Encapsulation of octenidine hydrochloride into bioresorbable polyesters for extended antimicrobial activity

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ABSTRACT

Nosocomial infections remain a serious threat even for patients of highly industrialized countries. These infections occur on surgical sites and wounds, on catheter entry sites, tubes and other indwelling devices. Medical devices providing local antiseptic action over an extended time period represent an opportunity to prevent such infections. One way to achieve this goal is the encapsulation of active molecules into bioresorbable polymer microparticles, which can locally release the compound of interest along a tunable time span depending on the polymer characteristic. In this work, spray drying is used to encapsulate the broad band antiseptic octenidine hydrochloride into a set of poly(D,L-lactide) (PDLLA) and poly(D,L-lactide-co-glycolide) (PLGA) carrier materials with different molecular weights and chain end-groups, forming redispersable powders. It is demonstrated that the carrier materials bearing acid end-groups provide a significantly larger entrapment efficacy comparing with their ester counterparts independently of carrier composition and molecular weight. The quantitative understanding of the release mechanism allows guiding the selection of suitable encapsulating polyesters to tune initial burst and long time release of a given drug. Finally, it is demonstrated on cultures of *Staphylococcus epidermidis* that the encapsulated and subsequently released OHC has conserved its antiseptic activity, which supports the potential applicability of the approach.

1. Introduction

Nosocomial infections (NI) concern yearly about 8% of patients in Switzerland, and out of 70,000 cases of NI, 2000 are fatal [1]. According to the European Centre for Disease Prevention and Control (ECDPC), these NI cases are either occurring on surgical sites during postoperative phase, or related to the insertion of invasive devices such as endotracheal tubes, vascular and urinary catheters [2,3]. Very often, these infections can be associated with increased bacterial resistance against antibiotics, which may be related to two factors. First, the abusive use of antibiotics in the past century has pressured microbial evolution towards the emergence of multiply resistant strains [4], such as the *Staphylococcus aureus* strains bearing resistance against methicillin (MRSA) in the late 50s and against vancomycin (VRSA) later in the 90s [5]. More recently, the global spread of rifampicin-resistant *Staphylococcus epidermidis* [6] lineages has been evidenced, indicating that the occurrence of such cases is on an increasing trend. Second, the ability of those pathogens to form communities on surfaces and interfaces, commonly called biofilms, further increases their resistance to antibiotics by a factor 1000 compared to the planktonic growth mode [7]. It has been shown that indwelling devices such as tubes, valves and catheters, provide a suitable habitat for biofilm formation and growth, which eventually leads to the device-associated infections [8].

According to Harding et al. [9], one promising approach to counteract this phenomenon will consist in the efficient aseptization of surgical sites over extended time.

Octenidine hydrochloride (OHC) is a bispyridinic cationic surfactant known for its antimicrobial activity since the late 80s. It shows a higher in vitro antimicrobial activity than chlorhexidine, its efficacy against both gram-negative and gram-positive bacteria has been demonstrated [10–12] and unlike the latter, no resistance effects have been observed yet [13]. In addition, its ability to reduce biofilm formation on orthopedical implants has been shown [14], and its in vitro impact against biofilms of vancomycin (VRSA) and methicillin (MRSA) resistant *Staphylococcus* strains has shown to be promising in view of inactivating such biofilms on indwelling devices [15]. For all these reasons it is source of high expectations against nosocomial infections [16].

In terms of administration mode, OHC in aqueous solution is

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sprayed or applied as a gel on open and chronical wounds, mucosae as well as on the skin of premature babies [17]. Nevertheless, in an *in vivo* study where this molecule was parenterally administered, some adverse effects could be evidenced with an extent that depended on the dosage applied [18,19]. Moreover, in its most commonly used aqueous solution, OHC is easily washed off and does not provide efficacy against microbial pathogens over an extended period of time. Indeed, regarding the antiseptics used in postoperative wound management, the treatment is considered successful if it is able to provide antimicrobial activity in a time window within 2–6 weeks. Therefore, the design of a suitable carrier system for this molecule should include two key characteristics: firstly, it should release OHC at a controlled and steady rate to keep its concentration above the minimum inhibitory concentration limit (MIC), and secondly, maintain it below its cytotoxic threshold.

For a longer lasting antiseptic effect on surgical sites, OHC-eluting, biodegradable suture materials made of polyglycolic acid (PGA) were proposed by Obermeier *et al.* [20]. In the same field of application, wound dressings containing solid formulations of OHC and bacterial nanocellulose [21], as well as a combinations of nanocellulose and amphiphilic copolymers [22] have been developed. For the control of the biofilm formation on orthopedic implants, some promising results were obtained by Weckbach *et al.* [23], who developed a formulation consisting of a combination of OHC and a poly(methyl metacrylate)-based bone cement. The authors demonstrated with an elution study over 24 h that the released amount was effective against *Staphilococcus aureus* and *Pseudomonas aeroginusa*.

In order to achieve extended release, bioresorbable aliphatic polyesters such as polylactic acid (PLA), poly(lactic-co-glycolic)acid (PLGA) or poly-ε-caprolactone (PCL) have been considered as carrier materials for active pharmaceutical ingredients (APIs) since 1967 because of their unique features [24]. These materials gained a leading role in this field, as they naturally degrade in the human body through hydrolysis, and since their repeating units are similar to naturally occurring metabolites, their hydrolytic degradation leads to biocompatible byproducts [25], which ensures safe and non-toxic bioresorption *in vivo* [26]. Besides their biodegradability and non-toxicity, they exhibit great versatility: indeed, tuning the polymer composition and molecular weight, and therefore its degradation kinetics and drug diffusivity, enables a properly engineered release that assures the attainment of therapeutic concentration over the desired time span. Furthermore, drug – eluting devices thereof allow to minimize the amount of administered drug, which prevents the development of pathologic resistances that would lead to the long term inefficacy of the antimicrobial agent [27]. Additionally, the release control allows to limit possible side effects due to concentrations exceeding the cytotoxic threshold. In this respect, the combination of drug agents with bioresorbable aliphatic polyesters has emerged as a major methodology to achieve controlled and targeted release [28], and as such, bioresorbable aliphatic polyesters have been comprehensively reviewed [29–31] and extensively employed in drug delivery applications [32–34].

In line with this idea, Baier et al. [35], developed a carrier system for OHC based on enantiopure poly(L-lactic)acid nanoparticles. The latter were subsequently incorporated into polyvinyl alcohol (PVA) fibers via electro spinning to form a drug delivering composite for wound dressings. Within the same study, the authors emphasized the role played by enzymatic degradation of the carrier material in the release kinetics of the API by using Proteinase-K as model enzyme.

Nevertheless, it has been pointed out that nanoparticles, due to their small size, tend to be washed away from the surgical sites and may accumulate in the liver and lymph nodes [36]. Therefore, a larger carrier size might provide some practical advantage when localized delivery is desired.

Numerous methods for the encapsulation of APIs into polyester microparticles have been proposed [29–31,37]. They can be classified into emulsion based methods [38,39], coacervation [40], and spray drying [41,42]. When dealing with hydrophobic components which however exhibit some water solubility, the use of an aqueous phase during the encapsulation process may cause a loss of the API during particle preparation [43]. Furthermore, surfactant molecules are needed in order to suppress aggregation, which requires additional care since their pharmacological compliance needs to be established. Moreover, the obtained products need to be dried in order to obtain a powder, which adds up to the complexity of the manufacturing process and can lead to product coagulation. Spray drying is a technique that bears several advantages: first, the encapsulation of APIs into microparticles can be done without going through an emulsion step, thus avoiding the addition of surfactants that would enhance product complexity. Secondly, the encapsulation process involves one single step, which reduces manufacturing complexity [44], and makes it more suitable to sterile operation [45]. Nevertheless, significant differences in encapsulation efficiency and release kinetics were observed, depending on the combination of API and carrier material used [46–48].

In this study, we apply spray drying for the encapsulation of OHC into biocompatible polyesters. As carrier material, we use a series of commercially available biodegradable aliphatic polyesters with different chain compositions, chain lengths and end group chemical structures (ester or carboxylic acid). For the sake of this study, we focus on racemic poly(D,L-lactide) and poly(D,L-lactide-co-glycolyde), which are known to be completely amorphous in contrast to their enantiopure counterparts, that bear crystalline domains [26]. The obtained products are subsequently evaluated with regard to their drug release behavior within the first 24 h and up to three weeks. Finally, the antimicrobial activity of the released drug is assessed via cultures of Staphylococcus epidermidis in view of the potential application of the product on surgical sites.

2. Materials and methods

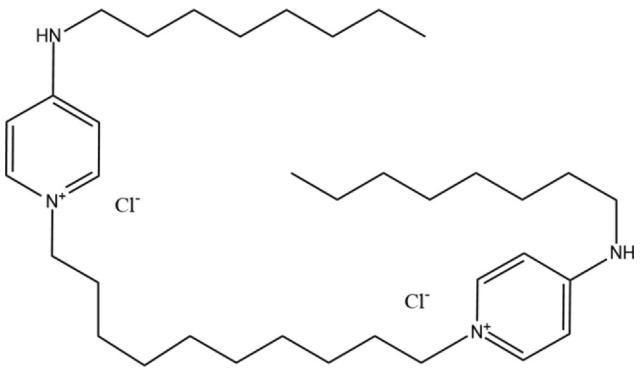
2.1. Materials

Octenidine hydrochloride, depicted in Scheme 1, was kindly provided by the manufacturer (Schülke & Mayr GmbH, Germany).

The solvents tetrahydrofuran and methanol were HPLC grade (VWR Int., Radnor PA, USA). As solvent for the particle preparation, HPLC grade dichloromethane was used (Fisher Scientific, Hampton NH, USA) whereas citric acid and the Tris-base were purchased from Sigma Aldrich (Sigma Aldrich, St Louis, USA) and Biosolve (Biosolve, Dieuze, France). The employed Proteinase-K was bought from ProSpec (ProSpec-Tany Techno Gene Ltd, Rechovot, Israel). The polyesters PDLLA and PLGA were purchased from Sigma Aldrich. The employed materials and manufacturer specifications are summarized in Table 1.

2.2. Spray drying

For each preparation, a solution of 1 g of polyester and 100 mg of OHC dissolved into 250 mL dichloromethane was used. The particle



Scheme 1. Chemical structure of Octenidine hydrochloride.

Table 1
Polyesters used in this study along with the manufacturer indicated weight average molecular weight and end-group structure.

Name	Commercial name	Polymer	Mw (kDa)	End group
PdLA small acid	Resomer R 202 H	Poly(D,L-lactide)	10–18	−COOH
PdLA small ester	Resomer R 202 S	Poly(D,L-lactide)	10–18	−COOEt
PdLA large acid	Resomer R 203 H	Poly(D,L-lactide)	18–24	−COOH
PdLA large ester	Resomer R 203 S	Poly(D,L-lactide)	18–24	−COOEt
PLGA small acid	Resomer RG 502 H	Poly(D,L-lactide-co-glycolide, 50/50)	7–17	−COOH
PLGA small ester	Resomer RG 502	Poly(D,L-lactide-co-glycolide 50/50)	7–17	−COOEt
PLGA large acid	Resomer RG 503 H	Poly(D,L-lactide-co-glycolide 50/50)	24–38	−COOH
PLGA large ester	Resomer RG 503	Poly(D,L-lactide-co-glycolide 50/50)	24–38	−COOEt

preparation was done on a Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with an inert loop for the solvent recovery. The liquid feed rate was set at 10 mL/min and the process was driven at an inlet temperature of 50 °C. The carrier nitrogen gas flow was set at 0.3 m^3/h while the aspirator flow rate was set at 37 m^3/h.

2.3. API release experiments

For the release experiments, a Tris-HCl buffer at pH of 7.4 was used. In the following, this buffer is referred to as release buffer. When specified, solutions of Proteinase-K with concentrations of 10 mg/mL in this same buffer were used as release buffer. Each experiment consisted in suspending ca. 10 mg of powder into 1.5 mL of release buffer and charged into 1.5 mL Eppendorf vials. The vials were then placed into an incubator at 37 °C and continuously shaken along there longitudinal axis. The sampling was achieved by first centrifuging each sample at 17,000 rcf for 2 min and subsequently collecting 1 mL of supernatant with the help of a syringe, and replacing the sampled volume by fresh release buffer. Each release experiment was done in triplicate.

2.4. Antiseptic activity

The antiseptic activity of the encapsulated formulations was measured on *Staphylococcus epidermidis*. The bacteria used were stored on a porous bead support at −80 °C. Bacterial suspensions were prepared in sterilized Tris-HCl at pH 7.4. These suspensions were mixed with 1 mL of suspensions containing encapsulated octenidine in Tris-HCl and 150 µL aliquots of the resulting suspensions were placed on irradiated agar plates (CT3P, Biomerieux, Marcy-l'Étoile, France) in triplicate. The plates were then stored in the incubator (37 °C, 5% CO_2) for 24 h to allow for bacterial growth and the resulting bacterial colonies were counted.

2.5. In vitro cytotoxicity test

In vitro cytotoxicity of OHC was assessed using the THP-1 cell line. The cells were routinely cultivated in RPMI medium 1640 (Termofisher, USA) with 10% v/v of FBS (Termofisher, USA). To evaluate the cytotoxicity, the cells were seeded on a 24-well plate. Different volumes of sterile solutions of OHC in Tris-HCl buffer (pH 7.4) were added in order to achieve the targeted concentrations of OHC inside the wells and subsequently placed in the incubator (37 °C, 5% CO_2) for 72 h. Then, cells were counted using CEDEX HIRES Analyser (Roche, Basel, Switzerland) to assess their viability. A control experiment was performed, where only sterile Tris-HCl buffer was added to the well. All experiments were performed in duplicate.

2.6. API concentration measurement

The OHC concentration was measured via reverse phase chromatography using a slight modification of a published method [35]. The column used was a Phenomenex Jupiter C18 (Phenomenex, Torrance, USA) and the HPLC system was an Agilent 1100 Series (Agilent Technologies, Santa Clara, USA). As mobile phase, a mixture of methanol and McIlvain buffer of pH 2.2 at a volumetric ratio of 75 : 25 was used. The system was operated at 1 mL/min and the detection of OHC was done via UV at 285 nm.

2.7. Polymer molecular weight distribution

The molecular weight of the polymer was measured via GPC by modifying a method already published elsewhere [49]. For the analysis, three PLGel columns were set up in series. The measurements were done on an Agilent 1200 Series HPLC system. As mobile phase, tetrahydrofuran was used, which was pumped at 1 mL/min and room temperature. The instrument was calibrated with polystyrene standards ranging from 0.162 to 6870 kDa purchased from Agilent Technologies. The detection of the polyester samples was performed via refractive index measurements.

2.8. SEM and STEM/EDX micrographs

Prior to the imaging via SEM, the conductivity of the samples was ensured by coating them with a 4 nm layer of Pt and Pd with the help of a CCU-010 HV sputter coater unit (Safematic, Bad Ragaz, Switzerland). The SEM micrographs were acquired on a Leo 1530 (Zeiss, Oberkochen, Germany) at an extraction voltage of 3 kV and an aperture size of 30 µm. Both in-lens and secondary electron (SE2) detection modes were used.

For the STEM/EDX micrographs, the particles were placed on the TEM grid, the extraction voltage was set at 200 kV, and an acquisition time of 10 min was applied for the detection of nitrogen and chlorine.

2.9. Particle size measurement

For the particle size determination, ca. 10 mg of powder was suspended in 100 mL of deionized water and subsequently placed into an ultrasonic bath for 2 min (Branson, Switzerland). The sizing was achieved via small angle static light scattering and the data were treated according to *Mie*-theory, using a refractive index of 1.44.

2.10. Composition analysis

The nitrogen content of the samples was analyzed via micro-elemental analysis on a TruSpec Micro (LECO, Saint Joseph MI, USA).

3. Results and discussion

3.1. Particle morphology and OHC content

The spray-dried products consisted in spheres characterized by a relatively broad size distribution, as it is seen in the SEM micrographs of the two acid and ester-ended polylactic particles, namely PdLA large acid and PdLA large ester, shown in Fig. 1a, b, d and e. Moreover, Fig. 2b and d indicate that some aggregation of the particles did also occur.

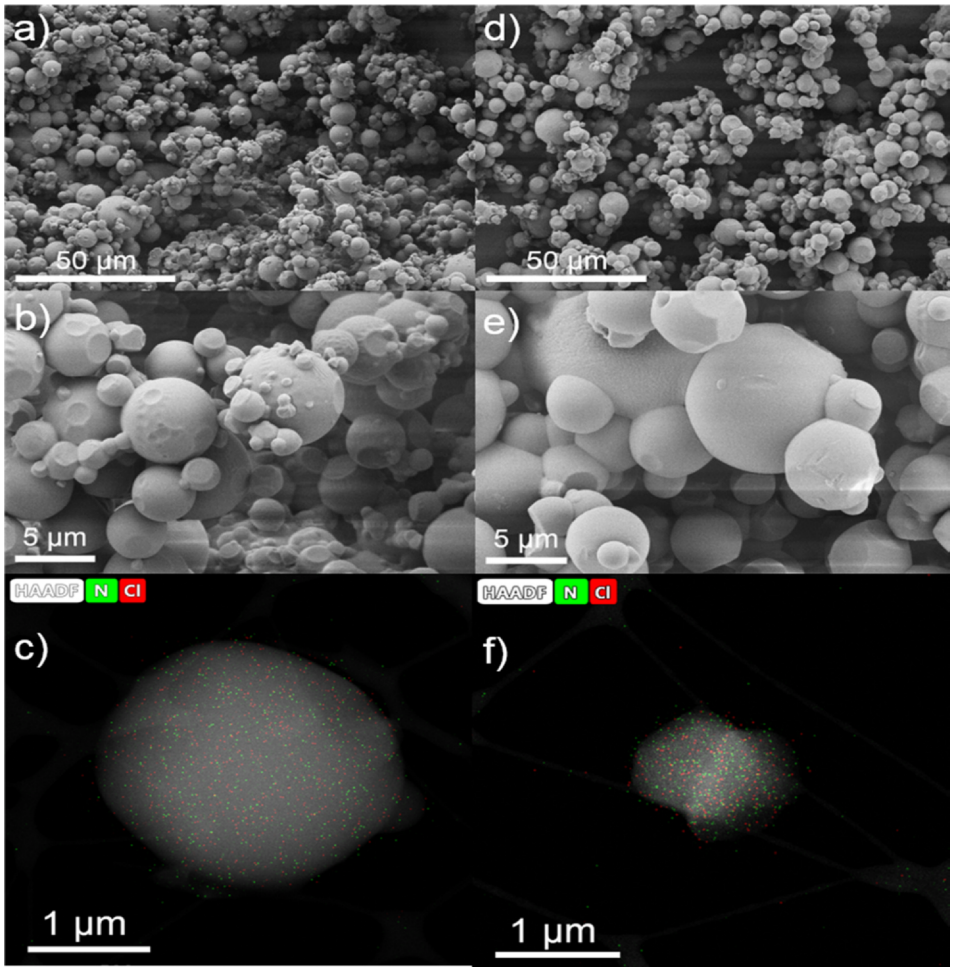


Fig. 1. SEM and STEM-EDX micrographs of the product obtained with PdLA large acid (a, b, c) and PdLA large ester (d, e, f).

These data are in line with the SASLS measurements, evaluated using *Mie*-theory and reported in Table 2 in terms of 50% (d_{50}) and 90% (d_{90}) cumulative diameters. It is seen that all samples exhibit d_{50} values around 15 µm except the two PLGA based materials (PLGA large acid and PLGA large ester) for which this is significantly smaller. On the other end, only small differences between the two acid or ester-ended analogues could be evidenced.

Samples of acid and ester-ended analogues (PdLA large acid and PdLA large ester) were also analyzed with respect to their internal content of chlorine and nitrogen in order to locate OHC inside the particles via STEM/EDX. The obtained results are shown in Fig. 1c and d. The distribution of both elements appears to be homogeneous throughout the particles, thus indicating a minor effect of the chain-end composition also on the particle morphology. Finally, the overall OHC content of the powders was evaluated through the determination of the nitrogen content and the corresponding results are summarized in the second column of Table 2. These correspond to a large OHC encapsulating efficiency after spray drying ranging from 88% to 99%.

3.2. Release mechanism

In general, the release of an API from a biodegradable microparticulate carrier involves different physico-chemical mechanism and very often the rate limiting one changes during the release process [28]. Accordingly, a three-step release process is most commonly observed [50]. Phase I is usually referred to as burst release. The duration of this phase is in the order of 24 h or a few days at most [50,51]. It is usually ascribed to the desorption of the API from the surface of the carrier, or

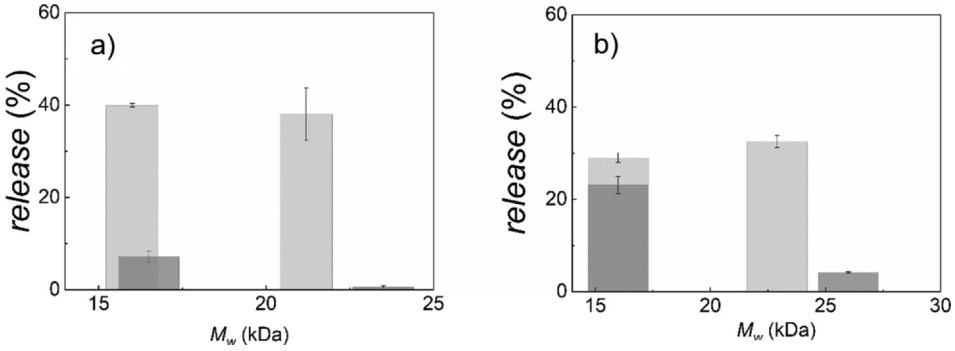


Fig. 2. Initial burst release for the tested poly (D,L-lactide) in (a) and poly(D,L-lactide-co-glycolide) carrier systems in (b) as a function of the initial weight average molecular weight of the carrier material. The dark bars refer to carrier materials bearing acid end groups, and the light grey bars to the ester ended ones. All tests were carried out for 24 h at 37 °C.

Table 2
OHC content, 50% and 90% cumulative diameters particle size and measured initial weight average molecular weight for the considered spray-dried powders and the corresponding relative amount of OHC released over the first 24 h.

Carrier material	OHC (% wt)	d_{50} (μm)	d_{90} (μm)	Mw (kDa)	Release over 24 h (%)
PdLA small acid	8.89	16.2 ± 1.3	38.8 ± 4.1	16.5 ± 0.8	7.1 ± 1.2
PdLA small ester	8.66	17.1 ± 6.1	41.4 ± 11.7	16 ± 3	39.9 ± 0.4
PdLA large acid	8.83	17.6 ± 0.4	38.2 ± 2.5	23.5 ± 0.9	0.6 ± 0.3
PdLA large ester	9.05	14.8 ± 1.2	34.1 ± 2.9	21.2 ± 0.2	37.7 ± 5.6
PLGA small acid	8.27	15.6 ± 0.6	35.6 ± 2.4	16 ± 2	23.1 ± 1.8
PLGA small ester	8.00	12.3 ± 0.9	34.0 ± 5.9	16 ± 1	29.1 ± 1.0
PLGA large acid	8.55	8.3 ± 0.4	25.0 ± 5.7	26 ± 1	4.2 ± 0.2
PLGA large ester	8.27	7.5 ± 0.2	21.6 ± 1.3	22.9 ± 0.2	32.5 ± 1.3

its diffusion from pores close or initially connected to the surface [52]. The amount released during this phase depends strongly on the combination of API and carrier material. Although no theory is available to quantitatively explain this process [46–48], it is expected that an increased density of polar functional groups within the carrier material increases its swelling in aqueous solutions [53], thus facilitating the API release [54]. On the other hand, in the case of API containing polar groups, their binding to increasingly polar materials becomes stronger [55]. Phase II consists in slow and almost no release, typically ascribed to intraparticle diffusion [56]. This phase is also often referred to as a lag-time [56], since the actual release is minimized by the steric hindrance between the high molecular weight polymer chains and API. Phase III is faster, and is promoted by the wider diffusive paths which are made available as a consequence of the carrier degradation and dissolution, as typically occurs for polyester in aqueous solutions. In the following, we analyse the release behavior of the prepared materials with reference to two phases, that is the initial burst and the long-time release.

3.2.1. Initial burst release

The weight percentage of OHC released during the first 24 h is reported in Table 2 for all materials and shown in Fig. 2 as a function of the initial weight averaged molecular weight of the respective polyesters. Fig. 2a refers to the poly(D,L-lactide) and Fig. 2b to the poly(D,L-lactide-co-glycolide) based carrier materials, in both cases, dark grey indicates acid end groups and light gray ester end groups. It is seen that in both cases the end group plays a significant role. In the case of poly (D,L-lactide) in Fig. 2a, the acid end-capped high molecular weight material exhibits a release of 0.6% whereas its ester-ended analogon reaches up to 37.7%. The same trend is observed in the case of poly(D,L-lactide-co-glycolide) in Fig. 2b, where the larger molecular weight samples exhibited 4.2% and 32.5% for the acid and ester-ended materials, respectively.

Since no significant difference in the particle morphology or size could be evidenced in the SEM micrographs in Fig. 1a, b, d, e and in the data in Table 2 for these two pairs of analogues, it can be concluded that the in initial burst releases cannot be related to the particle specific surface area. Also the mechanism proposed by Lee *et al.* [57,58] based on different concentration distributions of the API within the particles, can be excluded in this case. The two polymer end groups do infact exhibit similar homogeneous distributions of the API as observed above in the context of Fig. 1c and f.

Tzafriri [59], proposed a mechanism based on the coexistence of two pools of API, one where the API is able to freely diffuse from the pores to the outer medium, and the other where the API is either physically or chemically immobilized, and therefore not or less available for a burst release. Different interactions between the API and the carrier material [47], would then result in different distribution of the API in the two pools and then in different extent of the burst release. This effect is also suggested by Prior *et al.* [55], to explain similar differences in the initial burst release of gentamicine sulfphate from poly (D,L-lactide) and poly(D,L-lactide-co-glycolide) carriers. In our case, at the applied pH of 7.4, all carboxylic groups that are in contact with the release buffer are expected to be dissociated and negatively charged, since a pKa of about 3 is expected for terminal carboxylic acid groups on polylactic acid [60]. Since OHC is introduced in chloride salt form, it dissociates into cations leading to strong interactions with the carboxylic end groups of the carrier system leading to a better entrapment compared to the ester ended analogon.

The data in Fig. 2a and b also show that the molecular weight of the carrier affects significantly the initial burst for both PLGA and PDLA in the case of carboxylic acid ends, but not in the case of ester ends. This effect has already been reported in the literature [54,61] and is related to the extent of swelling with acid end groups in water, which is larger for smaller molecular weights as evidenced by Sharp *et al.* [53]. This leads to water-filled channels [62], which facilitate the transport of the drug to the surrounding liquid [54]. Conversely, the more hydrophobic ester end groups have a lower effect on swelling and consequently the burst release is less affected by their density, that is by the polymer molecular weight. On the whole they exhibit a larger initial burst release, since this is no more hindered by electrostatic interactions, which is slightly higher for PDLA, due to its higher hydrophobicity and thus decreased affinity to the hydrophilic API.

As a matter of comparison, considering the initial powder contents reported in Table 2, it is possible to compute the powder concentration in solution that would produce, right after the initial burst, the same OHC solution concentration of 0.1% wt as in the commercially available format Octenisept®. In the case of the material composed of PLGA large acid, a solution with a powder concentration of 28% wt would fulfill this condition, while for PdLA large acid, such a concentration cannot be achieved: a minimum dilution of this powder would in fact lead to a OHC concentration after the initial burst of 0.05% wt.

3.2.2. Long time release - up to three weeks

As mentioned above, in the case of polyester materials, the second and the third release phases rely on slow intraparticular diffusion and the hydrolytic degradation of the carrier material, respectively.

The release kinetics of the considered poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) systems up to three weeks are shown in Fig. 3a and b, respectively. Fig. 3a shows that, after the initial burst, the OHC encapsulated into poly(D,L-lactide) is not released further in the investigated time interval. Such a long lag-time was already observed by Johnson *et al.* [56], and it indicates that the intraparticle diffusion of OHC within this carrier is strongly hindered. Moreover, Thomasin *et al.* [46], observed that no significant degradation occurs before 50 days for unloaded poly(D,L-lactide) of 14 kDa molecular weight. Conversely, substantial release is observed in Fig. 3b for the case of acid-ended poly (D,L-lactide-co-glycolide), although not for the ester-ended analogon. This difference between the poly(D,L-lactide) and the poly(D,L-lactide-co-glycolide) acid-ended materials is due to their different hydrophilicity leading to different degradation kinetics.

Strikingly for both acid-ended poly(D,L-lactide-co-glycolide) carrier systems, a sigmoidal release profile is observed, indicating that both systems entered the third release phase. The strong difference in release

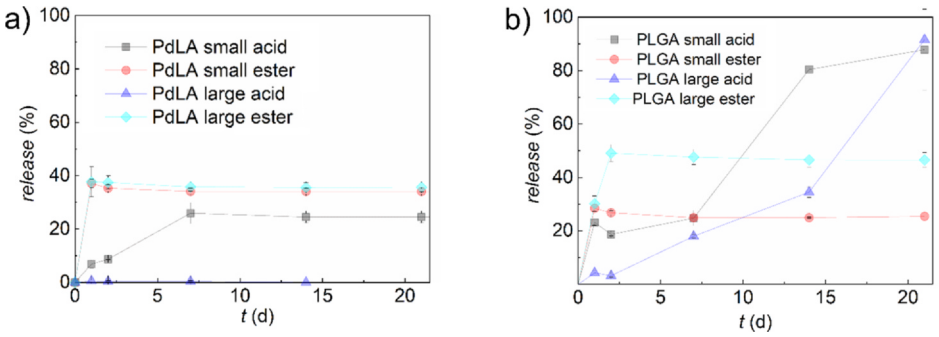


Fig. 3. OHC release during incubation in Tris-HCl at 37 °C over three weeks for the case of (a) poly(D,L-lactide) and (b) poly(D,L-glycolide).

rate between acid and ester-ended analogues was reported earlier by Ertle *et al.* [63] and essentially relies on the difference in degradation rates. This difference is caused by the catalytic effect exerted by the carboxylic end group on chain scission [64]. Both release kinetics are again in line with the findings of Thomasin *et al.* [46], on the degradation kinetic of unloaded poly(D,L-lactide-co-glycolide) of comparable molecular weight. Morever, the reported degradation time are comparable to the release time showed in Fig. 3b, thus confirming that these two systems entered indeed phase 3 of the release process.

3.2.3. Role of enzymes

The presence of suitable enzymes may strongly accelerate the polyester degradation kinetics, as for example in *in vivo* systems [25], and consequently the release rate of the carrier materials considered in this work. In the case of poly(D,L-lactide-co-glycolide), protease does not affect the release kinetics since it has been shown not to degrade this substrate [65]. On the other hand, the protease mediated degradation of poly(D,L-lactide) is quite significant [35]. The effect of Proteinase-K on the release kinetics of the poly(D,L-lactide) samples was assessed and the resulting profiles are shown in Fig. 4a. It is seen that in the presence of the enzyme the poly(D,L-lactide) samples with acid end groups release continuously over the entire investigated time period, while the corresponding material with ester end group does not release any drug after the first initial burst. This can be explained by considering the molecular weight distribution of the carrier materials before and after 4 weeks incubation time with Proteinase-K shown in Fig. 3b, c and d. While both acid bearing samples show significant degradation, attested by a major decrease in molecular weight, the sample bearing ester groups exhibits an unchanged molecular weight distribution. It was demonstrated by Kemme *et al.* [65], that the end groups affect the ability of lipases and esterases to hydrolytically degrade such materials, which could also be the case for Proteinase-K.

3.3. Antimicrobial activity and cytotoxicity

The minimal inhibitory concentration of OHC able to provide antimicrobial activity was evaluated on *Staphylococcus epidermidis*. Through the agar plate culture tests described in the experimental section, we found that a concentration of OHC down to $5{\cdot}10^{-4}$ mg/mL inhibited bacterial growth completely as can be seen by comparing the agar plate in Fig. 5a with the corresponding control in Fig. 5c which yielded 138 ± 24 colonies. This OHC concentration is below the values found by Van Meurs *et al.* [66], (0.033 mg/mL) and by Müller *et al.* [67], (0.0175 mg/mL on *Staphylococcus aureus*) but in the same order of magnitude of the values of antimicrobial efficacy reported by Hubner et *Al* (1 μg/ml). In the first two cases, the tested microorganisms were first allowed to grow in broth cultures for 24 h, whereas in the present work it and in the last example was not the case. These differences in the

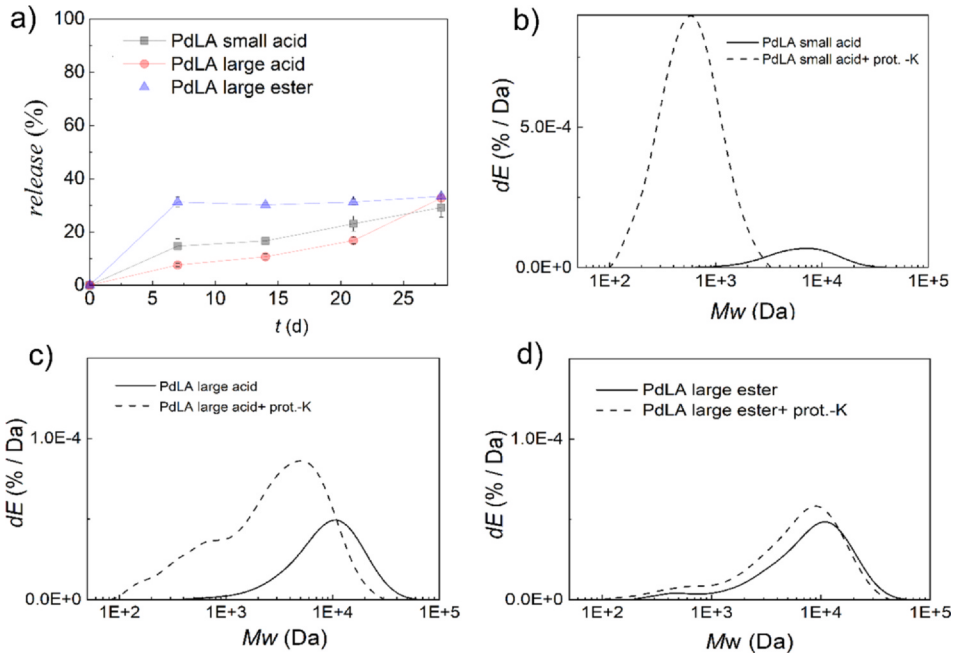


Fig. 4. (a) OHC release in solution with 10 mg/mL Proteinase-K over 4 weeks; (b, c and d) Molecular weight distribution before and after 4 weeks incubation in Tris-HCl with 10 mg/mL Proteinase-K for three poly(D,L-lactide) carrier systems.

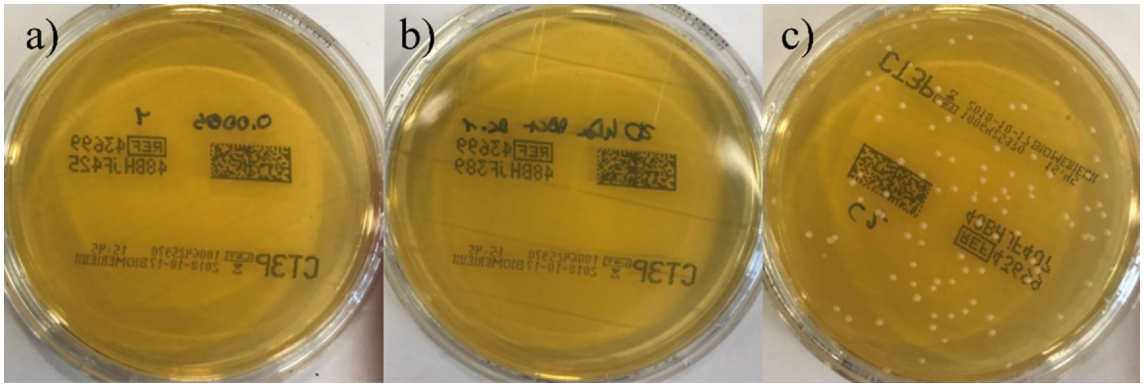


Fig. 5. Cultures of *Staphylococcus epidermidis* with (a) OHC solution of $5{\cdot}10^{-4}$ mg/mL (b) supernatant of the PdLA large acid after 5 weeks release time in Tris-HCl with 10 mg/mL Proteinase-K and (c) Tris-HCl (control).

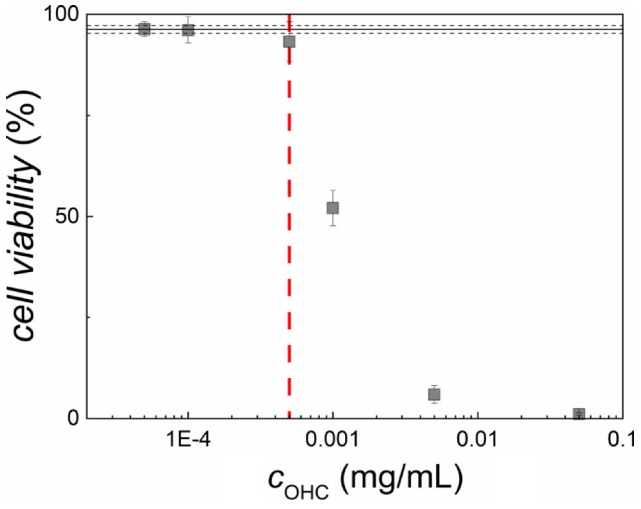


Fig. 6. Cytotoxicity curve of OHC on THP-1 cell lines in a range of concentration between $5{\cdot}10^{-5}$ mg/mL and $5{\cdot}10^{-2}$ mg/mL. The vertical red line represents the minimal concentration at which no bacterial growth was observed, while the horizontal lines shows cell viability with addition of Tris-HCl containing no OHC (control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

procedures may explain the observed disparity in the values. However, taking into account the different protocols of analysis the results described are aligned with the literature.

Next, we wanted to check that OHC did not loose any antimicrobial activity neither during the encapsulation process nor during the incubation time. For this, some powder sample was incubated in a release buffer containing 10 mg/mL Proteinase-K, whereby the buffer was replaced every week, as described above. After 5 weeks incubation time, the release medium was sampled, mixed with a suspension of *Staphylococcus epidermidis* and spread on an agar plate. After 24 h incubation at 37 °C, as shown in Fig. 5b, no bacterial growth was observed, in contrast to the control experiment in Fig. 5c. This demonstrates that the OHC released in the last week of incubation was sufficient to prevent bacterial growth, and therefore that OHC fully retains its antimicrobial activity upon encapsulation and storage over an extended time frame of five weeks.

The cytotoxicity test on THP-1 cell line was performed in order to evaluate the OHC concentration levels that do not affect the vitality of human cells. The choice of the cell line, was performed in order to gain information of the effect of the drug in the blood stream. The data in Fig. 6 show that at OHC concentration below $5{\cdot}10^{-4}$ mg/mL, the cell viability is comparable to that of the control (without OHC). This OHC concentration is lower than the concentration found by Van Meurs *et al.* [66]. The difference may be related to the cell type used to perform this test, as discussed by Niepel *et al.* [68], Nevertheless, the vertical line indicates the possibility to reach a concentration of OHC in the formulation able to provide significant antimicrobial activity without influencing the vitality of human cells.

4. Concluding remarks

In this work, a process for the encapsulation of OHC in biocompatible and bioresorbable polyesters is developed based on spray-drying with very high incapsulation efficiencies. The obtained product is demonstrated to provide delayed and extended release of OHC. The method is applicable using commercially available polyesters and, depending on the molecular structure, different percentages of the drug can be released in a day as an initial burst or for longer periods of time extending to tens of days. The understanding of the release mechanism allows determining the polyester characteristics in order to tune the duration of the long time release, as well as the fraction of drug released in this period compared to the one in the initial burst.

In particular, it is shown that acid bearing polyesters exhibit smaller initial burst release, which are up to 50 times lower than their ester-ended analogues, due to the strong interaction with the dissociated API in salt form. For long times, only poly(D,L-lactide-co-glycolide) bearing acid groups has been shown to provide steady release of this antiseptic. This is because this carrier system reached phase 3 of the release mechanism. This behavior can be tuned by properly tuning the molecular structure of this material so to change its degradation kinetics. It is shown that suitable enzymes can accelerate the release of the API, by promoting the degradation of the carrier material. For the case of Proteinase-K, this enzymatic degradation depends on the end group, and in particular the degradation of ester-ended polyester is negligible compared to the acid-terminated one at equivalent starting molar mass.

The antimicrobial activity of the final products has been verified using *Staphylococcus epidermidis* cultures. It resulted that the encapsulation does not alter the antimicrobial activity of octenidine, since the material released after five weeks incubation exhibited inaltered anti microbial activity. Finally, it was shown that the developed procedure can yield powders able to release an amount of OHC sufficient to provide antimicrobial activity without affecting human cell viability.

The findings of this work indicate that the produced encapsulated OHC might represent a valuable alternative to the commercial solutions containing OHC, for the topical treatment of mucosae and wounds since it proved to provide a prolonged sterility.

CRediT authorship contribution statement

Antoine Klaue: Conceptualization, Writing - original draft. **Matteo Maraldi:** Investigation, Writing - review & editing. **Camilla Piviali:**

Investigation. **Davide Moscatelli:** Project administration. **Massimo Morbidelli:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

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