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Research Paper

Nanoparticle-mediated delivery of the antimicrobial peptide plectasin against *Staphylococcus aureus* in infected epithelial cells

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ABSTRACT

A number of pathogenic bacterial strains, such as *Staphylococcus aureus*, are difficult to kill with conventional antibiotics due to intracellular persistence in host airway epithelium. Designing drug delivery systems to deliver potent antimicrobial peptides (AMPs) intracellularly to the airway epithelial cells might thus be a promising approach to combat such infections. In this work, plectasin, which is a cationic AMP of the defensin class, was encapsulated into poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles using the double emulsion solvent evaporation method. The nanoparticles displayed a high plectasin encapsulation efficiency (71–90%) and mediated release of the peptide over 24 h. The antimicrobial efficacy of the peptide-loaded nanoparticles was investigated using bronchial epithelial Calu-3 cell monolayers infected with *S. aureus*. The plectasin-loaded nanoparticles displayed improved efficacy as compared to non-encapsulated plectasin, while the eukaryotic cell viability was unaffected at the assayed concentrations. Further, the subcellular localization of the nanoparticles was assessed in different relevant cell lines. The nanoparticles were distributed in punctuate patterns intracellularly in Calu-3 epithelial cells and in THP-1 macrophages, whereas A549 epithelial cells did not show significant uptake of the nanoparticles. Overall, encapsulation of plectasin into PLGA-based nanoparticles appears to be a viable strategy to improve the efficacy of plectasin against infections in epithelial tissues.

1. Introduction

Bacterial infections constitute a global health threat and a burden to healthcare systems, primarily due to a rising incidence of nosocomial infections and the spreading of multidrug-resistant bacteria [1]. This serious threat recently compelled the World Health Organization to express its concern for the prospect of a post-antibiotic era in the 21st century [1]. *Staphylococcus aureus* (*S. aureus*) and its methicillin-resistant strains (MRSA) are the cause of a large fraction of infections in both hospital and community settings [2]. Moreover, an estimated 20% of the global population are persistent carriers of the pathogen while 60% are intermittent carriers [3]. *S. aureus* is the most common cause of soft tissue and skin infections [4] and other more severe infections such as endocarditis [5], osteomyelitis [6], bacteremia [7] and pneumonia [8]. Additionally, *S. aureus* is a prevalent pathogen in cystic fibrosis patients, in which it can colonize the lungs for prolonged periods [9]. The persistence of *S. aureus* infections has in recent years been linked to the ability of the bacteria to invade host epithelial cells and survive antibiotic treatment in the intracellular environment [10] as also found for well-known intracellular bacteria such as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Salmonella* spp. The exact mechanism of intracellular persistence of *S. aureus* is not fully understood and its elucidation is ongoing [10].

Notably, intracellular persistence of bacteria can decrease the therapeutic efficacy of antimicrobial compounds considerably due to poor uptake of many antibiotics into infected host cells. Furthermore, the increased enzymatic activity in the infected cells, higher clearance and/or altered antibacterial activity of antibiotics at the micro-environmental conditions (e.g. pH) inside the host cell may diminish their efficacy. Such reduced intracellular efficacy has been reported for many antibiotics commonly prescribed against *S. aureus* infections, e.g. azithromycin [11], vancomycin [12], oxacillin [13] and imipenem [13]. Thus, combined with the overall rapid rise in the occurrence of bacterial resistance, the need for novel treatment strategies is evident and this might be pursued by either

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developing new classes of antibiotics and/or improving drug delivery strategies.

Antimicrobial peptides (AMPs) constitute a promising naturally derived new class of potential antibiotics as they are a part of the innate immune system of virtually all organisms in which they form the first line of defense against foreign pathogens including viruses, fungi, yeast, protozoa and bacteria [14]. Many AMPs are highly potent and associated with reduced potential for development of drug resistance [15] as compared to conventional small-molecule antibiotics currently applied in the clinic. Plectasin, a defensin-class peptide, has proved to be efficacious against several Gram positive bacterial strains [16,17]. Unlike many classical antibiotics [18] its activity toward intracellular *S. aureus* has been reported to be lower than against extracellular bacteria [19]. To overcome such reduced efficacy and improve the therapeutic potential of these next-generation antimicrobials, the present work explored the use of poly(lactic-*co*-glycolic acid) (PLGA) polymeric nanoparticles to improve the cellular uptake of plectasin to treat *S. aureus* infections in epithelial cells. PLGA nanoparticles have been investigated for a variety of local drug delivery applications, i.e. topical [20], ocular [21] and pulmonary [22] delivery. Besides, nanoparticles have been explored for intracellular delivery of different therapeutic molecules including nucleic acids, proteins, peptides, small molecules and antimicrobial compounds such as gentamicin [23,24] and rifampicin [25].

Epithelia in the nasal cavity and lungs are both interesting targets for enhanced drug delivery. Nasal carriage is a known risk factor for secondary infections [26], and thus complete clearance of *S. aureus* in immunocompromised and post-surgical patients may be facilitated by a drug delivery system capable of eradicating intracellular bacteria. In the case of lung epithelia, pulmonary infections are the most common secondary nosocomial infection, and increased incidence of secondary infections has been linked to nasal carriage. This emphasizes the need for development of intracellularly active antibiotics.

In the present work, we investigated the use of PLGA nanoparticles as a drug delivery system for plectasin with a focus on potential application in drug delivery to the epithelia in the airways. The physicochemical properties of plectasin-loaded PLGA nanoparticles, prepared by using the double emulsion method, were investigated with regard to uptake of the nanoparticles, subcellular localization in human epithelial cells and macrophages, and their effect was tested against *S. aureus* in infected airway epithelial cell monolayers.

2. Materials and methods

2.1. Materials

Poly(L-lactide:glycolide molar ratio 75:25) (PLGA75) 20 kDa and polyvinyl alcohol (PVA) 403 were purchased from Kuraray Chemical Co. (Osaka, Japan). Plectasin GFGCNGPWDEDDMQCHNH CKSIKGYKGGYCAKGGFVCKCY; ~4.4 kDa was kindly provided by Novozymes A/S (Bagsværd, Denmark). Cell culturing materials and assay reagents, i.e. Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (pen/strep), L-glutamine, fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), human serum albumin (HSA), phosphate-buffered saline (PBS), F-12K medium, RPMI1640 medium, Mueller–Hinton broth (MHB), gentamicin, Triton X-100, sodium dodecyl sulfate (SDS) and trypsin–EDTA were acquired from Sigma–Aldrich (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) and HEPES buffering salt were purchased from Fisher Scientific (Slangerup, Denmark) and AppliChem (Darmstadt, Germany), respectively. Solvents for analysis were of analytical grade and purchased from Merck (Darmstadt, Germany), ultra-pure water was generated using a Barnstead™ Nanopure™ machine (Thermo Fischer Scientific, Rockford, IL, USA). Low protein-binding plastic ware was used whenever possible.

2.2. RP-HPLC analysis of plectasin

All concentration determinations of plectasin were performed using a Shimadzu RP-HPLC system (Shimadzu, Kyoto, Japan) with a C_{18} column (50 mm × 4.6 mm, 2.6 μm, Kinetex, Phenomenex, Allerød, Denmark) at a constant flow of 1.85 mL/min measuring UV-absorbance at 214 nm. The mobile phase was composed of solvent A (5:95 (v/v) acetonitrile:water with 0.1% (v/v) TFA) and solvent B (95:5 (v/v) acetonitrile:water with 0.1% (v/v) TFA). All samples were run on a gradient from 10% to 40% B over 2 min at 35 °C. Calibration curves were established ($n = 3$) and the limits of detection (LOD) and quantification (LOQ) were determined to 0.4 μg/mL and 1.4 μg/mL, respectively.

2.3. Nanoparticle preparation

Plectasin-loaded nanoparticles were prepared at a theoretical drug loading of 0.625%, 1.25% and 2.5% (w/w, plectasin:PLGA75) using a water-in-oil-in-water (w/o/w) double emulsion solvent evaporation method ($n = 3$). In short, 500 μL of an aqueous peptide stock solution or water (negative control) was added to 500 μL of a PLGA solution (60 mg/mL) in dichloromethane. The primary emulsion was formed by sonication for 60 s using a 600W UP100H ultrasonic processor (Hielscher Ultrasonics, Teltow, Germany) at 60% amplitude (samples kept on ice) after which 1 mL of 2% (w/v) PVA in water was added to the primary emulsion and whirl mixed for 1 min. Another round of sonication was performed to create the secondary emulsion (60 s at 60% amplitude), additional 5 mL of 2% (w/v) PVA in water was added, and the samples were stirred for 1 h at room temperature to complete solvent evaporation. Afterward, the nanoparticles were washed twice with water by subsequent centrifugation (22,000*g*, 12 min) and redispersed using whirl mixing and an ultrasonic water bath until complete dispersion was achieved. The nanoparticles were finally freeze-dried for 24 h after which they were stored at −20 °C until use. All experiments were conducted using the same batches of nanoparticles.

2.4. Physicochemical characterization of nanoparticles

2.4.1. Particle size and zeta potential

Particle size and zeta potential measurements before and after freeze-drying with subsequent redispersion were performed by dynamic light scattering (DLS) ($n = 3$). Freeze-dried nanoparticles were dispersed in water (0.2 mg/mL) by whirl mixing while non-freeze-dried particles were diluted 100-fold after final dispersion (0.3 mg/mL). The mean particle diameter (*Z*-average) and polydispersity index (PDI) were measured by intensity at $\lambda = 633$ nm at 25 °C by using a Malvern NanoZS (Malvern Instruments, Worcestershire, UK) at a 173° scattering angle.

Zeta potential measurements were performed using the Malvern NanoZS on the same batches of nanoparticle dispersions. The performance of the zeta cell and the instrument was verified before use, applying a zeta potential transfer standard purchased from the equipment manufacturer. Size measurements were verified regularly by using a polystyrene particle size standard. Data were analyzed using a standard *t*-test comparing plectasin-loaded nanoparticles against the non-loaded nanoparticles ($P < 0.05$).