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"Nano-Trojan Horses" of Apoptotic Bodies with an Antimicrobial Peptide for Targeting Intracellular Infections

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"NANO-TROJAN HORSES" OF APOPTOTIC BODIES WITH AN ANTIMICROBIAL PEPTIDE FOR TARGETING INTRACELLULAR INFECTIONS"

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"Thesis presented as part of the requirements for the title of DOCTOR IN BIOTECHNOLOGY, in the Graduate Program in Biotechnology of the Universidade Católica Dom Bosco - Field: Biotechnology"

This thesis is dedicated to those who have supported me throughout my education.

Thank you for allowing me this adventure in science.

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Biography

Valentina Nieto Marín was born in 1994 in Sevilla, Valle del Cauca, a small coffee-growing town in the Colombian Andean mountains. She is a biology professional with experience in mutagenesis, cancer, biotechnology and drug delivery.

Table of contents

Tables and figures 6
Vocabulary 7
Abstract 9
1. Introduction 11
1.1 Intracellular infections 11
1.2 *S. aureus* and its intracellular survival mechanisms 12
1.3 Treatments against *S. aureus* small colony variants 15
1.4 Antimicrobial peptides 16
1.5 Apoptotic bodies as Nano-Trojan Horses for intracellular drug delivery 19
2. General objective: 22
2.1 Specific objectives: 22
3. First chapter: "Nano-Trojan" horses of apoptotic bodies with an antimicrobial peptide for targeting intracellular infections 24
4. Appendix figures: Sumplemmentary material, chapter 1. 74
6. General discussion 97
7. Conclusions and perspectives 107
8. Appendix 2: articles and book chapters published in indexed journals during the Ph.D. program. 108
9. References 116

Tables and figures

Figure 1. *S. aureus* intracellular evasion strategies..... 13

Figure 2. The sequence of the batroxicidin, Btn region, and BotrAMP14. 18

Figure 3. Hypothetical affinity and interaction between apoptotic bodies (ApoBDs) membranes from cancer and normal cells with cationic peptides. 21

Figure 4. Origin and mechanism of action of the ApoBDs-AMP formulation for intracellular infection treatments. 21

Vocabulary

%EE: percentage encapsulation efficiency

A5: annexin V

AFM: atomic force microscopy

AMP: antimicrobial peptide

ApoBD: apoptotic body

ApoBD-AMP: apoptotic body conjugate with AMP

ApoBD-VANH: apoptotic body conjugate with vancomycin

Apocells: apoptotic cells

Aurora: *Staphylococcus aureus* strain isolated from cows with bovine mastitis

CFU: colony-forming unit

CLSI: Clinical & Laboratory Standards Institute

d(H): hydrodynamic diameter

DLS: dynamic light scattering

EV: extracellular vesicle

F-moc: 9-fluorenylmethylenoxy-carbonyl

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

FSC: forward scatter

HPLC: high-performance liquid chromatography

LPS: lipopolysaccharides

MALDI-ToF: matrix-assisted laser desorption/ionization-time of flight

MHB: Muller-Hinton broth

MHA: Muller-Hinton agar

MIC: minimum inhibitory concentration

MRSA: methicillin-resistant *S. aureus*

MS: mass spectrometry

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OD: optical density

PBS: phosphate-buffered saline

PDI: polydispersity index

PS: phosphatidylserine

ReApoBD: size reconstructed apoptotic body

ReApoDB-AMP: size reconstructed apoptotic body conjugate with AMP

ReApoDB-VANH: size reconstructed apoptotic body conjugate with vancomycin

ROS: reactive oxygen species

RP-HPLC: reversed-phase high-performance liquid chromatography

SCV: small colony variants

SSC: side scatter

STM: scanning tunneling microscope

VAN: vancomycin hydrochloride from *Streptomyces orientalis*

WAS: whole apoptotic sample

Abstract

Persistent intracellular infections caused by *Staphylococcus aureus*, particularly its small colony variant phenotype, are challenging to eradicate due to the poor intracellular penetration of conventional antibiotics. Antimicrobial peptides offer a promising alternative, but their therapeutic potential is limited by low stability, host toxicity, and inefficient delivery to infected cells. This study presents a novel intracellular delivery platform based on reconstructed apoptotic bodies (ReApoBDs) engineered to transport the cationic AMP BotrAMP14 into infected macrophages. ApoBDs were isolated from BV-2 and HeLa cells, size-remodeled, and successfully loaded with BotrAMP14 using freeze–thaw and extrusion techniques. The resulting ReApoBDs were characterized by dynamic light scattering and transmission electron microscopy, confirming their structural integrity and nanometric size. Encapsulation efficiency was around 70%, and confocal microscopy demonstrated effective macrophage uptake. Biodistribution studies confirmed the accumulation of ReApoBDs at infection sites *in vivo*. Functionally, the ReApoBD-BotrAMP14 nanoformulation significantly reduced intracellular *S. aureus* loads while showing lower cytotoxicity compared to the free peptide. A murine cutaneous abscess model reduced dermonecrosis by 31.7% and small colony variants prevalence by 72.9%, demonstrating superior efficacy compared to conventional treatments. These findings establish ReApoBDs as Nano-Trojan Horses for targeted AMP delivery, offering a promising strategy to overcome the limitations of current therapies in the treatment of persistent intracellular *S. aureus* infections.

Keywords: drug delivery; bacterial infections; nanoformulations; antimicrobials

Resumo

Infecções intracelulares persistentes causadas por *Staphylococcus aureus*, particularmente sua variante fenotípica de pequenas colônias (*small colony variant*, SCV), são difíceis de erradicar devido à baixa penetração intracelular dos antibióticos convencionais. Peptídeos antimicrobianos (AMPs) oferecem uma alternativa promissora, mas seu potencial terapêutico é limitado por baixa estabilidade, toxicidade para o hospedeiro e entrega ineficiente às células infectadas. Este estudo apresenta uma nova plataforma de entrega intracelular baseada em corpos apoptóticos reconstruídos (ReApoBDs), projetados para transportar o AMP catiônico BotrAMP14 para dentro de macrófagos infectados. Os ApoBDs foram isolados de células BV-2 e HeLa, remodelados em tamanho e carregados com sucesso com BotrAMP14 utilizando um método de congelamento-descongelamento e uma técnica de extrusão. Os ReApoBDs resultantes foram caracterizados por espalhamento dinâmico de luz e microscopia eletrônica de transmissão, confirmando sua integridade estrutural e tamanho nanométrico. A eficiência de encapsulamento foi de cerca de 70%, e a microscopia confocal demonstrou a captação eficaz pelos macrófagos. Estudos de biodistribuição confirmaram o acúmulo de ReApoBDs nos locais de infecção *in vivo*. Funcionalmente, a nanoformulação ReApoBD-BotrAMP14 reduziu significativamente a carga intracelular de *S. aureus*, apresentando menor citotoxicidade em comparação com o peptídeo livre. Em um modelo murino de abscesso cutâneo, reduziu a dermonecrose em 31,7% e a prevalência de variantes de pequenas colônias em 72,9%, demonstrando eficácia superior em comparação com tratamentos convencionais. Esses achados estabelecem os ReApoBDs como Cavalos de Troia Nano para a entrega direcionada de AMPs, oferecendo uma estratégia promissora para superar as limitações das terapias atuais no tratamento de infecções intracelulares persistentes por *S. aureus*.

Palavras-chave: entrega de fármacos; infecções bacterianas; nanoformulações; antimicrobianos

1. Introduction

1.1 Intracellular infections

The different types of infectious diseases caused by fungi, viruses, parasites, or bacteria can be classified as extracellular or intracellular infections (Thakur et al., 2019). The most common type is extracellular, which causes acute infections followed by the rapid development of clinical signs (Schorey & Harding, 2016). Acute infections can be resolved within days by the host's immune system or, in some cases, with antibiotic treatment (Thakur et al., 2019).

However, some pathogens can cause persistent rather than acute infections in which the immune system fails to eliminate the pathogen (Hobby et al., 1942; Y. Zhang, 2014). Persistent infections are known to cause high rates of mortality and morbidity in humans worldwide (Thakur et al., 2019). However, because the pathogens that cause persistent infections have lower densities in the bloodstream than extracellular pathogens and are not easily detected, mortality and morbidity rates are difficult to estimate (Drevets et al., 2004). In the development of diseases associated with this type of infection, the pathogen or molecules derived from it as DNA or RNA in the case of viruses, continue to replicate over long periods (Pacios et al., 2020). Persistent infections can be classified as chronic if they can eventually be resolved, or latent if they cannot. The pathogens that cause this type of infection have in common the ability to colonize the intracellular space, where they remain protected from the immune system and have access to nutrient sources (Eisenreich et al., 2021; Kim et al., 2018; Weiss & Schaible, 2015). Interestingly, these intracellular microorganisms primarily target phagocytic immune cells, such as macrophages and dendritic cells. These cells are responsible for recognizing and eliminating pathogens (Kaufmann, 2011). This adaptation is advantageous for the pathogen because it exploits the immune system's capacity for recognition and phagocytosis, enabling quicker entry into the intracellular space.

Throughout evolution, intracellular pathogens have developed various mechanisms that allow them to persist inside immune cells, causing periods of acute infection followed by dormancy and repeated periods of reactivation (Martinez et al., 2018; Omotade & Roy, 2019; Pizarro-Cerdá et al., 2016; Ray et al., 2009). Examples of such pathogens include

Mycobacterium tuberculosis (Srinivas et al., 2020a), *Treponema pallidum* (Fitzgerald, 1981), human immunodeficiency virus (HIV) (McDonald et al., 2002), and *Staphylococcus aureus* (Hébert et al., 2000).

According to a recent systematic review, methicillin-resistant *S. aureus* (MRSA) was the most dangerous pathogen in 2019, causing more than 100,000 deaths (Murray et al., 2022). This bacterium can infect both non-professional and professional phagocytes in the intracellular space. Although *S. aureus* has been shown to survive inside neutrophils and macrophages, evidence suggests that intracellular replication of the bacterium occurs inside macrophages (Flannagan et al., 2015; Jubrail et al., 2016; Lacoma et al., 2017; Surewaard et al., 2016; Tranchemontagne et al., 2016)

1.2 *S. aureus* and its intracellular survival mechanisms

Once *S. aureus* is internalized into the phagosome of the macrophage, it activates several cellular signaling pathways that allow this compartment to fuse with lysosomes and undergo several maturation steps until it becomes a phagolysosome.

The phagolysosome contains a cocktail of molecules designed to destroy the phagocytized bacterium, such as degradative lysosomal enzymes and antimicrobial peptides (AMPs). It is also bombarded with reactive oxygen species (ROS) (Flannagan et al., 2009). However, as mentioned above, there seems to be evidence that *S. aureus* has developed several mechanisms to resist the phagolysosomal environment and survive inside the macrophage (Fig. 1) (Hommes & Surewaard, 2022). Some examples related to ROS elimination are the superoxide dismutases SodA and SodM (Das et al., 2008; Karavolos et al., 2003), AhpCF, an alkyl hydroperoxidase reductase, and KatA, a catalase, which give bacteria the ability to resist peroxides and H_2O_2, respectively (Cosgrove et al., 2007; Mashruwala & Boyd, 2017). At the same time, a carotenoid pigment (staphyloxanthin) acts as an antioxidant (Pandey et al., 2019), and a flavonoid hemoglobin (Hmp) helps bacteria survive in high concentrations of nitric oxide (Nobre et al., 2008). Other *S. aureus* resistance molecules include lipoic acid (LA), which controls ROS production (Grayczyk & Alonzo, 2019), and the peroxide inhibitor SPIN, which blocks the enzyme myeloperoxidase (N. W. M. de Jong et al., 2017). Other important macrophage defense mechanisms against pathogens are the cationic AMPs located in the

phagolysosomal environment, for which *S. aureus* expresses another type of resistance molecule, VraFG, an AMP transporter (Li et al., 2007), and MprF. MprF is responsible for a reaction that binds a lysine to the negative lipids of its membrane, helping to reduce the electrostatic interaction with the cationic AMPs and other defensins (Kristian et al., 2003; Peschel et al., 1999, 2001).

As mentioned above, the fusion of the lysosome with the phagosome leads to the formation of the phagolysosome, and, as expected, its pH tends to be highly acidic due to the release of hydrogen ions by the v-ATPase. For this, the expression of GraXRS has been correlated with bacterial resistance to acidic pH environments (Flannagan et al., 2018; Villanueva et al., 2018). Finally, the most recognized macrophage mechanisms to eliminate phagocytized pathogens are the expression of digestive enzymes, such as lysozymes, that catalyze the hydrolysis of the bacterial cell walls, leading to the lysis of the pathogens. Lysozyme is present in the lysosomes and phagolysosomes, and as a protective and evasive mechanism, *S. aureus* expresses OatA, an O-acetyltransferase that acetylates the peptidoglycan bacterial wall, thus modifying the target of lysozyme (Bera et al., 2006; Shimada et al., 2010). All these reservoirs of *S. aureus* virulence factors, its intracellular survival ability, which is related to its antibiotic tolerance, and the problems of bacterial antimicrobial resistance make this bacterium one of the most dangerous for human health (Murray et al., 2022).

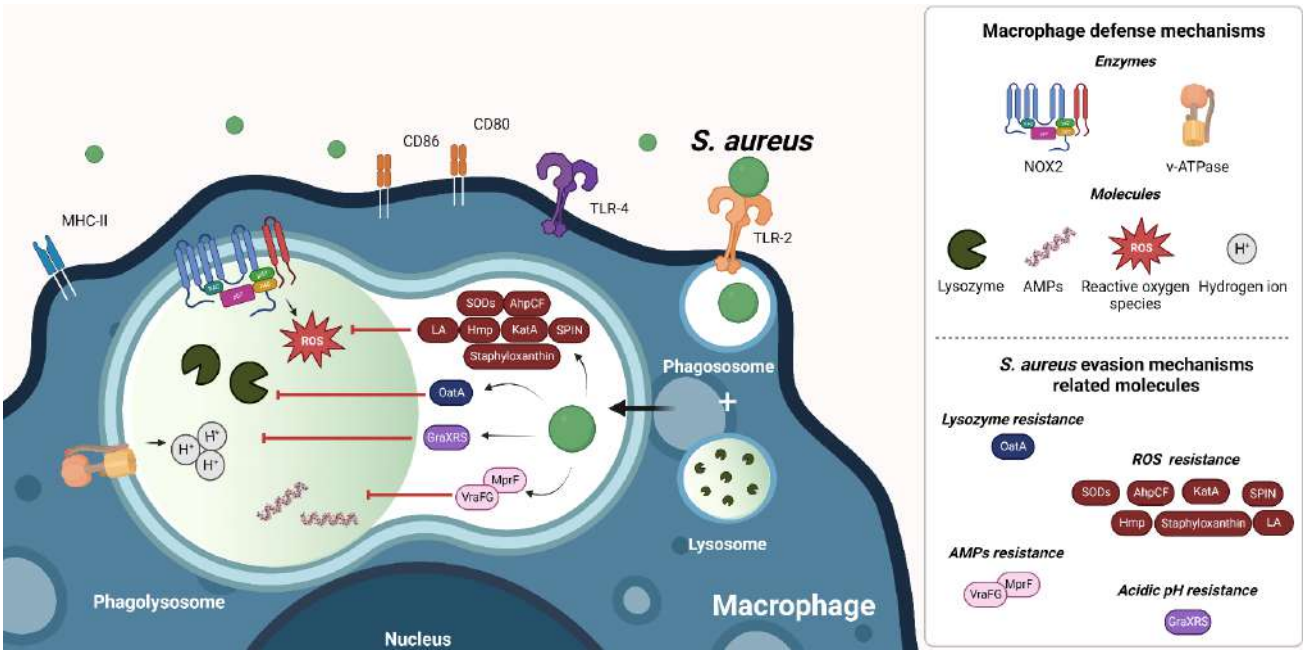

Figure 1. *S. aureus* intracellular evasion strategies. Once *S. aureus* is phagocytosed, phagosome and lysosome fusions occur. Phagolysosomes are generated where the pH is acidic, and they can contain some enzymes and molecules responsible for killing the phagocytosed pathogens. However, *S. aureus* turns on various resistance/evasion mechanisms to avoid being lysed by the macrophage antimicrobial response mechanisms. Created with BioRender.com.

One of the most critical adaptations of *S. aureus* that contributes to its persistence in intracellular environments is its ability to form small colony variants (SCVs) (Conlon et al., 2013; Garcia et al., 2013; Kaiser et al., 2014; Lechner et al., 2012). SCVs are a slow-growing, metabolically altered subpopulation characterized by reduced pigmentation, decreased electron transport activity, and an enhanced ability to persist under stressful conditions, including those within phagolysosomes (Delgado-Valverde et al., 2014). These variants display increased resistance to host immune defenses and conventional antibiotics, partly due to reduced metabolic activity and altered membrane composition. SCVs are often associated with chronic or relapsing infections, including osteomyelitis, endocarditis, and device-related infections, and have also been observed in skin and soft tissue infections (Libraty et al., 2012).

Importantly, SCVs can revert to the more virulent wild-type phenotype once environmental conditions become favorable, leading to renewed infection episodes

(Conlon et al., 2013; Garcia et al., 2013; Kaiser et al., 2014; Lechner et al., 2012)This phenotypic plasticity and its intracellular persistence pose a significant challenge for treating S. aureus infections and underscore the need for novel therapeutic strategies capable of targeting these elusive bacterial forms. In this context, developing drug delivery systems capable of reaching and eliminating intracellular *S. aureus*, including its SCV phenotype, is crucial for effectively managing persistent infections.

1.3 Treatments against *S. aureus* small colony variants

Treating *S. aureus* infections, particularly those involving SCVs, remains an ongoing clinical challenge (Brouillette, Martinez, et al., 2004; Garcia et al., 2013; Tuchscherr et al., 2010). Among the mainstay antibiotics used against *S. aureus* are vancomycin, linezolid, dicloxacillin, cefapirin, gentamicin, and β-lactams (Brouillette, Grondin, et al., 2004; Joosten et al., 2005; Miller et al., 1978; Sandberg et al., 2011). Each agent presents specific advantages and limitations when addressing persistent intracellular infections or SCVs.

Vancomycin, a glycopeptide antibiotic, is a frontline treatment for MRSA infections and functions by inhibiting peptidoglycan synthesis in the bacterial cell wall (Bose et al., 2020; Hemmati et al., 2024). However, its efficacy against SCVs is limited, especially in chronic infection models. For example, vancomycin-loaded hydroxyapatite bone cements used in rabbit models of chronic osteomyelitis required 42 days of sustained application at extremely high doses (80,000–240,000 $mg.kg^{-1}$) to achieve total eradication of SCVs. These findings highlight the antibiotic's poor intracellular penetration and limited efficacy against dormant or low-metabolism bacterial subpopulations (Joosten et al., 2005).

Linezolid, an oxazolidinone antibiotic, demonstrates intracellular activity and has shown potential in controlling both intra- and extracellular *S. aureus* (Sandberg et al., 2011). In a murine peritonitis model, linezolid at 17 $mg.kg^{-1}$ reduced bacterial burdens but failed to eliminate SCVs after a single dose. Similarly, dicloxacillin, another intracellular-active agent, displayed comparable limitations when SCVs were present (Sandberg et al., 2011).

In a mouse mastitis model, cefapirin (10–25 $mg.kg^{-1}$) showed limited efficacy against hemin-dependent SCVs (Brouillette, Grondin, et al., 2004), while combination therapies like

gentamicin and β-lactams effectively eliminated normal *S. aureus* phenotypes but remained ineffective against SCVs (Miller et al., 1978).

Overall, these conventional antibiotics exhibit partial success in reducing bacterial burden but generally fail to eradicate SCVs. This is particularly problematic as SCVs can revert to the wild-type phenotype under favorable conditions, leading to reinfection and therapeutic failure (Conlon et al., 2013; Garcia et al., 2013; Kaiser et al., 2014; Lechner et al., 2012). The limitations of these antibiotics highlight the need for alternative or adjunctive therapies capable of efficiently penetrating host cells and targeting dormant bacterial forms.

1.4 Antimicrobial peptides

Within the spectrum of microbial infections, those caused by intracellular pathogens have seriously impacted public health. This is due to the low specificity and availability of treatments, the development of drug resistance, and co-infections of these microorganisms (Thakur et al., 2019). The development of antimicrobial peptides has emerged as an alternative due to their broad spectrum of action to meet the challenge posed by these pathogens.

AMPs are peptides produced by all types of organisms and used by the innate immune system as a mechanism to eliminate pathogenic microorganisms (Ageitos et al., 2017; Guaní-Guerra et al., 2010; Kang et al., 2017). One advantage of AMPs over antibiotics is that, although AMPs are known to induce bacterial resistance, this is less likely than with conventional antibiotics (Lewies et al., 2019). This is because while antibiotics generally work by interfering with the metabolic processes of bacteria, most AMPs act directly on bacterial cell membranes or interact with more than one molecular target. The reason for this is that AMPs are generally positively charged and hydrophobic molecules and it is believed that this property makes it easier for some of them to be attracted to the negatively charged membranes of bacteria and interact with the fatty acids, e.g., binding to lipopolysaccharides (LPS) and causing membrane rupture (Brogden, 2005a; Hancock et al., 2016; Yeaman & Yount, 2003). Another attractive feature of AMPs is that, using bioinformatics tools, natural AMPs can be redesigned into simpler, smaller, or more potent molecules, making them, in some cases, cheaper to synthesize (Torres et al., 2019).

A bioinformatics strategy for the rational design of AMPs is based on the point substitution of amino acid residues (Irazazabal, 2016). Using this strategy, a novel cathelicidin-type AMP has already been designed. Cathelicidins are characterized as cationic and amphipathic molecules found in vertebrates, including humans (Pazgier et al., 2013). Several AMPs from snake venom gland cDNA libraries were identified, including the cathelicidin batroxicidin AMP from *Bothrops atrox* (Falcao et al., 2014)The batroxicidin peptide was tested and demonstrated antimicrobial activity against ATCC and clinical isolates of Gram-positive and Gram-negative bacteria, as well as moderate hemolysis properties.

Once the antimicrobial activity of batroxicidin was confirmed, the different regions of the AMP were analyzed. Thus, the last session of the C-terminal region, composed of amino acid residues rich in Lys/Arg and with a strong positive charge, was attributed to the antimicrobial activity and named Btn. After the Btn region responsible for the antimicrobial activity of batroxicidin was identified(Oliveira et al., 2020), they used it as a template for designing a new AMP, BotrAMP14. To this end, the Btn parent sequence was mapped onto a helical wheel diagram to identify the cationic and hydrophobic faces when folded into an α-helix. The goal was to improve the hydrophobic moment and amphipathicity by alternately localizing positively charged and hydrophobic residues while deleting residues unfavorable for the electrostatic surface. Thus, residues Btn K1, V4, P11, R12, G15, V16, T17, and P19 were removed. A Lys (K) and a Trp (W) amino acid residue were then inserted at positions 3 and 12, respectively (Oliveira et al., 2020). Since Lys is an amino acid containing an amino group, it imparts a positive charge to AMPs when protonated. It is often involved in forming hydrogen or ionic bonds. Therefore, Lys was introduced to improve electrostatic interactions with negatively charged bacterial membranes while avoiding toxicity to normal zwitterionic eukaryotic cells. In addition, the Trp residue in the peptide structure functions as a zwitterionic amino acid at physiological pH levels. It assists in the formation of α-helices that anchor transmembrane proteins to the plasma membrane, giving the AMP the ability to anchor deeply into bacterial membranes. Therefore, the physicochemical property-driven design focused on reducing the size while maintaining or improving the antibacterial properties and low toxicity of batroxicidin. This resulted in a 14 amino acid residue AMP [KRWKKFFRKVIKFF-NH_2] (Fig. 2) with a high net positive charge

and an increased hydrophobic moment. The antimicrobial activity of BotrAMP14 was confirmed against susceptible and resistant Gram-positive and Gram-negative bacterial strains, particularly against *S. aureus* (Oliveira et al., 2020).

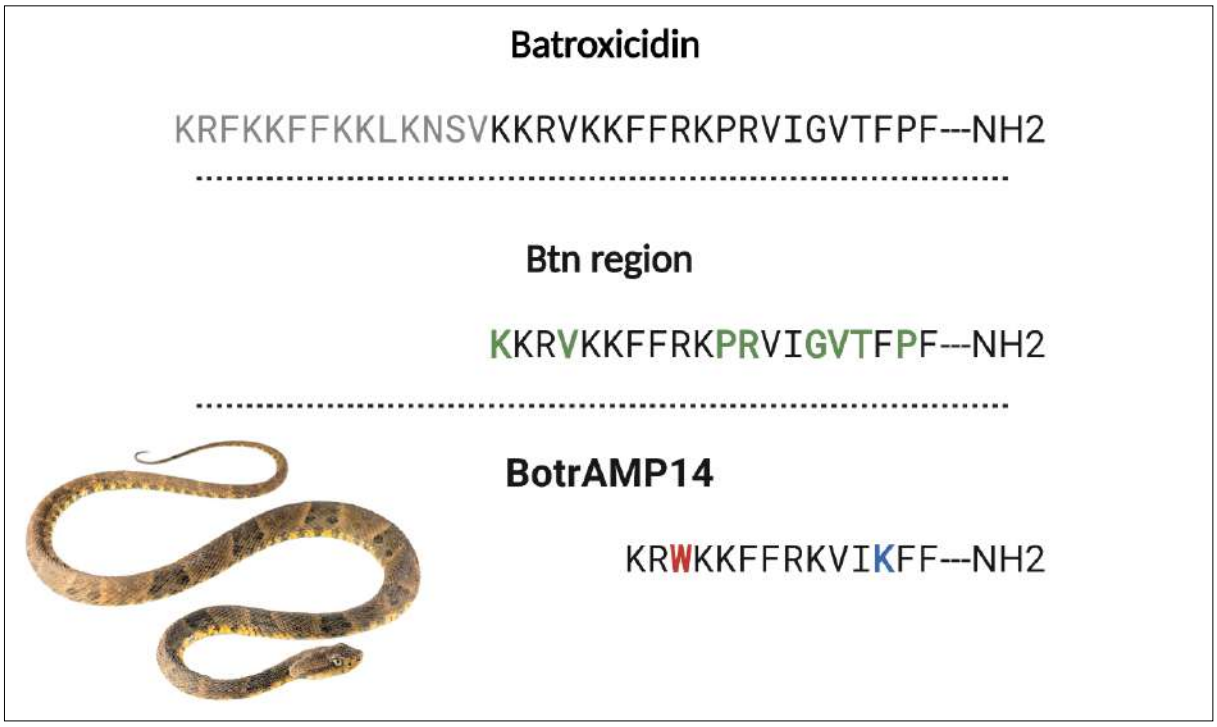

Figure 2. The sequence of the batroxicidin, Btn region, and BotrAMP14. The black letters represent the amino acid sequence of the antimicrobial region Btn. The green letters represent the deleted amino acids in the BotrAMP14 sequence. The red and blue letters represent the hydrophobic and cationic amino acid residues added to the BotrAMP14 sequence. Created with BioRender.com

This novel peptide could treat intracellular infection caused by *S. aureus* in macrophages. However, the main challenges in developing new AMPs, including BotrAMP14, for treating intracellular infections are their limited cell specificity, bioavailability, and delivery. Regardless of the type of pathogen being treated, the delivery vehicle may be more critical than the peptide for successful infection clearance (Allen et al., 1995; Elsharkasy et al., 2020). As mentioned above, *S. aureus* has evolved certain specialized characteristics to infect key cells of the immune system, such as macrophages, thus inhibiting the maturation of phagosomes once phagocytosed (Blander & Medzhitov, 2004; F. Zhu et al., 2015). Therefore, vehicles are important because they act as "Trojan

horses" loaded with bioactive molecules for therapeutic purposes (Nieto Marín et al., 2023).These vehicles can help stabilize AMPs, thereby preserving their biological activities for further action against intracellular pathogens in mammalian cells. This requires low toxicity to the host.

1.5 Apoptotic bodies as Nano-Trojan Horses for intracellular drug delivery

Among the most attractive and innovative vehicle strategies are nanovesicles, microvesicles (liposomes), and cell-derived extracellular vesicles (EVs). EVs are used to enhance immune modulation, cancer, and infectious disease treatment (Baek et al., 2019). In general, both systems (liposomes and EVs) offer advantages in terms of biocompatibility and biodegradability, allowing for better drug passage and targeting across various biological barriers (Mendt et al., 2019). This is because liposomes and EVs are phospholipid bilayer structures that are naturally compatible with the physiological structure of host cells, compared to synthetic nanoparticles (Akter et al., 2018). In addition, synthetic nanoparticles have been shown to exhibit some cytotoxicity and induce pro-inflammatory immune responses at the site of infection (Ahmadi, 2020; Labouta et al., 2019).

EVs appear to have an advantage over liposomes, as they are naturally produced and secreted by cells. The advantages of EVs include their ability to mediate cell-cell communication and leukocyte recruitment signals, their specific tissue recognition, and low host toxicity (Grant et al., 2019; Torr et al., 2012). Apoptotic bodies (ApoBDs) are large-diameter EVs secreted only by apoptotic cells. These ApoBDs are naturally configured to be recognized explicitly by tissue macrophages, which are responsible for the clearance of apoptotic cells (Wyllie et al., 1980). In addition, ApoBDs can be loaded with molecules using exogenous or endogenous strategies, making them manageable according to different needs (Nieto Marín et al., 2023).

However, a significant obstacle in developing ApoBDs-AMP conjugates is the potential disruption of the vesicles due to the strong interaction of AMPs with phospholipids in mammalian membranes. It has been shown that the membrane surface charge of EVs is highly variable. Therefore, using ApoBDs from regular cell lines may be more advantageous than using those from cancer cells. This is because cancer cell membranes,

in contrast to normal cells, have a higher exposure of phosphatidylserine (PS) on the outer side of their membrane and therefore a higher electrostatic attraction with cationic AMPs such as BotrAMP14 compared to normal cells (Fig. 3). However, some degree of electrostatic attraction between ApoBDs and AMPs is necessary for the latter to be loaded into the vesicle. Thus, using ApoBDs from normal cells could prevent the high electrostatic attraction that leads to vesicle rupture by AMPs.

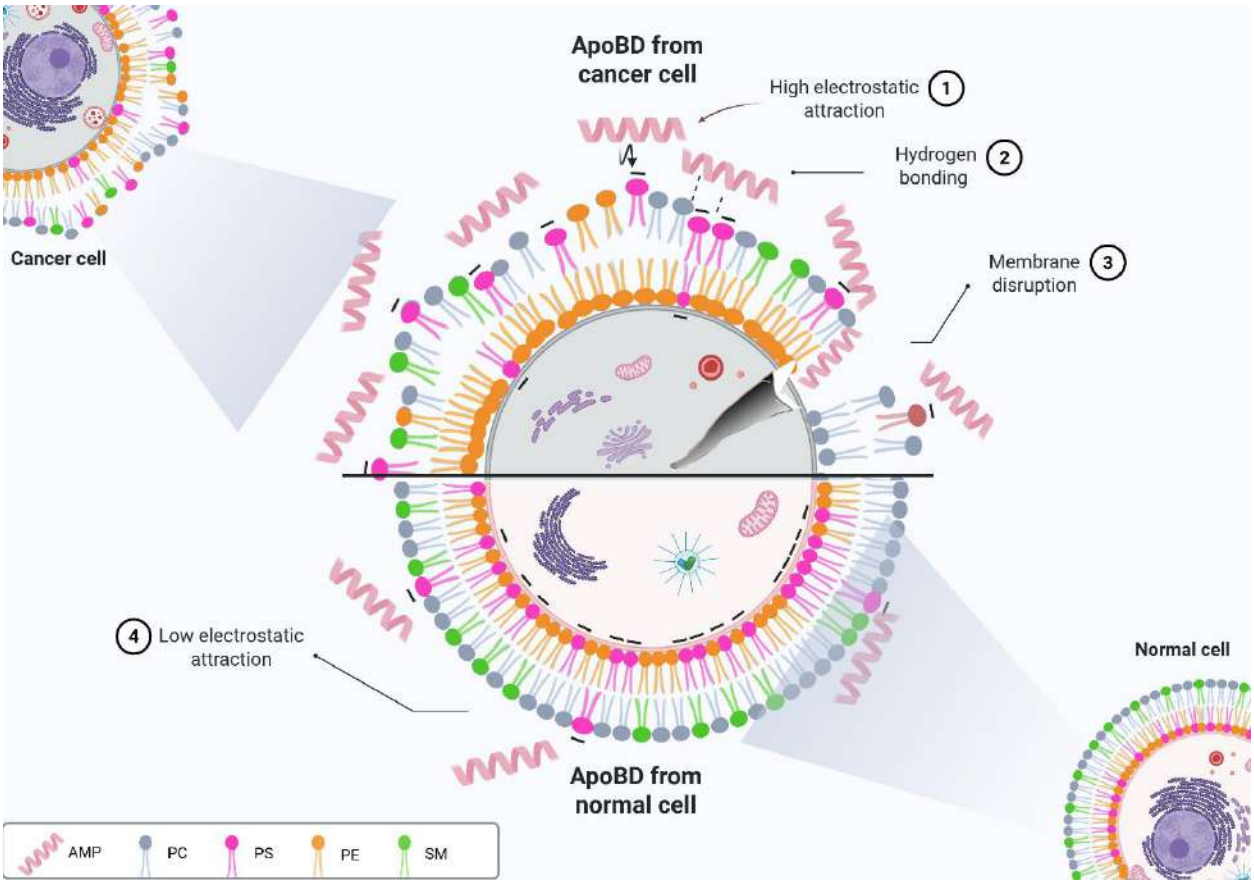

Figure 3. Hypothetical electrostatic affinity and physical interaction between apoptotic bodies (ApoBDs) membranes from cancer and normal cells with cationic peptides. Expanding therapeutic strategies for intracellular bacterial infections through formulations of ApoBDs–antimicrobial peptides (AMPs), Nieto Marin et al. (2023). *Drug Discovery Today*.

Therefore, the combination of AMPs and ApoBD-based delivery strategies represents a novel therapeutic avenue that may help overcome current limitations in the treatment of intracellular infections, particularly those caused by persistent forms of *S.*

aureus, such as SCVs (Fig. 4). This work introduces a novel therapeutic approach in which ApoBDs are repurposed as nano-Trojan Horses—biological delivery systems engineered to carry and protect the AMP BotrAMP14, facilitating its entry into infected macrophages. Combining selective targeting with enhanced intracellular delivery offers a new avenue for treating persistent *S. aureus* infections and overcoming the limitations of current therapies.

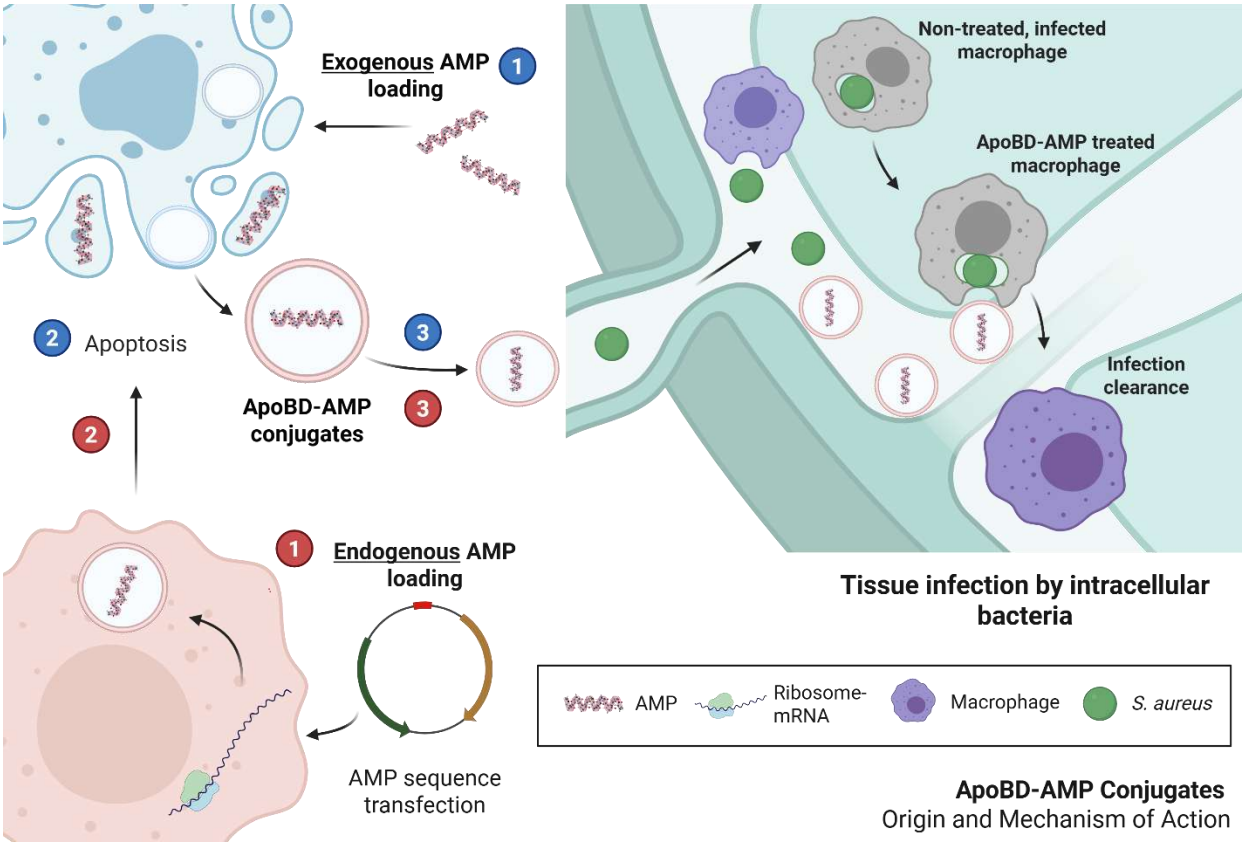

Figure 4. Origin and hypothetical mechanism of action of the ApoBDs-AMP formulation for intracellular infection treatments. Expanding therapeutic strategies for intracellular bacterial infections through conjugates of apoptotic bodies–antimicrobial peptides, from Nieto Marin et al. (2023). *Drug Discovery Today*.

2. General objective:

To develop and evaluate a novel antimicrobial strategy using ReApoBDs for the intracellular delivery of an engineered AMP (BotrAMP14), aiming to enhance the treatment of macrophage infections caused by *S. aureus*, including its SCV phenotype.

2.1 Specific objectives:

- To isolate and reconstruct apoptotic bodies (ApoBDs) from BV-2 and HeLa cell lines, and confirm their purity, size, and structural integrity by flow cytometry, transmission electron microscopy (TEM), and atomic force microscopy (AFM).
- To load and remodel ApoBDs with vancomycin hydrochloride (VAN) and BotrAMP14 using freeze–thaw cycles and extrusion, optimizing encapsulation efficiency and vesicle stability.
- To characterize the physicochemical properties of the ReApoBD conjugates, including particle size, polydispersity index (PDI), and ζ-potential, and verify drug encapsulation by RP-HPLC and mass spectrometry.
- To evaluate the antimicrobial activity of free drugs and ReApoBD-loaded formulations against *S. aureus* in planktonic cultures and in an *in vitro* macrophage infection model.
- To assess the cytotoxicity of free drugs and ReApoBD conjugates in RAW 264.7 cells, determining safe and effective concentration ranges.
- To analyze the internalization and intracellular distribution of ReApoBDs in infected and uninfected macrophages using confocal fluorescence microscopy.
- To evaluate the *in vivo* antibacterial efficacy of ReApoBD-BotrAMP14 in a murine model of *S. aureus* abscess, quantifying total bacterial load and SCV prevalence at the infection site.
- To assess the *in vivo* biodistribution of ReApoBDs and confirm their accumulation at the site of infection following systemic administration.

- To compare the intracellular antibacterial performance of ReApoBD-delivered drugs with their free forms, and determine whether ApoBD origin (normal vs. tumor cells) affects formulation stability and therapeutic outcomes.

3. First chapter: “Nano-Trojan” horses of apoptotic bodies with an antimicrobial peptide for targeting intracellular infections

This chapter corresponds to a scientific article submitted to Nature Communications on May 02, 2025.

Title

"Nano-Trojan Horses" of Apoptotic Bodies with an Antimicrobial Peptide for Targeting Intracellular Infections.

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Abstract

Intracellular infections caused by *Staphylococcus aureus* are challenging due to their ability to evade host defenses by developing small colony variants (SCVs) resistant to conventional therapies. This study yielded and evaluated size-remodeled apoptotic bodies (ReApoBDs) as nano-Trojan horses for the targeted delivery of BotrAMP14, an antimicrobial peptide, to treat *S. aureus*-infected macrophages. ReApoBDs demonstrated high encapsulation efficiencies (~70%), biocompatibility, sustained drug release over 12 h, colloidal stability, and improved intracellular delivery. The ReApoBDs-BotrAMP14 nanoformulation reduced intracellular bacterial loads while exhibiting lower cytotoxicity than the free peptide. Moreover, *in vivo* experiments demonstrated that ReApoBD-BotrAMP14 reduced dermonecrosis by 31.7% and SCV prevalence by 72.9%, more effectively than conventional treatments in a skin abscess model. Finally, the positive correlation between cell viability and bacterial survival highlights the challenge of designing treatments that effectively eliminate intracellular bacteria while preserving host cell integrity. As the first study to develop a ReApoBD-AMP formulation, these findings position ReApoBD-BotrAMP14 as a groundbreaking platform for treating persistent intracellular infections.

Introduction

Infectious diseases continue to challenge global health, particularly those caused by pathogens capable of intracellular invasion and persistence. *Staphylococcus aureus* is a common pathogen that causes persistent infections and was associated with more than one million deaths in 2019[1]. *S. aureus* prominently acts as an intracellular pathogen that often resides within macrophages, evades the host immune response, and complicates treatment, thereby driving resistance to traditional antibiotics[2–5]. This persistence stems from the pathogen's ability to transition into small-colony variants (SCVs), a slow-growing, metabolically altered phenotype that enhances survival under hostile conditions[6–9].

Upon entering macrophages, SCVs encounter a harsh phagolysosome environment, designed to attack and kill pathogens through a cocktail of antimicrobial peptides (AMPs), degradative lysosomal enzymes, reactive oxygen species, and an acidic environment[10]. However, *S. aureus* SCVs have multiple defense mechanisms that enable them to survive and persist in this hostile environment, expressing superoxide dismutases (e.g., SodA and SodM), catalases (e.g., KatA), and other resistance proteins that allow them to neutralize oxidants and modify their surface to evade immune detection[11–14]. SCVs pose a significant problem in chronic infections, as they revert to more virulent forms under favorable conditions, causing recurrent infections and treatment failures[15]. In addition, *S. aureus* SCVs are involved in chronic cases of endocarditis and osteomyelitis, where standard treatments often fail[16].

Since SCVs are persistent and resistant, innovative therapeutic strategies have been pursued. One is vancomycin, a glycopeptide antibiotic that inhibits bacterial cell wall synthesis and has been a cornerstone in treating MRSA infections[17, 18]. Additionally, ligand-targeted therapies have been developed to facilitate direct intracellular delivery of antimicrobials[19, 20] and bioengineered

peptides or small molecules, such as NP-6, that disrupt *S. aureus* survival mechanisms[21, 22] to target and eliminate these variants within macrophages effectively. Researchers have engineered nanoparticle systems to target macrophages selectively by modifying properties such as size, shape, stiffness, and charge, or by incorporating specific surface ligands, including phosphatidylserine (PS) and the macrophage-targeting peptide (M2pep)[20, 23]. Additionally, novel approaches have been developed to enhance intracellular drug delivery, such as the use of Coomassie blue-cholesterol conjugate (CB-tag), which enables the direct cytosolic translocation of proteins without endosomal entrapment, representing a promising strategy for improving therapeutic bioavailability[24].

Moreover, apoptotic bodies (ApoBDs), vesicles generated during apoptosis, represent a promising and unusual avenue for targeted drug delivery. ApoBDs induce chemokine expression to direct macrophages toward phagocytosis[25, 26], and these vesicles also correlate with local anti-inflammatory cytokines' (e.g., TGF-β and IL-10) expression at infection sites, which favorably modulate immune responses (27, 28). Macrophages internalize ApoBDs by recognizing specific "eat me" signals (e.g., PS, ICAM-3, annexin I, and calreticulin) on their surface, while the vesicles suppress "do not eat me" signals (e.g., CD47 and CD31) (29). More specifically, ApoBD phagocytosis is mediated by redundant pattern recognition receptors (PRRs), most of which directly recognize PS on the vesicle surface[30, 31]. By mimicking natural apoptotic processes, ApoBDs may provide a biocompatible, efficient means of delivering antimicrobial agents to infected macrophages while reducing immune rejection and systemic toxicity[32]. Once internalized, effective ApoBD-based antimicrobial strategies rely on the targeted release of encapsulated drugs within phagolysosomes to directly target intracellular *S. aureus* while minimizing cytotoxic effects on the macrophage. These properties make ApoBDs natural and

ideal nano-Trojan horses for various therapeutic agents, including AMPs, a new and elegant strategy never previously explored.

AMPs are essential to host defense systems and combat diverse pathogens, including bacteria, viruses, and fungi[33, 34]. Most AMPs disrupt microbial membranes, causing cell lysis and death[35–37]. Among these, BotrAMP14, an AMP inspired by *Bothrops atrox* venom, demonstrates significant antimicrobial activity against resistant Gram-positive and Gram-negative bacteria, particularly against *S. aureus*[38]. BotrAMP14's amphipathic structure enables it to integrate into bacterial membranes, making it a promising candidate for treating infections caused by *S. aureus* that are difficult to treat. However, AMPs face significant challenges in treating intracellular infections due to their limited ability to penetrate host cells and achieve therapeutic concentrations within macrophages[39]. This limitation underscores the need for advanced delivery systems, such as ApoBDs, which can facilitate targeted AMP delivery to infected macrophages and circumvent SCV-associated resistance mechanisms.

In this study, we pioneered ApoBDs conjugated to BotrAMP14 as a novel strategy to enhance the treatment of intracellular *S. aureus* infections. This approach aims to optimize AMP delivery to infected macrophages, increasing their bioavailability, preventing degradation, and minimizing systemic toxicity.

Results

Induction of apoptosis and isolation of apoptotic bodies

Apoptosis was induced in BV-2 (murine microglial) and HeLa (human cervical cancer) cell cultures by combining nutrient starvation and hydrogen peroxide (H_2O_2) treatment[40]. These cell lines were selected due to their distinct membrane charge properties, influencing apoptotic body composition and peptide encapsulation efficiency. Morphological changes indicative of apoptosis, such as membrane blebbing and apoptopodia formation, were observed as early as the third day after treatment. Using Hoechst 33342 dye to stain nuclei, DNA in viable cells produced a uniform blue fluorescence. In contrast, in apoptotic cells, the presence of fragmented DNA and condensed chromatin resulted in altered Hoechst staining patterns, often showing bright, condensed, or fragmented nuclei (Fig. 1A). In BV-2 cultures, the accumulation of ApoBDs was observed from day 3, indicating a significant increase in apoptotic cell death (Fig. S1). Similarly, HeLa cultures showed a significant increase in ApoBDs by day 4, reflecting the progression of apoptosis induced by the combined treatment method. After successful induction of apoptosis in BV-2 and HeLa cultures, samples were collected when approximately 95% of the cells exhibited an apoptotic morphology. Four samples were collected after sequential centrifugation cycles. The success of the differential centrifugation-based isolation approach was then validated by flow cytometry (FC). Five distinct subpopulations were identified using the annexin V apoptosis marker (A5) and forward (FSC) and side (SSC) scatter: viable cells characterized by $SSC^{intermediate/high}$, $A5^{low}$; early apoptotic cells characterized by $FSC/SSC^{intermediate/high}$, $A5^{low}$, and $FSC/SSC^{intermediate/high}$, $A5^{high}$; necrotic and late apoptotic cells characterized by $FSC/SSC^{intermediate/high}$, $A5^{high}$; debris characterized by FSC/SSC^{low}, $A5^{low}$; and ApoBDs characterized by FSC/SSC^{low}, $A5^{intermediate}$.

Fig. 1 B shows the A5/SSC scatter plot for the control sample gating, where black dot events represent predominantly viable cells with a minor presence of early apoptotic cells (-A5). Red dots indicate a mixture of debris, apoptotic cells, and ApoBDs. Notably, this separation was observed exclusively in the A5/SSC scatter plot for the control treatment, demonstrating distinct subpopulations defined by size, complexity, and apoptotic markers. Fig. 1 B also shows a mixture of events without clear differentiation in the A5/SSC and A5/FSC scatter plots for the whole apoptotic sample (WAS) gating. This reflects the proportional combination of different subpopulations within the sample, which is particularly evident in the A5/FSC scatterplot, where events representative of apoptotic cells and ApoBDs overlap significantly. In addition, this result also highlights a higher frequency of events associated with apoptotic cells compared to ApoBDs under WAS gating. Conversely, for the ApoBD target sample, a predominant presence of events characteristic of ApoBDs was observed compared to other subpopulations. The strategy of isolating ApoBDs by differential centrifugation successfully isolates vesicles distinct from other cellular subpopulations. This isolation facilitates subsequent characterization and loading steps, critical for further experimental analysis. Notably, we established that the flow cytometry-based validation approach does not require secondary markers (e.g., TO-PRO-3) when employed solely for validation purposes rather than for isolation.

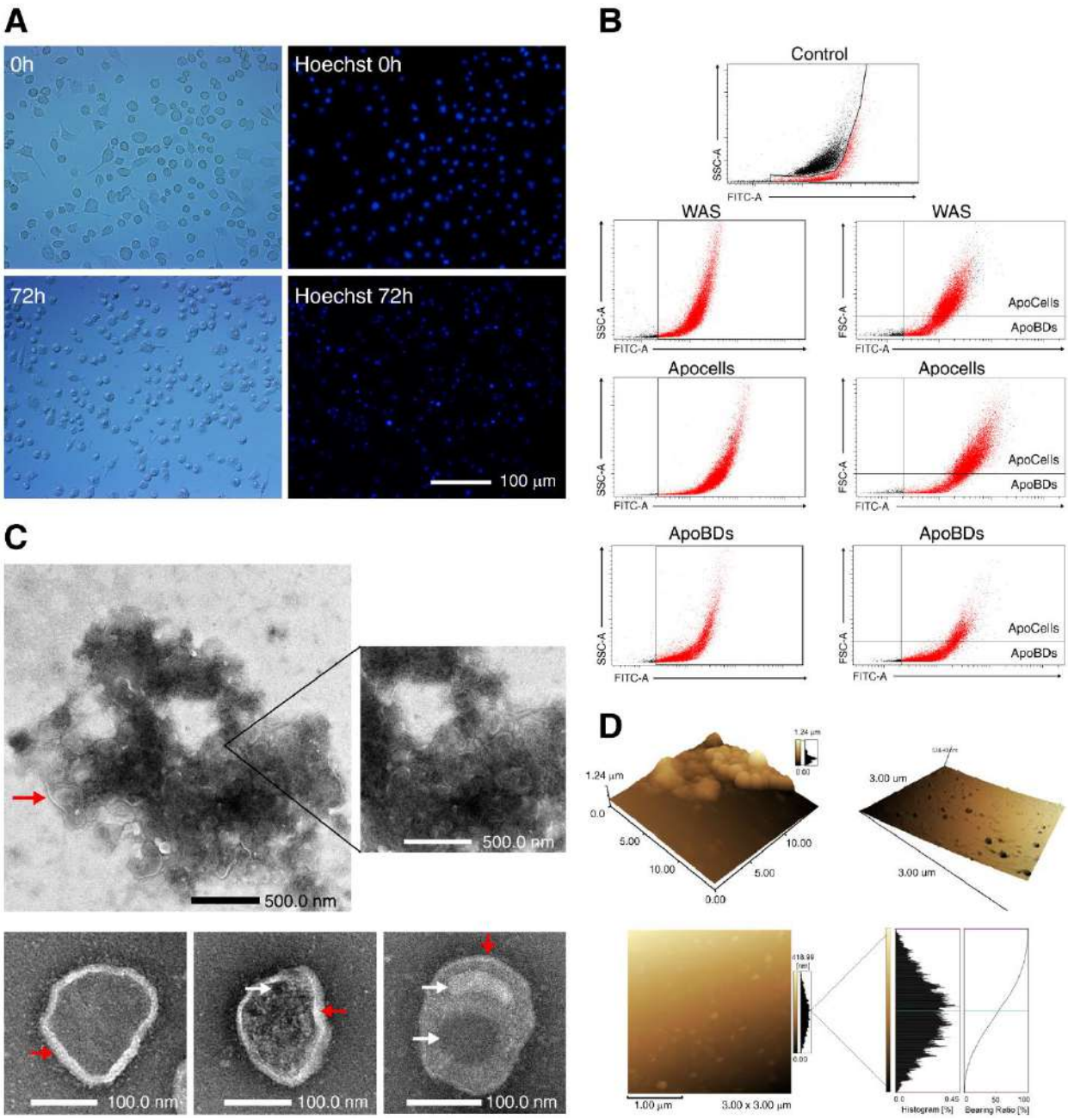

Fig. 1. Isolation and observation of apoptotic bodies. (**A**) Induction of BV-2 apoptosis and staining with Hoechst 33342 (bisbenzimide), a fluorescent nuclear stain to visualize DNA condensation and fragmentation. The figure shows the time-dependent apoptosis process of BV-2 cells as indicated by the fragmented and intensely stained nuclei (10×). (**B**) Flow cytometry dot plot of each sample. Fluorescein isothiocyanate (FITC)-A/SSC (annexin V/side scatter) dot plot of a control sample containing viable and apoptotic cells (Apocells). FITC-A/SSC and A5/FSC (annexin V/ V/forward scatter) were used as a whole apoptotic sample (WAS) control, consisting of a mixture of apoptotic cells and debris,

including extracellular vesicles (EVs). FITC-A/SSC and A5/FSC Apocells control with a mixture of apoptotic cells and EVs. FITC-A/SSC and FITC-A/FSC ApoBDs (apoptotic bodies) have a high concentration of these vesicles. (**C**) Transmission electron microscopy (TEM) micrographs, where negative staining electron microscopy was used to visualize an apoptotic cell (top), with some ApoBDs being formed, and (below) the ReApoBDs (size-remodeled apoptotic bodies). After apoptosis induction, the ApoBDs were collected from BV-2 cultures and used to construct the ReApoBDs. Red arrows indicate the vesicle membrane, and white arrows denote the presence of organelles or cellular debris within the vesicles. (**D**) Atomic force microscopy (AFM) micrographs of ApoBDs' and ReApoBDs' topography.

Structural and Morphological Characterization of ApoBDs

Isolated ApoBDs were size-remodeled (ReApoBDs) using an extrusion strategy as described in the methods section, and transmission electron microscopy (TEM) was used to analyze apoptotic cells and ReApoBD samples (Fig. 1C). The structure and size of apoptotic cells are clearly defined, with ApoBDs forming at the periphery as part of the apoptotic process. Additionally, ReApoBDs of approximately 100 nm in size, resulting from the extrusion process, can be observed. These have a round shape and contain cellular debris or even whole organelles, a characteristic of this type of extracellular vesicle (EV)[41]. Additionally, atomic force microscopy (AFM) topographic analysis was used to assess the physical structure, integrity, and size distribution of ApoBDs and ReApoBDs. As shown in Fig. 1D, ApoBD aggregates appeared round with heights of ~1.2 µm, consistent with sizes reported in the literature (0.5-5 µm)[26, 41]. Similarly, ReApoBDs were analyzed, showing rounded vesicles within a 3 µm x 3 µm area, with a height range of 100-200 nm. However, this range contrasts with the membrane pore used in the extrusion technique (100 nm), which should result in a single population with a diameter of ~100 nm. The size distribution of the vesicles exhibited a normal distribution, as shown in the histogram (Fig. 1D), although the broad curve suggests the possibility of agglomeration. The bearing ratio curve

further supports this, indicating homogeneity in size but within the 100-200 nm range. These findings align with issues described in another study, where it was noted that ApoBDs exhibit low stiffness and problematic adhesion, possibly due to their softness and lack of cytoskeleton[42]. While AFM confirmed the integrity of the vesicles, it does not appear ideal for detailed analysis of the size or shape of ApoBDs.

Encapsulation efficiency determination

After isolating the ApoBDs, the sample was used to prepare formulations using freeze-thaw or extrusion techniques. Vancomycin hydrochloride (VAN), a widely used glycopeptide antibiotic for treating *S. aureus* infections, was included as a control to compare the encapsulation efficiency of a conventional antimicrobial with the bioengineered peptide BotrAMP14. Two-fold dilutions of vancomycin hydrochloride (VAN) (86 μM to 5.4 μM) and BotrAMP14 (128 μM to 4 μM) were used to create regression equations (Fig. S2 A-D). MALDI-ToF analysis was conducted to confirm the presence of VAN and BotrAMP14 (Fig. S2E, F). Encapsulation efficiency (EE) was calculated by subtracting the free drug from the total drug mixed with the ApoBDs and verified by quantification of the encapsulated peptide after lysis of the nanoconjugate vesicles. First, the percentage of encapsulated VAN and BotrAMP14 tended to be higher when the extrusion strategy was used, and the vesicle size was remodeled (ReApoBDs) using HeLa and BV-2 ApoBDs (Table S1). For VAN, the results using the HeLa ApoBDs were consistent with another study[18] where encapsulation efficiencies between 40% and 60% were achieved using cancer cells as the source of ApoBDs (Fig. 2A). Thus, the freeze-thaw method resulted in an encapsulation efficiency of 21.5±0.7% for ApoBDs. In comparison, the freeze-thaw method supplemented with extrusion achieved a rate of 45.75 ± 0.63% (Fig. S3). In addition to

the difference in the encapsulation strategy used, the cell type also made a significant difference, with VAN entrapment tending to be higher in HeLa-derived ReApoBDs compared to BV-2 ReApoBDs. HeLa-derived ReApoBDs exhibited higher VAN EE, potentially due to the elevated cholesterol content in HeLa cell membranes[43]. Cholesterol-enriched vesicles have been associated with improved vesicle stability and pH gradient maintenance, which has been linked to successful VAN encapsulation[43, 44]. In contrast, for BotrAMP14, a higher percentage of EE (70.8 ± 3.39%) was obtained with extrusion and when BV-2 cells were used as the source of ApoBDs (Fig. 2B).

BV-2 cells are characterized by a predominantly neutral charge due to their lipid composition[45]. Their membranes consist mainly of glycerolipids that remain zwitterionic at pH 7, resulting in their ApoBDs having low negative charges, primarily due to the presence of the phosphatidylserine (PS) molecule[45]. In contrast, HeLa cells exhibit a more negatively charged outer leaflet membrane, resulting from O-glycosylation alterations in the membrane glycoproteins[46]. These modifications differ from those in non-cancerous cervical epithelial cells[46, 47]. This increased negative charge in HeLa membranes likely causes ApoBDs from HeLa cells to exhibit stronger electrostatic interactions with the cationic BotrAMP14 peptide, leading to vesicle destabilization and reduced EE.

In contrast, cationic peptide encapsulation in vesicles composed of neutral (zwitterionic) phospholipids, such as phosphatidylcholine (PC), is more stable because the interactions with the peptide are less likely to disrupt the membrane[48–51]. This stability is supported, for example, by studies demonstrating higher EE of a cationic AMP (nissin) in neutral lipid liposomes, such as PC, compared to those made with anionic phospholipids like phosphatidylglycerol (PG)[49–51]. Nisin EE was highest (34.6%) when using liposomes containing 85% neutral PC and only 1%

negatively charged phosphatidylinositol (PI), demonstrating the positive impact of neutral lipids on EE[49–51].

This finding aligns with our data, which shows a higher EE of BotrAMP14 in BV-2-derived ApoBDs. The lower negative charge and more stable membrane composition of BV-2-derived ApoBDs may contribute to their superior ability to encapsulate BotrAMP14. Additionally, reduced PS exposure in BV-2-derived ApoBDs may minimize nonspecific interactions, further enhancing peptide conjugation and intracellular bioavailability, consistent with descriptions from prior studies on biogenic vesicles[26, 30].

Drug release and stability

The drug release and stability profiles of the nanoformulations (ReApoBD-VAN and ReApoBD-BotrAMP14) were evaluated over a 48-hour period. Drug release and stability were measured at 0, 2, 4, 8, 12, 24, and 48 h (Fig. 2C, D). Both formulations showed a clear trend of increasing release over time. For ReApoBD-VAN, the release was minimal at 0 h but increased at 2 h (from 5.76% to 11.76%), reaching almost complete release (from 91.69% to 96.46%) by 48 h. Similarly, ReApoBD-BotrAMP14 had a minimal release at 0 h, increasing to 3.58% to 8.58% at 2 h and reaching 88.96% to 94.83% at 48 h. Stability decreased as drug release progressed. For ReApoBD-VAN, stability dropped from 1 at baseline to 0.3 by 12 h and 0.1 by 48 h. ReApoBD-BotrAMP14 exhibited a similar pattern, with stability decreasing from 1 at baseline to 0.5 at 4 h and 0.09 at 48 h. This decline suggests a loss of colloidal stability, likely due to weakened electrostatic repulsion between particles, promoting aggregation or structural rearrangements in the ReApoBDs as the drug was released[52].

Biophysical characterization of the particles

After extrusion, DLS and zeta potential analyses were performed to determine the hydrodynamic diameter (d(H)), ζ-potential, and stability of the vesicles and other cell populations in suspension. The DLS measurements provided a correlation curve that served as quality control for particle movement in suspension (Fig. S4A). Fig. 2E shows the particle size distribution based on their d(H). In our study, particle sizes were generally consistent across all distribution types, indicating homogeneity and high sample quality. However, as expected, ApoBD vesicles ranged from 1000 to 5000 nm (Fig. S5), as expected due to their physiological heterogeneity[41]. In contrast, ReApoBDs showed a uniform size of around 100 nm, confirming successful size control through extrusion. Moreover, the ReApoBD-VAN and ReApoBD-BotrAMP14 nanoformulations showed d(H)s values slightly higher and lower than those of the empty ReApoBDs, respectively. Additionally, the polydispersity index (PDI) values, below 0.7, confirmed that the samples were monodisperse and suitable for DLS analysis (Table S1)[53].

Zeta potential analysis further validated sample quality through a V-shaped phase diagram (Fig. S4B), which reflects the relationship between the particle's surface charge and the ionic strength of the surrounding liquid. Zeta potential measurements of ApoBDs, ReApoBDs, and their drug-conjugated formulations provide important insights into their colloidal stability and electrokinetic potential properties (Fig. 2F). A value greater than ±30 mV generally indicates stability due to electrostatic repulsion, preventing aggregation[54]. Pure ApoBDs exhibited mean values ranging from -27.1 mV to -23.4 mV (Table S1), indicating a lower negative charge compared to ReApoBDs (-36.16 mV to -31.45 mV), likely due to the natural heterogeneity and broader size range (1-5 μM) of the ApoBDs. This is because smaller particles tend to have higher absolute zeta potential values due to greater susceptibility to random motion in suspension[55]. The

negative zeta potential values of both ApoBD and ReApoBD populations are attributed to the presence of negatively charged molecules, such as PS, on their surface, which facilitates macrophage interaction and uptake[56–58]. For ReApoBD-VAN, the zeta potential mean values ranged from -28 mV to -25.56 mV (Table S1), which is consistent with previous reports on similar ReApoBD or liposomal VAN formulations[18, 59, 60]. These slightly less negative values, compared to empty ReApoBDs, may result from an increase in formulation size, partial shielding of surface charges, or the incorporation of positively charged molecules. Despite this reduction, moderate colloidal stability is maintained. ReApoBD-BotrAMP14 exhibited zeta potential values from -17.3 mV to -21.4 mV, representing the most significant reduction in negative charge. However, the absence of a shift to positive values suggests encapsulation rather than surface adherence. Given that BotrAMP14 is cationic, it likely introduces positive charges that neutralize some surface negativity, leading to a less negative zeta potential. This was further supported by measurements of empty ReApoBDs mixed with the peptide in suspension, which exhibited near-zero values (4.98 ± 4.63 mV), suggesting the presence of positive charges. This phenomenon has also been reported in studies on bacterial and liposomal suspensions treated with cationic peptides [61] and peptide antibiotic NK-2[62].

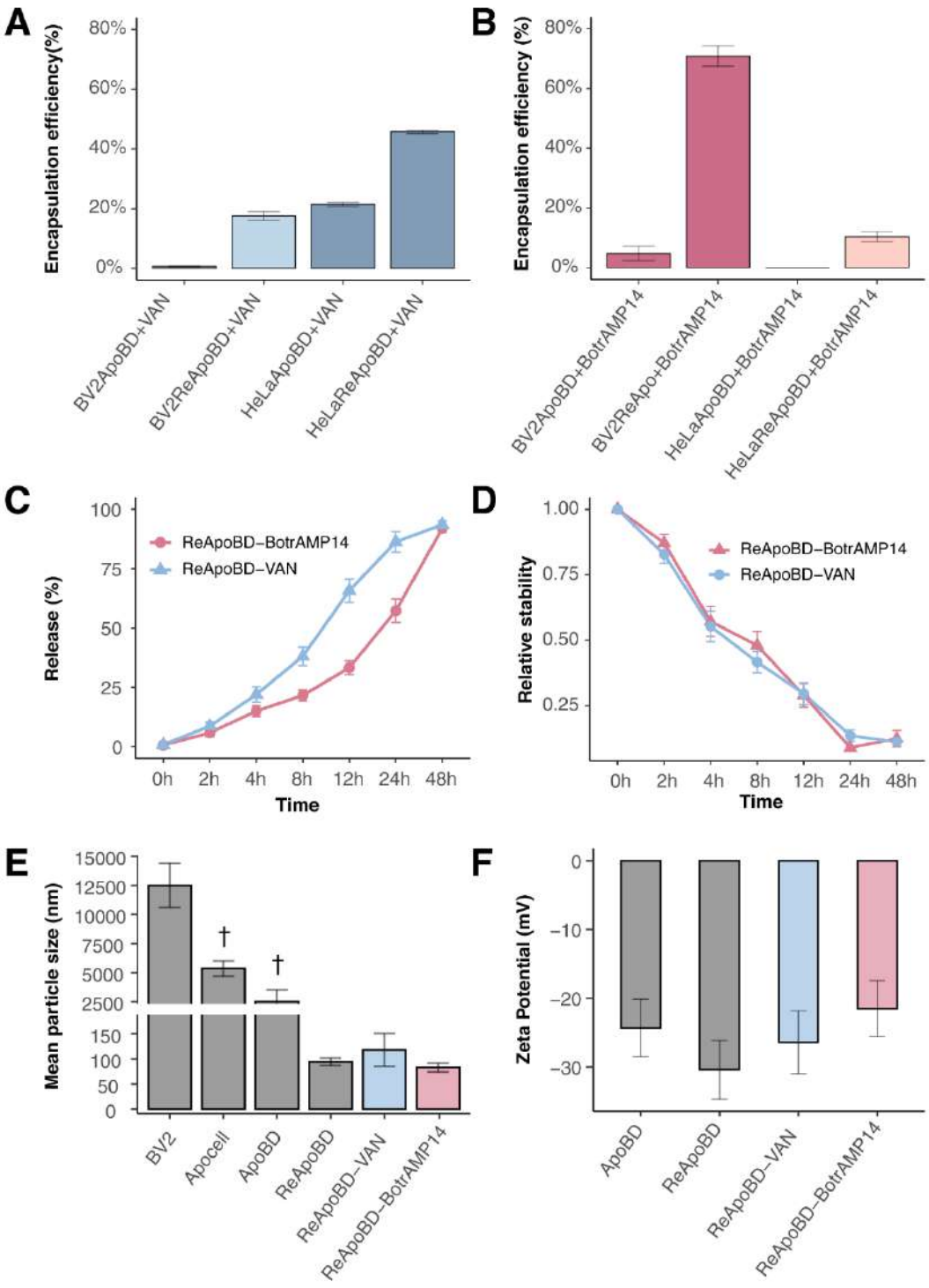

Fig. 2. Encapsulation and biophysical evaluation of formulations. Efficiency percentages for different cell types and encapsulation methods. Apoptotic bodies (ApoBDs) or size-remodeled ApoBDs (ReApoBD) from BV-2 (BV-2ApoBD and BV-2ReApoBD, respectively) or HeLa cultures (HeLaApoBD and HeLaReApoBD) were used to load (**A**)

vancomycin (ApoBD/ReApoBD-VAN) and (**B**) BotrAMP14 (ApoBD/ReApoBD-BotrAMP14). (**C**) Drug release percentage and (**D**) relative stability of ReApoBDs formulations after 48 h, and (E) Size frequency distribution of BV-2 vesicles/cell populations, including BV-2 cells (theoretical cell size), apoptotic cells (Apocells), and ReApoBDs formulations with VAN and BotrAMP14. (**F**) Results of ζ-potential parameters of particles and formulation suspensions from BV-2 culture cells. † Indicates the cell populations that are highly heterogeneous or outside the measurable size range, where only the two most similar technical replicate data were used. Data represent the mean ± SD of three replicates.

Cytotoxicity evaluation of the formulations

The cytotoxicity of the ReApoBD-VAN and ReApoBD-BotrAMP14 nanoformulations was assessed using the MTT assay to measure cell viability. Results for untreated RAW cells were compared with free drugs (VAN and BotrAMP14) and their nanoformulations using ApoBDs derived from BV-2 cells (Fig. 3A). Empty BV-2-ReApoBDs exhibited low cytotoxicity, with cell viability of approximately 90%, indicating biocompatibility and suitability as drug carriers. For VAN, both free and formulated drugs showed dose-dependent cytotoxicity, with the ReApoBD-VAN exhibiting slightly lower cytotoxicity at higher concentrations (24.6% to 20.6%) compared to free VAN (27.8% to 22%). Similarly, for BotrAMP14, the free peptide induced a dose-dependent decrease in viability, ranging from 45.35 ± 2.17 at 256 μM to 89.93 ± 2.09 at 2 μM. The ReApoBD-BotrAMP14 treatment also exhibited dose-dependent effects, with lower cytotoxicity at higher concentrations (40.65 ± 2.32 at 256 μM to 79.43 ± 2.12 at 2 μM). The formulation of VAN and BotrAMP14 slightly reduced cytotoxicity at higher concentrations, likely due to the controlled release mechanism of the vesicles over 12 h (Fig. 2C), as described in previous studies[63–65].

Designing antimicrobial treatments for intracellular delivery necessitates a careful balance in drug efficacy while minimizing cytotoxicity to host cells. The observed reduction in cytotoxicity upon formulation was similar to another nanostructured antimicrobial approach, such as the self-assembled peptides F3FT and N3FT, which demonstrated potent intracellular antibacterial activity with low cytotoxicity in RAW 264.7 cells (66). These peptides efficiently penetrated macrophages and eliminated intracellular *S. aureus* while maintaining IC_{50} values above 64 μM, in contrast to melittin, which exhibited high toxicity at just 4 μM[66]. These findings highlight the potential of engineered nanosystems to enhance the therapeutic index by optimizing intracellular drug delivery and cellular interactions.

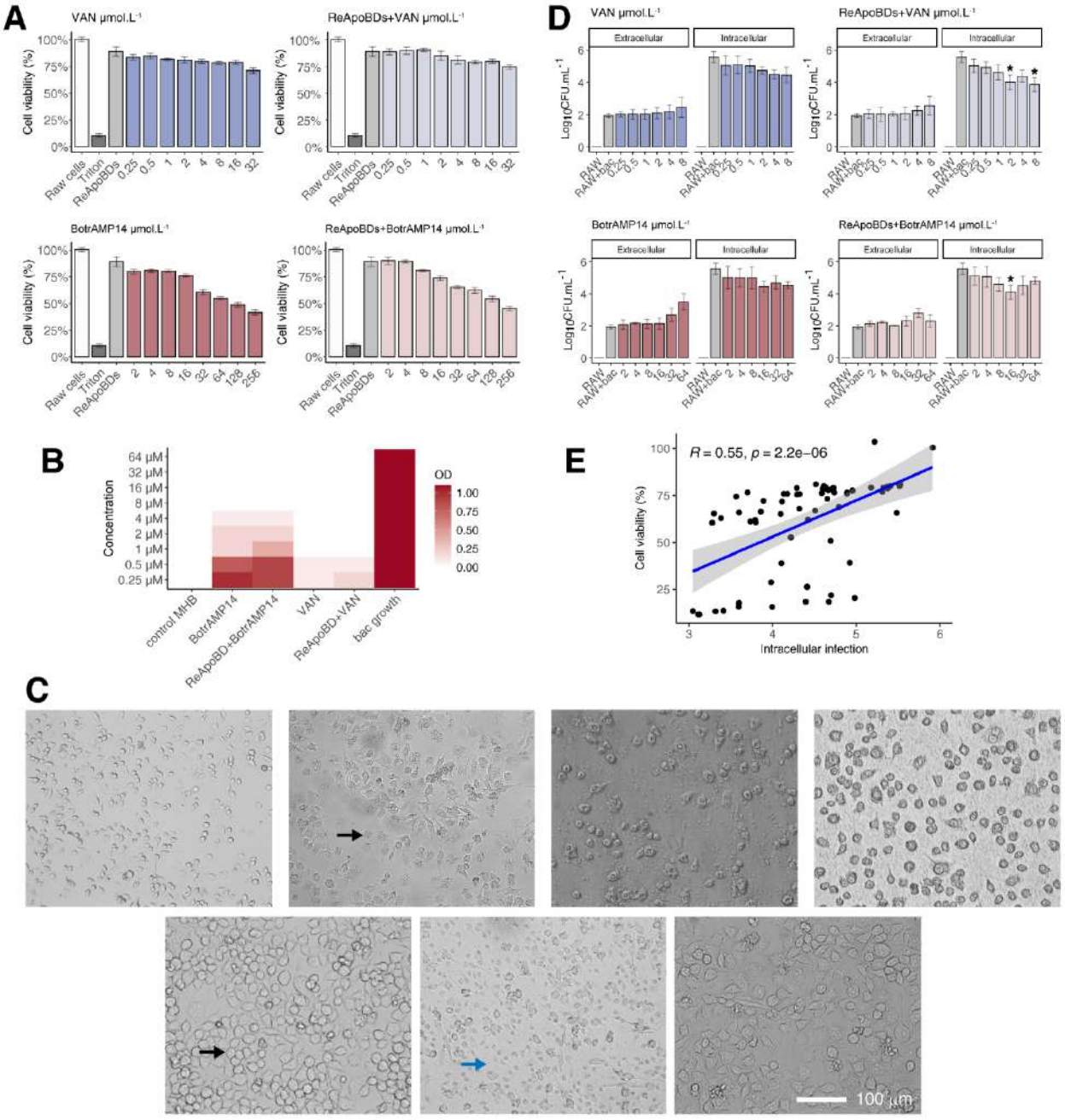

Fig. 3. Evaluation of the biological efficacy of the formulations. (**A**) Cytotoxicity of free compounds, formulations, and ReApoBDs (size-remodeled apoptotic bodies) against RAW 264.7 macrophage cells. (**B**) Minimum inhibitory concentration (MIC) assay to evaluate the antibacterial activity of vancomycin (VAN, BotrAMP14, and their formulations against the *S. aureus* strain. (**C**) Images of intracellular bacterial killing assays in macrophages. (first, top) RAW cell morphology in cultures without *S. aureus* and co-culture of (second right, top) RAW cells with *S. aureus* Aurora after 24 h. Infected RAW cells were treated after 24 h with (third right, top) ReApoBDs 78 µg.mL^{-1},

(last, top) VAN 256 μM, and (first, bottom) BotrAMP14 256 μM or (middle, bottom) ReApoBD-VAN 8 μM and (last bottom) and ReApoBDs-BotrAMP14 16 μM. Cultures were observed at 40× magnification using an inverted microscope. The black arrow indicates the presence of bacteria in the intracellular space, and the blue arrow indicates the presence of bacteria in the extracellular space. (**D**) Quantifying bacterial infection in a co-culture of RAW cells with *S. aureus* Aurora after 24 h of treatment, expressed as the Log10 of CFU recovered from the extracellular and intracellular spaces. (**E**) Pearson correlation between the cytotoxicity of the treatments on RAW cells and the quantification of intracellular *S. aureus* infection (*p*-value indicates the result of a Student's t-test). * Indicates significant effects (*p*-values < 0.05) from ANCOVA, using macrophage viability as a covariate. All data are median ± SD of three replicates.

In vitro antibacterial evaluation

The susceptibility of the *S. aureus* Aurora strain to VAN and BotrAMP14 and ReApoBD-VAN and ReApoBD-BotrAMP14 nanoformulations was evaluated *in vitro* using planktonic and macrophage infection models. MIC tests confirmed the susceptibility of the Aurora strain to VAN (MIC = 0.25 μM) based on CLSI standards (≤1.4 μM). BotrAMP14, in contrast, exhibited an MIC of 8 μM. In addition, the ReApoBD-VAN and ReApoBD-BotrAMP14 nanoformulations also exhibited MIC values similar to the free drug, indicating the successful release of the drug during the first 24 h (Fig. 3B).

In the *in vitro* macrophage infection model, intracellular bacterial loads in untreated cells reached 5.7 ± 0.3 Log_{10}CFU, while extracellular levels stabilized around 2 ± 0.1 Log_{10}CFU. Free VAN reduced intracellular loads to an average of 4.4 ± 0.5 Log_{10} CFU with a maximum reduction of 1.7 Log_{10}CFU at the highest concentration (8 μM) (Fig. 3C, D). The ReApoBD-VAN nanoformulation at the same concentration further reduced intracellular bacterial loads to an average of 3.9 ± 0.4 Log_{10}CFU, with reductions of up to 2.2 Log_{10}CFU observed, underscoring

its enhanced efficacy in penetrating macrophages. These findings are consistent with reconstructed ApoBDs loaded with VAN, which achieved a two-Log10 reduction in intracellular bacterial loads within RAW 264.7 cells[18]. Similarly, free BotrAMP14 achieved intracellular decreases to an average of 4.4 ± 0.3 Log_{10}CFU at 16 μM, resulting in efficacy comparable to VAN (Fig. 3C, D). Thus, the ReApoBD-BotrAMP14 nanoformulation showed superior activity, achieving an average of 4.1 ± 0.3 Log_{10}CFU at 16 μM, with reductions extending to 2.1 Log_{10}CFU, demonstrating the advantages of encapsulation for intracellular delivery.

These findings align with recent research on antimicrobial nanoformulations for intracellular *S. aureus* eradication[66]. Another study developed self-assembling nanopeptides (F3FT and N3FT) with dual antibacterial and cell-penetrating properties[66]. That study demonstrated that these nanopeptides eliminated up to 98.3% of intracellular *S. aureus* in RAW 264.7 cells at 32 μM, significantly outperforming vancomycin, which achieved only 32.6% reduction at the same concentration. The higher efficacy of F3FT and N3FT was attributed to their efficient cell penetration and their dual mechanism of membrane disruption and ROS accumulation. While our ReApoBD-VAN and ReApoBD-BotrAMP14 nanoformulations also improved intracellular bacterial clearance, their effectiveness remains dependent on antibiotic release and macrophage uptake, highlighting a complementary but distinct strategy compared to self-assembled nanopeptides.

Notably, our study quantified extracellular bacterial loads (intracellular bacteria released after the total elimination of bacteria that did not achieve intracellular infection) without using antibiotics in the medium. This distinguishes intracellular bacterial reductions caused by antibacterial activity and cytotoxic effects. Thus, for extracellular bacteria, any treatment significantly reduced bacterial loads, which remained stable at ~2 Log_{10}CFU. Pearson correlation

analysis (Fig. 3E) revealed a moderate positive correlation (R = 0.55, *p*-value = 2.2×10^{-6}) between macrophage viability and intracellular bacterial loads. This indicates that higher cell viability was associated with reduced antimicrobial efficacy, suggesting that cytotoxic effects play a key role in enhancing bacterial clearance. These results underscore the delicate balance between cytotoxicity and antimicrobial activity. ReApoBD-VAN and ReApoBD-BotrAMP14 nanoformulations that achieved a higher antimicrobial-to-cytotoxicity ratio yielded more favorable outcomes (Fig. S6).

In vivo antimicrobial efficacy of the ReApoBD-BotrAMP14 nanoformulation

The antimicrobial efficacy of the ReApoBD-BotrAMP14 nanoformulation was evaluated using a subcutaneous abscess model in mice infected with the *S. aureus* Aurora strain (Fig. 4A). The ReApoBD-BotrAMP14 nanoformulation treatment significantly reduced abscess size by 31.7%, with an average area of 43.59 mm² compared to 63.83 mm² in the saline-treated group (Fig. 4B, C). The *in vivo* model proved to be well-suited to this study, as it induced the appearance of SVC phenotypes. The identification of SCVs was based on direct observation of colony morphology on Mueller-Hinton agar. SCVs were distinguished from normal *S. aureus* colonies by their significantly smaller size, reduced pigmentation (appearing pale or translucent instead of golden or yellow-cream), rough and dry texture, and prolonged incubation time required for visible growth (>24 h compared to 18–24 h for normal colonies). These characteristics are consistent with previously described SCV phenotypes in the literature[15, 67]. The ability to detect and reduce SCVs in this model underlines the ReApoBD-BotrAMP14's potential to target these difficult-to-eradicate bacterial subpopulations. Our results showed that the ReApoBD-BotrAMP14 treatment effectively reduced SCV prevalence, with treated animals exhibiting a

relative frequency of 0.271 compared to the control (Fig. 4D). This underscores its capacity to target intracellular *S. aureus*, overcoming a key limitation of conventional strategies. Additionally, in the total bacterial load analysis, the treated groups showed a reduction of 0.97 Log10 CFU ± 0.37 per abscess compared to the control (Fig. 4E). This efficacy against intracellular bacteria, while controlling bacterial growth, positions the ReApoBD-BotrAMP14 nanoformulation as a promising therapeutic strategy for persistent *S. aureus* infections. Its significant impact on SCVs and control of total bacterial burden addresses the complexity of targeting intracellular and extracellular compartments.

Previous studies have highlighted the difficulty of eradicating *S. aureus* SCVs, particularly in models of chronic infection. For instance, in a rabbit model of chronic osteomyelitis, vancomycin-hydroxyapatite-loaded cements effectively targeted SCVs but required prolonged administration (42 days) and extremely high doses (80,000–240,000 $mg.kg^{-1}$) for complete eradication[68]. Similarly, in the mouse peritonitis model, intracellular-active antibiotics such as linezolid (17 $mg.kg^{-1}$) and dicloxacillin (60 $mg.kg^{-1}$) controlled intra- and extracellular infections but failed to eliminate SCVs after a single dose[69]. In a mouse mastitis model, cefapirin (10–25 $mg.kg^{-1}$) showed limited efficacy against hemin-dependent SCVs (70), while combination therapies like gentamicin and β-lactams effectively eliminated normal *S. aureus* phenotypes but remained ineffective against SCVs[71].

In contrast, our study demonstrated that a single subcutaneous dose of ReApoBD-BotrAMP14 (0.3 $mg.kg^{-1}$) significantly reduced SCV prevalence compared to the control, achieving a notable effect with a markedly lower dose and without the need for prolonged treatment. This result highlights the potential of ReApoBD-BotrAMP14 as a highly effective and less intensive strategy for targeting SCVs in persistent *S. aureus* infections. Similar results were

observed with the self-assembled peptides F3FT and N3FT, which showed high antimicrobial efficacy in a mouse model of *S. aureus* peritonitis-sepsis[66]. A single intraperitoneal injection of F3FT or N3FT (10 mg.kg-¹) showed greater efficacy in eliminating intracellular bacteria in peritoneal macrophages compared to VAN, probably due to their better cellular penetration. These findings support the efficacy of nanoscale antimicrobial strategies in controlling intracellular *S. aureus* infections, offering a promising alternative to conventional antibiotics that often fail to eradicate SCVs and persistent bacterial populations.

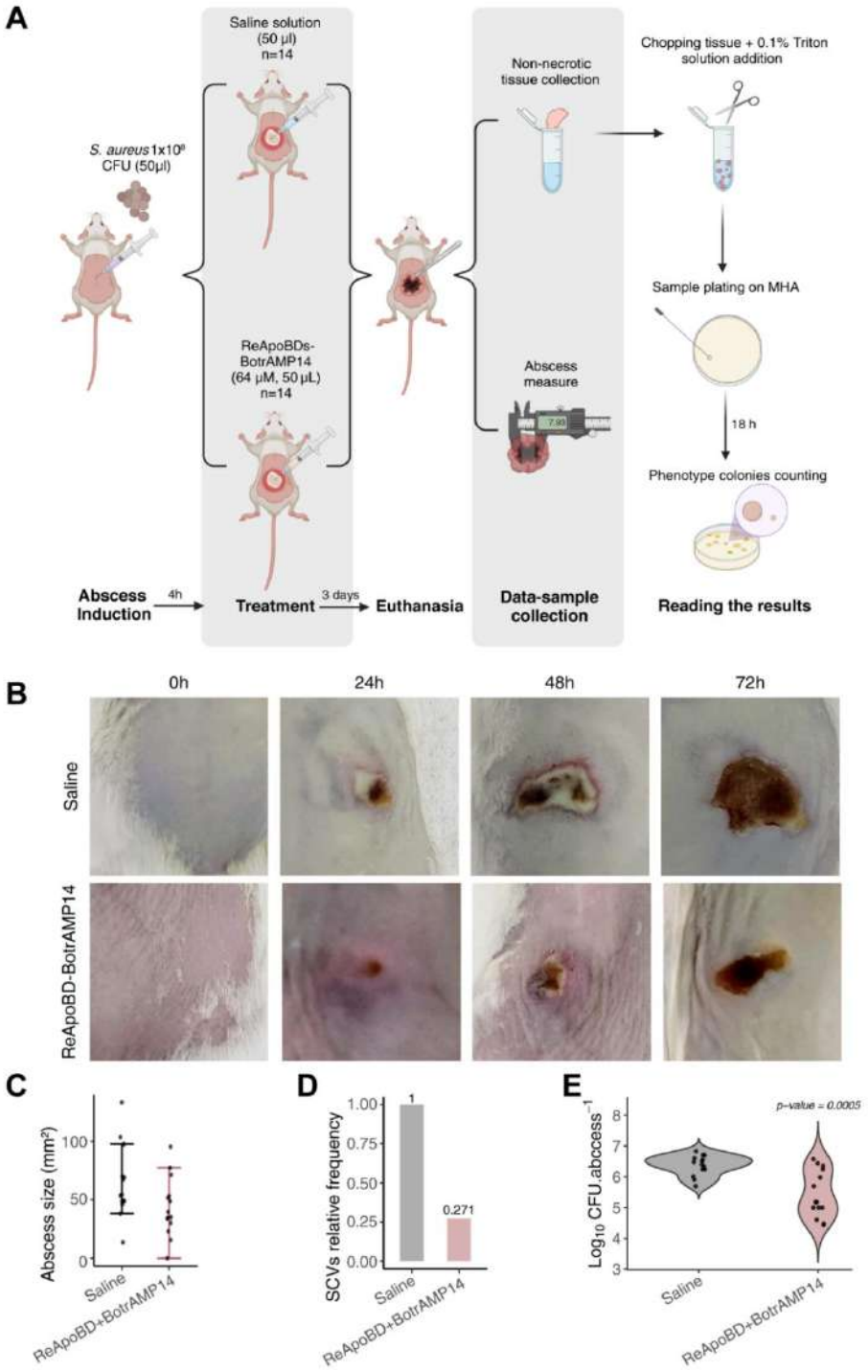

Fig. 4. *In vivo* evaluation of ReApoBD-BotrAMP14 nanoformulation. (**A**) Experimental design outlining the timeline of mouse treatment conditions, including the use of the *S. aureus* Aurora strain to induce abscess formation, peptide nanoformulation treatments, and sample collection. Created in BioRender. (**B**) Images of Aurora *S. aureus* cutaneous infection on Swiss Webster mice over three days. (**C**) Abscess area expressed in mm^2 for saline and nanoformulation-

treated groups. (**D**) Relative frequency of presence of *S. aureus* Aurora small colony variants (SCVs) in Mueller-Hinton agar (MHA) plaques of plated samples collected from non-necrotic abscess tissue of animals treated with the peptide nanoformulation compared to the saline-treated group. (**E**) Effect of ReApoBD-BotrAMP14 nanoformulation on total bacterial concentration recovered from non-necrotic tissue of *S. aureus*-induced abscess. The *p*-value indicates the result of a Student's t-test. All data represent three biological replicates with at least 4-5 mice group.

Intracellular uptake assay using confocal microscopy

Confocal microscopy was used to evaluate the intracellular uptake of ReApoBDs-BotrAMP14 and its interaction with *S. aureus* in RAW 264.7 macrophages (Fig. 5A, B). Three experimental conditions were designed to compare the intracellular behavior of free BotrAMP14 and ReApoBD-BotrAMP14 treatments, with a focus on uptake dynamics and localization at varying incubation times. First, RAW cells were incubated with free BotrAMP14 for 20 min. The absence of 5-TAMRA fluorescence indicated that the free peptide could not penetrate the cellular membrane or localize in intracellular compartments, highlighting the peptide's limited capacity for intracellular delivery (Fig. 5A, Fig. S7A).

In the second treatment, RAW cells infected with *S. aureus* were incubated with the ReApoBD-BotrAMP14 nanoformulation. The FITC channel revealed green fluorescent bacterial particles distributed within the intracellular space, while the 5-TAMRA signal, representing the ReApoBD-BotrAMP14 treatment, was predominantly observed at the cell periphery. This distribution suggests initial interactions between vesicles and the cell membrane, indicating early stages of internalization. The merged image demonstrated clear colocalization between bacterial particles (green) and the 5-TAMRA signal (orange), confirming the proximity of the peptide to bacteria, either at the cell membrane or within early intracellular compartments. Volumetric

rendering (Fig. 5B) provided a three-dimensional perspective that confirmed the spatial proximity of the peptide to bacterial particles without overlapping, suggesting localization within distinct cellular compartments.

In the third treatment (60 min treatment), the 5-TAMRA signal transitioned from the cell periphery to colocalize with bacterial particles, indicating successful intracellular delivery of the peptide (Fig. 5A). Partial overlap of 5-TAMRA and FITC signals, as revealed by volumetric analysis, indicated localized peptide interaction with bacteria without cytoplasmic distribution. In the merged image, the intense green fluorescence from FITC partially masked the 5-TAMRA signal; however, faint yellow regions indicated localized interactions between the peptide and bacterial particles (Fig. 5A, Fig. S7B).

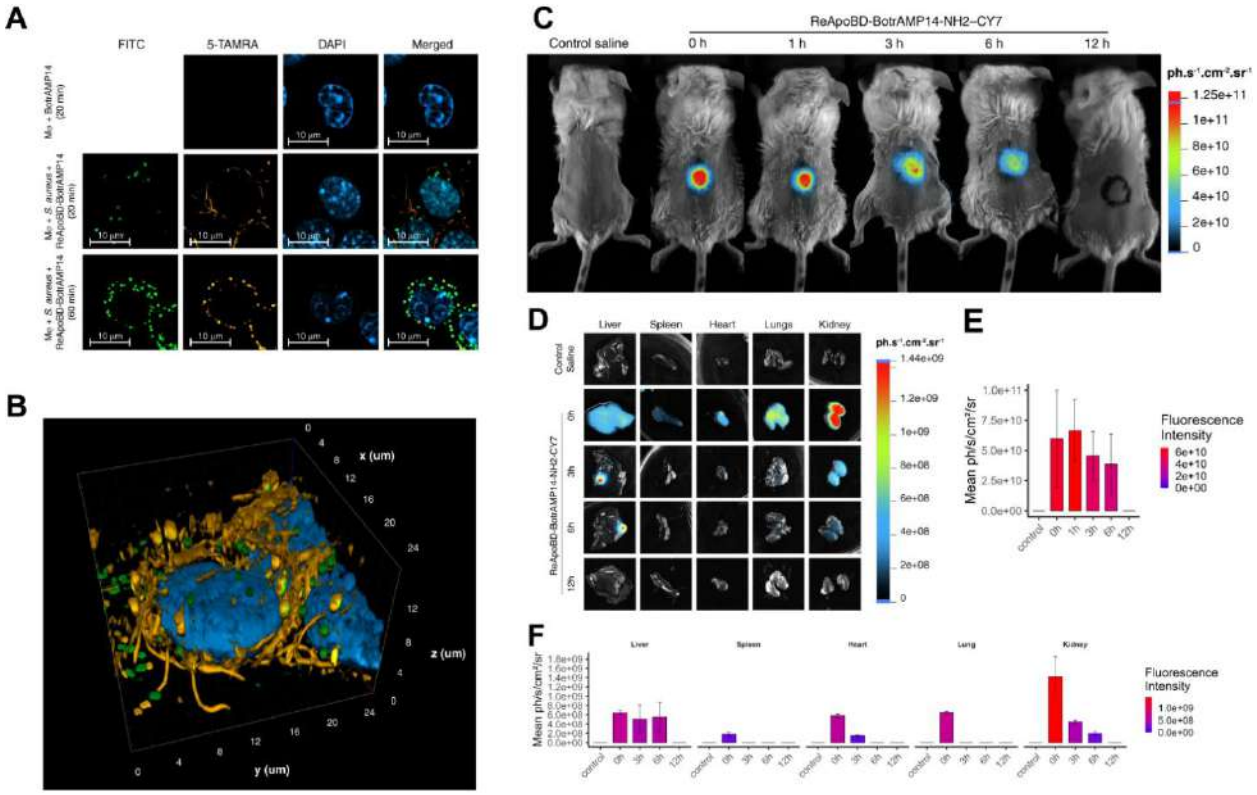

Fig. 5. Intracellular uptake and *in vivo* 2D biodistribution of the ReApoBD-BotrAMP14 nanoformulation. (**A**) Confocal microscopy images showing fluorescence channels for FITC (*S. aureus*, green), 5-TAMRA (free and nanoformulated peptide, orange), DAPI (nuclei, blue), and merged images. Treatments include free peptide and ReApoBD-BotrAMP14 at different incubation times. (**B**) Volumetric rendering of fluorescence signals from confocal microscopy, providing a three-dimensional view of the signal distribution of the 20 min ReApoBD-BotrAMP14 treatment. (**C**) *In vivo* 2D fluorescence imaging of BALB/c mice treated with the ReApoBD-BotrAMP14 nanoformulation, showing fluorescence at the injection site over time. (**D**) *Ex vivo* 2D imaging of major organs (liver, spleen, heart, lungs, and kidneys) was performed at various time points to assess the biodistribution of the ReApoBD-BotrAMP14 nanoformulation (5 mice). 2D fluorescence intensity pattern of nanoformulation between 0 and 12 h of injection for (**E**) mice and (**F**) various organs.

In vivo biodistribution of ReApoBD-BotrAMP14 nanoformulation

The *in vivo* biodistribution of the ReApoBDs-BotrAMP14-NH2-Cy7 nanoformulation was evaluated using a subcutaneous injection model in BALB/c mice. Fluorescence monitoring at the injection site, in significant organs, and over time provided insights into the ReApoBDs-BotrAMP14's systemic distribution and tissue accumulation (Fig. 5 C-F).

In vivo 2D fluorescence imaging and intensity pattern of nanoformulation between 0 and 12 h post-injection in BALB/c mice demonstrate the temporal dynamics of fluorescence at the injection site (Fig. 5 C, E). Initially, a high-intensity fluorescence signal was observed at the injection site immediately after administration (0 h), confirming the successful delivery of the ReApoBDs-BotrAMP14 nanoformulation. This signal steadily decreased over time, and by 12 h, fluorescence at the injection site was undetectable, indicating complete dispersion or clearance from the delivery site. Fig. 5 D, F provide complementary insights into the accumulation of ReApoBD-BotrAMP14 treatment in major organs. Quantitative analysis reveals a redistribution of fluorescence from the injection site to the systemic circulation, peaking in key metabolic organs, such as the liver and kidneys, by 3–6 hours (Fig. 5F). These organs play central roles in metabolism and excretion, confirming their involvement in the clearance and processing of the ReApoBDs-BotrAMP14 nanoformulation. Fluorescence in the lungs peaked at 6 h, likely due to transient circulation through the pulmonary vasculature. Minimal fluorescence was detected in the spleen and heart, suggesting limited interaction with these organs.

For instance, in rabbit models of endocarditis and osteomyelitis, SCVs persisted in the kidneys and spleen despite oxacillin treatment, highlighting the difficulty of eradicating these bacterial subpopulations in systemic infections[72]. Notably, our biodistribution analysis revealed renal accumulation of the ReApoBD-BotrAMP14 nanoformulation, an essential advantage given

that SCVs are known to persist in the kidneys. This targeted accumulation, combined with the demonstrated potency of ReApoBD-BotrAMP14 against SCVs, reinforces its potential as a therapeutic strategy for persistent *S. aureus* infections. Furthermore, the nanoformulation's dynamic redistribution and gradual clearance suggest a favorable pharmacokinetic, particularly when compared to conventional antibiotic treatments that often fail to effectively clear SCVs from organs such as the kidneys and spleen[69, 72]. This improved biodistribution profile supports its potential for systemic application in persistent *S. aureus* infections.

This study introduces a novel nanoformulation using ReApoBDs conjugated with the antimicrobial peptide BotrAMP14, representing a pioneering approach to tackling persistent intracellular infections. Designing antimicrobial treatments for intracellular infections requires a careful balance between the efficacy of the drug and its cytotoxicity to host cells. While direct intracellular delivery of antimicrobial agents enhances therapeutic outcomes, it also carries the risk of off-target effects or disruption of essential cellular functions. Our findings highlight the importance of assessing cytotoxicity when developing intracellular antimicrobial treatments. Compared to conventional free-drug administration, nanoformulations such as ReApoBDs offer a promising alternative by modulating drug release and reducing toxicity. Future studies should explore the long-term effects of ReApoBD-based formulations on host cell function and their potential for targeted intracellular delivery mechanisms that optimize therapeutic efficacy while minimizing adverse effects.

The *in vitro* antimicrobial efficacy of ReApoBDs was significant, with ReApoBDs-VAN and ReApoBDs-BotrAMP14 achieving substantial intracellular bacterial clearance. The proposed mechanism of action (Fig. 6) provides a possible explanation for these effects: (i) fusion of the endosome containing the nanoformulation with the phagolysosome, allowing direct interaction

with intracellular bacteria, and (ii) direct cytosolic action of the nanoformulation following bacterial escape from the phagolysosome. The first hypothesis suggests an efficient antimicrobial response by targeting *S. aureus* within its intracellular niche. In contrast, the second mechanism could explain the observed cytotoxic effects, as the formulation's presence in the cytosol might inadvertently impact host cell viability. Given that SCVs persist intracellularly by adapting to the phagolysosomal environment, these mechanisms of action could enhance their eradication by directly disrupting these adaptations (Fig. 6).

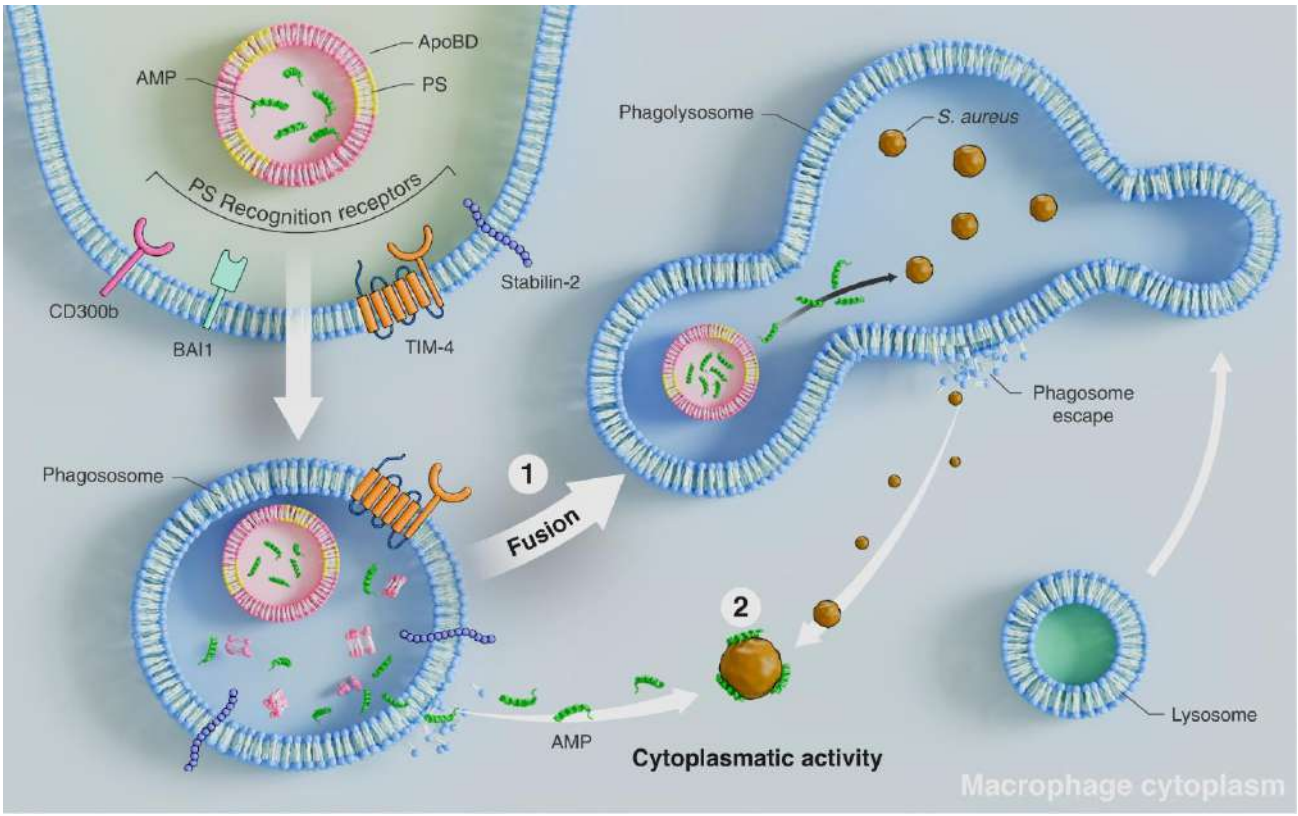

Fig. 6. Scenario of a macrophage with intracellular infection caused by *S. aureus* and treatment mechanism of action with a nano-Trojan horse formulation based on apoptotic bodies-antimicrobial peptide (e.g., ApoBDs-BotrAMP14). The mechanism of internalization of ApoBDs is illustrated, where they release their content inside phagosomes, followed by two hypotheses regarding the mechanism of action of ApoBDs-AMP formulations. The first hypothesis is based on the

fusion between the endosome containing the formulation and the phagolysosome. The second is based on the direct action of the formulation in the cytosol once the bacteria have escaped from the phagolysosome. Both hypotheses could co-occur.

While our study provides valuable insights into the potential of ApoBD-based nano-Trojan horse formulations for delivering antimicrobial agents, several limitations must be acknowledged. The variability in encapsulation efficiency and drug release profiles, influenced by donor cell type and vesicle characteristics, underscores the need for further optimization. Differences in membrane composition, such as cholesterol content and surface charge, affected drug entrapment and stability, with HeLa-derived ApoBDs favoring vancomycin encapsulation due to their higher cholesterol levels, while BV-2-derived ApoBDs were more efficient for BotrAMP14 encapsulation due to their lower negative charge. These factors must be considered to ensure consistent therapeutic outcomes. The lack of direct evidence for drug-release mechanisms within phagolysosomes also limits our understanding of the intracellular targeting process. Future studies should further validate the findings in expanded animal models of *S. aureus* infections to address these challenges, with a focus on pharmacokinetics, biodistribution, and long-term efficacy. Moreover, the scalability and reproducibility of ApoBD production need to be established, as these factors are critical for clinical translation. Investigating the combination of ApoBD-based formulations with other therapeutic strategies, such as immune modulation or combinatorial antibiotic therapies, could further enhance efficacy and broaden the application of this approach. These steps will be essential for bridging the gap between laboratory findings and real-world clinical applications, ultimately advancing treatment strategies for persistent bacterial infections.

These findings position the ReApoBD-BotrAMP14 nanoformulation as a promising candidate for intracellular antimicrobial therapy, particularly against persistent *S. aureus* infections, acting as a nano-Trojan horse. Integrating sustained release, enhanced stability, and targeted delivery offers significant therapeutic advantages, laying a foundation for future clinical exploration.

Material and Methods

Cell culture and bacterial strain

BV-2, HeLa, and RAW 264.7 cell lines were purchased and cultured according to the protocols of the Rio de Janeiro Cell Bank (BCRJ) and the Instituto Adolfo Lutz (IAF). BV-2 cells, derived from mouse microglial cells immortalized with v-raf/v-myc oncogenes, and HeLa cells from human cervical cancer, were maintained in DMEM or RPMI medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. *S. aureus* strain Aurora 457 (Universidade Federal de Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil), a clinical isolate from dairy cows with mastitis symptoms, was used to infect RAW 264.7 mouse macrophage cells to assess the antibiotic effects of treatments (Fig. S8).

Induction of apoptosis

BV-2 and HeLa cell lines were thawed, grown in culture flasks to approximately 80% confluence, and then detached. Next, ~2 $\times 10^6$ viable cells per well were plated into a 6-well culture plate and incubated at 37 °C for 24 h to allow adherence. Subsequently, the culture medium was removed, and each well was washed three times with 1 × phosphate-buffered saline

solution (PBS) (pH 7.4). Finally, RPMI culture medium without FBS[73] and 0.09% hydrogen peroxide (H_2O_2)[40] were added to each treatment well to induce the apoptosis process. Using an inverted Zeiss microscope, the cell culture was monitored every 12 h, recording the presence of apoptotic morphologies such as blebbing, apoptopodia, and ApoBD formation at 40× magnification. Additionally, Hoechst 33342 dye (Thermo Fisher Scientific, USA) was used to stain nuclei, enabling the visualization and confirmation of apoptotic cells by fluorescence microscopy.

Isolation of ApoBDs

Following the confirmation of apoptosis induction, ApoBDs were isolated from other cellular particles and debris present in the culture suspension, commonly achieved via differential centrifugation. We followed a previously described protocol for detecting and separating high-purity ApoBDs with some modifications[74]. Briefly, the contents of all apoptosis-inducing wells were collected, and three tubes were prepared. The first tube contained the control sample of viable cells, the second one contained all apoptosis-induced cells, and the third one contained $1/10^{th}$ of the whole apoptotic sample (WAS). The first and third tubes were centrifuged at 3000 × g for 6 min. The pellets were resuspended in 2 mL of 1 × PBS and kept on ice. The second tube was centrifuged at 300 × g for 10 min to separate apoptotic cells from other extracellular vesicles (EVs) and debris. The supernatant was transferred to a new tube labeled "ApoBDs," and the remaining pellet-containing tube was labeled "apocells." The pellet was resuspended in 3 mL of 1 × PBS and stored on ice alongside the WAS and viable cells as controls. Finally, the ApoBD tube was centrifuged at 3000 × g for 20 min, and the supernatant was discarded. The formation of the pellet was visually confirmed, and it was resuspended in 3 mL of 1 × PBS. The three control

and the ApoBD tubes were used to verify the separation of different populations via flow cytometry.

Confirmation of apoptotic bodies isolation by flow cytometry

FC was used to confirm the separation of ApoBDs after the initial application of the differential centrifugation method[74] with some modifications. Briefly, 100 µL of the previously separated samples were taken and placed into four new round-bottomed polystyrene FC tubes. Then, 100 µL of a staining solution containing 2 × A5 binding buffer and 1:100 A5-FITC was added to each tube. The tubes were then incubated for 10 min at room temperature in the dark. The samples were measured on a FacsCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) using FACS Diva V6.0 software (BD Biosciences). The cytometer machine was previously calibrated using BD™ Cytometer Setup and Tracking beads (BD Bioscience) to adjust the fluorescence and light scattering detectors. Once calibrated, the equipment was configured with a 100 µm nozzle and drop delay, ensuring a stable flow rate and setting the acquisition speed to ~1000 events.s^{-1}. After adjusting the FSC, SSC, and FITC (A5) voltages, events were positioned within each plot, and 20.000 events were recorded per run.

Field emission transmission electron microscopy

For sample TEM analysis, carbon-coated 300 square copper mesh grids were glow-discharged using an EMS GloQube, dual chamber glow-discharge system (Electron Microscopy Sciences, Hatfield, PA, USA) in negative mode at a plasma current of 15 mA for 120 s. A 10 µL drop of the apoptotic cell or ReApoBD suspensions was applied to the grids for 1 min, and the

excess sample was removed with absorbent paper. A 10 µL drop of 1% phosphotungstic acid (PTA) was added to the grids, and the excess was blotted off. The grids were examined using a JEOL JEM-1400 Flash transmission electron microscope (JEOL, Tokyo, Japan) at 120 kV acceleration voltage.

Peptide synthesis and validation

The BotrAMP14 [KRWKKFFRKVIKFF-NH2] AMP (1957.51 $g.mol^{-1}$) was synthesized using F-moc solid-phase chemical synthesis by AminoTech Research & Development Ltda., São Paulo, Brazil. Its purity, confirmed to be over 95%, was verified through reverse-phase high-performance liquid chromatography (RP-HPLC) (LC system with an LC-20AR pump, SIL-10AF auto-injector, and SPD-M40 photodiode array detector Shimadzu Corp., Japan) and mass spectrometry (MALDI-ToF Ultraflex III, Bruker Daltonics, USA).

Loading of therapeutic agents

Both vancomycin hydrochloride (VAN) (from *Streptomyces orientalis*, Sigma-Aldrich, USA) and BotrAMP14 at a 1:1 v/v ratio (250 $\mu g.mL^{-1}$) were mixed with isolated ReApoBDs (78 $\mu g.mL^{-1}$ protein equivalent quantified by Bradford, (1976). Moreover, they were subjected to three freeze-thaw cycles of -20 °C/20 min and 45 °C/10 min. A sequential extrusion process was performed using an Avanti Mini Extruder to ensure uniform vesicle sizing through 0.8, 0.4, 0.2, and 0.1 µm pore polycarbonate membranes[18]. This resulted in the final nanoformulated ReApoBDs (~100 µm ApoBDs) with VAN and BotrAMP14 or ReApoBDs-VAN and ReApoBDs-BotrAMP14, respectively.

Encapsulation efficiency of therapeutic agents

The encapsulation efficiency (EE) of VAN and BotrAMP14 on the ApoBDs was quantified using RP-HPLC. Chromatographic separation was achieved on a Venusil ASB C18 column (250 mm × 4.6 mm × 5 μm particle size, Bonna-Agela Technologies) using a mobile phase gradient of water and acetonitrile. The flow rate was set at 1.0 $mL.min^{-1}$, and VAN and BotrAMP14 were detected at 230 nm and 216 nm, respectively. Calibration curves were generated for both compounds (each time a quantification assay was performed) using known concentrations of each to allow quantification of the amounts encapsulated in the ApoBDs. Only equations with R^2 greater than or equal to 0.9 were used for quantification each time. The %EE was calculated using equation 1, where the 'free compound' is the amount of unencapsulated drug or peptide determined in the supernatant post-ultrafiltration (Amicon® Ultra-4 centrifugal filters, ten kDa). After filtration, the ReApoBD-VAN and ReApoBD-BotrAMP14 fractions were mixed with a solution containing 0.1% Triton X-100 in PBS to lyse the vesicles, and the amount of peptide released was quantified to determine the total amount of formulated peptide. The amount of encapsulated peptide was determined, and both methods yielded values with differences of less than 5%.

Eq.1 $$\% EE = \left(\frac{free\ compound}{Total\ compound\ added}\right) X\ 100\%$$

The mass of the recovered VAN and BotrAMP14 was confirmed by mass spectrometry using matrix-assisted laser desorption ionization time of flight (MALDI-ToF).

Drug release of therapeutic agents

The drug release was evaluated using the RP-HPLC quantification methods described above. Formulations were prepared at the highest quantifiable concentration using the calibration curve equation for each drug and stored at room temperature. Aliquots were taken at 0, 2, 4, 8, 12, and 48 h, centrifuged (Amicon® Ultra-4 centrifugal filters, 10 kDa), and the volume was adjusted not to alter the concentration measurement. RP-HPLC was used to determine the amount of drug released at each time point analyzed.

Dynamic light scattering and zeta potential measurement

The size distribution and electrokinetic potential of the formulated ReApoBDs were analyzed using a Zetasizer Pro (Malvern Instruments, UK). The pure empty ReApoBDs and formulated suspensions were centrifuged, washed 2 times, and diluted in 0.1 × PBS. DTS0012 and DTS1070 cells were used for DLS and ζ potential measurements. Measurements were performed in triplicate per sample of 10 to 100 runs each, at 25 °C, pH 7.4, and a voltage level of 150 V for the ζ potential. The ζ potential was measured at different time points (0, 2, 4, 8, 12, and 48 h) to evaluate the relative colloidal stability of the drug formulations by comparing the value at each time point (t_x) with that at time 0 (t_0) according to equation 2. The data were processed using the absorption and refractive indices of the liposomes, 0.001 and 1.45, respectively, and analyzed using ZS Xplorer software v. 2.2.0.147, which provides information on the mean particle size (hydrodynamic diameter), polydispersity index (PDI), and ζ potential of the formulates.

Eq2. $$Relative\ stability = 1 - \left(\frac{|\overline{t_x} - \overline{t_0}|}{\overline{t_0}}\right)$$

Atomic force microscopy

An SPM-9700 Atomic force microscope in dynamic mode (Shimadzu Corp., Japan) was used for topographic imaging of the vesicles. Clean cover glass slides were attached to AFM/scanning tunneling microscope (STM) magnetic stainless-steel specimen discs using double-sided tape. At room temperature, the top surface of the slides was treated with a 0.5% gelatin solution. After 24 h, the ApoBD and ReApoBD samples were diluted 200-fold in ultrapure water, and a 50 µL drop of the solution was applied to the gelatin surface. The STM discs with the samples were incubated for ~18 h at 18 °C in a glass desiccator. Before imaging, the sample surfaces were rinsed 3 times with ultrapure water and allowed to dry. The non-contact cantilevers (Nanoworld, Switzerland) used for imaging had dimensions of 4 µm thickness, 125 µm length, and 30 µm width, and a resonant frequency of 320 kHz and a force constant of 42 $N.m^{-1}$ were used. Trace and retrace scans and topographic heights were imaged at a 0.5 to 1 Hz scanning rate, with images recorded at 512×512 pixels and processed using SPM analysis software (Shimadzu Corp., Japan).

Determination of minimum inhibitory concentration

The VAN and BotrAMP14 MIC were assessed using a broth microdilution method by the Clinical & Laboratory Standards Institute (CLSI) guidelines (protocol M07-A10). To the mid-log phase*, S. aureus* Aurora was cultured in Mueller-Hinton broth (MHB). Serial two-fold dilutions of VAN (32 to 0.25 µM) and BotrAMP14 (256 to 2 µM) were prepared in 96-well plates. Bacteria were added to each well, and the plates were incubated at 37 °C for 18 h. Finally, the lowest concentration that inhibited bacterial growth was reported as the MIC and determined by reading the plates at OD_{600}.

Cytotoxicity essays

The MTT assay determined the lethal and sublethal concentrations of VAN, BotrAMP14, empty ReApoBDs, and nanoformulations against RAW 264.7. Macrophages were grown in 96-well plates at a density of 3.5×10^3 cells per well. Serial two-fold dilutions of VAN/ReApoBDs-VAN (32 to 0.25 μM), BotrAMP14/ReApoBDs-BotrAMP14 (256 to 2 μM), and empty ReApoBDs (78 $\mu g.mL^{-1}$) were added to the wells and incubated at 37 °C for 24 h. Then 10 μL of MTT (5 $mg.mL^{-1}$) was added to each well, and the plate was incubated at 37 °C for 6 h with constant shaking and protected from light. A hundred μL of isopropanol was added to each well to solubilize the formed formazan crystal. Finally, the OD_{570} nm was read, and the cell viability was calculated according to equation 3:

Eq 3. $$Cell\ viability\ \% = \frac{optical\ density\ of\ treated\ cells}{optical\ density\ of\ untreated\ cells}\ x\ 100\ \%$$

***In vitro* antibacterial efficacy testing**

RAW 264.7 macrophages were cultured and infected with *S. aureus* at a multiplicity of infection (MOI) of 10 (i.e., ten bacterial cells per macrophage). The culture plates were centrifuged at 600 × g for 5 min to allow bacterial cells to settle, followed by a 2 h incubation period for phagocytosis. Macrophage cells were washed 10 times with 1× PBS and treated with 200 $\mu g.mL^{-1}$ gentamicin (Sigma-Aldrich, USA) to eliminate extracellular bacteria for 6 h (Bose et al., 2020; Lehar et al., 2015). The culture medium was then changed back to an antibiotic-free medium. Control groups were established, using uninfected cells to determine baseline cell health

and for statistical comparison. Infected, untreated cells served as a comparison for treatment efficacy. The efficacy of free and formulated VAN and BotrAMP14 was evaluated over a concentration range of 256 to 0.25 μM and 256 to 2 μM, respectively. After 24 h of treatment, 100 μL of medium aliquots were carefully collected to avoid detaching the macrophages and plated on Mueller-Hinton agar (MHA) to determine the extracellular bacterial concentration. The remaining samples with macrophages were then lysed with a solution containing 0.1% Triton X-100 in PBS to release intracellular bacteria. To remove cellular debris, the lysates were centrifuged at 300 × g for 5 min. The bacteria were then separated by centrifugation at 5000 × g for 5 min and resuspended in 1× PBS[77]. Serial dilutions of the bacterial samples were then plated on MHA to determine the total *S. aureus* concentration. Finally, the extracellular and reduced intracellular bacterial load (total bacteria – extracellular bacteria) attributable to each treatment was compared to the infected, untreated control.

Intracellular uptake assay using confocal microscopy

RAW 264.7 cells were seeded on 6-well plates and grown on sterilized glass slides for 24 h. *S. aureus* bioparticles (*S. aureus* Wood strain without protein A BioParticles®, fluorescein-FITC conjugate, Thermo Fisher Scientific, USA, catalog #S-2851) at a MOI of 100 were added to the 6-well plate. The plate was centrifuged at 600 × g for 5 min at room temperature to allow the particles to settle, followed by a 2 h incubation period to facilitate phagocytosis. After bioparticle incubation, the cells were washed 3 times, and ReApoBDs-BotrAMP14-NH2-TAMRA (78 $\mu g.mL^{-1}$-64 μM) was added to the wells to evaluate the phagocytosis of the vesicles for peptide internalization, and incubated for 20, and 60 min. The cells were washed 5 times with pre-warmed PBS, fixed with 4% formaldehyde, washed 3 times, and permeabilized with 0.1% Triton X-100.

Finally, DAPI staining was performed with a 0.25 μg.mL^{-1} solution for ~3 min, followed by 3 PBS washes. Slides were analyzed using a Zeiss LSM 900 microscope with a 63× objective, and the images were analyzed using the ZEISS arivis Pro software (version 4.1.2) to evaluate peptide localization.

In vivo bacterial killing assay

To assess the antimicrobial efficacy of the ReApoBD-BotrAMP14 nanoformulation, an *in vivo* cytotoxicity assay was first performed to determine the maximum concentration that could be used without causing tissue toxicity. For this, increasing concentrations of BotrAMP14 and ReApoBD-BotrAMP14 (8 to 64 μM), as well as 78 μg.mL^{-1} of vesicles (ReApoBDs), were injected subcutaneously into the back of mice. After 24 h, the tissue condition was evaluated to confirm that the selected concentrations were well tolerated before proceeding with the antimicrobial efficacy study.

The *in vivo* antimicrobial efficacy of the peptide formulation was evaluated using a mouse abscess model (78). All animal studies were conducted using a protocol approved by the University of Otago Animal Ethics Committee under approval number AUP-19-125. Swiss Webster (SW) male and female mice (6-8 weeks old) purchased from the University of Otago Biomedical Research Facility were used to evaluate the antibacterial effect of the ReApoBDs-BotrAMP14 nanoformulation. A skin infection model was used with 14 mice per group, divided into three biological replicates conducted at different times. The mice were shaved before being injected subcutaneously in their back with 1x10^{8} CFU *S. aureus* Aurora to induce abscess formation. After 4 h of infection, the animals were treated with either saline or the formulation (78 μg.mL^{-1}-64 μM, 50 μL). Mice were euthanized by an inhalant anesthetic overdose followed by cervical

dislocation after 3 days. The abscess area was measured, the surrounding non-necrotic skin was collected, homogenized, and a solution containing 0.1% Triton X-100 in PBS was added to release intracellular bacteria. Serial dilutions of the bacterial samples were plated on MHA to determine the total *S. aureus* concentration.

In vivo biodistribution

All animal studies were conducted in accordance with a protocol approved by the Universidade Católica Dom Bosco Animal Ethics Committee, under approval number 017/2021. BALB/c female mice (6–8 weeks old), purchased from the Universidade Estadual de Campinas (UNICAMP, Brazil), were used to evaluate the biodistribution of the ReApoBDs-BotrAMP14-NH2-Cy7 nanoformulation[79, 80]. Five mice were used: one untreated control injected with saline solution and four treated with the ReApoBD-BotrAMP14-CY7 nanoformulation, each corresponding to a specific time point (1, 3, 6, and 12 h) for organ extraction. The mice were shaved and injected subcutaneously with the nanoformulation (78 μg·mL^{-1}, 64 μM, 50 μL) into the back before imaging. After 1, 3, 6, and 12 h of treatment, organs (i.e., liver, spleen, kidneys, heart, and lungs) were collected from the treated mice and compared with the corresponding organs from the untreated control animal. Fluorescence imaging was performed using the Newton FT500 with an X54276 camera and a 24 mm motorized lens. Calibration steps included evaluating signal linearity using known ReApoBD-BotrAMP14-CY7 concentrations (256 to 8 μM), focusing on reproducibility and sensitivity. The system was optimized for Cy7 detection, with the excitation source set to 780 nm and the emission set to 800 nm, thereby eliminating autofluorescence. Fluorescence intensity was expressed in physical units of radiance (photons per second per square centimeter of the sample and per steradian of solid angle detected by the

system). These units enable a quantitative comparison of fluorescence signals between samples while accounting for variations in illumination and detection geometry. The exposure and aperture were adjusted to ensure accurate detection without oversaturation, and these parameters were maintained at constant levels throughout the experiment to facilitate direct comparisons between samples. The images were processed using Kuant software (version 2.3) to quantify fluorescence signals. Quantification was performed by integrating the fluorescence intensity over a region of interest (ROI) corresponding to each organ, normalized to the control fluorescence background to account for potential autofluorescence.

Statistical analysis

The statistical analyses were performed in R (R core team, Version 4.4.2). Data distribution was determined by the Shapiro–Wilk test. Students' T-tests evaluated the differences between treatments and controls. The reduction in the *in vitro* intracellular bacterial load was analyzed using ANCOVA, with macrophage viability as a covariate to account for the potential cytotoxic effects of the treatments. Statistical significance was set a priori at $p < 0.05$.

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4. Appendix figures: Sumplemmentary material, chapter 1.

Supplementary Materials for

"Nano-Trojan Horses" of Apoptotic Bodies with an Antimicrobial Peptide for Targeting Intracellular Infections

This PDF file includes:

Figs. S1 to S8

Tables S1

Fig. S1.

Induction of apoptosis in BV-2 cell cultures (**A**). Before and (**B**). 72 h after induction of apoptosis by starvation and H2O2 treatment. Images show the presence of ApoBDs (floating particles, black arrow), apoptotic cells (red arrow), and other cell debris and EVs.

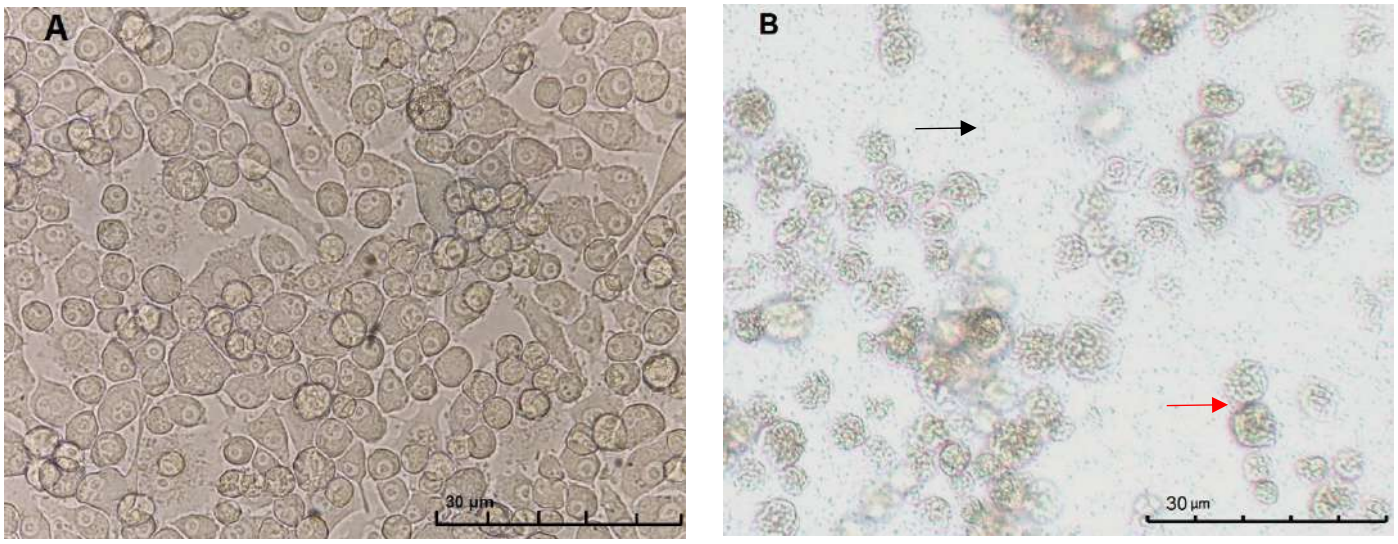


Fig. S2.

Detection and quantification of vancomycin and BotrAMP14 by RP-HPLC and MALDI-ToF. Chromatograms of drug detection (64 μM), calibration curves, and equations examples for %EE quantification of (**A, B**) vancomycin and (**C, D**) BotrAMP14, respectively. The %EE was determined by subtracting the amount of free VANH or BotrAMP14 from the total amount initially mixed with the ApoBDs and verified by quantification of the encapsulated peptide after lysis of the nanoconjugate vesicles. (**E**). Mass spectrum of vancomycin and BotrAMP14 stocks and post-RP-HPLC recovery fractions from one of the experiments, showing an ion mass of approximately 1471 Da and (**F**) 1957.51 Da, respectively.

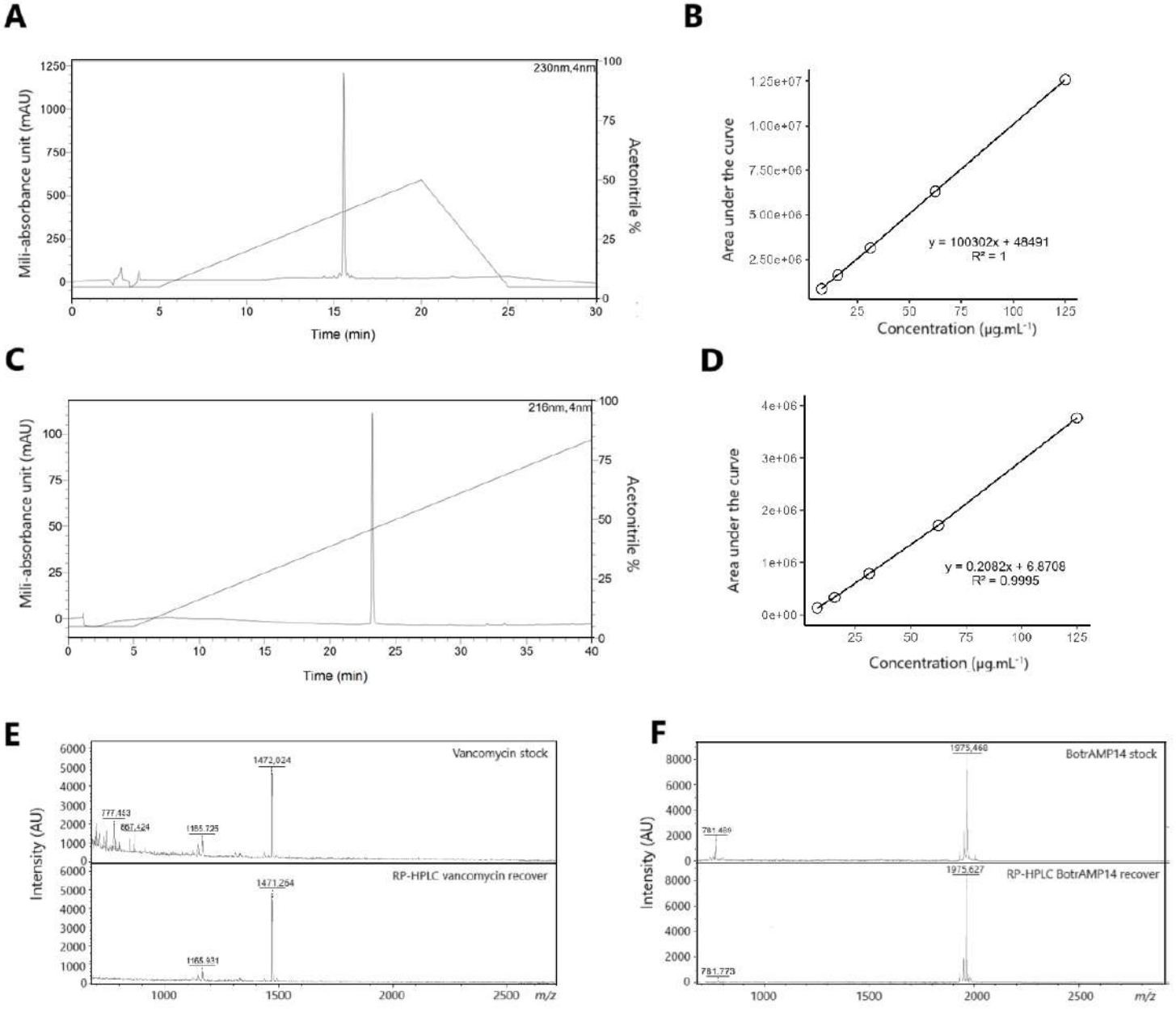


Table S1.

Dynamic light scattering (DLS) and encapsulation efficiencies of ApoBDs and ReApoBDs nanoformulations with VAN or BotrAMP14. The nanoformulations were prepared from HeLa and BV-2 cell cultures using freeze-thaw and freeze-thaw combined with extrusion as encapsulation methods. * Indicates cell populations that are highly heterogeneous or outside the measurable size and zeta potential range, where only the data from the two most similar technical replicates were used. Data represent the mean ± SD of at least three replicates.

Particle/Formulation	Hydrodynamic diameter (nm)	Surface charge (mV)	Polydispersity index (PDI)	Encapsulation efficiency (%EE) w/wo extrusion
HeLa Apocells *	5049 ± 4165	ND	ND	
Empty HeLa ApoBDs *	2911 ± 1732	-27.1 ± 5.83	0.452	
Empty HeLa ReApoBDs	97.5 ± 4.7	-35 ± 3.84	0.14	
HeLa ReApoBDs+VAN	117.9 ± 11.16	-28 ± 6.13	0.14	45.75 ± 0.63 / 17.53 ± 2.11
HeLa ReApoBDs+BotrAMP14	93.74 ± 12.35	-17.3 ± 6.22	0.457	22.25 ± 0.89 / 2.23 ± 0.67
BV-2 Apocells *	5275 ± 4054	ND	ND	
Empty BV-2 ApoBDs *	2999 ± 1962	-23.4 ± 4.17	0.484	
Empty BV-2 ReApoBDs	94.44 ± 3.1	-31.45 ± 4.28	0.181	
BV-2 ReApoBDs+VAN	119.73 ± 13.12	-25.46 ± 4.57	0.224	17.56 ± 1.41 / 0.63 ± 0.2
BV-2 ReApoBDs+BotrAMP14	86.86 ± 6.1	-21.4 ± 4.05	0.202	70.8 ± 3.39 / 5.32 ± 2.55

Fig. S3.

Percent encapsulation efficiency for HeLa and BV-2 formulations using freeze-thaw and freeze-thaw combined with extrusion as encapsulation methods. ApoBDs or size-modified ApoBDs (ReApoBD) from BV-2 or HeLa cultures were used for vancomycin (VAN) loading. Data represent mean ± SD of three replicates.

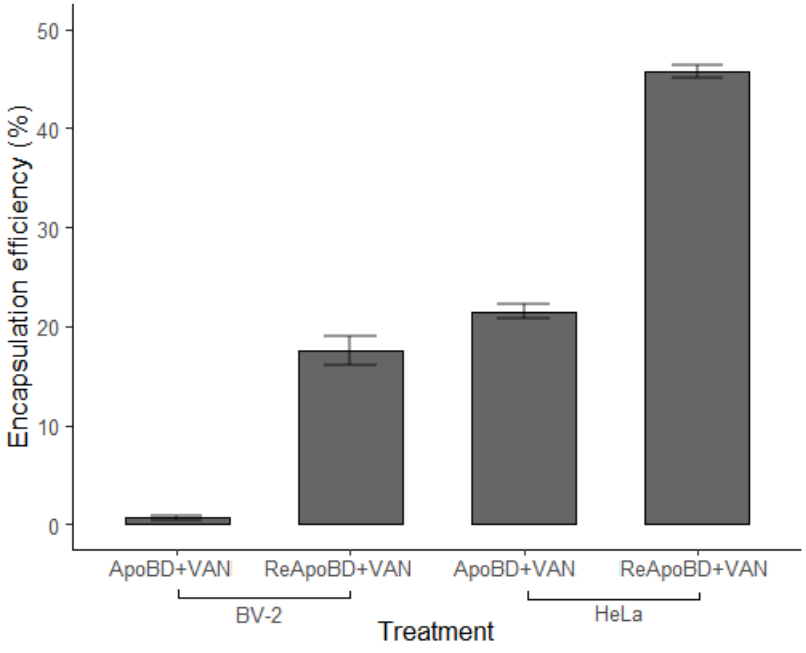


Fig. S4.

Correlation and phase plot from (**A**). size and (**B**). Zeta potential measurements.

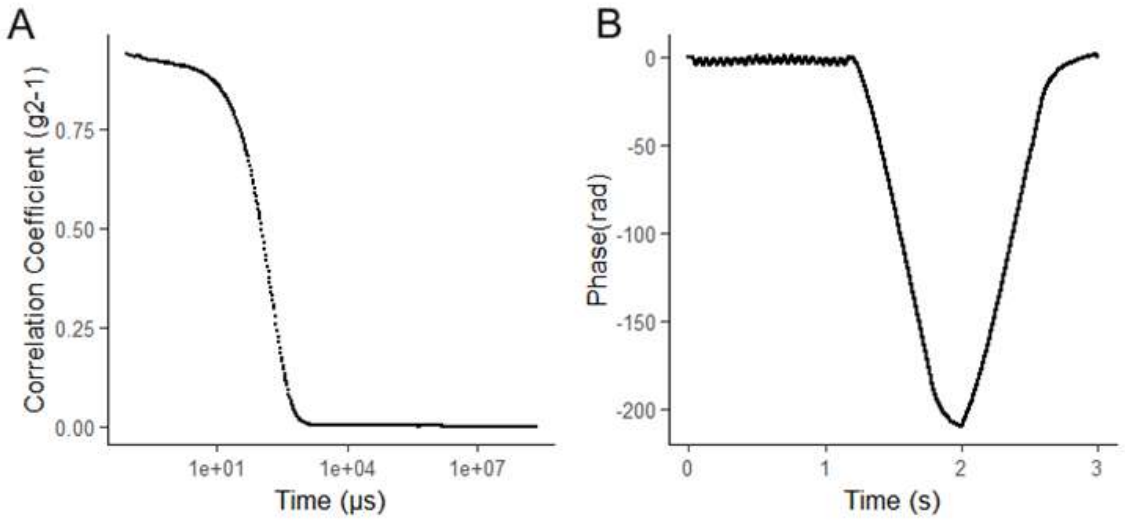


Fig. S5.

Size frequency distribution graph of different vesicles/cell populations by intensity, number, and volume. The plots represent the data distribution of three replicates.

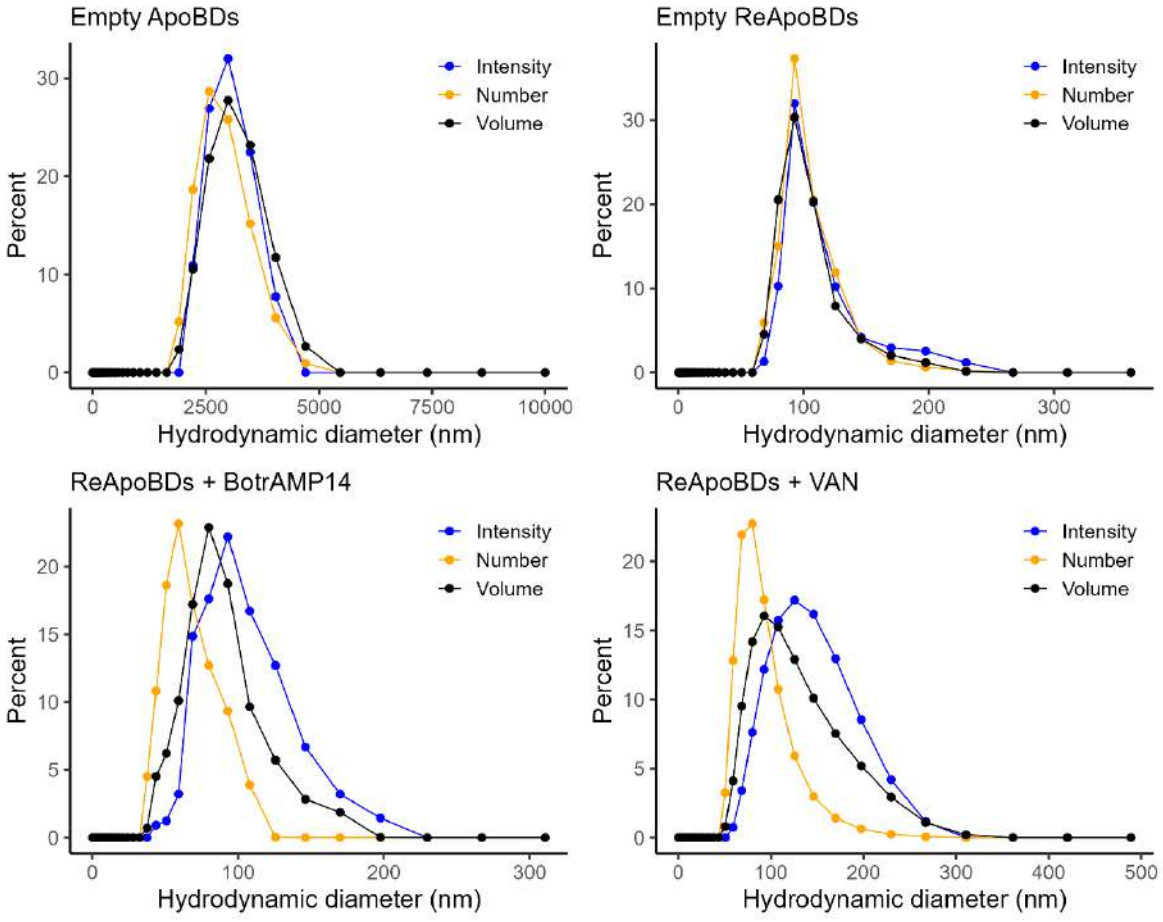


Fig. S6.

Theoretical response of macrophages to intracellular drug treatments based on cytotoxicity and antimicrobial activity. The figure illustrates how treatment outcomes vary depending on the balance between intracellular cytotoxic effects and intracellular antimicrobial efficacy.

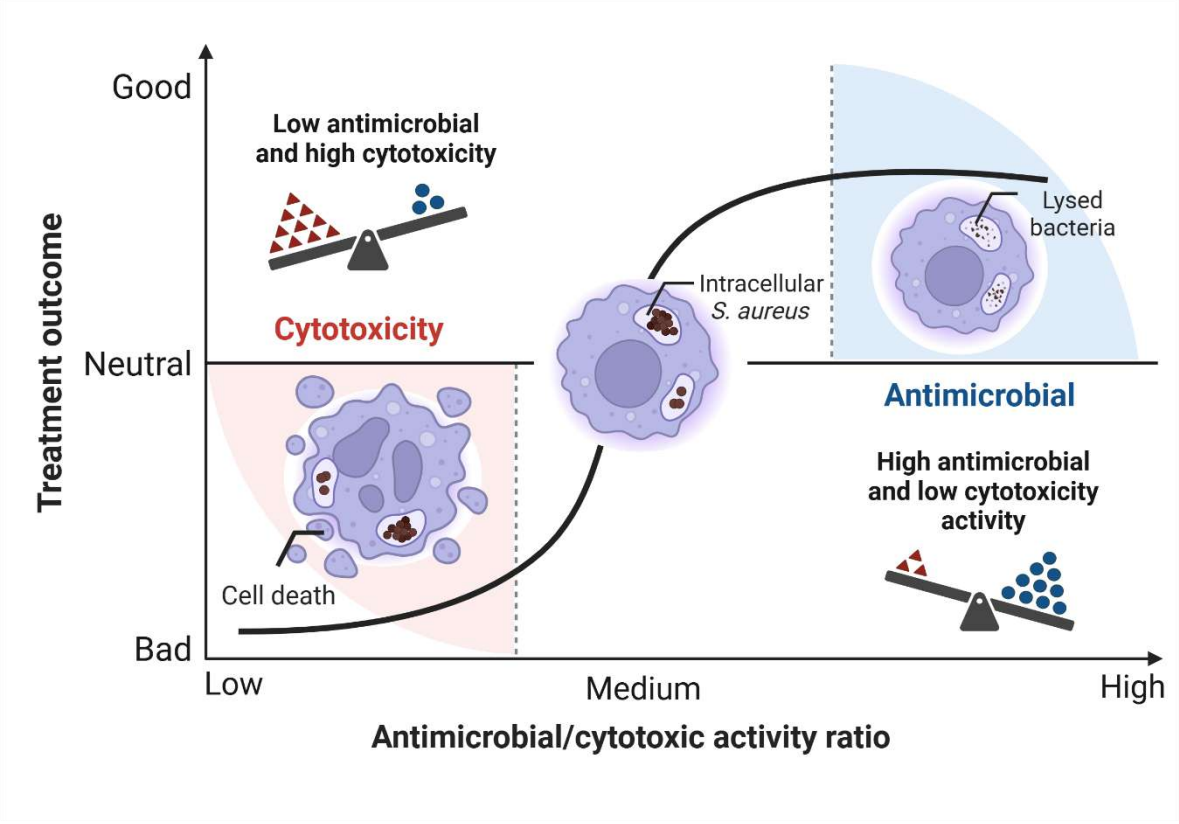


Fig. S7.

Three-dimensional visualization of the intracellular uptake of the antimicrobial peptide and its nanoformulation using confocal microscopy. (**A**). 3D reconstruction of RAW 264.7 cells treated with BotrAMP14-NH2-TAMRA for 20 min with no detectable TAMRA signal, indicating that the non-nanoformulated peptide fails to internalize into the macrophages. (**B**). 3D reconstruction of RAW 264.7 cells treated with ReApoBDs-BotrAMP14-NH2-TAMRA for 60 min. The image highlights successful peptide internalization, as evidenced by a high number of fluorescent vesicles (green and yellow) that are colocalized within the cytoplasm. This analysis underscores the effectiveness of the nanoformulation in promoting cellular uptake.

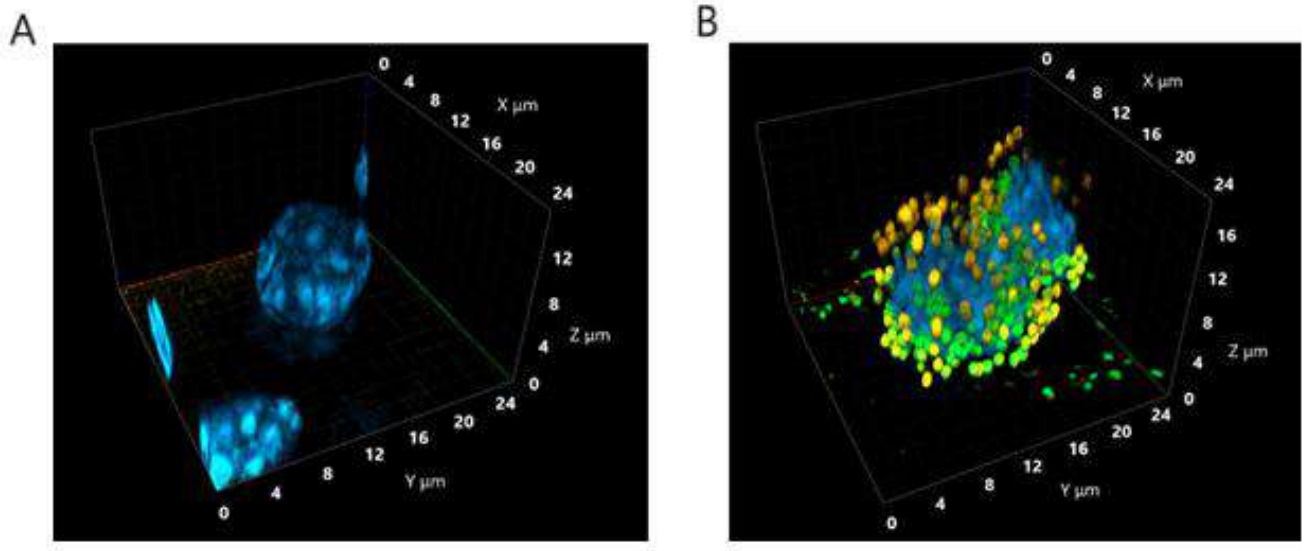
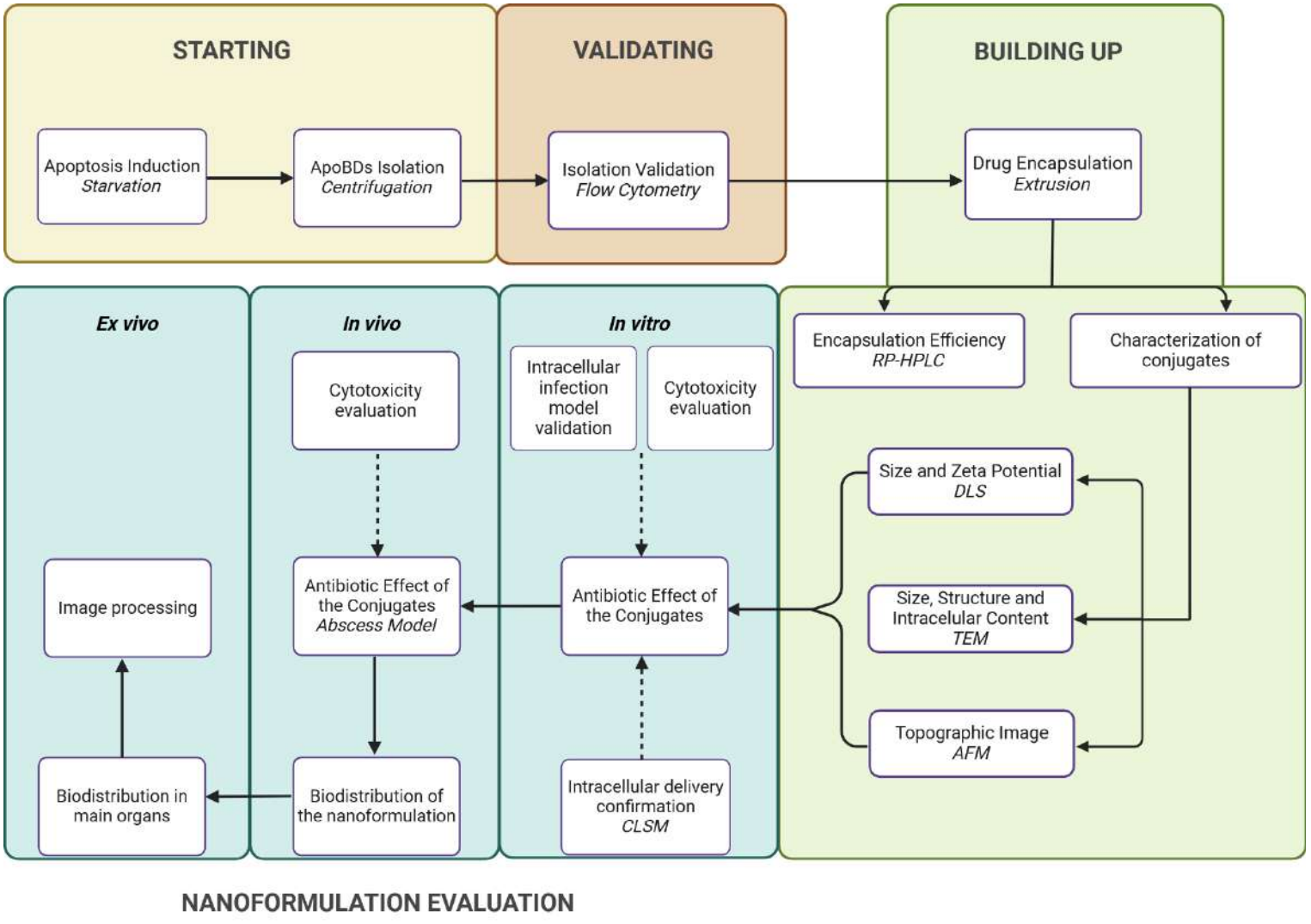


Fig. S8.

Flow chart of the experimental design. The diagram outlines the experimental steps for the production and purification of apoptotic bodies (ApoBDs) from HeLa and BV-2 cells, as well as the production, characterization, and antimicrobial evaluation of a reconstructed BV-2 ApoBD (ReApoBD) nanoformulation with vancomycin (VANH) and BotrAMP14.



5. Second chapter: Expanding Therapeutic strategies for intracellular bacterial infections through conjugates of apoptotic body–antimicrobial peptides

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REVIEWS

Expanding therapeutic strategies for intracellular bacterial infections through conjugates of apoptotic body–antimicrobial peptides

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Macrophage intracellular infections are difficult to treat because conventional antibiotics tend to have poor penetration of mammalian cells. As a consequence, the immune response is affected and bacteria remain protected inside macrophages. The use of antimicrobial peptides (AMPs) is one of the alternatives developed as new treatments because of their broad spectrum of action. To improve drug delivery into the intracellular

space, extracellular vesicles (EVs) have emerged as an innovative strategy for drug delivery. In particular, apoptotic bodies (ApoBDs) are EVs that exhibit attraction to macrophages, which makes them a promising means of improving AMP delivery to treat macrophage intracellular infections. Here, we review important aspects that should be taken into account when developing ApoBD–AMP conjugates.

Keywords: apoptotic bodies; antimicrobial peptides; drug delivery; intracellular infection; macrophages

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Introduction

Within the spectrum of microbial infections, those caused by intracellular pathogens have a severe impact on public health. This is because, unlike extracellular pathogens, which lead to the development of acute infections, intracellular pathogens are often associated with chronic or persistent infections. Persis- tent infections, unlike chronic infections, are not eliminated and may remain latent in the host for life.[1] Intracellular pathogens have evolved specific characteristics that have enabled them to evade elimination by the host immune system.[2,3] A large number of these intracellular pathogens are particularly efficient at infecting and thriving within key cell populations of the immune response, such as macrophages and dendritic cells. The advantages of this type of infection for the pathogens are that, by remaining inside the host cell, they are protected, avoid- ing opsonization processes or recognition by other cells of the immune system, in addition to having access to sources of nutrients.

To meet the challenge posed by this type of pathogen, the development of AMPs has emerged as an alternative to conventional antibiotics. AMPs are small protein biomolecules produced by the innate immune system as a response to antigen presence. Given their broad spectrum of action, natural and synthetic AMPs have been used for the treatment of bacterial, fungal, par- asitic, and viral infections.[4] In bacterial infections, AMPs gener- ally act directly on the membrane of the microorganism and in a variety of metabolic pathways.[5] These mechanisms of action are normally correlated with cationic and amphipathic AMP properties, which result in electrostatic attraction and hydrophobic interactions generated between AMPs and negatively charged pathogenic microorganism membranes. However, the most relevant challenges regarding the design of new AMPs for treating intracellular infections are their limited cell specificity, bioavailability, and delivery. Regardless of the type of pathogen to be treated, the vehicle used for administration might be more crucial than the peptide itself for successful clearance of infection.[6,7] Therefore, such vehicles act as 'trojan horses' loaded with bioactive molecules for therapeutic purposes. These vehicles could assist AMP stabilization, thus preserving their biological activities for further action on intracellular pathogens in mammalian cells, which requires low toxicity to the host.[8,9]

One of the most attractive and innovative vehicle strategies includes nanovesicles, microvesicles (liposomes) and cell-derived EVs. EVs are used to enhance immunomodulation and cancer and infectious disease treatments by packaging different types of molecule in or onto them to improve their tissue-specificity delivery (Table 1).[10,11] In general, both liposomes and EVs exhibit greater biocompatibility and biodegradability and are better at allowing the passage and targeting of drugs through different biological barriers compared with synthetic vesicles.[10,12] This is because, compared with nanoparticles (NPs) comprising synthetic materials, liposomes and EVs are phospholipid bilayer constructs that are naturally compatible with the physiological structure of the cells of the host. Additionally, synthetic NPs exhibit some degree of cytotoxicity, also triggering proinflammatory immune responses at the infection site.[13–15]

EVs appear to have an advantage over liposomes, being naturally generated and secreted by cells. Among the advantages of

TABLE 1

EV classification and properties.

EV type	Approximate size range (nm)	Membrane origin	Content	Function	Refs
Exosomes	30–150	Endosomal pathway	Cytosolic or endosomal proteins and glycoproteins	T cell stimulation Antigen transfer to dendritic cells Eliminate endosomal and lysosomal proteins	101
Microvesicles	50–1000	Plasma membrane	Cytosolic proteins, membrane proteins	Cell–cell communication	102
ApoBDs	1000–5000	Plasma membrane	Intact organelles, nucleic acids, proteins	Promote homeostasis and normal apoptotic cells clearance	20

EVs are their ability to mediate cell–cell communication and leukocyte recruitment signaling, their recognition of specific tissues, and low host toxicity.[16,17] ApoBDs are a type of large- diameter EV exclusively formed and secreted by apoptotic cells to carry out the cellular disassembly process in a safe and orga- nized manner to maintain homeostasis. These vesicles pack cellu- lar debris that might cause damage to neighboring cells or extracellular matrix in the tissue where apoptosis is occurring.[17] Therefore, ApoBDs are naturally configured to be specifically rec- ognized by tissue macrophages, responsible for the clearance of apoptotic cells.[18,19] This feature makes ApoBDs attractive for improving specific molecule delivery to treat macrophage intra- cellular infections caused by highly pathogenic bacteria, includ- ing *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Salmonella enterica*, and *Chlamydia trachomatis,* among others. Thus, AMP packing into transport vesicles (e.g., ApoBDs) appears a promising strategy for developing new treat- ments against intracellular bacterial infections. Here, we review the nature of ApoBDs and their macrophage-mediated interaction and recognition resulting from the apoptotic cell clearance pro- cess. We also focus on the perspectives and hypotheses relating to loading AMPs in ApoBDs according to the characteristics of the vesicle membranes and their possible interaction with AMPs.

Origin and physiological function of ApoBDs

During cell death, the apoptotic cell packs all types of biomole- cule and intact organelle into minor divisions of its membrane (ApoBDs) to facilitate its phagocyte-mediated clearance (Table 1).[18] This is the result of an orderly process that avoids both tissue damage and the activation of a proinflammatory immune response that, in turn, favors the detection and elimination of apoptotic cell residues.[18,19] Some of the distinguishing features of the apoptotic cell are the secretion of 'find me' signals and the alteration of its membrane to express 'eat me' signals to different phagocytes. These chemoattraction and membrane signals are also inherited by ApoBDs and are differential according to parent cell types and vesicle size.[20,21]

Cell signals such as those associated with lysophosphatidyl- choline (LPC), uridine triphosphate (UTP), and ATP (mediated by pannexin 1 channels) and CX3CL1 (released by ApoBDs of B lymphocytes) represent general soluble factors or lipids involved in the recruitment of phagocytes to the site of cell death.[22–25] However, some signals can be precise and even inhi- bit phagocyte migration, including lactoferrin, which induces only the differential migration of mononuclear phagocytes.[26] Other signals, such as ICAM-3 (CD50), present in the ApoBDs of apoptotic leukocytes appear to have attractant and differential signaling effects on the recruitment and recognition of macro- phages, expressing specific recognition receptors (PRRs) on their surface.[27] These attractive properties of ApoBDs have been tested in *in vitro* and *in vivo* models. Once phagocytes are attracted to the apoptosis site, the combination of 'eat me' signals and altered 'don't eat me' signals help selective phagocytosis of cellular debris.[28] For this, the apoptotic cell must undergo different mem- brane changes. The aminophospholipid translocases activity is lost, and a nonspecific phospholipid flip-flop occurs, exposing some molecules on the outer leaflet that were hidden during the previous stages of the cell cycle on the inner leaflet.[29,30]

Among these 'eat me' molecules are phosphatidylserine (PS), ICAM-3, annexin I, and calreticulin, which are recognized by scavenger, ICAM-3, FPR2, and LRP macrophage receptors, indicating that the cell is undergoing apoptosis and should be rapidly removed from the tissue.[27,31,32] Thus, once a cell dies and has packed its internal material into small membrane-derived ApoBDs, as reported for apoptotic cells, the expression of those specific signals on the outer leaflet is observed. In particular, PS expression is vital for phagocyte-mediated clearance of apoptotic cells. First, PS expression is a crucial factor in the specific recogni- tion by CD36 receptors of tissue macrophages responsible for apoptotic cell clearance by phagocytizing their debris and elimi- nating them in their phagolysosome.[33,34] In addition, the appearance of PS activates some molecules, such as the comple- ment system and b2GPI. This PS-dependent opsonizing molecule activation, together with the activation of other opsonizing molecules, including thrombospondin I, immunoglobulins, CRP, among others, leads to opsonization of both apoptotic cells and ApoBDs.[35] Thus, the opsonized cell debris could be recog- nized by different receptors, such as CR1, CR3, TLR, CRIg, and FccRI, which may enhance macrophage-mediated recognition.[35] All these cell membrane changes for the expression of 'eat me' signals lead to the alteration or silencing of 'don't eat me' signals, such as the expression of CD47 and CD31. These two signals are normally expressed on the membrane of viable cells as signals that prevent their phagocytosis mediated by immune system cells.

Isolation of ApoBDs and AMP loading

Since apoptosis is common to all cell types, ApoBDs can be extracted from all types of samples, including fluids, blood, or cell cultures.[36–39] The most common is the use of cell lines because they are easy to handle. Apoptosis can be triggered by different types of internal and external stimulus. However, whether intrinsic or extrinsic, both ultimately converge to cas- pase pathway activation and the formation of ApoBDs. Extrinsic apoptosis induction can be activated by transmembrane recep- tors, such as TNFR-1, TNFR-2 (CD120a and CD120b) and CD95, associated with TNF and Fas recognition, and TRAIL (Apo2L)-activated DR receptors.[40] By contrast, induction of apoptosis by the intrinsic pathway can occur through different methods, such as induction of DNA damage mediated by ultravi- olet (UV) radiation, cell stress, use of staurosporine, and starving by serum-free culture media.[41,42] The time required for the apop- totic process to start based on the starving method varies from 3 to 72 h.[43] It occurs in response to the difference between the maintenance needs of the different types of cell line. Once the cell death process begins, it can present some morphological changes, including: (i) blebbing formation; (ii) formation of membrane protrusions followed by karyorrhexis; and, finally,

(iii) the complete separation of these invaginations into indepen dent membranes (i.e., ApoBDs).[18]

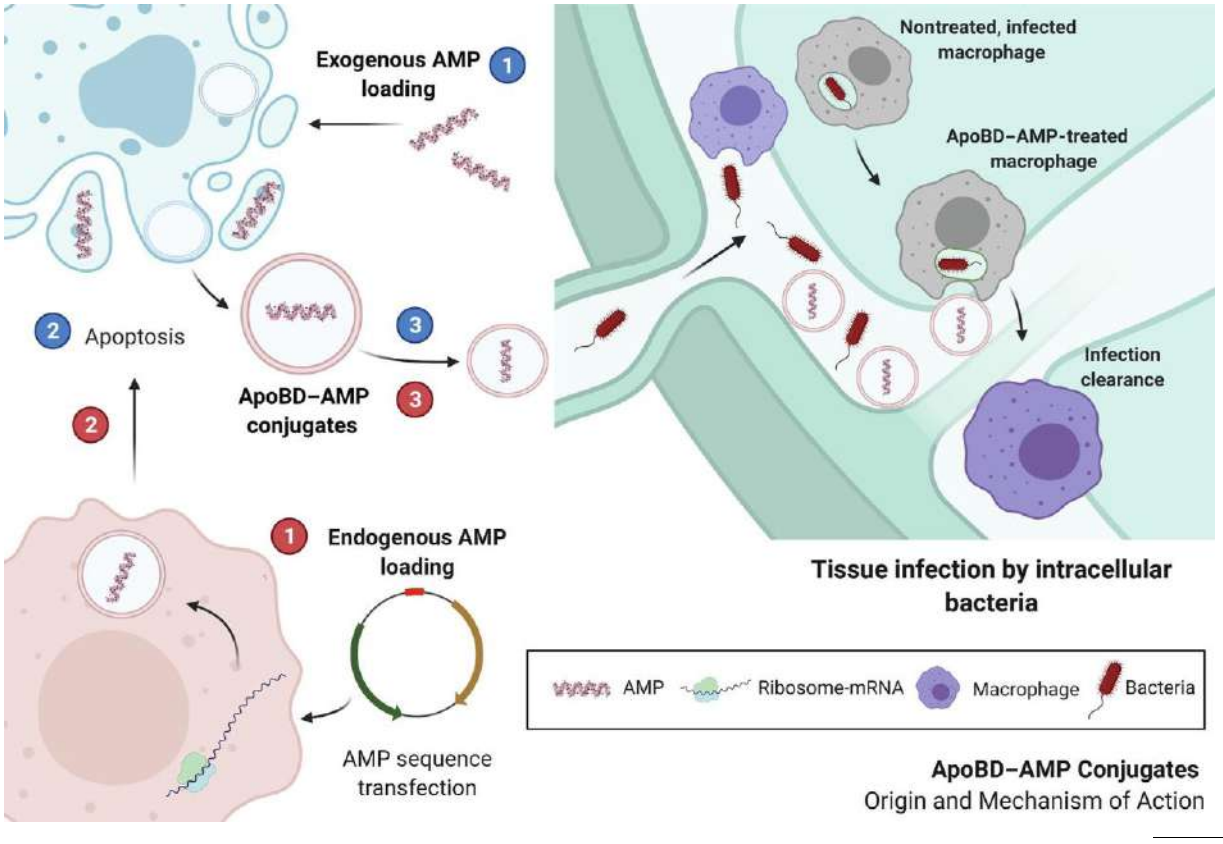

FIG. 1

Schematic of the origins of apoptotic body (ApoBD)–antimicrobial peptide (AMP) conjugates and their specific interaction with bacterially infected macrophages. (1) Endogenous and exogenous strategy for the isolation/loading of ApoBDs/AMPs. (2) Induction of apoptosis in ApoBD precursor cells. (3) Obtaining ApoBDs loaded with AMPs. Mechanism of action of ApoBD–AMP conjugates by administration and specific interaction with macrophages infected by the affected tissue.

Considering its progressive nature, four cellular subtypes or stages and two different particles are expected to be found in solution, including viable cells, early apoptotic cells, late apoptotic cells, necrotic cells, ApoBDs, and other EVs and debris. Sepa- ration of such populations or subtypes can be done by cytometry and centrifugation. Separation via fluorescence-activated cell sorting (FACS) is recommended when, in addition to a separation with a purity of ~99%, a clear distinction between the different cell stages and particles in solution is desired.[41,44] Nevertheless, centrifugation is a fast and reliable method when only ApoBDs are intended to be isolated from other cell stages, with a purity of ~97%.[41]

Given the different EV sizes, exosomes and microvesicles can also be separated by a more complex series of ultracentrifugation cycles because they are smaller vesicles.[45,46] However, there are other methods for their separation, such as density-gradient separation and the use of microfluidic techniques, including nanowire-on-micropillar and acoustic sorting.[47] There are also other separation techniques based on the differential expression of specific proteins on their surface, such as immunoaffinity-based isolation.[47]As described above, ApoBDs reflect the cell of origin. There- fore, ApoBDs from cancer cell lines can carry oncogenes, includ- ing *h-ras* and *c-myc*.[48] These oncogenes could be responsible for developing new tumors in the case of p53-deficient recipient cells. Additionally, another attractive feature of ApoBDs derived from cancer cell lines is that they have a higher concentration of cholesterol in their membrane.[48] This characteristic stabilizes the membrane fluidity and appears to have a positive effect on the loading of complex glycopeptides (e.g., vancomycin).[49,50]

Once ApoBDs have been successfully isolated, the next step for the construction of customized vehicles is the loading of these molecules into the vesicle. Two major pathways have been described for this purpose: the endogenous and exogenous pathways (Fig. 1). The endogenous loading process uses the cellular machinery for the transgenic production of the target molecule (e.g., AMP).[7] This occurs by the transfection and induction of gene expression in target mammalian cells. Later, the apoptosis process is induced and, finally, endogenously loaded ApoBDs are obtained. However, the final yield from this pathway might not be high because of the low specificity of the packaging between the different EVs, the low effectiveness of the transfec- tion method, and because endosomal proteases can degrade pep- tides during

the biogenesis of the EVs.[17,51] By contrast, the exogenous packing pathway is well known and is the easiest to implement. For this, it is enough to induce the apoptosis process in the donor cells, isolate the empty ApoBDs, and, later, load them with the molecule of interest.

There are several alternatives already described for the exoge- nous packing process, including extrusion and freeze and thaw,[52,53] electroporation,[54] and simple incubation.[55] As described above, according to the membrane characteristics, some methods mgiht be more efficient than others. The method that appears to have the highest efficiency is the freeze and thaw method, followed by sonication of the vesicles in the presence of the molecule of interest. This method reduces the lamellarity of the vesicle to generate less polydispersion or disruption of the bilayer, thus assisting the uptake of molecules inside of the vesi- cle.[56] Additionally, some methods for ApoBD remodeling, aim- ing at decreased diameters from the micro- to nano-size, have been described.[57] This remodeling is expected to facilitate macrophage-mediated phagocytosis by decreasing the size of the vesicle. However, the difference in phagocytosis efficiency using ApoBDs and remodeled ApoBDs with a smaller diameter has not yet been evaluated.

AMP–mammalian cell membrane interactions

Numerous conventional antibiotics are used to counter infection processes by blocking proteins essential for bacterial metabolism. Unlike these, AMPs usually act by different mechanisms of action, generally based on the formation of pores in the bacterial membrane, causing its disruption, fusion, or translocation.[58,59] Regardless of the mechanism of action, interaction with the membrane is an essential step for most of these molecules. Therefore, AMP behavior will depend on both the nature of the pep- tide itself (e.g., physicochemical properties and environment-dependent structural profile) and the nature of the biological membrane it interacts with.[58] Most AMPs are cationic and might present a specific selectivity toward bacterial membranes because of their negatively charged phospholipids, thus facilitating electrostatic interactions for peptide attachment and reorientation.[60,61] By contrast, mammalian membranes are zwitterionic and enriched with cholesterol, which is more likely to impair AMP attachment.[62,63]

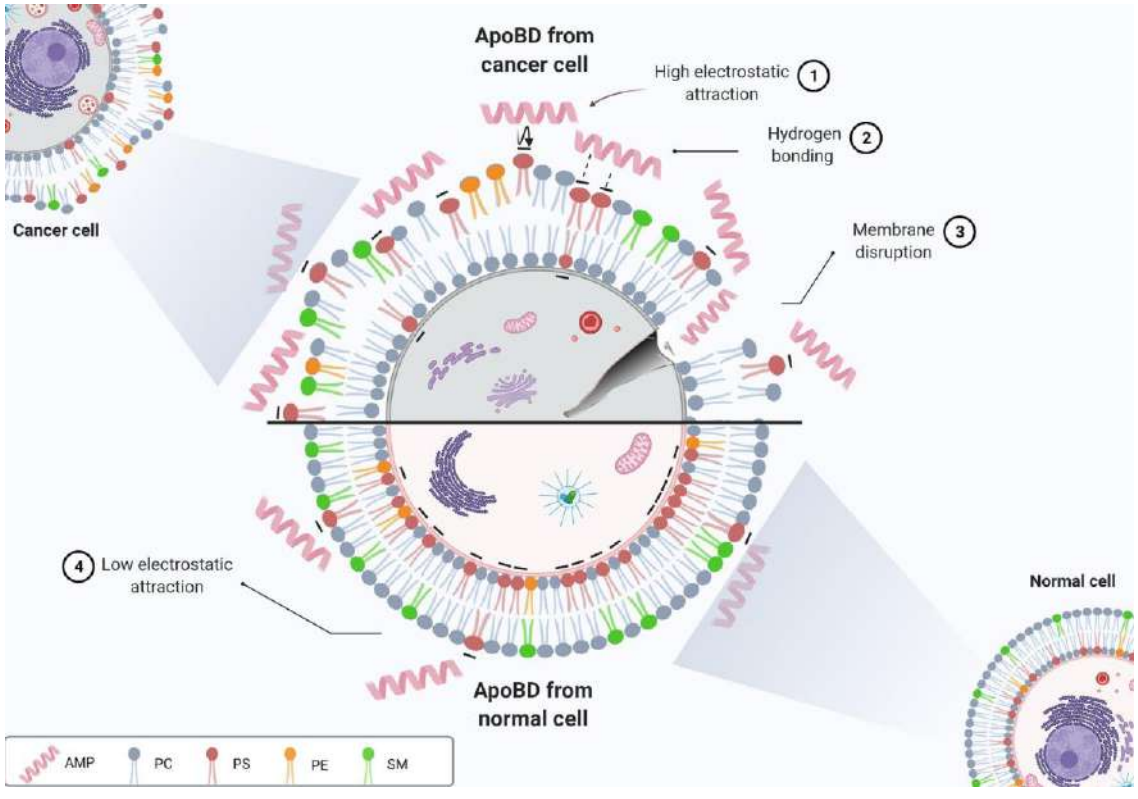

FIG. 2

Hypothetical schematic of the symmetry differences between cancer and normal cell membranes and possible implications for the phospholipid constitution, distribution, and ratio of the resulting apoptotic body (ApoBD) membranes. (1–3) High-affinity electrostatic and physical interactions between ApoBD membranes from cancer cells with cationic peptides, which could result in membrane disruption. (4) Low or medium affinity between ApoBD membranes from normal cells with cationic peptides, resulting in a high stability in solution. Abbreviations: AMP, antimicrobial peptide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

Peptide–membrane charge interactions also depend on the lipid distribution and the different proteins embedded in the bilayers. Mammalian cell membranes are highly variable depend- ing on the cell line or tissue they belong to. However, phos- phatidylcholine (PC) and sphingomyelin (SM) are commonly found on the outer leaflet of the cell membrane, and the amino phospholipids, phosphatidylethanolamine (PE) and phos- phatidylserine (PS) on the inner leaflet.[62] In membranes that comprise ApoBDs, when flip-flop occurs, there is a translocation of PS and other proteins of the innermost face of the mem- brane,[30] which probably affects the increase in the negative charge of the outer leaflet of the vesicle. This might be related

to an increased electrostatic attraction between cationic AMPs and ApoBDs. Nevertheless, modification of the outer membrane leaflet charge in ApoBDs compared with its precursor cell and the interaction of ApoBDs with cationic AMPs have not yet been evaluated. This encourages further studies of ApoBD–AMP conjugates as new treatments for intracellular infections.

In cancer, the cellular change process is well described and highlighted as one of the main factors why many AMPs present cytotoxic effects on tumor cells. In this case, the tumor cell membrane loses its asymmetry by activating several enzymes, including aminophospholipid translocase and scramblase, which mediate the translocation of PS to the outer leaflet.[64,65] In addition, there are changes in the glycosylation processes of membrane glycoproteins, which increase the net negative charge on the outer leaflet of the tumor cell compared with a normal cell of the same lineage.[66,67] Given this net charge modification, cationic AMPs can establish hydrogen bonding with the tumor cell membrane surface after electrostatic attraction.[68,69] Once the AMPs are bound to the membrane, the hydrophobic face facilitates their insertion into the lipid core, causing membrane disruption and, ultimately, cell death.[69] However, the original cell type from which ApoBDs are obtained can be strongly linked to the interaction between AMPs and the vesicle membrane.

So far, it has been shown that the membrane surface charge of EVs is highly variable.[70] Therefore, it might be more advantageous to use ApoBDs from fetal cell lines than from cancer cells. As mentioned above, cancer cell membranes, unlike normal cells, already have higher expression of PS on the outer side of their membrane and, therefore, a higher electrostatic attraction with cationic AMPs compared with normal cells (Fig. 2). However, a certain degree of electrostatic attraction between ApoBDs and AMPs is necessary, because the latter can be loaded in and onto the vesicle.[71,72] Thus, using ApoBDs from normal cells could prevent the high electrostatic attraction leading to vesicle rupture by AMPs.

Different studies have achieved the encapsulation of different specific AMPs in liposomes with a high encapsulation effi- ciency.[73–75] These synthetic vesicles are comparable, in part, to mammalian cell membranes. However, as mentioned above, both the complexity of mammalian cells and their variability between different cell lineages, including tumor cells, signifi- cantly affect the outcome of the peptide–vesicle interaction. The insertion of glycopeptide antibiotics (e.g., vancomycin) into liposomes and ApoBDs from different cell sample types has also been achieved, but there are discrepancies in encapsulation effi- ciency. Some studies have found low entrapment of such water- soluble antibiotics in the lipid core of the vesicle, whereas others have achieved relatively high efficiency (from 40% to 60%).[50,76] Moreover, such an increase in glycopeptide encapsulation effec- tiveness appears to be directly related to the increase in the cholesterol concentration in the vesicle membrane.[50,77] The use of cancer cell lines in these cases could be more advantageous, because they have more cholesterol in their membranes cells.

Biological activities of conjugates of ApoBDs/peptide- based antibiotics

The use of NPs for drug delivery enhancement has been tested using different compounds, giving rise to synthetic and natural NPs derived from cell membranes. Lehar *et al.* developed a new therapy that effectively eliminates intracellular infections in mammalian cells caused by methicillin-resistant *S. aureus* (MRSA).[78] This therapy comprises an antibody–vancomycin (peptide antibiotic) conjugate that is activated after being deliv- ered specifically into the phagolysosome of the infected cell. This again underscores the importance of the delivery vehicle having the ability to come into direct contact with the phagolysosome membrane where the intracellular bacterium is harbored. Although the ability of ApoBDs to fuse with the membrane of the infected phagolysosome has so far not been proven, Bose *et al.* found evidence to support the existence of ApoBD– phagolysosome fusion.[50] Increased intracellular infection clear- ance was confirmed using ApoBD–vancomycin conjugates in both *in vitro* and *in vivo* models of intracellular MRSA-type infec- tion. In addition, synthetic particles, including silicon NPs or natural terpenes (farnesyl and geranyl) loaded with cyclic pep- tides conjugated with antibiotic or antibiotic alone, respectively, have also been tested.[79,80] Both studies with *in vivo* models found that intracellular infections of *S. aureus* were suppressed com- pared with antibiotic monotherapy, reducing bacterial replica- tion by up to 99%.[79] These studies highlight the importance of the transport vehicle for protecting antimicrobial drugs.

By contrast, antibiotic loading in membrane-derived vesicles has gained much attention and, recently, cholesterol-enriched platelet[77] and red blood cell[81] membrane-derived vesicles have been developed to improve targeted delivery of doxorubicin and vancomycin. These studies found that these vehicles showed high affinity for breast cancer cells and MRSA. This affinity for cancer cells and macrophages with intracellular MRSA-like infec- tions was also found when developing remodeled conjugates of apoptotic bodies (ReApoBDs) with vancomycin-derived cancer cells (SKBR3, MDA-MB-231, HepG2, U87-MG, and LN229).[50] In this *in vitro* and *in vivo* model, a two-log-order reduction in total *S. aureus* colony-forming units (CFU) recovered from intracellularly infected RAW-264.7 cells was achieved. Additionally, a statistically significant difference in U87-MG (p53-wt) and LN229 (p53-mt) cancer cell killing was found when comparing ReApoBDs-

vancomycin conjugates with antibiotic monother- apy.[50] Other studies have found similar results, whereby the development of cancer cell-derived nanocarriers appears to express a high affinity for tumor cells, rendering them a promis- ing option for developing new anticancer therapies.[82] Finally, the use of liposomes continues to be one of the most explored strategies, where different conjugates have been developed with antibiotics, including vancomycin[76] or synthetic AMPs.[73–75,83] This shows the feasibility of encapsulating peptides within compartmentalized environments delimited by lipid monolayers or bilayers. It could ultimately protect the peptide from degradation, increasing its bioavailability, specificity, and penetration, as well as decreasing the necessary doses to be administered and, thus, reducing cytotoxicity.

ApoBDs-AMPs as innovative therapeutic conjugates?

Once ApoBDs have been isolated and loaded with AMPs, they can be administered as a treatment by intravenousinjection. So far, dis- crepant results have been seen regarding the stability of such vehicles in the bloodstream. Some studies have found them to be highly stable,[84] whereas others have found that they appear to be eliminated with liposome-like kinetics.[85] This could be explained by differences in the ApoBD origin cells and the integrity of the particles, which might be affected by the isolation technique used.[86,87]

The orientation or tropism properties of ApoBDs can be highly variable.[88] Some characteristics of the vesicle membrane, including variation in lipid and protein composition (e.g., different types of integrin), significantly affect their accumulation in the tissue, organ, or host cell membrane and their subsequent uptake.[89,90] Different receptor cell types will take up some particles at a higher rate than others, as is the case of macrophages, where PS expression mediates the uptake of ApoBDs.

When it comes to macrophages, which are found only in tissues, an alternative route of administration includes direct injec- tion into the affected tissue. Thus, the 'find me' and 'eat me' signals of ApoBDs could rapidly aid the recruitment of both infected and healthy macrophages, but only when infected macrophages do not lose their ability to uptake these signals. There are important differences in the interaction of the receptor cells with the vehicle when liposomes and ApoBDs are compared. Liposomes appear to have high aggregation in the membrane of the recipient cells (e.g., macrophages).[91–95] However, their inter- nalization rate is low, which it is correlated with the immune sup- pression of macrophages, preventing bacterial clearance by them. By contrast, when EVs are used, the interaction with the mem- brane is similarly high, but aggregation at the host cell membrane is low.[90–92,96,97] This is because of the high rate of vesicle internal- ization, apparently via the endocytic pathway and the specific ligand–receptor interaction.[96] In macrophages, such internaliza- tion occurs by phagocytosis with the formation of tight phago- somes, or a 'zipper-like' phagocytosis mechanism that tightly envelops the ApoBD.[90] This internalization mechanism has been related to ligand–surface receptor interactions; thus, the phago- some membrane must be tightly bound to the surface of the ApoBDs. However, successful recognition, membrane interaction, and subsequent internalization of the apoptotic body by the zip- per mechanism requires integrity of the ApoBD membrane.[90,98]

Once ApoBDs are phagocytosed by macrophages, they enter the cellular interior and remain inside the phagosome, which is the second membrane surrounding them. After this, two events can occur: the first is when the macrophage is a healthy cell. In this case, a natural clearance of ApoBDs takes place and AMPs are then degraded. In this process, the phagosome fuses with the lysosome, giving rise to the phagolysosome, leading to cell digestion. However, when macrophages are already infected, intracellular bacteria are protected in immature phagolysosomes.[99,100] So far, it is unclear by which mechanism or how the phagolysosome where the ApoBDs are placed comes into contact or fuses with the immature phagolysosomes where the infection is located to discharge its contents. Nevertheless, there is increased clearance of intracellular infections when using these vehicles loaded with some antibiotics.[50] Therefore, it would be expected that ApoBDs loaded with AMPs could also reach these intracellular targets and release the peptides into the immature phagolysosomes, so that the bacteria that were protected inside the macrophage could be eliminated (Fig. 1). This represents an alternative and innovative strategy to counter difficult-to-treat intracellular bacterial infections.

Concluding remarks

ApoBDs and other EVs are innovative and promising alternatives for improved drug delivery because of their advantages over synthetic encapsulation systems. However, many details about their biological roles, recognition, and action mechanisms remain unknown. Therefore, it is necessary to explore this field further, opening doors to study and better understand the mechanisms and processes of intercellular communication mediated by ApoBDs as messenger vesicles. This would help to achieve communication between specific cells, tissues, and organs with transport vehicles for AMPs and other types of drug to treat intracellular macrophage infections. There is still much to be explored in the field, specifically regarding the issues related to the interaction between the ApoBD membrane and AMPs. Finally, the use of ApoBDs loaded with AMPs has potential for the delivery of biotherapeutics because of their advantages in increasing biosafety and enhancing their ability to cross biological barriers. Therefore, the development of new studies with preclinical models should be encouraged. To achieve this, some barriers must be overcome, including the improvement of vesicle isolation, packaging, and proper storage techniques to ensure efficient and stable production to improve the use of ApoBDs as AMPs nanocarriers for the treatment of intracellular infections.

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6. General discussion

This thesis explored apoptotic bodies (ApoBDs) as novel drug delivery vehicles to overcome the limitations of treating persistent intracellular infections caused by *Staphylococcus aureus*. The original research presented in Chapter 1 demonstrates the design, characterization, and therapeutic application of reconstructed ApoBDs (ReApoBDs) loaded with the antimicrobial peptide BotrAMP14 or vancomycin, highlighting their superior intracellular targeting and reduced cytotoxicity *in vitro* and *in vivo*. Complementing these findings, Chapter 2 presents a review of the state of the art regarding ApoBD-AMP formulations, discussing critical factors that influence their efficacy (such as vesicle origin, surface charge, and drug compatibility) and positioning this strategy within the broader landscape of emerging intracellular antimicrobial therapies. Notably, it underscores that despite promising *in vitro* and *in vivo* outcomes, challenges such as AMP-induced vesicle destabilization, variable biodistribution, and limited standardization of vesicle isolation techniques must still be addressed to ensure clinical translatability (Miron et al., 2024). Together, these chapters contribute to the growing field of biologically derived nanocarriers by showcasing ReApoBDs as promising nano-Trojan horses capable of addressing the therapeutic challenges posed by intracellular *S. aureus*. Combining experimental innovation and critical field synthesis, this dual approach establishes ReApoBDs as a feasible delivery platform and tool for analyzing the interplay between vesicle structure, drug characteristics, and intracellular infection dynamics.

A central contribution of the original study lies in the successful generation and biophysical characterization of ReApoBDs derived from BV-2 and HeLa cells, which were engineered to achieve a nanometric size through extrusion and validated by TEM, AFM, and DLS. These vesicles exhibited homogeneous size distributions (~100 nm), moderate polydispersity, and negative zeta potentials, particularly in BV-2-derived formulations. Such surface charge facilitates uptake by phagocytic cells through phosphatidylserine-mediated recognition and contributes to colloidal stability (Gao et al., 1998; Grimsley & Ravichandran, 2003; Kitano et al., 2008; Lemke, 2019; Mevorach, 2000; Szondy et al., 2017). In the context of intracellular drug delivery, this negative surface charge is not only relevant for immune recognition but also for maintaining electrostatic repulsion between particles, minimizing aggregation, and promoting biodistribution (Matsumoto et al., 2017).

These results align with design principles highlighted in the literature reviewed in Chapter 2, where optimal nanocarriers for intracellular delivery are characterized by small, uniform size, negative surface charge, and biocompatibility (Baek et al., 2019; Mendt et al., 2019; Wiklander et al., 2019). Notably, while synthetic nanoparticles often require additional

surface modifications (e.g., PEGylation or ligand attachment) to mimic these properties, ReApoBDs inherently express 'eat-me' signals like PS and ICAM-3, enhancing macrophage uptake without the need for artificial targeting moieties (Alavi et al., 2022; Elena de Souza et al., 2021; Moffatt et al., 1999; Myers & Comolli, 2023; Torr et al., 2012b; Zomorodian et al., 2018). In contrast to synthetic nanoparticles, ReApoBDs also benefit from endogenous membrane components that reduce immunogenicity and enhance biological compatibility, as supported by comparative analyses with liposomes and exosomes discussed in the review (Baek et al., 2019; Mendt et al., 2019; Wiklander et al., 2019). Furthermore, the presence of residual organelles or cytosolic content, sometimes seen as a limitation in EV-based systems, may confer an advantage for therapeutic applications by enhancing the vesicle's capacity for bioactive cargo and mimicking natural apoptotic processes more closely (Jan & Chaudhry, 2019). Thus, the structural attributes of ReApoBDs position them favorably among bioinspired vesicle systems for antimicrobial drug delivery.

The original data presented in Chapter 1 revealed that encapsulation efficiency (EE) and formulation stability were strongly influenced by both the therapeutic agent's physicochemical nature and the apoptotic bodies' source. Vancomycin showed higher EE when encapsulated in ReApoBDs derived from HeLa cells, potentially due to their elevated membrane cholesterol content (Pumerantz et al., 2011), which is known to enhance vesicle stability and facilitate the retention of hydrophilic drugs (De Meyer & Smit, 2009; Pumerantz et al., 2011). In contrast, BotrAMP14, a cationic antimicrobial peptide, was more efficiently encapsulated into ReApoBDs obtained from BV-2 cells. This finding is especially relevant given the amphipathic and positively charged nature of BotrAMP14, which can induce membrane destabilization through electrostatic interactions with negatively charged vesicles, a known challenge in AMP delivery systems (Brogden, 2005).

This difference is best understood by examining the net surface charge of the vesicles. While HeLa cells possess intrinsically more negatively charged membranes due to O-glycosylation alterations in the membrane glycoproteins (Reis et al., 2010) and increased phosphatidylserine (PS) exposure upon apoptosis (Wyllie et al., 1980; Yoon et al., 1996), BV-2 cells have a more neutral lipid composition and generate ApoBDs with lower surface negativity (Marschallinger et al., 2020). Consequently, the reduced electrostatic repulsion between BotrAMP14 and the BV-2-derived vesicles likely contributed to improved membrane stability and higher encapsulation. Moreover, this reduced electrostatic mismatch likely prevents premature vesicle disruption or peptide leakage, which are common drawbacks of encapsulating cationic AMPs in highly anionic systems (Wang et al., 2016). These findings reinforce one of the key messages of the

review in Chapter 2: optimizing drug-vesicle interactions, particularly charge compatibility, is essential for developing stable and effective ApoBD-based antimicrobial formulations.

This trend aligns with prior studies cited in Chapter 1, where stable encapsulation of cationic AMPs such as nisin was more successful in neutral or slightly negatively charged vesicles than in highly anionic systems (Taylor et al., 2008; Were et al., 2003). For example, liposomes rich in phosphatidylcholine (PC) and minimal anionic components achieved higher EE and greater formulation stability than those composed of phosphatidylglycerol (da Silva Malheiros et al., 2010; Taylor et al., 2008; Were et al., 2003). In our study, the superior EE of BotrAMP14 in BV-2 ApoBDs also translated into more stable release profiles, as evidenced by sustained drug liberation over 48 h and reduced colloidal destabilization. Notably, this prolonged release is crucial for maintaining therapeutic concentrations within infected cells, particularly when targeting intracellular pathogens that reside in compartments with limited drug accessibility and slow replication rates, such as SCVs (Brouillette, Martinez, et al., 2004; Tuchscherr et al., 2010; C. Zhang et al., 2019).

These findings emphasize the importance of selecting appropriate donor cell types to tailor the electrostatic environment of ApoBDs, particularly when working with cationic peptides whose interaction with membrane surfaces can determine both encapsulation success and biological performance. This cell-source dependency underscores a broader principle discussed in Chapter 2: biologically derived nanocarriers, while offering inherent biocompatibility, demand rigorous optimization at the physicochemical interface between cargo and vesicle to ensure stability, controlled release, and efficient delivery. Failure to align these parameters can result in reduced therapeutic efficacy and increased cytotoxicity or premature degradation of the active agent.

The *in vitro* infection model presented in Chapter 1 clearly demonstrated the superior performance of ReApoBD-loaded formulations in eradicating intracellular *S. aureus* within macrophages, while maintaining low cytotoxicity. Notably, ReApoBD-BotrAMP14 reduced over 2 Log_{10} CFU in intracellular bacterial load, exceeding the efficacy of free BotrAMP14, and displaying a better antimicrobial-to-cytotoxicity ratio. This enhanced efficacy is likely attributable to both improved cellular uptake via PS-mediated recognition and sustained drug release, which ensures prolonged intracellular exposure without overwhelming host cells (Matsumoto et al., 2017; Miron et al., 2024; Pujol-Autonell et al., 2015; C. Zhang et al., 2019). A moderate positive Pearson correlation between cell viability and intracellular bacterial survival suggested that increased cytotoxicity, while enhancing bacterial clearance, may compromise host cell integrity (highlighting the delicate balance required in intracellular antimicrobial therapies). By providing controlled release and reduced exposure

peaks, the encapsulated formulations helped mitigate this effect. These findings are particularly relevant in the context of SCV-mediated persistence, where treatment failure is often linked to insufficient intracellular drug retention or host cell damage that disrupts immune responses (Brouillette, Grondin, et al., 2004; Joosten et al., 2005; Miller et al., 1978; Sandberg et al., 2011).

Comparatively, Chapter 2 discusses alternative approaches such as self-assembling peptides (e.g., F3FT and N3FT), which demonstrated potent antimicrobial activity but, in some cases, posed higher cytotoxic risks or required higher doses (Y. Zhu et al., 2025). While these peptide-based systems offer advantages in membrane permeation and dual mechanisms of action (e.g., ROS generation and membrane disruption), their lack of targeting specificity can lead to off-target effects, especially in immune cell populations (Y. Zhu et al., 2025). The ReApoBD strategy thus emerges as a more balanced platform, offering effective bacterial clearance with reduced host toxicity, an advantage critical for the treatment of chronic infections where prolonged intracellular residence and immune evasion are major therapeutic hurdles (Leimer et al., 2016; Libraty et al., 2012). By leveraging the natural clearance pathways of apoptotic debris, ReApoBDs enhance delivery precision while preserving the functional integrity of macrophages, an essential feature for achieving long-term control of intracellular infections (Kaufmann, 2011; Le et al., 2017).

Among all the findings presented in this thesis, the *in vivo* efficacy of the ReApoBD-BotrAMP14 nanoformulation represents the most significant and clinically promising outcome. In a murine abscess model, a single subcutaneous dose (0.3 $mg \cdot kg^{-1}$) significantly reduced lesion size by 31.7%, decreased total bacterial burden by nearly 1 Log_{10} CFU, and most notably, reduced the prevalence of *S. aureus* SCVs by over 70%. This is particularly remarkable considering that SCVs are often recalcitrant to conventional antibiotics, and achieving such therapeutic efficacy typically requires prolonged treatment regimens and high-dose systemic delivery (Brouillette, Martinez, et al., 2004; Garcia et al., 2013; Leimer et al., 2016; Vesga et al., 1996). These effects were achieved within a short 72 h without the need for high systemic doses or sustained administration. SCVs are notoriously challenging to treat due to their metabolic dormancy, reduced susceptibility to antibiotics, and their role as reservoirs for recurrent infections. (Conlon et al., 2013; Garcia et al., 2013; Kaiser et al., 2014; Lechner et al., 2012). Their intracellular localization within macrophages and capacity to revert to the virulent wild-type phenotype further complicate therapeutic approaches, making them a critical target for next-generation antimicrobial strategies (Leimer et al., 2016; Tuchscherr et al., 2010).

The ability of ReApoBD-BotrAMP14 to substantially target both the bulk infection and the SCV subpopulation places this strategy at the forefront of emerging intracellular therapies. Moreover, these results suggest that leveraging ApoBD-based delivery enhances drug bioavailability at infection sites and facilitates the disruption of bacterial niches where immune evasion and antibiotic tolerance are maximized. This positions ReApoBDs as a unique therapeutic platform capable of acting like Trojan horses by modulating the infection microenvironment while delivering potent antimicrobials directly to intracellular reservoirs, an innovation with clear translational potential.

This performance contrasts sharply with conventional approaches. Vancomycin-loaded hydroxyapatite, for instance, required prolonged local administration (42 days) at doses ranging from 80,000 to 240,000 $mg \cdot kg^{-1}$ to achieve SCV clearance in chronic osteomyelitis models (Joosten et al., 2005). Similarly, linezolid and dicloxacillin, although capable of reducing intracellular burden, failed to eliminate SCVs in murine peritonitis following single doses (Sandberg et al., 2011). Other agents, such as cefapirin, showed limited efficacy against hemin-dependent SCVs in mastitis models, and combinations like gentamicin with β-lactams remained ineffective against phenotypically persistent forms (Brouillette, Grondin, et al., 2004; Miller et al., 1978). These examples highlight a key therapeutic limitation of conventional antibiotics: their poor intracellular penetration and lack of sustained bioactivity within the hostile environment of the phagolysosome, where SCVs are known to persist (Brouillette, Martinez, et al., 2004; Garcia et al., 2013; Leimer et al., 2016; Tuchscherr et al., 2010). Even antibiotics with documented intracellular activity, like linezolid, are constrained by rapid clearance, limited tissue accumulation, or dose-limiting toxicity, which collectively undermine their ability to eradicate dormant subpopulations fully (Sandberg et al., 2011). Moreover, many of these agents were not designed to bypass endosomal entrapment or exploit natural cellular uptake pathways, a gap that ReApoBDs effectively bridge by mimicking physiological clearance mechanisms. Thus, the efficacy observed with ReApoBD-BotrAMP14 reflects successful antimicrobial delivery and underscores the need to rethink drug distribution paradigms, favoring vehicles that navigate biological barriers rather than relying solely on passive diffusion or extracellular exposure. This shift is particularly urgent given the increasing prevalence of persistent infections, for which existing treatment guidelines often fail to account for intracellular bacterial reservoirs or the pharmacokinetic mismatch between drug and target site (Jalife, 2014; Lechner et al., 2012; Peyrusson et al., 2020; Srinivas et al., 2020b; Y. Zhang, 2014b).

Notably, ReApoBD-BotrAMP14 also compares favorably to other peptide-based nanoformulations. Self-assembling antimicrobial peptides such as F3FT and N3FT achieved impressive intracellular clearance rates (eliminating up to 98.3% of bacteria in

peritonitis-sepsis models at 10 mg·kg^{-1}), outperforming vancomycin due to their superior cellular penetration and dual antimicrobial mechanisms involving membrane disruption and ROS generation (Y. Zhu et al., 2025). However, these peptides still required intraperitoneal delivery and relatively high doses, while the ReApoBD system achieved similar qualitative results with significantly lower peptide concentrations and systemic exposure. This difference is critical when considering clinical translation, as high-dose systemic or intraperitoneal administration of cationic peptides may raise concerns about toxicity, immunogenicity, and off-target effects, issues that are minimized by using biologically derived, macrophage-targeted carriers like ReApoBDs (Yeung et al., 2011; Y. Zhu et al., 2019). Moreover, while F3FT/N3FT act through intrinsic penetrability, ReApoBD-BotrAMP14 achieves targeting via natural macrophage recognition, offering a more modular and potentially tunable delivery strategy (Y. Zhu et al., 2025).

Notably, the modularity of the ReApoBD platform allows not only for drug encapsulation but also for potential surface functionalization, controlled release kinetics, and adaptation to different infection models or therapeutic agents, an advantage not easily achievable with self-assembling peptides whose structure and function are often tightly coupled (Jan & Chaudhry, 2019). This comparative analysis illustrates that while self-assembling peptides hold promise, especially for rapid intervention, they may lack the selectivity and adaptability required for precision therapies targeting persistent intracellular pathogens (Yang et al., 2023).

These comparisons emphasize that the ReApoBD-BotrAMP14 nanoformulation not only meets but exceeds the performance of many current and experimental strategies by effectively targeting intracellular *S. aureus* and its SCV forms with minimal toxicity and logistical burden. As the first formulation to demonstrate such efficacy using remodeled apoptotic vesicles, this nano-Trojan horse opens new avenues for the treatment of persistent infections that have long eluded conventional therapeutics. Nonetheless, important questions remain regarding the scalability, long-term stability, and regulatory acceptance of ApoBD-based systems. Standardizing vesicle production and ensuring batch-to-batch reproducibility will be essential for clinical translation (Nieto Marín et al., 2023b). Moreover, although promising in murine models, the immune dynamics and biodistribution in larger animals or humans may differ substantially, requiring further validation (Bjornson-Hooper et al., 2022; Mestas & Hughes, 2004; Takao & Miyakawa, 2015). Future work should also explore the capacity of ReApoBDs to co-deliver multiple agents (e.g., AMPs and immune modulators) or to respond to microenvironmental triggers for enhanced precision. In this context, ReApoBDs represent a delivery innovation and a

potential platform technology for addressing diverse intracellular pathologies beyond *S. aureus* infections, including other bacterial, parasitic, or even oncologic targets.

A key strength of the ReApoBD-BotrAMP14 formulation lies in its *in vivo* biodistribution profile, which reveals a dynamic and therapeutically relevant pattern of organ targeting. Fluorescence imaging performed between 0 and 12 post-injection showed a clear transition from the strong localized signal at the injection site to systemic redistribution, with peak accumulation in the liver and kidneys at 3–6 h post-administration. This time-dependent redistribution not only reflects the controlled release of the nanoformulation but also underscores the vesicles' ability to remain stable in circulation and evade rapid clearance by the mononuclear phagocyte system, a standard limitation of many nanoparticle-based delivery systems (Mills et al., 2022).

Notably, the formulation exhibited renal accumulation, which may hold particular clinical significance. SCVs of *S. aureus* persist in the kidneys following systemic infections, as shown in rabbit models of endocarditis and osteomyelitis, where these subpopulations remained even after prolonged oxacillin treatment (Bates et al., 2003; Sandberg et al., 2011). These renal reservoirs are notoriously difficult to eliminate due to the immune-privileged microenvironment of the kidneys and the reduced metabolic activity of SCVs, which decreases their susceptibility to antibiotics (Bates et al., 2003; Sandberg et al., 2011). Therefore, the detection of ReApoBD-BotrAMP14 in the kidneys not only confirms its systemic bioavailability but also suggests that this delivery system may reach and act upon critical bacterial reservoirs typically inaccessible to conventional therapies (Bates et al., 2003; Sandberg et al., 2011). This is particularly promising given the growing concern over chronic and relapsing *S. aureus* infections associated with biofilm formation in renal tissues, where SCVs can evade both immune responses and drug action.

Moreover, the minimal accumulation in immune-related organs such as the spleen and strong hepatic and renal signals reflect a favorable clearance profile while avoiding immune overload (Cataldi et al., 2017). This selective organ distribution minimizes the risk of immune activation and off-target inflammatory responses, a known limitation of several synthetic delivery platforms (Mills et al., 2022). The biodistribution pattern aligns with apoptotic bodies' natural biological recognition mechanisms, which exploit "eat-me" signals like phosphatidylserine for targeted uptake by phagocytic cells (Mevorach, 2000). This endogenous targeting capacity is inherently safer and more efficient than receptor-targeting strategies involving antibodies or ligands, which often face limitations such as immunogenicity, reduced receptor availability, or competition with endogenous ligands (Lee et al., 2021; Sousa et al., 2017; Valcourt et al., 2018). This contrasts with synthetic

nanoparticles that require surface modifications to achieve similar specificity (Guerrini et al., 2018). The combined evidence supports the view that ReApoBDs not only effectively deliver their payload into infected macrophages at local sites but also navigate systemically to organs where bacterial persistence is known to occur. In this context, the biodistribution of ReApoBDs is a pharmacokinetic advantage and a strategic alignment with infection pathophysiology, particularly for intracellular pathogens like *S. aureus*, which exploit tissue niches such as the liver and kidneys to establish chronic infections. This targeted delivery, together with the proven intracellular activity of BotrAMP14, reinforces the clinical promise of ReApoBD-BotrAMP14 as a multifocal therapeutic strategy for both local and systemic *S. aureus* infections, including those driven by difficult-to-treat phenotypes such as SCVs. The dual benefit of cell-specific uptake and organ-specific accumulation positions this system as a robust platform for next-generation antimicrobial therapies.

Despite the promising results presented, several limitations of this study must be acknowledged. First, the complexity and heterogeneity of ApoBD preparations may pose challenges for reproducibility and scalability. While the use of differential centrifugation (Phan et al., 2018) and extrusion allowed for size-controlled remodeling of ApoBDs (ReApoBDs) (Bose et al., 2020), batch-to-batch variability in vesicle composition, surface charge, and protein content, primarily between cell types, could impact encapsulation efficiency, stability, and biological performance (Kumar et al., 2022). This variability could also influence interactions with recipient immune cells, potentially altering biodistribution, cellular uptake, and therapeutic outcomes. Moreover, the lack of standardized protocols for ApoBD isolation across laboratories adds a layer of technical variability that could hinder large-scale application or regulatory approval (Aksamitiene et al., 2022; Phan et al., 2020). Additionally, although the study compared BV-2 and HeLa cell-derived vesicles, further exploration of other cell sources, including primary cells, may be needed to assess safety and compatibility in translational settings. Primary human-derived ApoBDs may more closely mimic clinical scenarios, potentially improving immunotolerance and therapeutic predictability in human hosts. However, their scalability and ethical sourcing remain significant hurdles that must be addressed in future research (Aksamitiene et al., 2022; Phan et al., 2020).

Another limitation is the exclusive use of murine models for *in vivo* validation. While the abscess model recapitulates key aspects of localized *S. aureus* infection, it does not fully capture the systemic complexity of chronic or disseminated infections, nor the immunological diversity seen in human patients (Hesketh et al., 2017; Sheet, 2002; Siddiqui & Bernstein, 2010; Syed-Ahmed & Narayanan, 2019). Murine immune systems differ significantly from humans in terms of cytokine expression profiles, macrophage subtypes,

and tissue architecture, which can lead to discrepancies in drug efficacy and host response (Bjornson-Hooper et al., 2022; Mestas & Hughes, 2004; Takao & Miyakawa, 2015). Furthermore, although the biodistribution results suggested targeting of infection-relevant organs (i.e., liver and kidneys), direct *in vivo* quantification of intracellular bacterial loads within these organs was not performed, leaving some uncertainty regarding functional antimicrobial activity at distal sites (Hussain et al., 2018). Future studies incorporating bacterial load measurements in specific organs, alongside imaging and immunohistochemistry, would provide a more complete picture of the nanoformulation's therapeutic action in systemic infection models.

Finally, while this study focused on the encapsulation of a single AMP (BotrAMP14) and one conventional antibiotic (vancomycin), it remains to be determined how broadly applicable the ReApoBD system is to other therapeutic molecules, particularly those with different physicochemical profiles or mechanisms of action. The electrostatic interactions that favor BotrAMP14 encapsulation may not translate equally to peptides or drugs with neutral or anionic charges, hydrophobic cores, or high molecular weights, potentially affecting loading efficiency, vesicle integrity, and intracellular delivery (Schweizer, 2009; Yeung et al., 2011). Moreover, the compatibility of ReApoBDs with biologics such as RNA, proteins, or CRISPR components remains unexplored and could open new therapeutic avenues beyond antimicrobial delivery. Standardized protocols for ReApoBD production, drug loading, and stability assessment will be essential for ensuring consistency and advancing toward clinical-grade formulations. This includes defining critical quality attributes such as vesicle size distribution, surface marker expression, residual apoptotic content, and encapsulation yield, parameters necessary to meet regulatory expectations for safety and efficacy (W. H. De Jong et al., 2008; Ibrahim et al., 2022; Rao & Geckeler, 2011; Zwaal et al., 2005).

Addressing these limitations will translate this bioinspired delivery strategy into a robust and widely applicable therapeutic platform. With further optimization and validation across different drug classes and disease models, ReApoBDs could emerge as a versatile solution for the targeted treatment of intracellular diseases, including infections, cancer, and inflammatory disorders.

This thesis presents compelling evidence supporting reconstructed apoptotic bodies (ReApoBDs) as a novel and effective platform for intracellular antimicrobial delivery. Through the encapsulation of BotrAMP14 and vancomycin, the system demonstrated enhanced physicochemical stability, efficient macrophage uptake, and superior antimicrobial efficacy against *S. aureus*, including its SCV phenotype. The findings from *in*

vitro and *in vivo* models highlight the therapeutic advantage of controlled, targeted drug release and the translational value of leveraging biologically derived vesicles for infection site-specific delivery. Moreover, the use of naturally occurring apoptotic pathways for vesicle targeting offers a biologically coherent mechanism that reduces the need for artificial targeting ligands, potentially minimizing immunogenicity and off-target effects (Lee et al., 2021; Sousa et al., 2017; Valcourt et al., 2018). Integrating original experimental data with a comprehensive field review advances the conceptual and practical foundation for ApoBD-based therapeutics. While limitations remain regarding scalability, *in vivo* complexity, and the generalizability of the platform to other molecules, the overall results strongly support continued development and optimization of ReApoBDs as nano-Trojan horses for the treatment of persistent intracellular infections. Future research expanding this approach to other intracellular pathogens and therapeutic classes could further consolidate ReApoBDs as a flexible and high-impact strategy in nanomedicine.

7. Conclusions and perspectives

This thesis proposes a biologically inspired strategy for intracellular antimicrobial therapy, introducing reconstructed apoptotic bodies (ReApoBDs) as a delivery platform with strong potential against *Staphylococcus aureus*, including phenotypically resistant forms such as small colony variants (SCVs). By combining experimental development with a critical review of the current landscape, this work contributes to the design logic and therapeutic application of cell-derived vesicles loaded with both conventional antibiotics and engineered peptides.

However, several scientific challenges must be addressed to translate these findings into clinically viable therapies. One key issue is the inherent heterogeneity of apoptotic vesicles, which may vary in membrane composition, surface charge, and protein content depending on the donor cell type and apoptotic stimuli. These variables can influence not only drug encapsulation but also biodistribution, immune recognition, and therapeutic efficacy. Future work should aim to define standard markers and biophysical parameters that correlate with optimal vesicle performance, enabling more predictable and reproducible formulations. Additionally, while biodistribution data suggested favorable targeting of infection-relevant organs such as the kidneys, functional validation of antimicrobial action at these distal sites remains an open question. More sophisticated infection models, including chronic, biofilm-based, or disseminated infection systems, will be required to fully evaluate the therapeutic range of these vesicles.

Looking forward, the modular nature of ReApoBDs opens multiple avenues for exploration. Can apoptotic vesicles be functionalized without losing their biological stealth properties? What is the optimal balance between stability and release kinetics for different types of intracellular pathogens? Could these vesicles be used to co-deliver immune modulators or to “re-educate” infected macrophages in diseases where host response contributes to pathogenesis? Addressing these questions will require interdisciplinary efforts bridging cell biology, nanotechnology, and infectious disease pharmacology.

In conclusion, this work provides a foundational step toward redefining intracellular antimicrobial delivery using biologically derived vesicles. While proof-of-concept has been established, the path forward lies in systematizing production, deepening mechanistic understanding, and adapting this strategy to clinically relevant infection scenarios.

8. Appendix 2: articles and book chapters published in indexed journals during the Ph.D. program.

Nanoinformatics applied to smart nanomaterials

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Abstract

Nanotechnology advances have enabled the development of many nanomaterials. Nevertheless, these nano-sized particles may present some limitations related to their therapeutic properties. To overcome these obstacles, the study and development of smart nanomaterials have grown exponentially. Smart nanomaterials can respond to environmental stimuli and, therefore, could be applied as biosensors, antimicrobials, bioimaging, and drug delivery. Above all, drug delivery systems have shown promising results when it comes to transporting one or more therapeutic agents to their target sites. Considering nanomaterials' importance in the biomedical field, a new research area, called nanoinformatics, has emerged. Nanoinformatics is defined as an artificial intelligence applied to nanomedicine, representing a very promising biotechnological strategy to fine-tune smart nanomaterials. Additionally, nanoinformatics uses the available computational tools to build new approaches for the design of safer and more efficient smart nanomaterials for the successful delivery of diverse classes of therapeutics. In this chapter, we will discuss how nanoinformatics has been applied for smart nanomaterials development.

Keywords: Nanoinformatics, Smart nanomaterials, Stimuli, Drug delivery

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

Antimicrobial Chemotherapy | Full-Length Text

Employment of mastoparan-like peptides to prevent *Staphylococcus aureus* associated with bovine mastitis

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AUTHOR AFFILIATIONS See affiliation list on p. 16.

ABSTRACT Bovine mastitis is a frequent infection in lactating cattle, causing great economic losses. *Staphylococcus aureus* represents the main etiological agent, which causes recurrent and persistent intramammary infections because conventional antibiotics are ineffective against it. Mastoparan-like peptides are multifunctional molecules with broad antimicrobial potential, constituting an attractive alternative. Nevertheless, their toxicity to host cells has hindered their therapeutic application. Previously, our group engineered three mastoparan-L analogs, namely mastoparan- MO, mastoparan-R1, and [I[5], R[8]] MP, to improve cell selectivity and potential. Here, we were interested in comparing the antibacterial efficacy of mastoparan-L and its analogs against bovine mastitis isolates of *S. aureus* strains, making a correlation with the physicochemical properties and structural arrangement changes promoted by the sequence modifications. As a result, the analog's hemolytic and/or antimicrobial activity was balanced. All the peptides displayed α-helical folding in hydrophobic and mem- brane-mimetic environments, as determined by circular dichroism. The peptide [I[5], R[8]] MP stood out for its enhanced selectivity and antibacterial features related to masto- paran-L and the other derivatives. Biophysical approaches revealed that [I[5], R[8]] MP rapidly depolarizes the bacterial membrane of *S. aureus*, causing cell death by subsequent membrane disruption. Our results demonstrated that the [I[5], R[8]] MP peptide could be a starting point for the development of peptide-based drugs for the treatment of bovine mastitis, with the advantage of no residue in milk, which would help reduce the use of classical antibiotics.

IMPORTANCE *Staphylococcus aureus* is a leading cause of mastitis, the world´s most important dairy cattle disease. The multidrug resistance and zoonotic potential of *S. aureus*, besides the likelihood of antibiotic residues in milk, are of critical concern to public and animal health. Antimicrobial peptides offer a novel antimicrobial strategy. Here, we demonstrate that [I[5], R[8]] MP is a potent and selective peptide, which acts on
S. aureus by targeting the bacterial membrane. Therefore, understanding the physico- chemical determinants and the modes of action of this class of antimicrobials opens novel prospects for peptide development with enhanced activities in the bovine mastitis context.

KEYWORDS mastoparan-like peptides, bovine mastitis, *Staphylococcus aureus*

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The authors declare no conflict of interest.

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Chapter 8

Molecular Farming of Pharmaceutical Proteins in Different Crop Systems: A Way Forward

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Abstract Molecular farming is commonly associated with the exploitation of plant cell cultures, parts of or whole plants, aiming to obtain products with commercial value. Genetic engineering has made it possible to obtain transgenic plants for agribusiness, with the capability to synthesize biomolecules with high added value and great pharmaceutical interest. Advances in molecular techniques have enabled the large-scale cultivation of plant cells in bioreactors. Besides, genetic engineering has made it possible to use different parts or the whole plant, to produce numerous biomolecules. The production of heterologous protein can be for a short period (tran- 14 sient gene expression) or for a prolonged period (stable gene expression). 15 Historically, plant engineering has been successfully applied to pharmacological protein production utilized for human and veterinary health, as well as in the use of adjuvants for drugs and medical supplies. Over the years, molecular farming products will become increasingly available in the marketplace, and here the use of dif- 19 ferent crop systems and their potential for the production of pharmaceutical proteins will be discussed.

Keywords Plant expression system · Antimicrobial peptides · Heterologous expression · Recombinant DNA · Biofactories

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ACS OMEGA

Article

Anti-Staphy Peptides Rationally Designed from Cry10Aa Bacterial Protein

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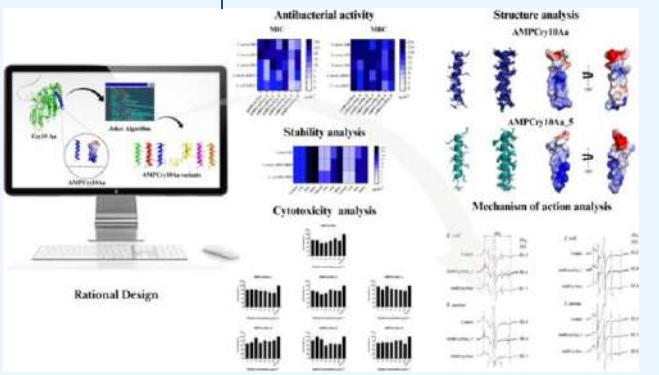

ABSTRACT: Bacterial infections pose a significant threat to human health, constituting a major challenge for healthcare systems. Antibiotic resistance is particularly concerning in the context of treating staphylococcal infections. In addressing this challenge, antimicrobial peptides (AMPs), characterized by their hydrophobic and cationic properties, unique mechanism of action, and remarkable bactericidal and immunomodulatory capabilities, emerge as promising alternatives to conventional antibiotics for tackling bacterial multidrug resistance. This study focuses on the Cry10Aa protein as a template for generating AMPs due to its membrane-penetrating ability. Leveraging the Joker algorithm, six peptide variants were derived from α-helix 3 of Cry10Aa, known for its interaction with lipid bilayers. In vitro, antimicrobial assays determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) required for inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baummanii*, *Enterobacter cloacae*, *Enterococcus facallis*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. Time-kill kinetics were performed using the parental peptide AMPCry10Aa, as well as AMPCry10Aa_1 and AMPCry10Aa_5, against *E. coli* ATCC, *S. aureus* 111 and *S. aureus* ATCC strains showing that AMPCry10Aa_1 and AMPCry10Aa_5 peptides can completely reduce the initial bacterial load with less than 2 h of incubation. AMPCry10Aa_1 and AMPCry 10Aa_5 present stability in human serum and activity maintenance up to 37 °C. Cytotoxicity assays, conducted using the MTT method, revealed that all of the tested peptides exhibited cell viability >50% (IC50). The study also encompassed evaluations of the structure and physical-chemical properties. The three-dimensional structures of AMPCry10Aa and AMPCry10Aa_5 were determined through nuclear magnetic resonance (NMR) spectroscopy, indicating the adoption of α-helical segments. Electron paramagnetic resonance (EPR) spectroscopy elucidated the mechanism of action, demonstrating that AMPCry10Aa_5 enters the outer membranes of *E. coli* and *S. aureus*, causing substantial increases in lipid fluidity, while AMPCry10Aa slightly increases lipid fluidity in *E. coli*. In conclusion, the results obtained underscore the potential of Cry10Aa as a source for developing antimicrobial peptides as alternatives to conventional antibiotics, offering a promising avenue in the battle against antibiotic resistance.

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Research Article

LETTERS IN APPLIED MICROBIOLOGY

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Evaluating virulence features of *Acinetobacter baumannii* resistant to polymyxin B

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Abstract

The increasing resistance to polymyxins in *Acinetobacter baumannii* has made it even more urgent to develop new treatments. Anti-virulence compounds have been researched as a new solution. Here, we evaluated the modification of virulence features of *A. baumannii* after acquiring resistance to polymyxin B. The results showed lineages attaining unstable resistance to polymyxin B, except for Ab7 (*A. baumannii* polymyxin B resistant lineage), which showed stable resistance without an associated fitness cost. Analysis of virulence by a murine sepsis model indicated diminished virulence in Ab7 (*A. baumannii* polymyxin B resistant lineage) compared with Ab0 (*A. baumannii* polymyxin B susceptible lineage). Similarly, downregulation of virulence genes was observed by qPCR at 1 and 3 h of growth. However, an increase in *bauE*, *abaI*, and *pgAB* expression was observed after 6 h of growth. Comparison analysis of Ab0, Ab7, and *Pseudomonas aeruginosa* suggested no biofilm formation by Ab7. In general, although a decrease in virulence was observed in Ab7 when compared with Ab0, some virulence feature that enables infection could be maintained. In light of this, virulence genes *bauE*, *abaI*, and *pgAB* showed a potential relevance in the maintenance of virulence in polymyxin B-resistant strains, making them promising anti-virulence targets.

Impact Statement

Acinetobacter baumannii's significant impact on health stems from its high resistance levels, necessitating the identification of novel treatment approaches for infections caused by this bacterium. Thus, anti-virulence therapy has been studied as a new way to combat infections caused by important pathogens. Evaluating virulence features in *A. baumannii* after it has acquired polymyxin B resistance makes it possible to develop new treatments against strains resistant to this last-resort option. Here, we evaluated potential virulent targets using mortality percentage, virulence expression, and biofilm production analysis.

Keywords: bacteria; anti-virulence; targets; resistance; evolutionary trajector

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Applicability of mouse models for induction of severe acute lung injury

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ABSTRACT

Acute lung injury (ALI) is a significant clinical challenge associated with high morbidity and mortality. Worldwide, it affects approximately 200.000 individuals annually, with a staggering 40 % mortality rate in hospitalized cases and persistent complications in out-of-hospital cases. This review focuses on the key immu- nological pathways underlying bacterial ALI and the exploration of mouse models as tools for its induction. These models serve as indispensable platforms for unraveling the inflammatory cascades and biological responses inherent to ALI, while also facilitating the evaluation of novel therapeutic agents. However, their utility is not without challenges, mainly due to the stringent biosafety protocols required by the diverse bacterial virulence profiles. Simple and reproducible models of pulmonary bacterial infection are currently available, including intratracheal, intranasal, pleural and, intraperitoneal approaches. These models use endotoxins such as commercially available lipopolysaccharide (LPS) or live pathogens such as *Pseudomonas aeruginosa*, *Mycobacte- rium tuberculosis*, and *Streptococcus pneumoniae*, all of which are implicated in the pathogenesis of ALI. Combining murine models of bacterial lung infection with in-depth studies of the underlying immunological mechanisms is a cornerstone in advancing the therapeutic landscape for acute bacterial lung injury.

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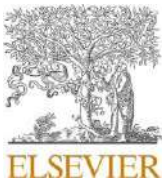

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Nano Trends

Nanoformulations of bioactive compounds derived from essential oils with antimicrobial activity

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ABSTRACT

Nanomaterial-based platforms for the formulation of bioactive compounds derived from essential oils (dEOs) have attracted considerable attention due to their potential to enhance the stability, controlled release, and antimicrobial efficacy of these natural compounds. This review analyzes the various nanomaterial platforms used for dEOs, including polymeric nanoparticles, liposomes, cyclodextrin and chitosan complexes, and inorganic nanosystems. The synthesis methods, physicochemical properties, and characterization techniques associated with these nanomaterial platforms are reviewed to elucidate their impact on the formulation and stability of dEOs. Furthermore, the antimicrobial activity of dEO formulations against a wide range of pathogenic micro-organisms is described, highlighting the potential applications of these platforms in the treatment of infections and food preservation. The review also discusses the current challenges and prospects in the field, aiming to provide valuable insights for researchers and industry professionals involved in the development of nanomaterial-based dEO delivery systems.

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