Effect of the enterocin AS-48 on planktonic cells and biofilms. Advantages of the combination with biomimetic magnetic nanoparticles

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Antibiotic resistance in bacteria is a phenomenon that can emerge and spread quickly, and it is specially problematic when it appears in biofilm-forming bacteria. Thus, it is a main concern in public health worldwide. Among the different venues to reduce this problem, discovery of new compounds remains an attractive solution.

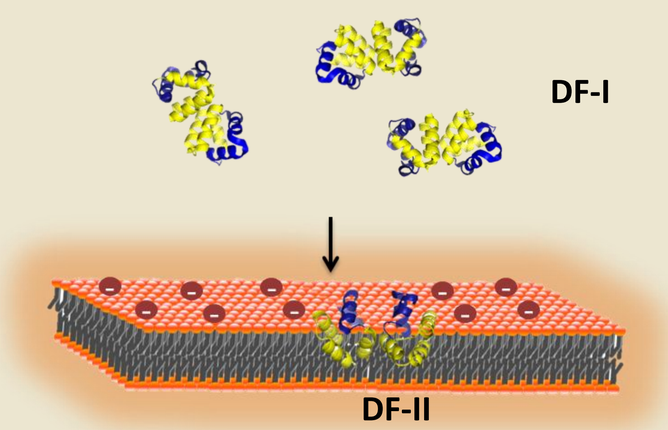
Head-to-tail cyclized peptides are bacteriocins with a circular backbone and a potent antimicrobial activity that may be useful in a clinical setup. The **enterocin AS-48** is the prototype of this compound class. Head-to-tail cyclization has an enormous impact in the structure and biological activity of AS-48 even in harsh environments. It is structured as 5 alpha helices (3 hydrophobic and 2 cationic) with an asymetric charge distribution and pI 10.5. In aqueous solution, AS-48 is arranged in dimers that interact through the hydrofobic regions (DF-I). Thus, the cationic helices are exposed and drive the attraction to the negatively charged bacterial membrane. In the acidic environment at the membrane, the DF-I form undergoes a transition to the DF-II dimer, in which the molecules interact through the hydrophilic parts and the hydrophobic helices insert in the lipid bilayer, creating pores **(Figure 1)**. This mechanism of action is independent of any receptor binding, which can reduce stable resistance appearance**.**

AS-48 is active at the micro- to nanomolar range against, mainly, Gram-positive bacteria. Gram-negative species are intrinsically more resistant due to the barrier function of their outer membrane. However, combined treatments that alter the outer membrane and that can grant AS-48 access to the cytoplasmic membrane enable sensitization to this compound.

Nanoparticles are part of a technology that, among other functions, aims at drug delivery at specific sites within the organism. Magnetic nanoparticles can be driven and concentrated using a magnetic field. Magnetosomes from the magnetotactic bacterium *Magnetococcus marinus* have inspired the production of **biomimetic magnetic nanoparticles (BMNPs)** with a net negative charge at physiological pH and pI aprox. 4.8. Thus, cationic compounds can bind at physiological pH and be released in the acidic environment of cells. Moreover, when the BMNPs are immersed in an alternating magnetic field (AMF), they rotate according to the field. Using an AMF with suitable frequency this rotation achieves a local temperature increase that facilitates drug release and can have a synergistic effect with AS-48.

In this work we analyze the interaction of AS-48 with BMNPs, drug release and the effect of the combined treatment on Gram-negative and Gram-positive species. In addition, we study a cheap medium-throughput system for biofilm establishment that can serve as a platform to test antimicrobial compounds in an economic and reproducible manner.

Figure 1. Structure and mechanism of action of AS-48.
Dimer DF-I displays a cationic surface to the solvent (blue regions). It is attracted to the cell surface due to electrostatic interaction. Local pH causes a transition to DF-II in which the hydrophobic helices (yellow regions) get inserted in the membrane creating pores.



We have studied the interaction between AS-48 and BMNPs **(Fig. 2A)**. First, BMNP saturation over time was controlled **(Fig. 2B)**. Saturation kinetics indicates that AS-48 interacts with BMNPs due to the charge difference and an additional interaction between AS-48 molecules **(Fig. 2C)** allows a cooperative binding according to a Langmuir-Freundlich model **(Fig. 2D)**. The AS-48-BMNP nanoassembly is stable over time and less than 7% is released during storage in a 96 h experiment at pH 7.4.

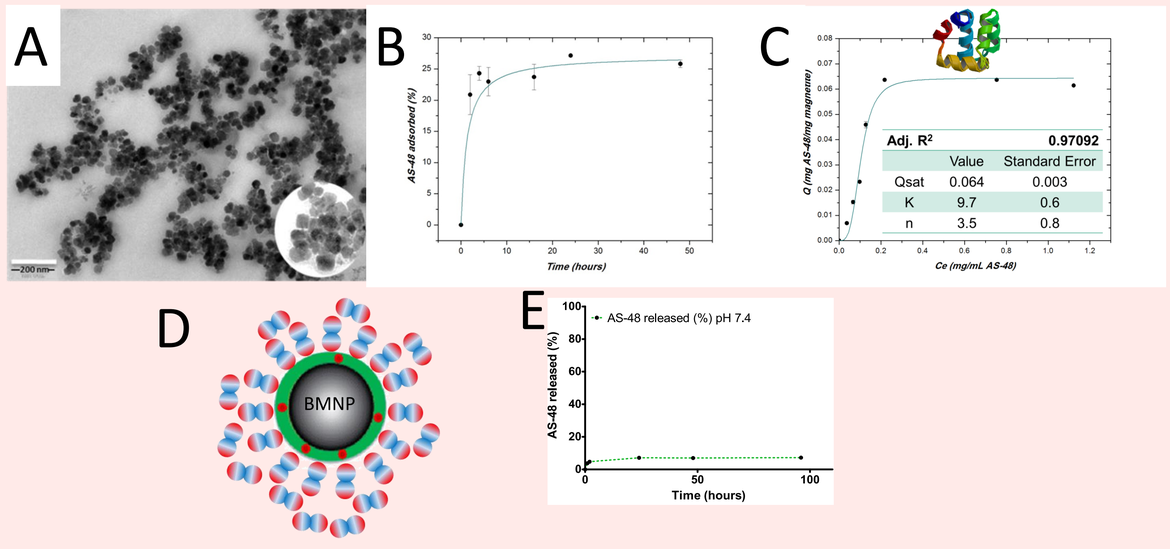


Figure 2. Characterization of AS-48 and BMNPs binding.
A) Electron microscopy of BMNPs. B) Adsorption kinetics of AS-48 to BMPs. C) saturation kinetics of BMNPs using AS-48. D) Schematic representation of AS-48 binding to BMNPs. E) AS-48 release during storage at pH 7.4.

Antimicrobial susceptibility tests for biofilms are not standardized. The Calgary biofilm device is a costly medium-throughput system that is one of the most broadly tested, in which the biofilm grows on pegs on the lid. Using glass beads (**Figure 3A**) enables a cheap and reproducible platform for biofilm production **(Fig. 3B and 3C)**, minimizing the amount of antimicrobials that are required for the tests.

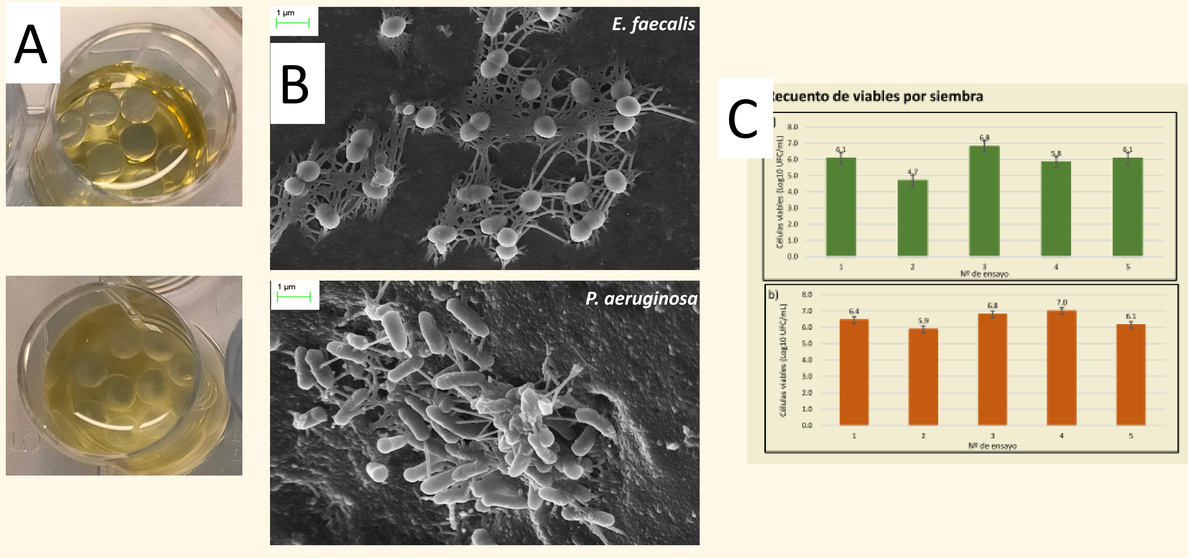


Figure 3. Characterization of biofilm growth on glass beads.
A) Image of the glass beads in the medium (up) and a 48 h biofilm (down). B) SEM of *E. faecalis* (up) and *P. aeruginosa* biofilms (down). C) Viable cell count of *E. faecalis* (up) and *P. aeruginosa* (down) biofilms (48 h biofilm growth). Average of 5 independent experiments.

AS-48-BMNPs have been assayed against Gram-negative and Gram-positive species. AS-48-BMNPs is active at either 37 or 45 ºC against Gram-positive species and *E. coli*, but not *K. pneumoniae* or *P. aeruginosa*. When an AMF is used to generate therapeutic hyperthermia (45 ºC) there is a potent synergy that drastically reduces the number of viable cells within 15 min in all cases, including Gram-negative bacteria.

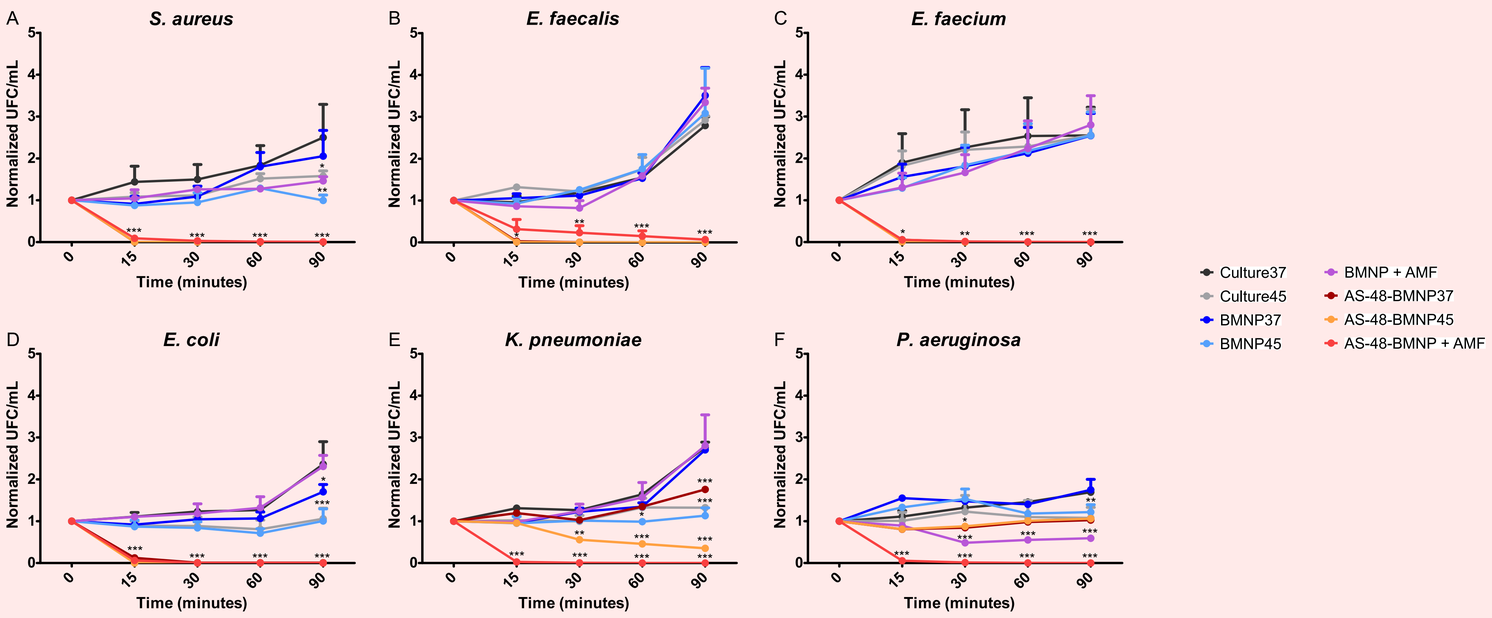


Figure 4. Activity of AS-48-BMNPs against planktonic bacteria. Cultures were maintained at 37 or 45 ºC without (controls culture37 and culture45) or with BMNPs (controls BMNP37 and BMNP45). AS-48-BMNPs were tested in a water bath at 37 and 45 ºC (AS-48-BMNP37 and AS-48-BMNP45). Last, the effect of an AMF with naked BMNPs (BMNP+AMF) or AS-48-BMNPs (AS-48-BMNP+AMF) on the cultures was assayed.

Conclusions and perspectives

The enterocin AS-48 adsorbs on BMNPs surface and the nanoassembly is stable along time. This binding fits into the cooperative binding model of Langmuir-Freundlich.

AS-48-BMNPs are active against planktonic cells, indicating that the bacterial surface enables AS-48 release and membrane insertion. The combined effect of mechanical damage induce by BMNP rotation, hyperthermia, and released AS-48 achieves a synergy in short time that can affect Gram-positive and, remarkably, Gram-negative pathogens.

Spheric glass beads represent a suitable surface for biofilm growth. These biofilms are reproducible with the bacterial species tested.

Availing such a simple, cheap and reproducible model for biofilm production enables testing different compounds and their combinations. We will explore the combination of AS-48 and several antimicrobials to provide treatments that can specifically target biofilm-embedded bacteria.

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