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Research paper

Release behavior and antibiofilm activity of usnic acid-loaded carboxylated poly(L-lactide) microparticles

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ABSTRACT

The use of controlled drug delivery systems could give a significant contribution to the improvement of therapies against biofilm-based infections. The aim of this study was to develop polymer microparticles, based on carboxylated poly(L-lactide)s, to be employed as carriers for usnic acid (UA), a poorly soluble drug possessing antiviral, antiproliferative and wide spectrum antimicrobial activity. Thanks to polymer surfactant-like structure, 2.4 μm-in-size microparticles were obtained by a surfactant-free oil-in-water emulsion/evaporation method. UA was encapsulated into these microparticles with a high loading efficiency (80%). The drug release kinetics was found to be temperature dependent (the released dose increasing with temperature) and showed bimodal release behavior. By polarized optical microscopy observations and the application of kinetics models, the initial burst effect was attributed to the delivery of the drug amorphous fraction while the slower release occurring for longer times to the crystalline one, both entrapped in the polymer amorphous phase. UA-loaded microparticles were able to promote the killing of a 24 h-old *Staphylococcus epidermidis* biofilm more efficaciously than free UA.

1. Introduction

Microbial biofilms are known to be implicated in a large variety of human infections, such as medical device-associated infections, chronic lung infections in cystic fibrosis patients, endocarditis, caries, and chronic wounds infections [1,2].

Biofilms exhibit distinct features with respect to the extreme resistance to antibiotics and antimicrobial agents as well as capacity to evade the host defense mechanisms. The high antibiotic tolerance of bacteria in biofilm has been attributed to various reasons [3], such as (i) the interaction of antibiotics with biofilm matrix components reducing drug diffusion through the biofilm; (ii) the presence of slowly growing or dormant bacterial cells that are not efficiently eradicated by antibiotics targeting cell metabolism (e.g., β-lactam); and (iii) the bacterial mutation frequency in biofilms much higher than in planktonic state due to the endogenous oxidative stress generated by bacterial cells upon exposure to antibiotics.

Therefore, there is an urgent need of either novel antifouling surfaces [4,5] or new antibiofilm drugs [3] as well as more efficacious dosage forms (topical or oral) for improving activity of drugs in the biofilm.

The application of micro- and nano-technology to drug delivery could give a significant contribution to the improvement of therapies against biofilm-based infections. Indeed, drug-loaded nano- or micro-particles could protect the drug, diffuse into the exopolysaccharide (EPS) matrix surrounding bacterial colonies in biofilm, and release the drug in a controlled manner thus increasing drug residence time and efficacy [6–8]. In addition, nano- or micro-particles could permit the use of novel efficacious drugs having limited therapeutic applications due to unfavorable physicochemical properties, such as poor water solubility.

This is the case of usnic acid (UA), a dibenzofuran originally isolated from lichens, poorly soluble in water but well known for its

Abbreviations: UA, usnic acid; PHA, poly(α-hydroxy acid); PLLA, poly(L-lactide); CPLLA, carboxylated poly(L-lactide); DMPA, 2,2-bis(hydroxymethyl)propionic acid; ROP, ring opening polymerization; ATR, attenuated total reflection; DSC, differential scanning calorimetry; SEM, scanning electron microscopy; DLS, Dynamic Laser Diffraction Particle Size Analyzer; POM, polarized optical microscopy; PBS, phosphate buffer solution; MIC, minimum inhibitory concentration; MH, Muller Hinton; RT, room temperature; UAA, usnic acid aggregates; PS, spherulite-like structures; VTF, Vogel–Tamman–Fulcher.

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antimicrobial, antiviral, antiproliferative and anti-inflammatory activities [9]. As for the antimicrobial activity, UA is active against methicillin-resistant *Staphylococcus aureus* [10], *Staphylococcus epidermidis* [11], *Enterococcus faecalis* and *Enterococcus faecium* [12], *Candida* species [13], and *Mycobacterium tuberculosis* [14]. UA has also been reported to control biofilm formation by staphylococcal species [15,16] that are known to be among the most leading causative agents of nosocomial infections [17]. In addition, recently, Gupta et al. [18] showed that UA exerts its antibacterial activity against methicillin-resistant *S. aureus* by disruption of the cell membrane, highlighting a potential UA activity also toward slowly growing or dormant cells present in the biofilm.

The few investigations so far available on UA entrapping in micro- or nano-carriers have underlined the applicative potential of controlled UA-releasing systems since a modulation of UA release kinetics [19] and a reduction of drug hepatotoxicity [20] were found.

In this study, novel microparticulate polymer systems, based on carboxylated poly(L-lactide)s, were developed and employed as carriers for usnic acid.

Poly(α-hydroxy acid)s (PHAs), which include polylactide, polyglycolide and their copolymers, are among the most studied and used synthetic polymers for drug delivery system. In fact, beside the good processability, they do not elicit adverse body tissue reaction and are able to degrade into non-toxic products [21]. Depending on polymer and drug features, different strategies, including drug entrapment, absorption or grafting, have been proposed to obtain drug-loaded PHA nano- or micro-particles [22–24]. Particularly, thanks to simplicity, good loading efficiency and flexibility, the water-in-oil emulsion and the water-in-oil-in-water double emulsion/evaporation methods are the most applied for hydrophilic and lipophilic drugs, respectively [25]. The performance of the release systems obtained by these methods is affected by several factors including the volume ratio between phases, rate of solvent evaporation, surfactant concentration as well as the concentration, molecular weight and structural properties (crystallinity) of the polymer carrier [26–29].

In semicrystalline polymers, as the stereoregular poly(L-lactide) (PLLA), the degree of polymer crystallinity, which can be tuned during microparticles preparation by modulating solvent evaporation rate, can affect not only the microparticle morphology but also the kinetics of drug release [27]. Similarly, Liggins and Burt [26] have found that the diffusion coefficient of Paclitaxel through PLLA microparticles depended on both polymer molecular weight and crystallinity. Also surfactants, routinely used to stabilize the polymer/drug emulsion during microparticles preparation, can influence the pharmaceutical properties of the release system such as biodegradability, biodistribution, drug release behavior, and intra-cellular uptake [30]. Therefore, surfactant removal is often mandatory, especially when toxic [31]. To avoid the use of surfactants, the use of amphiphilic copolymers based on hydrophobic poly(α-hydroxy acid) and hydrophilic blocks has been proposed [32]. Alternatively, Carrio et al. [33] used poly(DL-lactic acid) oligomers endowed with a polar head and a long hydrophobic tail (surfactant-like structure) as stabilizers during the preparation of high molecular weight poly(DL-lactic acid-co-glycolic acid) microparticles by emulsion/solvent evaporation method. Zhang and Feng [34] have used *d*-α-tocopheryl polyethylene glycol 1000 succinate as initiator for PLLA ring opining polymerization to prepare a self-emulsifying copolymer. It has been used to fabricate Paclitaxel-loaded nanoparticles.

In this work, carboxylated poly(L-lactide)s (CPLLAs) were synthesized by ring opining polymerization of L-lactide and 2,2-bis(hydroxymethyl)propionic acid (DMPA), this latter providing the polymer with a polar head. We hypothesized that the surfactant-like structure of the synthesized CPLLAs could allow the preparation of microparticles by water-in-oil emulsion/evaporation method without the use of surfactants. Particularly, a series of CPLLA oligomers with different molecular weight, ranging from about 1500 to 15,000 g mol^{-1}, was prepared. The CPLLA with 3300 g mol^{-1} molecular weight showed the optimal surfactant-like behavior to obtain UA-loaded microparticles. A possible application of these microparticles is in skin wound healing. Indeed, there is increased evidence that microbial biofilms are responsible for the chronic state of venous leg ulcers, diabetic foot ulcers, and pressure ulcers [35]. A number of wound dressings are available nowadays for local wound treatment most of which are based on silver [36,37]. To eradicate the biofilm, drug-releasing dressings should provide a long-term sustained release of drug. To this aim, a strategy consists in embedding drug-loaded microparticles in specific fibers or gels for wound dressing to be placed onto the wound bed in order to provide local and controlled drug release [38,39]. In this study, the kinetics of drug release from UA-loaded microparticles was investigated at different temperatures and the antimicrobial activity of the microparticles was assessed against a strain of *S. epidermidis* both in planktonic and in biofilm state.

2. Material and methods

2.1. Synthesis and characterization of carboxylated polylactides

Carboxylated poly(L-lactide)s with different molecular weight were prepared by bulk ring opening polymerization (ROP) of L-lactide by using 2,2-bis(hydroxymethyl)propionic acid (DMPA, Aldrich) as an initiator and stannous octoate (Aldrich) as a catalyst, according to the procedure reported by Kayaman-Apohan and Akdemir [28]. L-lactide (kindly supplied by Purac Biomaterials, The Netherlands) was purified by crystallization from ethyl acetate (Aldrich) solution. Bulk polymerization was carried out in glass vials by using different L-lactide/DMPA molar ratios (Table 1). After adding the catalyst (1:1000 M ratio with respect to the monomer), the glass vials were flame sealed under vacuum and immersed in a silicone oil bath at 120 °C for 20 h. At the end of polymerization, the product was dissolved in chloroform, precipitated in *n*-hexane (Aldrich) and dissolved again in chloroform. Then, the polymer solution was filtered to eliminate the catalyst solid residues and dried under vacuum at 40 °C. The chemical structure of the synthesized carboxylated poly(L-lactide)s is reported in Scheme 1. Samples were named CPLLA*n*, where *n* is the experimental number average polymerization degree found by NMR analysis.

^{1}H NMR spectra of polymers were recorded in chloroform by using a Varian XL300 (300 MHz) spectrometer. FT-IR spectra were acquired in attenuated total reflection mode (ATR) by using a Thermo Nicolet 6700 instrument equipped with a Golden Gate diamond single reflection device (Specac). Measurements were done at a resolution of 4 cm^{-1} and by co-adding 200 scans.

DSC analysis was carried out by using a Mettler DSC822e apparatus on 3–4 mg of sample under nitrogen atmosphere and at a heating rate of 10 K min^{-1}. The explored temperature range was −30 to +170 °C.

Table 1
Polymerization feed composition, theoretical and experimental number-average polymerization degrees (n_{th} and n_{NMR}) and number-average molecular weights ($M_{n,th}$ and $M_{n,NMR}$).

Sample	L-lactide/DMPA molar ratio	n_{th}	n_{NMR}	$M_{n,th}$	$M_{n,NMR}$
CPLLA*87*	93:1	93	87	13,540	12,673
CPLLA*39*	45:1	45	39	6620	5755
CPLLA*16*	22:1	22	16	3300	2440
CPLLA*6*	11:1	11	6	1720	999