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Research paper

Chasing bacteria within the cells using levofloxacin-loaded hyaluronic acid nanohydrogels

E. Montanari [a], G. D'Arrigo [a], C. Di Meo [a], A. Virga [b], T. Coviello [a], C. Passariello [b], P. Matricardi [a,*]

[a] Dept. Drug Chemistry and Technologies, Sapienza University of Rome, Rome, Italy
[b] Dept. of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy

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ABSTRACT

In the present work, an innovative approach based on the delivery of levofloxacin (LVF) from polysaccharide nanohydrogels for the treatment of bacterial intracellular infections is described. The nanohydrogels (NHs) were obtained by self-assembling of the hyaluronic acid-cholesterol amphiphilic chains in aqueous environment. LVF, a fluoroquinolone antibiotic scarcely efficient in intracellular infections, was entrapped within such NHs by nanoprecipitation, thus forming a drug delivery system (LVF-NHs) that was tested for its activity on different bacteria strains. The MIC values of levofloxacin-loaded nanohydrogels were determined for *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains and compared to those obtained using free LVF. The intracellular antimicrobial activity of LVF-NHs and free LVF was compared on HeLa epithelial cell line infected by the above mentioned bacteria, and the increase in antibacterial efficacy of LVF-NHs with respect to that of free LVF was evidenced. The obtained results allow to conclude that this new approach can be considered as really promising method for intracellular infection treatments.

1. Introduction

Antimicrobials inhibit or kill bacteria by selectively binding to some components of the prokaryotic cell, thereby inhibiting the synthesis of vital functional or structural biomolecules, or impeding normal cellular activities. Since the discovery and introduction of antimicrobial drugs in the sixties [1], many diseases of bacterial origin have been routinely successfully treated. Nevertheless, despite many different classes of antimicrobials have been developed in only a few decades, bacteria have rapidly "learned" to resist to these molecules or to escape from their lethal activity in vivo [2–5].

As a consequence, the treatment of common infections induced by both pathogenic or opportunistic bacteria can now be challenged with limited availability of clinically effective drugs.

Moreover, one of the most efficient strategies evolved by bacteria to resist antibiotics is actually represented by internalization into phagocytes and/or epithelial cells of the host, which leads to survival and multiplication [6–8], as the intracellular compartment is not accessible to the immune system. Moreover, most antimicrobials are not able to diffuse through cell membranes due to their physico-chemical features, [9]. Bacteria that are able to penetrate inside host cells, as a consequence, lead to infections that are frequently silent or minimally symptomatic, and recurrent. In these situations cells, including phagocytes, are unable to eradicate the intracellular bacteria and act as reservoirs that contribute to the spreading of the infection to other cells and organs [10].

In this context, the development of antibiotic delivery systems targeting the intracellular compartment is a very promising item to improve treatment outcomes of intracellular infections [11,12]. Drug delivery systems able to carry antibiotics within the intracellular sites of infection can increase the therapeutic index of antimicrobials in intracellular niches, while avoiding problems associated with the prolonged systemic administration of high doses of antibiotics.

Many antibiotic delivery systems are well suited as vehicles to deliver antimicrobial agents. Liposomes have shown good potential in improving the efficacy and tolerability of antibiotics of current use, even though problems concerning their stability during storage and administration require rigorous attention [13–15].

Polymeric microparticles and nanoparticles were developed as an alternative to liposomes in order to solve their stability problems during storage and after administration in biological fluids. Synthetic biodegradable and biocompatible polymers were shown to be effective in encapsulating a great variety of antibiotics and are suitable for intracellular delivery of antibacterial agents

* Corresponding author. Dept. Drug Chemistry and Technologies, Sapienza University of Rome, P.le Aldo Moro, 5, 00185 Rome, Italy. Tel.: +39 06 49913226; fax: +39 49913133.
E-mail address: pietro.matricardi@uniroma1.it (P. Matricardi).

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[16–19]. Lately, nanoparticles based on a squalene–penicilline derivative were developed, their antibacterial activity was tested against macrophage intracellular infection by *Staphylococcus aureus*, and the cell uptake mechanism of such nanosystems along with their intracellular localization was deeply studied [20].

In recent years, encapsulation of antimicrobial drugs in nanohydrogel systems has emerged as an innovative and promising alternative that enhances therapeutic effectiveness and minimizes undesirable side effects of the drugs. Nanohydrogels are hydrogel structures having a mean dimensions in the range of 10–1000 nm [21], showing the advantages of hydrogel features at a nano-length scale. Because of their soft and rubbery consistency, nanohydrogel can be reversibly deformed. Furthermore, they usually show good compatibility with physiological fluids without dissolving and, in some cases, can be spontaneously formed by self-assembling [22]. Drug-loaded nanohydrogel can be loaded with hydrophobic as well as hydrophilic drugs. They can enter host cells through endocytosis [23] and then release the drug payloads to treat microbe-induced intracellular infections.

In the present work, hyaluronic acid cholesterol nanohydrogels (HA-CH NHs) were obtained by self-assembly of the polymer chains, after an appropriate hydrophobic chemical derivatization of hyaluronic acid with cholesterol moieties [24]. NHs were then loaded with the antibiotic levofloxacin (LVF), a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. This fluoroquinolone antibiotic acts by inhibiting DNA gyrase, a type II topoisomerase, which is an enzyme necessary to separate replicated DNA, thereby inhibiting cell division [25]. The efficacy of fluoroquinolone antibiotics led to their use for the treatment and prophylaxis of different bacterial diseases: therapy for the respiratory tract, skin structure, and bone and gastrointestinal infections, as well as urinary tract infections. The extracellular and intracellular antimicrobial activity of the LVF-loaded NHs (LVF-NHs) was studied *in vitro* on *S. aureus* and *P. aeruginosa*, two microorganisms causing a number of difficult to treat, easily recurrent infections, and compared with those of free LVF in aqueous solution.

LVF-NHs represent the first example in literature of polysaccharide self-assembled nanohydrogel loaded with an antibiotic; thus, thank to the advantages of polysaccharide nanohydrogel and their ability to penetrate the host cell by endocytosis, LVF-NHs can represent an innovative strategy for the treatment of intracellular infections.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Hyaluronan tetrabutylammonium salt (HA, $M_\eta = 2 \times 10^5$) was kindly provided by Fidia Advanced Biopolymers, Abano Terme (PD), Italy. Cholesterol (CH), levofloxacin (LVF), N-Methyl-2-pyrrolidone (NMP) N-(3-dimethylaminopropyl)-N′-(ethylcarbodimide hydrochloride) (EDC·HCl), 4-(Dimethylamino) pyridine (DMAP), were Sigma products. Other chemicals were reagent grade and were used without further purification.

Media and reagents for bacterial cultures were Sigma products. The RPMI1640 medium for HeLa cell monolayers cultivation was from PAA Laboratories.

2.1.2. Bacterial strains and cell line

One reference strain of *P. aeruginosa* (PAO1) and two reference strains of *S. aureus* (the methicillin susceptible (MSSA) ATCC 6538P and the methicillin resistant (MRSA) USA300-0114) were used. Strains were preserved deep frozen at −80 °C in Luria Bertani medium (LB, Sigma Chemical Co.) containing 20% glycerol and were isolated for experiments on plates of LB agar.

The human ovarian cancer cell line HeLa was used for experiments. Cell monolayers were cultivated in RPMI-1640 medium containing 1% glutamine and 10% inactivated fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Methods

2.2.1. Hyaluronic acid-cholesterol nanohydrogels preparation and characterization

Cholesterol was chemically conjugated to the carboxylic groups of hyaluronic acid, as described elsewhere [24,26]. Briefly, cholesterol (CH) was previously derivatized with 4-bromo-butyric acid in the presence of EDC·HCl and DMAP in order to obtain a bromo-butyric derivative of CH; then, HA (200 mg) was dissolved in N-methyl-2-pyrrolidone (NMP) (10.0 ml) and the bromo-butyric derivative of CH (34.3 mg) solubilized in 2 ml of NMP was added. The reaction was kept under magnetic stirring for 48 h at 38 °C. An exhaustive dialysis against distilled water (Visking tubing, cut-off: 12,000–14,000) was then carried out and the hyaluronic acid-cholesterol derivative (HA-CH) was finally recovered by freeze-drying. An HA derivatization degree of 20% mol/mol (moles of cholesterol/moles of HA repeating units) was assessed by ^{1}H NMR as previously described [24].

In a typical preparation of HA-CH NHs, 3.0 mg of the obtained polymer (HA-CH) was solubilized in 1.0 ml of NMP (3.0 mg/ml) and then added dropwise to 1.0 ml of filtered distilled water under slow magnetic stirring (120 rpm), thus allowing the polymer self-assembling and the nanohydrogel formation. The resulting NHs suspension was stirred for 10 min at room temperature and then dialyzed (Visking tubing, cut-off: 12,000–14,000) against distilled water for 3 h in order to remove NMP (final NHs concentration in water: 0.5 mg/ml).

NHs size and polidispersity index (PDI) were measured by means of a Dynamic Light Scattering (DLS) using Submicron Particle Sizer Autodilute Model 370 (Nicomp).

2.2.2. Levofloxacin-loaded nanohydrogels preparation and characterization

NHs were loaded with LVF (LVF-NHs). In a typical preparation, 3.0 mg of the polymer (HA-CH) was solubilized in 1.0 ml of NMP (3.0 mg/ml) and then added dropwise to 1.0 ml of filtered distilled water containing 1.0 mg of LVF (w/w 3:1). The resulting NHs suspension was stirred for 10 min at room temperature and then purified from the unloaded LVF and from residual NMP by dialysis (Visking tubing, cut-off: 12,000–14,000) against distilled water for 3 h. At the end of the process, the volume increases up to 6 ml, obtaining a final NHs concentration of 0.5 mg/ml.

Both NHs and LVF-NHs suspensions in distilled water were sterilized by an autoclaving process (121 °C, 1.10 bar, 20 min) using a Juno Liarre autoclave (230 Vac, 50/60 Hz, 12A, 2000 W) [24]. In a typical preparation, 3 ml of NH or LVF-NH suspensions were poured in a closed vial and then autoclaved. The size and polydispersity of sterile NHs were measured by means of a Dynamic Light Scattering (DLS) using Submicron Particle Sizer Autodilute Model 370 (Nicomp). The stability of sterile NHs in water was followed for 30 days at $T = 4$ °C and for 7 days at $T = 37$ °C.

The NHs stability studies in RPMI-1640 medium supplemented with 10% of inactivated FBS were performed at 37 °C. NHs were prepared as above described and the RPMI 1640 medium was added to the NH suspensions in order to obtain the NHs final concentration of 0.5 mg/ml. The NHs size was monitored for 24 h at 37 °C by DLS measurements.