme 1) 1 mM in ethanol in a humid chamber during 2 h at RT. Substrates were transferred to a 6-well plate to perform the following immersions at RT: i) In MQ-water during 5 minutes (x2); ii) In HEPES buffer

solution for 10 minutes (x1), iii) In 10 mM NiCl2 in HEPES for 30 minutes; iv) In HEPES for 2 minutes (x3).

Then, substrates were incubated with the His-tagged protein (His6-GFP or antimicrobial protein in soluble (20 μM) and insoluble IB form (23 μM for evaluation against E. Coli or 500 μM for evaluation against KPC) in a humid chamber during 1 hour at RT.

For the preliminary trials, 45 μL of 0.735 mg/mL of His-GFP was used and the petri dish was covered with aluminium foil to avoid photobleaching. For the antimicrobial proteins, 50 μL of 0.735 mg/mL and 50 μL of 1.24 mg/mL for soluble antimicrobial protein and antimicrobial IBs, respectively, were used.

Samples were immersed in HEPES during 5 min (x2). For the negative control samples, a further treatment with EDTA was performed. EDTA acts as a competitive chelator for NTA because it cleaves the chelated Ni from NTA to form a Ni-EDTA complex. The effectiveness of this competition lies on the higher stability constant of the Ni-EDTA complex (18.56 in logarithmic) than the Ni-NTA complex (11.26 in logarithmic). As a consequence, the His-tagged protein can no longer bind to the **S-NTA**. The detailed steps were the following: i) Substrates were immersed in 100 mM EDTA solution (or 10 mM) for 20 minutes and rinsed again with HEPES afterwards; ii) If they were not characterized straight away, substrates were left immersed in HEPES in the fridge at 4 °C. The pH of both HEPES and EDTA solutions were adjusted before their use to values of 8 and 7.31, respectively, using 0.1 M NaOH and 0.1 M H_2SO_4 solutions for the adjustment.

For non-patterned substrates no μCP was performed, and instead the entire area of the surface was functionalized with SH-NTA.

Immunostaining. The immunostaining steps performed were the following: 1) After protein immobilization, substrates were incubated with a solution of primary antibody $sPLA_2$ (E-9, Santa Cruz Biotechnology, Inc.) in a ratio of 1:400 in BSA solution (1% in PBS). Each substrate was incubated with 50 μL in a humid chamber for 1 hour. 2) Substrates were rinsed in PBS on the shaker at 50 rpm for 10 minutes. 3) Substrates were incubated with a solution of a secondary antibody, Alexa goat anti-mouse 488 IgG (Thermo Fisher Scientific), in a ratio of 1:100 in BSA solution. Again, each substrate was incubated with 50 μL in a dark humid chamber for 45 minutes. 4) Substrates were rinsed in PBS on the shaker at 50 rpm for 10 minutes. 5) Substrates were mounted on glass slides with 50 μL of ProLong Gold Antifade Reagent (Thermo Fisher Scientific). They were left to dry overnight in a dark chamber.

Fluorescence microscopy. The instrument employed for visualization of the samples was an Olympus BX51 microscope equipped with a CCD camera Olympus DP20 for **S-NTA-Ni-GFP** samples and an Axio Observer Z1m optical microscope (ZEISS) for the rest of the samples. The software Image J was used to extract the intensity profile of the striped pattern. The substrates observed were the following: patterned **S-NTA-Ni-GFP**, immunostained patterned **S-NTA-Ni-Sol**, immunostained patterned **S-NTA-Ni-IB**, together with the negative controls.

Cyclic voltammetry. A solution of 5 mM of $[Ru(NH_3)_6)]^{3+}$ was prepared in an electrolyte solution consisting in a 50 mM KCl aqueous solution. A platinum wire was employed as the CE and an Ag/AgCl electrode as the RE. The WE was, depending on the measurement, the bare gold substrate, **S-NTA**, **S-NTA-Ni-Sol**, **S-NTA-Ni-IB** or **S-NTA-Ni-GFP**. The potentiostat used was an AUTOLAB 204 and the software used for the data acquisition and analysis was Nova 2.3. The scan rate used was 0.1 V/s and the area of the WE immersed into the electrolyte solution was around 1.5 cm^2.

Atomic force microscopy. Surface topography and film thickness were examined by a 5500LS SPM system from Agilent. Images were processed with the Gwyddion software. The samples analyzed had 2 μm striped pattern of (A) **S-NTA-Ni-Sol**, (B) **S-NTA-Ni-Sol**-Ctrl (by treatment with EDTA 10 mM) (C) **S-NTA-Ni-IB**, (D) **S-NTA-Ni-IB**-Ctrl (by treatment with EDTA 100 mM), (E) **S-NTA-Ni** and (F) **S-NTA**. It is important to mention that measurements were conducted in dry conditions and that the analyzed surfaces are biological samples, so the protein on the surface is dehydrated.

X-ray photoelectron spectroscopy (XPS). Measurements were performed with a Phoibos 150 analyzer (SPECS GmbH) under ultrahigh vacuum conditions (base pressure 5◊10-10 mbar) with a monochromatic aluminium K alpha X-ray source (1486.74 eV). The energy resolution measured by the FWHM of the Ag 3d5/2 peak for a sputtered silver foil was 0.6 eV. The spot size was 3.5 mm by 0.5 mm. Compositional survey and detailed scans (N 1s, S 2p, Ni 2p and O 1s,) were acquired. The samples measured were **S-NTA-Ni-Sol**, **S-NTA-Ni-Sol**-Ctrl (by treatment with EDTA 10 mM), **S-NTA-Ni-IB**, **S-NTA-Ni-IB**-Ctrl (by treatment with EDTA 100 mM), **S-NTA-Ni** and **S-NTA**. In all substrates, the prepared **S-NTA** was not patterned, so the incubation with **SH-NTA** was done directly without previous μCP of **SH-PEG**.

Biofilm formation assay. Bacterial strains were grown O/N at 37 °C and 250 rpm. Before adding bacteria to a 24-well sterile plate for biofilm formation, either His-tagged protein Ni-NTA SAMs or Ni-NTA SAMs were placed under sterile conditions in each well. After that,

bacteria from the O/N culture were diluted 1:200 in fresh medium supplemented with 0.2 % (w/v) glucose. A total of 400 μL of diluted bacterial culture supplemented with glucose to each well, for incubation at 37 °C for 24 h in static conditions. After the incubation, the supernatant was removed and wells were washed 3 times with 500 μL NaCl 0.9 %, then fixated with 500 μL methanol for 10 min at RT. Methanol was removed and the plate was dried for 15 min at 37°C. The remaining biofilm cells in the well were stained with crystal violet 1% (v/v) for 15 min at RT and washed 3 times with sterile ddH_2O. Finally, stained cells were diluted in 33 % (v/v) acetic acid and the absorbance was measured at 595 nm.[24] All measurements were done by triplicate and in sterile conditions

Statistical analysis. For all assays, each condition was performed in triplicate or quintuplicate (Figures 14-15) and represented as the mean ± standard error of the mean. All data were checked for normality. *p*-values correspond to *t*-test analyses for the biofilm formation assay.

Results and discussion

Production of a novel antimicrobial protein.

We used a multidomain antimicrobial polypeptide (AMP) named JAMF1, which combines the AMP human α-defensin-5 (HD5), a bacterial binding domain (gelsolin), and an enzymatic antimicrobial peptide ($sPLA_2$), flanked by two aggregation-seeding domains (a fragment of c-Jun and c-Fos leucine zippers at N- and C-terminal) (Figure 2). The AMP was produced recombinantly as IBs which were used directly as antimicrobial material or as a source of soluble protein. Both formats have previously shown activity against Gram-positive and Gram-negative bacteria[11] in solution. JAMF1 IBs also showed antibiofilm activity when these IBs were used to decorate plastic surfaces.[11] However, the activity of this protein anchored in surfaces has not been explored so far.

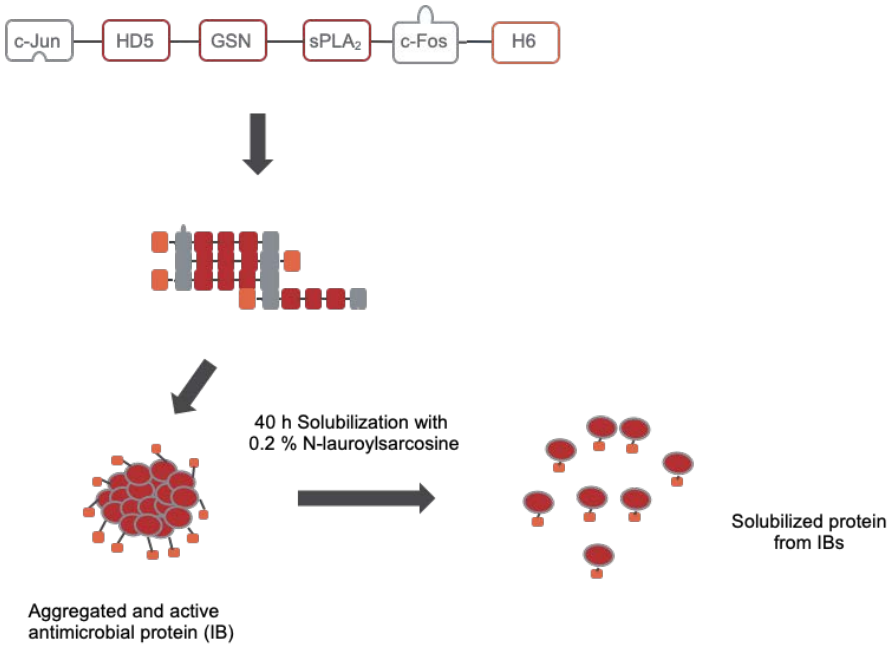


Figure 2. Diagrammatic representation of the antimicrobial protein JAMF1 construct forming IBs and in its soluble form after a solubilization process. From N-terminal to C-terminal, JAMF1 contains amino acids 257-318 from c-Jun leucine zipper, the sequence of Human α-defensin-5 (HD5), aminoacids 188-196 from gelsolin (GSN), group-XIIA secretory phospholipase A2 ($sPLA_2$), amino acids 118-210 from c-Fos and a six-histidine tag (H6).

His-tagged AMPs anchoring on micropatterned Ni-NTA SAMs

In order to better characterize the AMP anchoring process, patterned surfaces of the protein anchoring have been prepared using the μCP technique through a spatial control of the distribution of the SH-NTA and SH-PEG mixed SAMs. Before using the AMP JAMF1, a His-tagged green fluorescent protein (mGFPH6) has been used for surface protein immobilization using the SH-NTA and SH-PEG mixed SAMs in order to easily visualize the pattern with fluorescent microscopy and optimize its spatial distribution (Figure 3).

Briefly, a PDMS stamp inked with an SH-PEG ethanolic solution (1 mM) was put in contact with the clean gold surface and peeled off carefully. Thereafter, they were incubated with an SH-NTA ethanolic solution (1 mM) to backfill the non-patterned areas, obtaining a patterned S-NTA. Afterwards samples were incubated in a $NiCl_3$ solution, obtaining S-NTA-Ni. Negative control samples were prepared in the same way but finally treated with EDTA, obtaining again S-NTA. EDTA acts as a competitive chelator for NTA, cleaving the chelated Ni from NTA to form a Ni-EDTA complex (Figure 3). For the experimental details see Materials and Methods.

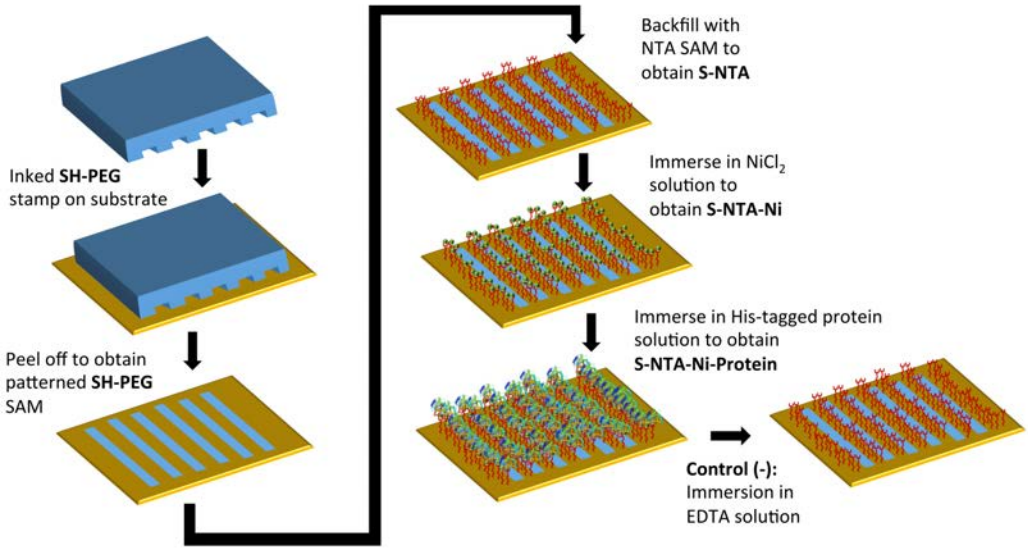


Figure 3. Schematic representation of the experimental procedure followed to prepare patterned SH-NTA and SH-PEG mixed SAMs, using the μCP technique. Subsequent protein immobilization via their His-tag termination led to the protein anchoring. Negative controls were prepared by immersing the substrates in an EDTA solution (10 or 100 mM).

Patterned anchoring of mGFPH6 as model surface.

Patterned S-NTA-Ni-mGFPH6 samples were prepared following the procedure depicted in Figure 3. A clear fluorescent striped pattern was obtained as visualized in Figure 4 showing a homogeneous coverage of the stripes, thus, demonstrating that the mGFPH6 is correctly immobilized on the S-NTA-Ni samples. These results showed that the spatial control of the SAM formation was successfully optimized via the μCP technique and that the His-tag of the produced proteins showed a high affinity for the Ni-NTA complex.

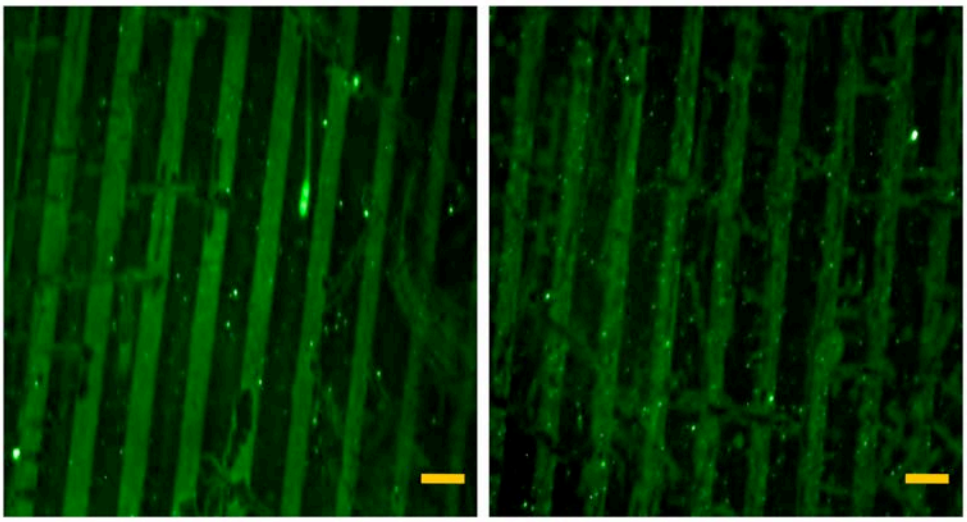


Figure 4. Fluorescence microscopy images of patterned mGFPH6 using the Ni-NTA SAM strategy, obtaining **S-NTA-Ni-GFP**. Micropatterns observed were created using the μCP technique with a PDMS stamp with a pattern of 20 μm. Scale bars correspond to 30 μm.

The absence of fluorescent pattern in the negative controls demonstrates that Ni is kidnapped from the NTA and chelated by EDTA, inducing the removal of the His-tag and, therefore, of the protein from the surface.

Cyclic voltammetry (CV) has also been used to examine the current barrier properties of the functionalized surface and further corroborate the integrity of the SAM and correct anchoring of mGFPH6. For this experiment non-patterned surfaces were used. A redox probe was used in order to study the electron transfer reactions between the redox probe and the gold surface under the functional SAM. As a redox probe it has been used hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$) because it is reported that can detect even the smallest defects in SAMs, which are invisible for other markers such as ferrocyanide.[25] The greater sensitivity of the $[Ru(NH_3)_6]^{3+}$ probe can be explained by the location of its excess charge, located on the metal core in the ion center, whereas, in the case of hexacyano metal complexes, it is located on the terminal nitrogen atoms of cyano ligands.[26] Thus, as a consequence, $[Ru(NH_3)_6]^{3+}$ species can penetrate further into SAMs and diffuse along the SAM chains, so despite forming a very compact SAM, with the probable presence of some collapsed sites, current signal can still appear in the recorded voltammogram. Several studies have confirmed that the thicker the SAM the higher the blocking properties.[27]

When performing CV experiments with mGFPH6 functionalized substrates (Figure 5) the current decreases when the NTA SAM is formed on the gold surface and decreases even more when the mGFPH6 is immobilized. This fact is indicative of a higher blocking effect which hinders electrons to travel from the redox probe to the gold surface and vice versa. The anchoring of mGFPH6 on the surface hinders even more the access of electrons to the gold surface, not only because they imply a larger separation distance between the gold and redox probe acting like a new layer of impedance for the electrons to overcome, but because their bigger size and volume in comparison to the SAM molecules make the electron transfer even harder. Therefore, a relevant decrease of both the oxidation and reduction peaks can be observed (Figure 5). In view of these results, we can conclude that a densely packed NTA SAM has been assembled and that mGFPH6 is correctly immobilized.

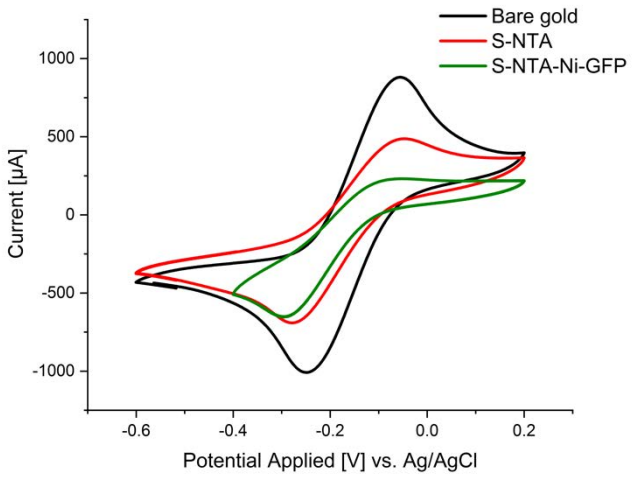


Figure 5. Cyclic voltammograms of bare gold, S-NTA SAM and S-NTA-Ni-GFP SAM. The electrolyte used is an aqueous KCl solution (50 mM) containing the redox probe $[Ru(NH_3)_6)]^{3+/2+}$ (5 mM). Scan rate used is 0.1 V/s.

Patterned anchoring of AMP JAMF1

The correct immobilization of the JAMF1 active antimicrobial polypeptide using SAMs was monitored using i) immunostaining and subsequent fluorescent microscopy; ii) atomic force microscopy (AFM) and iii) Cyclic voltammetry and iv) X-ray Photoelectron Spectroscopy (XPS). To produce the functional surfaces to be characterized by these techniques, first a PEG-SAM is µcontact printed on the substrate (20 µm stripes) and then the mixed PEG-NTA-Ni SAM back filled the empty stripes. Finally, the substrate is immersed in the JAMF1 protein solution for its spatially controlled anchoring (Figure 3 and Materials and Methods).

Immunostaining and Fluorescence microscopy

In this case proteins are depleted of fluorescence, thus, in order to visualize the pattern, immnunostaining had to be performed using a primary and secondary antibody as described in Materials and Methods. Following the procedure described in Figure 3, a homogeneous functionalization of the antimicrobial protein onto patterned S-NTA-Ni samples was achieved, both with the soluble and IB form of the protein (Figure 6). The fluorescent stripes appear well delimited, and a uniform coverage of the protein is found along the pattern. The absence of fluorescent pattern in the negative controls, prepared by immersing the substrates in EDTA (10 mM), as shown in Figures 6 (C) and (D), indicate the reversibility of the union. Moreover, the dark images obtained from the generic negative control (Figure 6 (E)), which consisted in the immunostaining of a patterned S-NTA sample, is a proof that the

immunostaining technique worked correctly and that the fluorescence is specific for the immunostained proteins.

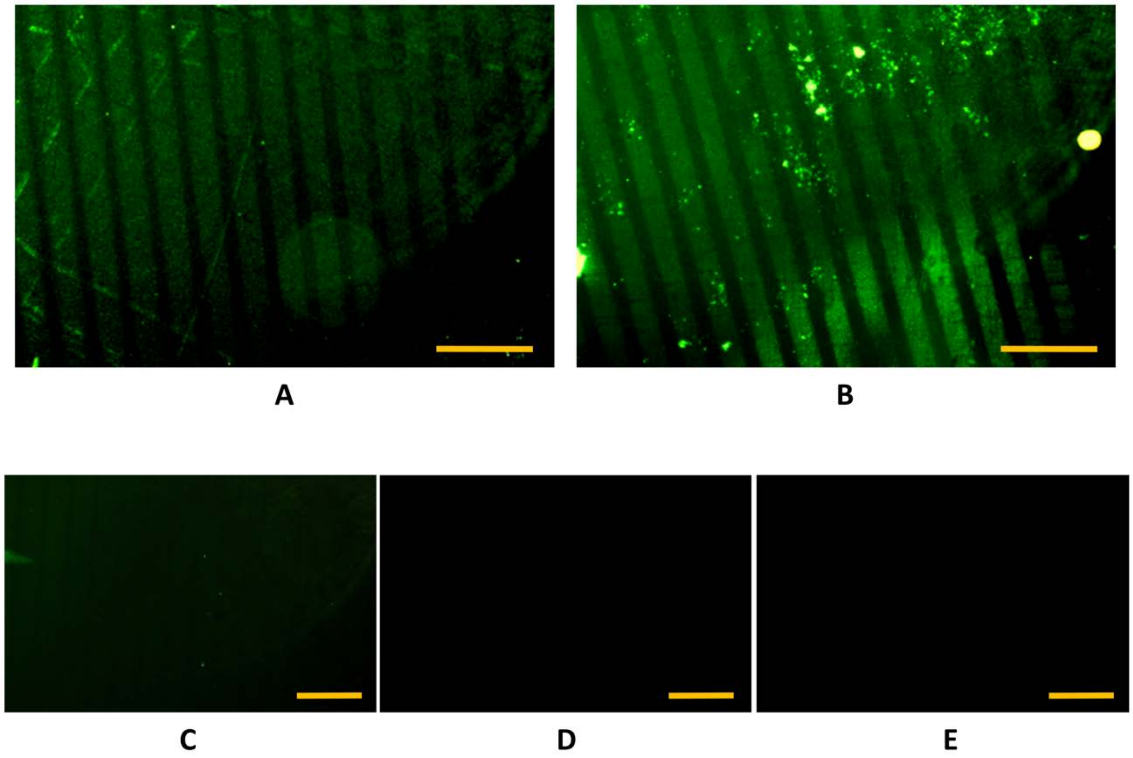


Figure 6. Fluorescence images of a 20 µm striped pattern of (A) **S-NTA-Ni-JAMF1** in soluble form and (B) **S-NTA-Ni-JAMF1** as IB nanostructured form. Fluorescence images of the negative controls (by immersion in EDTA (10 mM)) of a 20 µm striped pattern of (C) **S-NTA-Ni-Sol**-Ctrl and (D) **S-NTA-Ni-IB**-Ctrl. (E) A generic negative control of the immunostaining technique, which consisted in patterned **S-NTA**. The scale bars correspond to 100 µm.

In addition, the fluorescence intensity profiles (Figure 7) for the patterning of the soluble and IB formats of JAMF1 protein, show peaks with practically the same intensity in both cases, indicating a homogeneous coverage of the protein on the desired areas. A periodicity in the intensity peaks is observed: there is low or no fluorescence intensity between peaks, implying that there are no luminescent antibodies and therefore, no antimicrobial protein. Hence, the antimicrobial His-tagged protein is specifically attached only onto the Ni-NTA groups, and the **SH-PEG** molecules form a protein repelling surface, as desired. These results also confirm that µCP allows a correct spatial control of the protein immobilization.

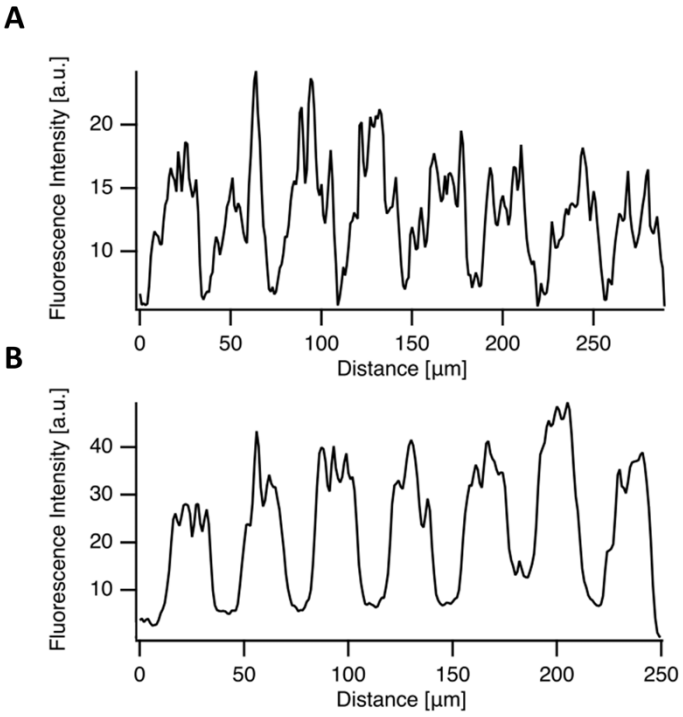


Figure 7. Fluorescence profiles extracted from fluorescence images of immobilized (A) soluble protein and (B) IB after an immunostaining treatment. Specifically, these profiles correspond to the images portrayed in Figure 6 (A) and (B).

Atomic force microscopy

In order to match with the lateral resolution of the AFM technique, in this case, 2 µm wide striped patterns were printed (Figure 3) for both soluble and IB forms of the antimicrobial protein. Both topographical (Figure 8 (A) and Figure 9 (A)) and phase shift images (Figure 8 (B) and Figure 9 (B)), confirmed the correct protein immobilization.

We observed that the stripes of the soluble protein are better delimited than the ones of the IBs pattern, and in addition, the stripes of the IBs pattern are wider than the ones of the soluble protein pattern. The height of the IBs is greater than the one from soluble protein, indicative of the successful immobilization of both soluble and IB proteins.

The antimicrobial protein in both soluble and IB forms can be detected in the negative phase shift regions, appearing darker, because **SH-PEG** and **SH-NTA** form a compact SAM with intermolecular forces between the thiolated chains. Therefore, the areas functionalized with **SH-PEG** appear stiffer than the areas with immobilized protein, which are only attached to the SAM by the His-tag, and therefore, are more likely to deform. In negative controls, their stiffness and viscoelastic properties are likely to be very similar and thus, the phase shift scan does not make a distinction between the two molecules and no pattern is appreciated. The

difference in height between the negative controls and the ones with bound protein is the height of the immobilized antimicrobial protein. The measured value of their height is not comparable with other reported protein sizes, because normally these are measured in aqueous conditions.

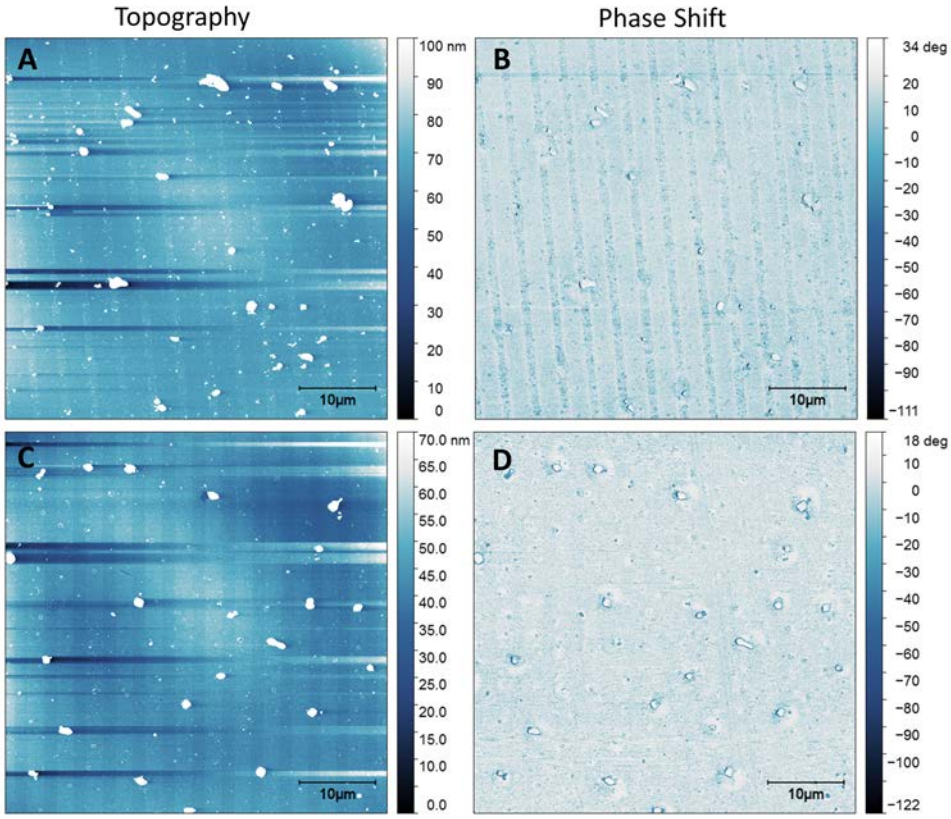


Figure 8. (A)(C) Topographical and (B)(D) phase shift AFM images of the 2 µm wide striped pattern of **S-NTA-Ni-Sol** (A)(B) and their negative controls treated with EDTA (100 mM), **S-NTA-Ni-Sol**-Ctrl (C)(D).

For analysis purposes, the average measurements of the height and the width of three topography peaks displayed in Figure 10 for each sample are recorded in Table 1.

The difference in height between the negative controls (Figure 10 (C) and (D)) consisting in a patterned S-NTA-Ni sample, and the ones with bound protein (Figure 10 (A) and (B)), is in fact, the height of the immobilized antimicrobial protein. It is important to note that measurements were conducted in dry conditions and thus, the protein on the surface is dehydrated. By analyzing the results portrayed in Table 1, this height is around 1 nm for the soluble protein and around 2.3 nm for the IBs. We have to take into account the dry conditions of the experiment, because protein hydration is very important for their 3D structure, dynamics and activity. Regarding the protein size, the aqueous media around the protein provides a hydration layer which can enlarge the measured protein size up to 15 Å

from the protein surface. In view of this implication, the removal of water from our substrates may have provoked a reduction in protein size and probably even the loss of their 3D structure. Therefore, the measured value of their height is not comparable with other reported protein sizes, because normally these are measured in aqueous conditions (e.g. using dynamic light scattering). Nonetheless, these measurements give an insight regarding the protein mass immobilized on the surface, since when comparing the height of the soluble protein and the IBs, the height of the IBs is greater than the one from soluble protein, indicative of the successful immobilization of both soluble and IB proteins. The width differences observed between peaks may be due, again, to the aggregated nature of the IBs, which when immobilized on the edge of the patterned NTA, can surpass in extension the pattern resulting in wider peaks compared to those obtained with the soluble protein.

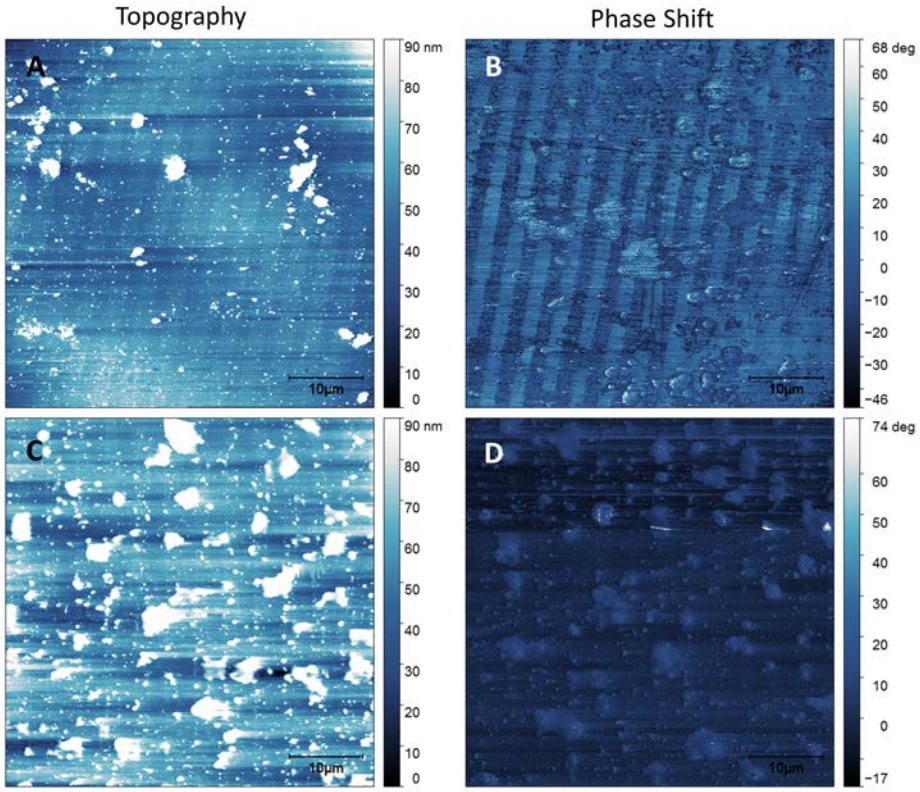


Figure 9. (A)(C) Topographical and (B)(D) phase shift AFM images of the 2 µm wide striped pattern of **S-NTA-Ni-IB** (A)(B) and their negative controls treated with EDTA (100 mM), **S-NTA-Ni-IB**-Ctrl (C)(D).

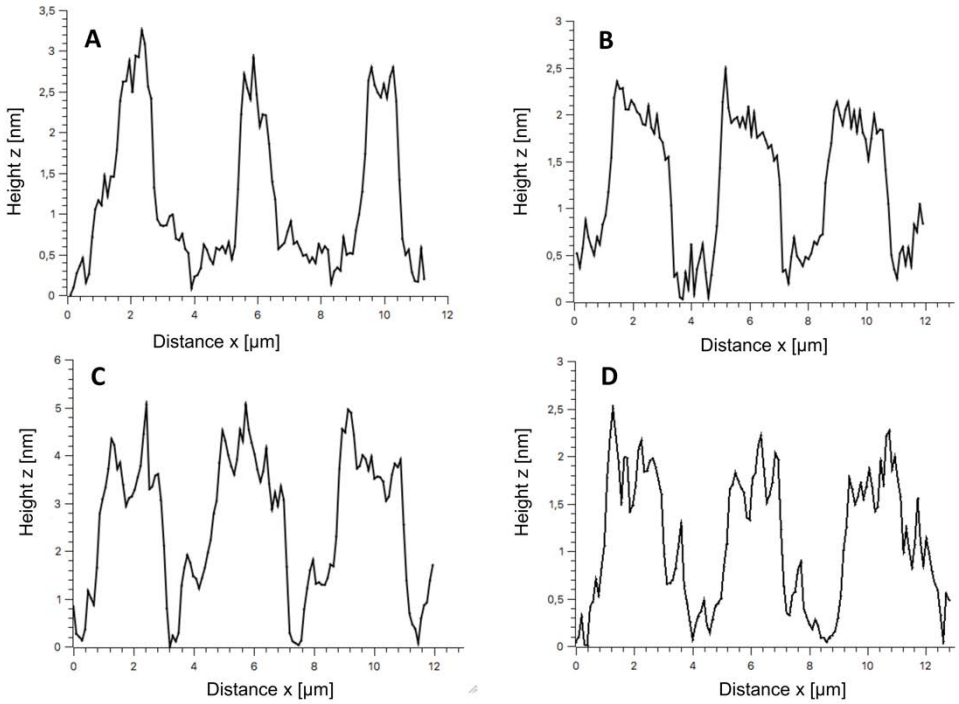


Figure 10. Topographic profiles of (A) patterned **S-NTA-Ni-Sol**, (B) patterned **S-NTA-Ni- Sol**-Ctrl, (C) patterned **S-NTA-Ni-IB** and (D) patterned **S-NTA-Ni-IB**-Ctrl.

Table. 1. Values corresponding to the average peak width and height extracted from the AFM images

Sample	S-NTA-Ni-Sol	S-NTA-Ni-Sol Ctrl	S-NTA-Ni-IB	S-NTA-Ni-IB-Ctrl
Mean peak width [µm]	1.40	2.15	2.20	2.05
Mean peak heigth [µm]	2.56	1.64	4.08	1.74

Phase imaging provides a map of stiffness variations on the sample surface: a stiffer region has a more positive phase shift than a less stiff region and, hence, appears brighter in a phase image. In soft materials, the phase shift is highly dependent on the viscoelastic properties of the material. The antimicrobial protein in both soluble and IB forms can be detected in the negative phase shift regions, appearing darker, because SH-PEG and SH-NTA form a compact SAM with intermolecular forces between the thiolated chains. Therefore, the areas functionalized with SHPEG appear stiffer than the areas with immobilized protein, which are only attached to the SAM by the His-tag, and therefore, are more likely to deform.

In negative controls the pattern is not detected in the phase shift images, as seen in Figures 8 (D) and 9 (D) since both SH-NTA and SH-PEG form together a compact SAM, with their aliphatic chains interacting between each other. As a result, both types of thiols are subjected

to practically the same interactions, forces, restrictions and the same degrees of freedom. Therefore, their stiffness and viscoelastic properties are likely to be very similar and thus, the phase shift scan does not make a distinction between the two molecules and no pattern is appreciated.

Cyclic voltammetry

As explained before, we have used CV to study the correct formation of S-NTA we study the immobilization of the antimicrobial protein. Analyzing the CV obtained when using SAM modified substrates as WE (Figure 11), a decrease in current is observed after NTA SAM formation, as expected. Protein immobilization decreases the current, but only slightly, for the oxidation peak. This means that this protein has a higher blocking effect on the electrons when these go from the redox probe to the gold substrate, in other words, the proteins on surface hinder more the access of electrons when they have to penetrate the SAM, than when they come from the gold substrate to reduce $[Ru(NH_3)_6)]^{3+}$, which is to say, when they are leaving the SAM.

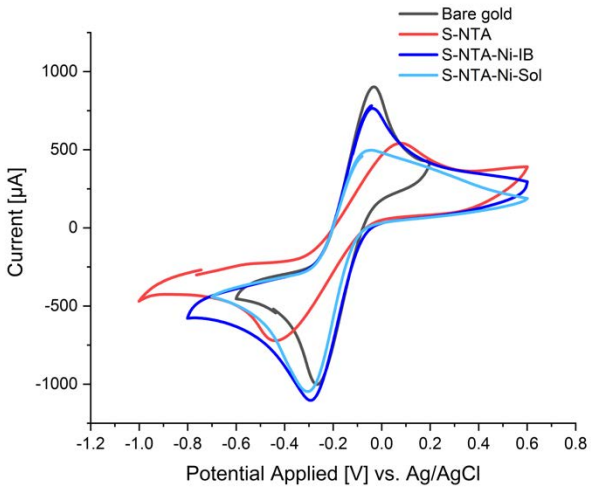


Figure 11. Cyclic voltammograms of bare gold, **S-NTA**, **S-NTA-Ni-IB** and **S-NTA-Ni-Sol**. The electrolyte used was an aqueous KCl solution (50 mM) containing the redox probe $[Ru(NH_3)_6)]^{3+/2+}$ (5 mM). Scan rate used is 0.1 V/s.

Additionally, the reduction of current observed is not as large as in the case of mGFPH6 (Figure 5). The reason may be that GFP is much smaller than the antimicrobial protein: GFP has 238 amino acids, while the novel antimicrobial protein has 487 amino acids, more than double. Therefore, the attachment of the GFP to Ni-NTA is easier and with a stochiometric relation of protein:SH-NTA more equal than in the case of the larger antimicrobial protein. Also, due to its size, steric hindrance may play a role, making it more difficult to occupy all

the Ni-NTA units with a His-tag belonging to the protein. Hence, the coverage of the S-NTA-Ni-Sol, even if efficiently performed, as seen before, is not as homogeneous as with the smaller GFP: it presents more defects, including probably small holes.

Regarding the voltammogram obtained after immobilization of the IBs (S-NTA-Ni- IB) an increase of current with respect to the S-NTA is observed. From other characterization techniques we already know that IBs are successfully immobilized by S-NTA-Ni samples (Figure 6), but the voltammogram does not show a clear decrease in the current peaks, as we would have expected. The cause may reside in the structure of the IB: the number of His-tags exposed on the outer face of the IBs is not predictable and controlled, and thus, it can be very low. Consequently, the amount of immobilized IBs is lower than for immobilized soluble protein.

X Ray Photoelectron Spectroscopy

XPS measurements were performed for 6 different samples: (A) **S-NTA-Ni-Sol**, (B) **S-NTA-Ni-Sol**-Ctrl (treatment with EDTA 10 mM), (C) **S-NTA-Ni-IB**, (D) **S-NTA-Ni-Sol**-Ctrl (treatment with EDTA 100 mM), (E) **S-NTA-Ni** and (F) **S-NTA**. Nitrogen, sulfur, nickel, oxygen and carbon were analyzed and their spectra deconvoluted.

XPS measurement of N 1s, S 2p, Ni 2p and O 1s, followed by the spectra treatment, showed peaks, shifts and intensity relations that are in agreement with the expected results, demonstrating as well successful protein immobilization.

For the N 1s spectra, two peaks can be observed, one at 400-401 eV and another one at 402 eV. The peak at around 400-401 eV corresponds to the N-C bond found in the NTA, in amino acids, and also attributed to 'pyrrole-like nitrogen' (typically assigned to peaks around 400.4 eV). Also, amines and secondary amides are attributed to peaks with energies of 400.5 eV. Positive samples present the His-tag, which in turn has imidazole groups that contain 'pyrrole-like' nitrogen, and the amino acid tryptophan, which also has 'pyrrole-like' nitrogen. Negative controls which have the NTA exposed on surface present N-C bonds and secondary amides and therefore, also present this peak.

The other peak clearly observed in positive samples but absent in negative controls is found at 402 eV. This peak is assigned to various oxidized nitrogen configurations, Pyridine-N-

oxide being the most frequently suggested one. 'Pyridine-like' nitrogen is present in the His-tag and therefore, in the positive samples containing protein.

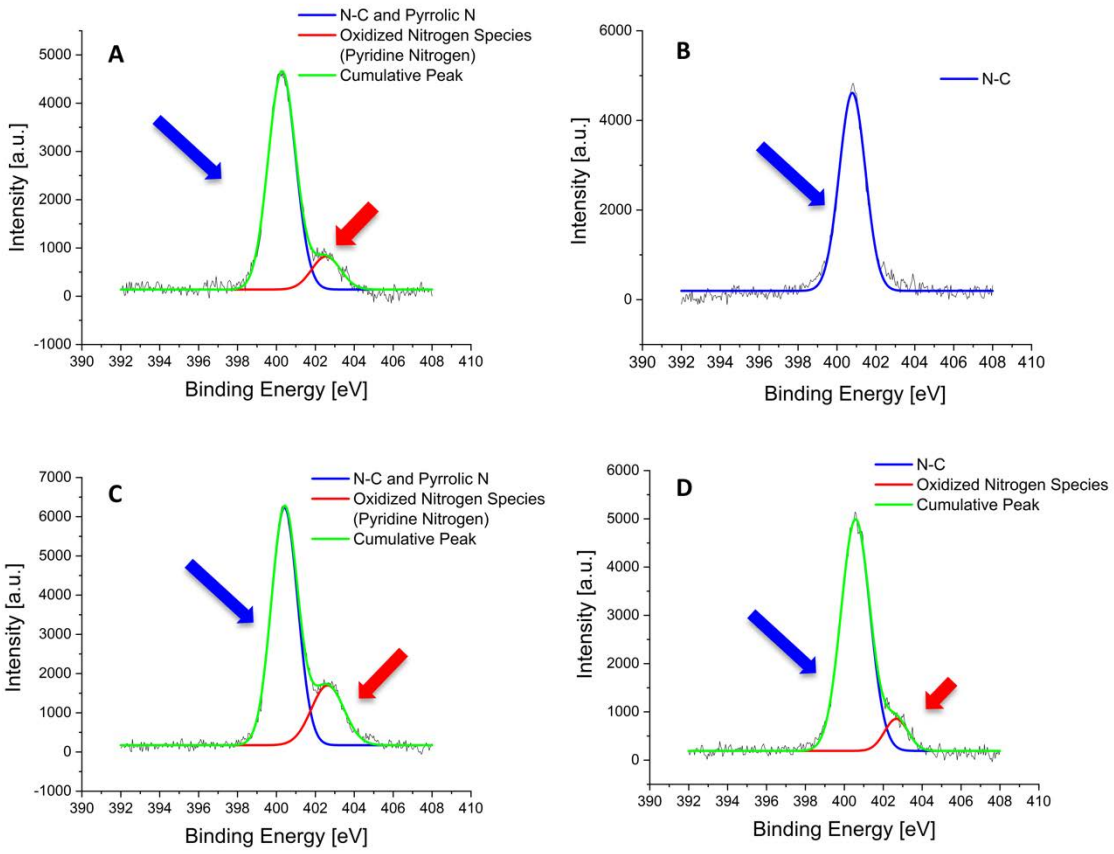
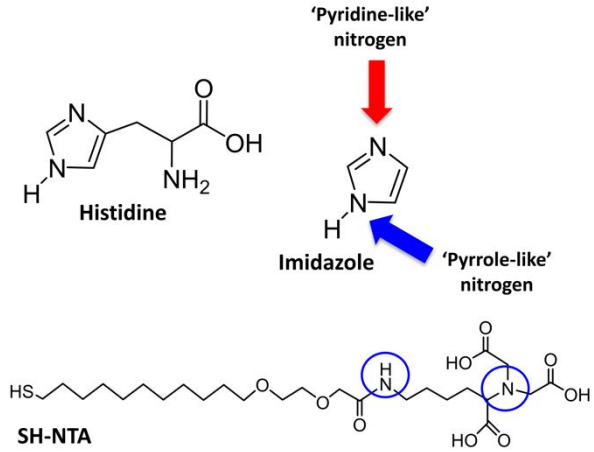


Figure 12. XPS deconvolutions of N 1s spectra for (A) S-NTA-Ni-Sol, (B) S-NTA-Ni-Sol- Ctrl (treatment with EDTA 10 mM), (C) S-NTA-Ni-IB and (D) S-NTA-Ni-IB-Ctrl (treatment with EDTA 100 mM). The red arrow indicates the contribution of 'pyridine-like' nitrogen and blue arrow of 'pyrrole-like' nitrogen.



Scheme 2. Representation of the different attributions to the nitrogen spectra: 'pyrrolelike' nitrogen, 'pyridine-like', N–C and secondary amides.

Regarding the analysis of sulfur, the S 2p spectra presents four peaks. From the analysis of the deconvoluted peaks, the peak at 161.9 eV and the peak at around 162.8-163.4 eV are

attributed to the split of the orbital 2p into the doublet 2p3/2 and 2p1/2, respectively. These peaks come from the thiol bond (S-Au) of the thiolated NTA chains to the gold surface, present in all samples.

On the other hand, there are two more peaks, one at 168.5 eV and the other at around 169.7-171.8 eV, both corresponding to oxidized sulfur species, which can come from the thiol groups in the alkanethiols or from cysteine amino acids present in the antimicrobial proteins. For this reason, these peaks appear in protein sample. Since the negative control does not have protein immobilized, these peaks are less intense in its spectrum.

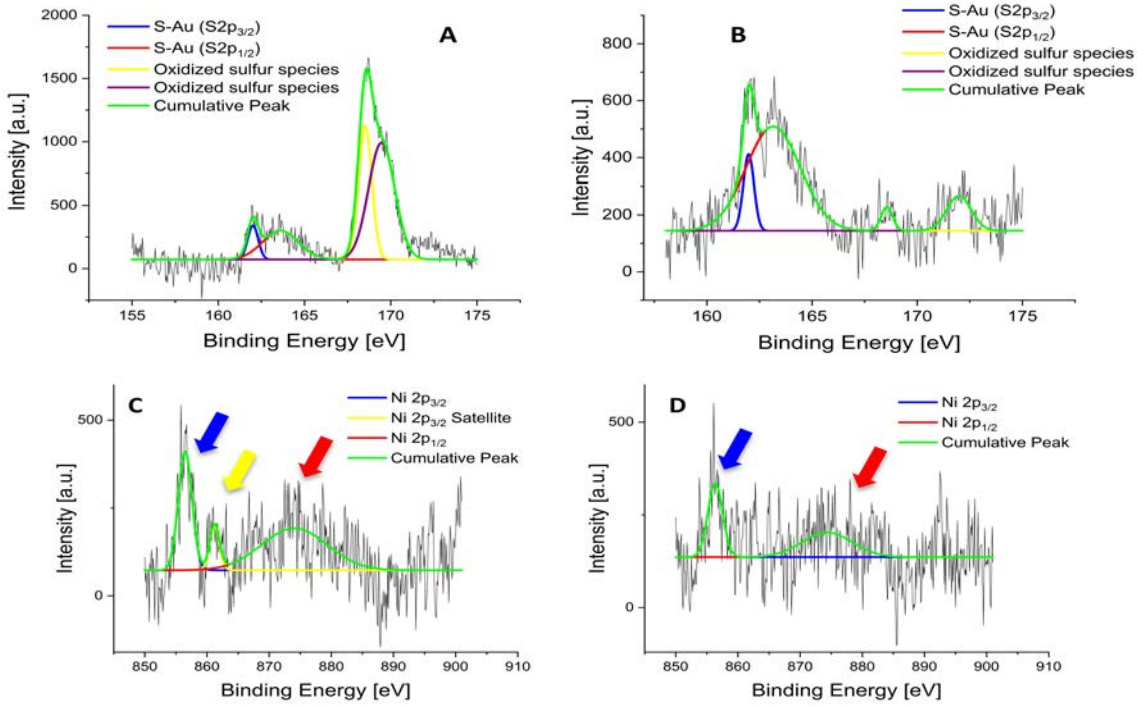


Figure 12. XPS deconvolutions of S 2p spectra for (A) **S-NTA-Ni-Sol** and (B) **S-NTA-Ni- Sol**-Ctrl (treatment with EDTA 10 mM). XPS deconvolutions of Ni 2p spectra for (C) **S-NTA-Ni-IB** and (D) **S-NTA-Ni- IB**-Ctrl (treatment with EDTA 100 mM).

In the O 1s spectra, the samples present two common peaks. The peak at 532 eV corresponds to the C–O bond and the one at around 532.7-532.9 eV corresponds to the C=O bond. Besides, except for the negative control **S-NTA-Ni-Sol-Ctrl**, the other samples also show a peak at 537 eV. The latter peak is attributed to either adsorbed water, acetate species or oxygen species interacting with Ni. Therefore, it can be associated either to the presence of –COOH groups in the amino acids or to the interaction of oxygen and Ni in the chelating complexes. Both cases demonstrate the presence of His-tag and soluble protein/IBs, also confirmed by the fact that there is no peak in the **S-NTA-Ni-Sol-Ctrl**.

The peak present in the negative control **S-NTA-Ni-IB-Ctrl** can be attributed to undesired oxidation due to the aging of the sample, which was analyzed three days after preparation. The ratio difference between the C–O and C=O peaks in the positive samples and may be due to the differences between the soluble protein and IBs.

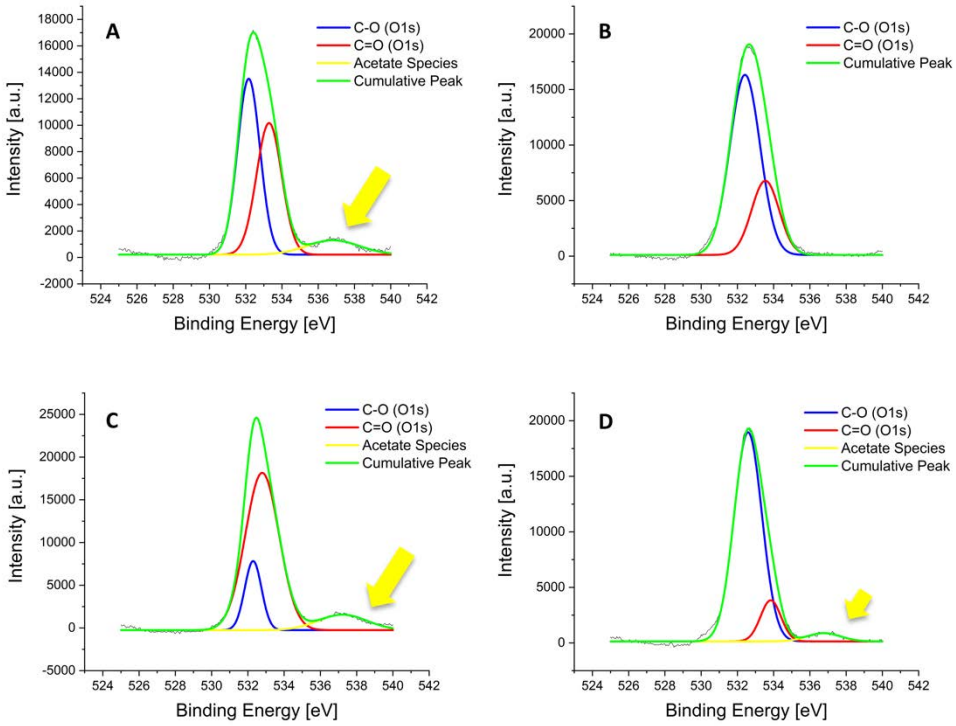


Figure 13. XPS deconvolutions of O 1s spectra for (A) **S-NTA-Ni-Sol**, (B) **S-NTA-Ni-Sol**- Ctrl (treatment with EDTA 10 mM), (C) **S-NTA-Ni-IB** and (D) **S-NTA-Ni-IB**-Ctrl (treatment with EDTA 100 mM).

Biofilm assay

The antibiofilm assay showed that the antimicrobial protein in both soluble and IBs forms is able to significantly reduce the biofilm formation (Figure 14). S-NTA-Ni-Sol and S-NTA-Ni-IB surfaces reduces *E. coli* survival up to 38 % and 34 %, respectively, showing that the strategy used to anchor the protein to the surface is highly promising. The same behavior is observed when S-NTA-Ni-IB surfaces are used against an antibiotic-resistant *Klebsiella pneumoniae* strain (Figure 15). Thus, this result proves that JAMF1 is active also against bacterial strains that do not respond to standard antibiotic treatments.

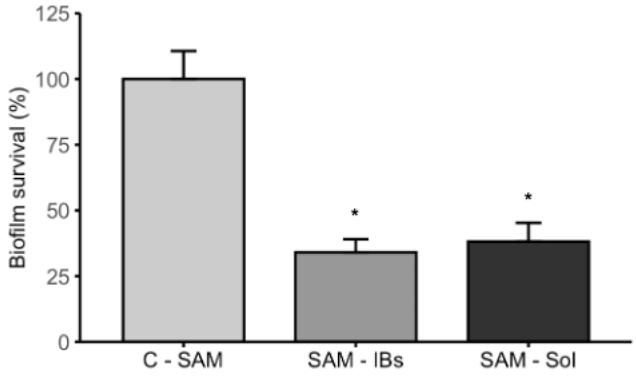


Figura 14. Biofilm formation ability (%) of *E. coli* DH5α after treating substrates with Ni and soluble (SAM – Sol) or insoluble (IBs) JAMF1antimicrobial protein. * indicates significant differences ($p \leq 0.05$).

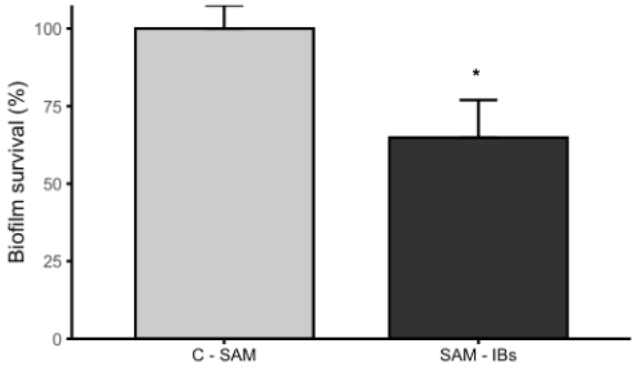


Figura 15. Biofilm formation ability (%) of KPC after treating substrates with Ni and insoluble JAMF1 antimicrobial protein or treating plastic well with JAMF1 IBs. * indicates significant differences ($p \leq 0.05$).

HD5 and $sPLA_2$ are the domains that have antimicrobial activity in JAMF1. The mechanism of action of the α-defensin HD5 is based on its cationic and amphipathic nature. HD5 is attracted by electrostatic forces to the negatively-charged lipid bilayer forming the bacterial membrane, which induces a change on the membrane structure and, consequently, a pore is formed.[28] $sPLA_2$ is also an enzyme from the innate immunity, which effectively hydrolyses the phospholipids components of the bacterial membrane.[29] Thus, the combination of both HD5 and $sPLA_2$ domains make JAMF1 protein a promising antimicrobial candidate against Gram-negative (Figure 14 and 15) and Gram-positive bacteria.[11] The gelsolin domain, also included in JAMF1, is a bacterial binding domain whose role is to increase the efficiency of JAMF1 molecule binding to the pathogen. Although after the immobilization process of JAMF1 using SAMs the activity of these molecule is slightly decreased compared to those proteins with any specific binding to the surface,[11] the remaining activity is still high, which proves that the strategy presented in this study is an approach for the design of materials with antimicrobial properties with great potential.

Conclusions

Micropatterns of AMPs were successfully formed using the μCP technique through the NTA functionalization of gold surfaces assisted by a thiol group, combined with the His-tagged antimicrobial protein JAMF1, both in soluble form and nanostructured as IBs. The successful pattern formation not only was verified by fluorescence microscopy, but also by AFM and XPS measurements. This functionalization strategy was then applied to fully coated surfaces, which demonstrated their effectiveness in preventing biofilm formation against *E. coli*. Specifically, the immobilized soluble and IB forms reduced bacteria survival up to a 38% and 34%, respectively. Moreover, the IB surfaces also inhibited biofilm formation in *Klebsiella pneumoniae* strains, which are unresponsive to standard antibiotic treatments. In conclusion, novel biofunctionalized surfaces with AMP were developed and characterized in response to the need of new antimicrobial agents to overcome the antibiotic crisis, which could be applied to coat medical devices (i.e., catheters) or be incorporated into food packaging materials, among others.

Acknowledgements

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specifically by the Biomaterial Processing and Nanostructuring Unit (U6), Unit of the CIBER in Bioengineering, Biomaterials & Nanomedicine (CIBER-BBN) located at the Institute of Materials Science of Barcelona (ICMAB-CSIC).

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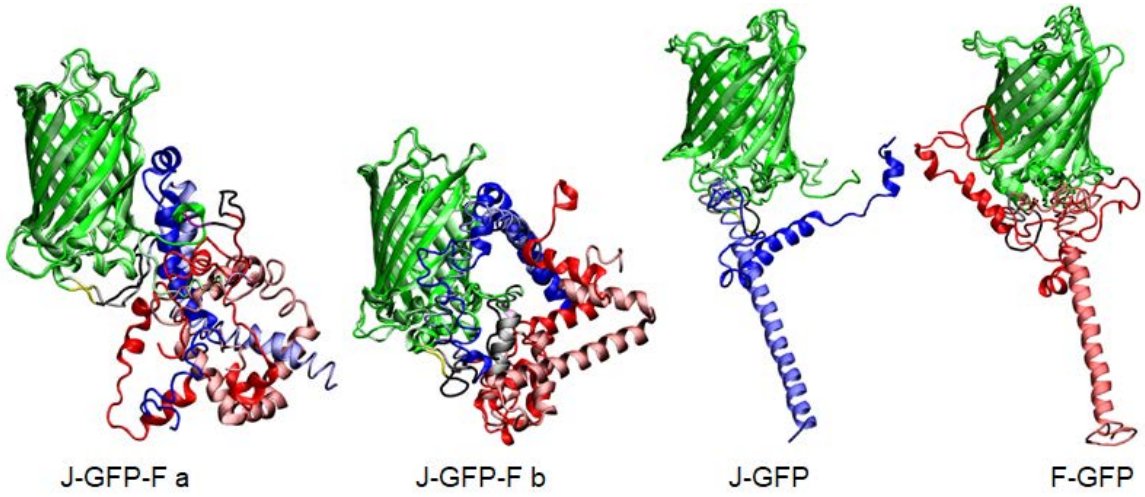
Annex 2: Supplementary material in Study 1

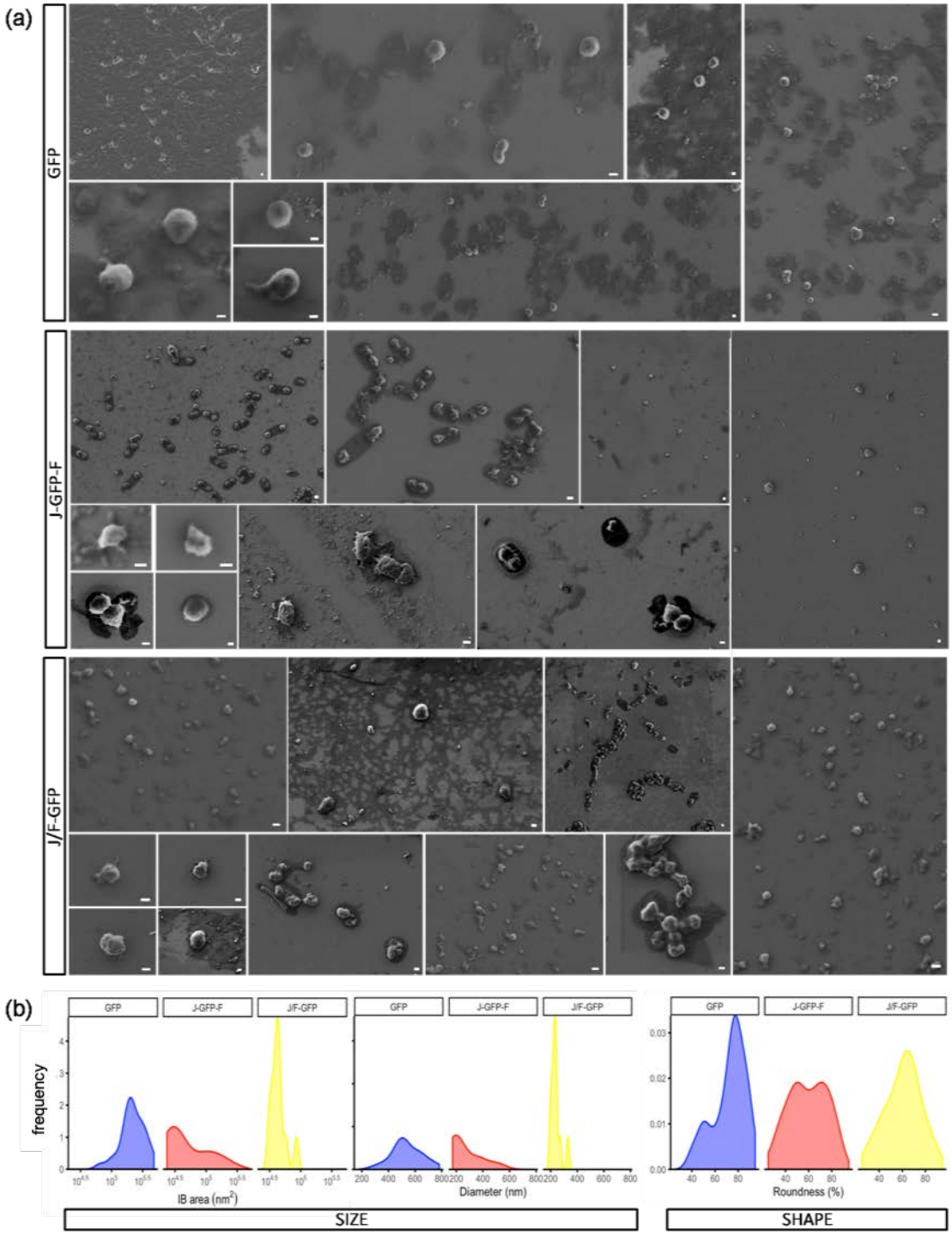
Exploring the use of leucine zippers for the generation of a new class of inclusion bodies for pharma and biotechnological applications

Ramon Roca-Pinilla, Sara Fortuna, Antonino Natalello, Alejandro Sánchez-Chardi, Diletta Ami, Anna Arís and Elena Garcia-Fruitós

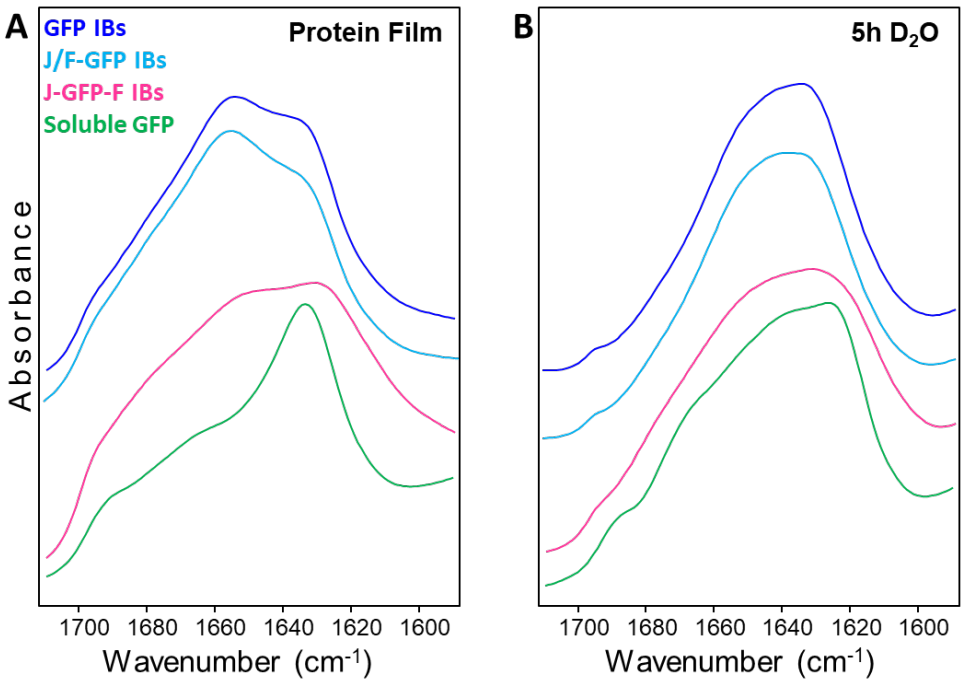
Figures



Supplementary Figure S1. Overlap between starting model (lighter shades) and final configuration (darker shades) of the J-GFP-F (two models: a and b) and J/F-GFP constructs (one model each), after 250 ns of molecular dynamics simulation. The generated models were minimized, placed in a cubic water box, minimized again, equilibrated and, for each construct, 250ns of molecular dynamics simulation were performed. Large rearragementsof the Jun/Fos domains were observed. Construct domains are color coded as follow: GFP (green), Jun (blue), Fos (red).



Supplementary Figure S2. (a) Representative FESEM images of the isolated IBs for each construct: GFP IBs, J-GFP-F IBs and J/F-GFP IBs. Bars size represent 200 nm. (b) Frequency distribution of IBs ultrastructural morphometry quantification for each construct: size (area (nm^2) and diameter (nm)) and shape (roundness (%)).



Supplementary Figure S3. A) FTIR absorption spectra of the protein films. B) FTIR absorption spectra collected after re-hydration of the protein films with D_2O for 5 h. GFP and J/F-GFP IBs displayed similar absorption spectra both as film and after re-hydration, while J-GFP-F IBs showed distinct spectral features. As a control, the absorption spectra of the soluble GFP are also shown.

Tables

Supplementary Table 1. Statistics for the protein aggregation ratio (%) for each construct over time. (a) Aggregation ratio (%) differences between the three constructs and (b) aggregation ratio (%) differences for each construct over time. Different letters mean statistically significant difference (Post-hoc Tukey HSD (THSD) comparisons).

(a)

Protein	Aggregation ratio (%)	*p*-value
GFP	44.57 ± 7.71 [a]	
J-GFP-F	52.56 ± 7.36 [a, b]	0.0189
J/F-GFP	73.55 ± 3.59 [b]	

(b)

Protein	**GFP**			**J-GFP-F**			**J/F-GFP**			p-value
Time (h)	**1**	**3**	**5**	**1**	**3**	**5**	**1**	**3**	**5**	Time
Aggregation ratio (%)	29.18 ±17.15 [a]	53.12 ±18.85 [a]	51.40 ±3.80 [a]	49.78 ±26.98 [a]	41.43 ±30.11 [a]	66.46 ±2.73 [a]	69.71 ±15.15 [a]	70.23 ±10.89 [a]	70.23 ±9.14 [a]	0.057

Annex 3: Supplementary material in Study 2

A new generation of recombinant polypeptides combines multiple protein domain for effective antimicrobial activity

Ramon Roca-Pinilla, Adrià López-Cano, Cristina Saubi, Elena Garcia-Fruitós and Anna Arís

JAMF1 construct domain aminoacidic sequences:

D1 Human α-defensin-5:

MRTIAILAAILLVALQAQAESLQERADEATTQKQSGEDNQDLAISFAGNGLSALRTSGSQARATCYCRTGRCATRESLSGVCEISGRLYRLCCR

D2 Gelsolin (Binding): QRLFQVKGRR

D3 Group XII-A $sPLA_2$:

MALLSRPALTLLLLLMAAVVRCQEQAQTTDWRATLKTIRNGVHKIDTYLNAALDLLGGEDGLCQYKCSDGSKPFPRYGYKPSPPNGCGSPLFGVHLNIGIPSLTKCC
NQHDRCYETCGKSKNDCDEEFQYCLSKICRDVQKTLGLTQHVQACETTVELLFDSVIHLGCKPYLDSQRAACRCHYEEKTDL

AD1 c-Jun Leucine zipper:

MRKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNHVNS

AD2 c-Fos Leucine zipper :

GRAQSIGRRGKVEQLSPEEEEKRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAAHRPACKIPDDL

H6 6x Histidine tag :

HHHHHH

Linker: SGGGSGGS

Figure S1. Aminoacidic sequence of JAMF1 for each of the construct domains.

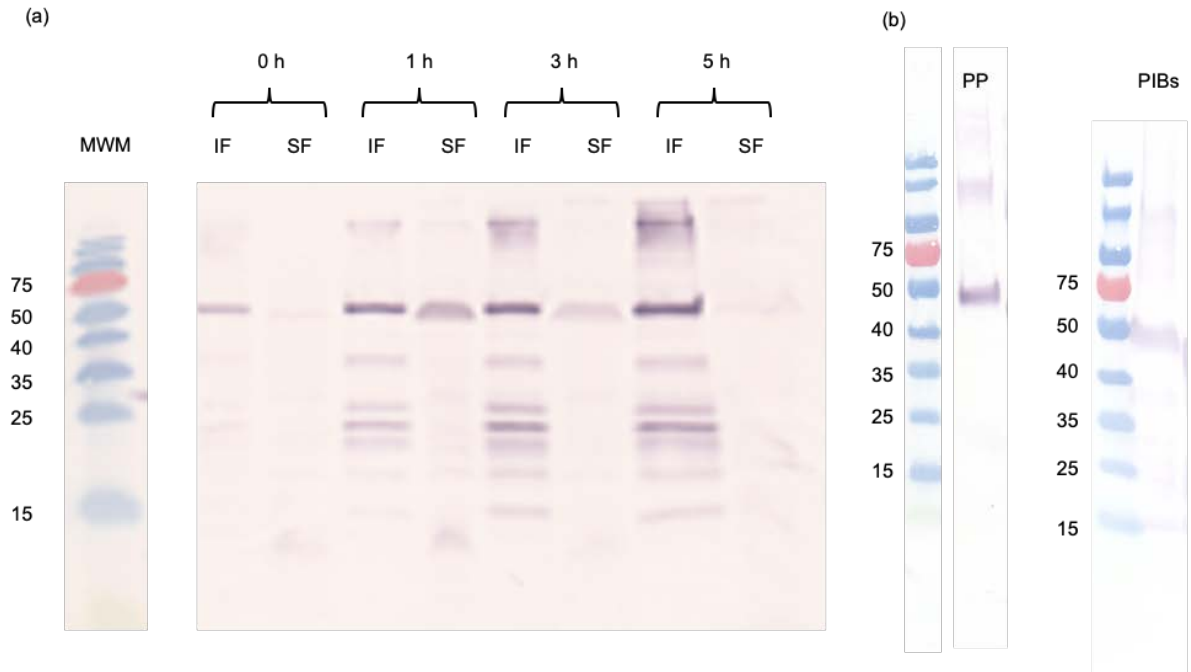


Figure S2. Western Blots of JAMF1. (a) Expression time course of JAMF1 at 0, 1, 3 and 5 h post-induction with IPTG in the insoluble and soluble fractions. MWM = Molecular Weight Marker (KDa), IF = Insoluble fraction, SF = Soluble fraction. (b) Western blot of the purified JAMF1 by IMAC (PP) and the purified IBs (PIBs).

Additional file 2. DNA coding sequence of JAMF1.

ATGCGTAAACGTATGCGTAATCGCATTGCAGCAAGCAAATGTCGTAAACGCAAACTGGAACGTATTGCACGTCTGGAA
GAAAAAGTTAAAACCCTGAAAGCACAGAATAGCGAACTGGCAAGCACCGCAAATATGCTGCGTGAACAGGTTGCACA
GCTGAAACAGAAAGTTATGAATCATGTTAATAGCAGCGGTGGTGGTAGCGGTGGTAGTATGCGTACCATTGCAATTC
TGGCAGCAATTCTGCTGGTTGCACTGCAGGCACAGGCAGAAAGCCTGCAAGAACGTGCAGATGAAGCAACCACACAG
AAACAGAGCGGTGAAGATAATCAGGATCTGGCAATTAGCTTTGCAGGTAATGGTCTGAGCGCACTGCGTACCAGCGG
TAGCCAGGCACGTGCGACCTGTTATTGTCGTACCGGTCGTTGTGCAACCCGTGAAAGCCTGAGCGGTGTTTGTGAAAT
TAGCGGTCGTCTGTATCGTCTGTGTTGTCGTAGTGGTGGTGGTTCAGGTGGTAGCCAGCGCCTGTTTCAGGTTAAAG
GTCGTCGCAGTGGTGGCGGTAGTGGCGGTTCAATGGCACTGCTGAGCCGTCCGGCACTGACCCTGCTGCTGCTGTTA
ATGGCAGCAGTTGTTCGTTGTCAAGAACAGGCACAGACCACCGATTGGCGTGCGACCCTGAAAACCATTCGTAATGGT
GTGCATAAAATCGACACCTATCTGAATGCAGCACTGGATCTGCTTGGTGGTGAAGATGGTCTGTGTCAGTATAAATGT
AGTGATGGTAGCAAACCGTTTCCGCGTTATGGTTATAAACCGAGTCCGCCTAATGGTTGTGGTAGTCCGCTGTTTGGT
GTTCATCTGAATATTGGTATTCCGAGCCTGACCAAATGTTGCAATCAGCATGATCGTTGTTATGAAACCTGCGGCAAAA
GCAAAAACGATTGCGACGAAGAATTTCAGTATTGCCTGAGCAAAATTTGCCGTGATGTTCAGAAAACCCTGGGTCTGA
CACAGCATGTTCAGGCATGTGAAACCACCGTTGAACTGCTGTTTGATAGCGTTATTCATCTGGGTTGTAAACCGTATCT
GGATAGCCAGCGTGCAGCCTGTCGTTGTCATTATGAAGAAAAAACCGATCTGTCAGGTGGTGGATCTGGTGGCAGCG
GTCGTGCACAGAGCATTGGTCGTCGTGGTAAAGTTGAACAGCTGAGTCCGGAAGAAGAAGAAAAACGTCGTATTCGT
CGTGAACGCAATAAAATGGCAGCCGCAAAATGCCGTAATCGTCGTCGCGAACTGACCGATACGCTGCAGGCCGAAACC
GATCAGCTGGAAGATGAAAAATCAGCACTGCAGACCGAAATTGCAAATCTGCTGAAAGAAAAAGAGAAACTGGAATT
TATCCTGGCAGCACATCGTCCGGCATGTAAAATTCCGGATGATCTGCATCATCACCATCACCACTAA

Additional file 3. DNA coding sequence of pET22b-JAMF1. (Top strand)

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACA
CTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCT
CTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGAT
GGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGA
CTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGC
CTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTCAGGT
GGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGA
GACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTAT
TCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAG
TTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGT
TTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTC
GGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATG
ACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGA
GGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCT
GAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAAC
TGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCT
GCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTG
CAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAA
CGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATAC
TTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATC
CCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCT
GCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAAC
TCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCAC
CACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATA
AGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTC
GTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCAC
GCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCT
TCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATG
CTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTT
TGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTC
GCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTT
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GGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCAC
CGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCC
TGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGG
GCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAA
CGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACT
GGCGGTATGGATGCGGCGGGACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTG
TTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCA
GACTTTACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAGTCGCTTC
ACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACA
GGAGCACGATCATGCGCACCCGTGGGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCG
GGACCAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCT
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GGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTT
CGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCA
GCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAAC
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GCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAA
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ACAGAATAGCGAACTGGCAAGCACCGCAAATATGCTGCGTGAACAGGTTGCACAGCTGAAACAGAAAGTTATGAATC
ATGTTAATAGCAGCGGTGGTGGTAGCGGTGGTAGTATGCGTACCATTGCAATTCTGGCAGCAATTCTGCTGGTTGCA
CTGCAGGCACAGGCAGAAAGCCTGCAAGAACGTGCAGATGAAGCAACCACACAGAAACAGAGCGGTGAAGATAATCA
GGATCTGGCAATTAGCTTTGCAGGTAATGGTCTGAGCGCACTGCGTACCAGCGGTAGCCAGGCACGTGCGACCTGTT
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GAGCCTGACCAAATGTTGCAATCAGCATGATCGTTGTTATGAAACCTGCGGCAAAAGCAAAAACGATTGCGACGAAGA
ATTTCAGTATTGCCTGAGCAAAATTTGCCGTGATGTTCAGAAAACCCTGGGTCTGACACAGCATGTTCAGGCATGTGA
AACCACCGTTGAACTGCTGTTTGATAGCGTTATTCATCTGGGTTGTAAACCGTATCTGGATAGCCAGCGTGCAGCCTG
TCGTTGTCATTATGAAGAAAAAACCGATCTGTCAGGTGGTGGATCTGGTGGCAGCGGTCGTGCACAGAGCATTGGTC
GTCGTGGTAAAGTTGAACAGCTGAGTCCGGAAGAAGAAGAAAAACGTCGTATTCGTCGTGAACGCAATAAAATGGCA
GCCGCAAAATGCCGTAATCGTCGTCGCGAACTGACCGATACGCTGCAGGCCGAAACCGATCAGCTGGAAGATGAAAA
ATCAGCACTGCAGACCGAAATTGCAAATCTGCTGAAAGAAAAAGAGAAACTGGAATTTATCCTGGCAGCACATCGTCC
GGCATGTAAAATTCCGGATGATCTGCATCATCACCATCACCACTAACTCGAGCACCACCACCACCACCACTGAGATCCG
GCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCC
TCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAT

Additional file 4. JAMF1 amino acid sequence.

MRKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNHVNSSGGGSGGSMRTIAILAAI
LLVALQAQAESLQERADEATTQKQSGEDNQDLAISFAGNGLSALRTSGSQARATCYCRTGRCATRESLSGVCEISGRLYRLC
CRSGGGSGGSQRLFQVKGRRSGGGSGGSMALLSRPALTLLLLLMAAVVRCQEQAQTTDWRATLKTIRNGVHKIDTYLNAA
LDLLGGEDGLCQYKCSDGSKPFPRYGYKPSPPNGCGSPLFGVHLNIGIPSLTKCCNQHDRCYETCGKSKNDCDEEFQYCLS
KICRDVQKTLGLTQHVQACETTVELLFDSVIHLGCKPYLDSQRAACRCHYEEKTDLSGGGSGGSGRAQSIGRRGKVEQLSP
EEEEKRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAAHRPACKIPDDLHHHH
HH

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