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Bioadhesive and biocompatible films as wound dressing materials based on a novel dendronized chitosan loaded with ciprofloxacin

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ABSTRACT

The bioadhesive polymeric films as topical drug delivery systems are interesting alternatives to improve the pharmacotherapy and patient compliances. New derivate biomaterials based on weisocyanate- dendronized PVP- crosslinked chitosan and loaded with ciprofloxacin (CIP), as model drug, were used to prepare bioadhesive films. Relevant *in vitro*/*in vivo* attributes to define main physicochemical and biopharmaceutical characteristics for topical wound-healing applications were evaluated. A high proportion of CIP, uniformly dispersed along throughout the film, was loaded. An extended release of CIP and different behaviors of release profiles, depending on the presence of dendron, were observed. The films loaded with CIP were effective in inhibiting the growth of both Gram positive and Gram negative bacteria. In addition, biocompatibility and bioadhesion into conjuntival-sacs of the rabbits suggests that these films have good properties to be applied over skin wounds for topical applications, allowing a reduction of the frequency of administration and improving the residence time of the films.

1. Introduction

Skin plays a key role in prevention of infections caused by microorganisms and simultaneously maintaining the homeostasis of the body. Once a trauma is suffered, the damaged skin should immediately be covered with a dressing to maintain a moderately moist environment for regeneration of the skin, prevention of infection, alleviation of pain, to allow gaseous exchange and to remove excessive exudates while the wound healing takes place (Díez-Pascual & Díez-Vicente, 2015).

The ability of a range of materials to accelerate wound healing and to control infection has been widely studied (Boateng, Matthews, Stevens, & Eccleston, 2008). Both synthetic and natural hydrophilic polymers could act as helpful agents to accelerate the regeneration of damaged dermal and epidermal tissues. Most of these polymeric materials are able to mimic some of the physical and biological characteristics of native tissues, due to their high water content and biocompatibility (Díez-Pascual & Díez-Vicente, 2015).

The natural polymers investigated for wound healing applications include polysaccharides (alginates, chondroitin, chitosan and chitin, cellulose, dextran and heparin), proteoglycans, and proteins (collagen, gelatin, fibrin, keratin and silk fibroin) (Chattopadhyay & Raines, 2014; Hu et al., 2014; Jayakumar, Prabaharan, Kumar, Nair, & Tamura, 2011; Voicu et al., 2016).

When the skin is damaged, opportunistic bacteria can cause infections and these can require intensive and long-term treatment. This can sometimes be a consequence of burn injuries or other serious skin lesions, and in other cases reveal immune deficiencies, affecting both skin (epidermis, dermis and subcutaneous connective tissue) and mucous surfaces (buccal, ocular or vaginal). Classical topical dosage forms, such as creams, ointments, lotions and sprays, present insufficient residence time, a lack of accurate dosing, variability in their therapeutic performance, or are easily wiped away from the applied site. These problems often create poor patient compliance and compromise the efficacy of the over-

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all therapy. Mucoadhesive gels, hydrogels, tablets and thin films have been designed to overcome these shortcomings (Boddupalli, Mohammed, Nath, & Banji, 2010). In addition, several approaches have been developed for controlled release of antimicrobial agents at the specific site of application to prevent bacterial growth (Cloutier, Mantovani, & Rosei, 2015; Gorrasi, 2015; Zilberman et al., 2015).

In particular, polymeric films containing an antimicrobial agent constitute one of the most suitable therapeutic alternatives applicable to topical administration, since they produce more prolonged effects and are easily and conveniently applied, avoiding the systemic adverse effects caused by drug absorption or the use of high levels of drug concentration (Anisree, Ramasamy, & John, 2012; Karki et al., 2016). An ideal film should be based on a biocompatible and non-toxic polymer, be flexible, elastic, and soft, but be adequately resistant to breakage due to stress in the application site.

In recent decades, several types of biocompatible carriers have been explored for bioadhesive thin-film design (Karki et al., 2016; Pereira, Guterres, Balducci, Colombo, & Sonvico, 2014). In particular, chitosan (Ch), a natural polysaccharide derivative of chitin, a linear polymer of β-(1 → 4)2-amino-2-deoxy-D-glucose units (D-glucosamine) has been explored for this purpose (Thakur & Thakur, 2014; Zargar, Asghari, & Dashti, 2015). Ch-based biomedical materials have been paid great attention because of their distinctive properties, such as biodegradability, biocompatibility, non-toxicity, antibacterial activity and wound-healing acceleration ability (Ahmed & Aljaeid, 2016; Kong, Chen, Xing, & Park, 2010; Raafat & Sahl, 2009). On the other hand, the modification of the surface of biomaterials has been of major interest for many years, due to the surface of these materials first coming into contact with the biological surroundings (Tangpasuthadol, Pongchaisirikul, & Hoven, 2003). Dendronization is one of the surface modification techniques which would lead to structural features within the nano-dimension range (Paez, Martinelli, Brunetti, & Strumia, 2012). The main reason for the use of dendritic structures in biology and medicine is their multivalency, caused by the presence of a large number of surface end groups within the same molecule that can interact with biological receptors or with other chemical entities to a greater extent, generating hierarchical complexes with high binding affinities. Modified nanostructured surfaces can be obtained with the ability to control the morphology and other surface properties of the dendrons, such as wettability, roughness, chemical reactivity, and hardness. Additionally, dendronization of the surface of biopolymers can therefore be used to introduce specific properties for drug delivery and tissue engineering (Khandare & Calderón, 2015; She et al., 2013; Wang, Huang, Chang, Xiao, & Cheng, 2016).

In a previous work, we used the dendronization process on a cross-linked biopolymer to obtain modified-hybrids Ch-weisocyanate (ChW) under microspheres (Aldana, Strumia, & Martinelli, 2016) and thin films (Aldana, Toselli, Strumia, & Martinelli, 2012) forms were used. In particular, bioadhesive films, based on this dendronized Ch, showed improved physical, chemical and mechanical properties, such as thermal stability, hydrophilic/hydrophobic balance, permeability, elastic modulus, and degree of swelling. These properties are the result of topographical and chemical changes introduced by dendrons on one side of the Ch film (Aldana, Barrios, Strumia, Correa, & Martinelli, 2016; Aldana, Strumia et al., 2016; Aldana, Toselli et al., 2012). In addition, these films showed interesting biological properties. Antibacterial activity *per se,* and activation of an alternative profile of L-arginine metabolism in macrophages, which enhanced cell proliferation and wound healing were demonstrated; properties of these biomaterials highly relevant to their topical application as carriers or potential adjuvants in antimicrobial agent delivery systems (Aldana, Barrios et al., 2016).

To take advantage of the intrinsic properties of these films, the incorporation of antimicrobial agent was proposed to enhance an active role of these films in wound healing and to prolong its use. Ciprofloxacin (CIP) was selected as the model drug. This is a fluoroquinolone with broad-spectrum antibacterial effects that is approved for prophylaxis and used for a variety of topical applications, such as skin, eye, nose and ear infections (García et al., 2016; Heggers et al., 1998; Kevadiya et al., 2014). Currently, topical pharmaceutical products containing CIP are available as hydrogel, aqueous solutions and ointments only for ophthalmic or otic administration (DrugBank, 2005; García et al., 2016), but a commercial formulation under the film form for skin application has not been yet approved. In the field of research several efforts have been focused in the development of Ch composites and nanoparticles loaded with different antimicrobial agent for application in wound healing, because of their capability to accelerate the wound closure and healing over conventional formulations (Elgadir et al., 2015); nevertheless, none of them are in clinical stage of development or approved as commercial products.

Our interest is focused in the film form, because it would be the more suitable pharmaceutical form for topical delivery in the treatment of skin wounds, because of its easy application, biocompatibility and bioadhesive properties, which allow it to behave as dressing material by itself.

On the other hand, the combination of organic synthesis and pharmaceutical technology would allow the design of a material with potential capabilities as a wounds dressing for high performance (Kamoun, Kenawy, & Chen, 2017).

Dendritic polymer shows significant advantages over linear polymers for different pharmaceutical applications. According the amount of reports published in the last decades, it is evident that a substantial part of dendrimer research has been focused for the development of biomedical tools. The synergy between the multifunctionality and nanoscale size that offers the dendritic architecture enables a chemical smartness fundamental for pharmaceutical applications (Abbasi et al., 2014; Khandare & Calderón, 2015).

The modification with dendritic structure of the classical polymer in the form of film loaded with antimicrobial drug for potential wounds dressing represents one of the most successful forms to exhibit the unique properties of dendrimers. In addition, the final wounds dressing would represent a patient-friendly material (Kamoun et al., 2017; Khandare & Calderón, 2015).

We hypothesize that the whole properties of the films as wound dressing materials are improved by incorporation of CIP as antimicrobial agent.

In this context, the goal of this work was to develop films based on Ch and ChW as functional carriers, using CIP as the model drug. The evaluation of relevant physicochemical characteristics, *in vitro* biopharmaceutical properties by drug release studies and antimicrobial activity, and *in vivo* biocompatibility and bioadhesive assays were conducted in order to define their potential use in the design of a scaffold for topical controlled drug delivery focused on wound healing therapy.

2. Materials and methods

Ciprofloxacin (CIP) free base was obtained by neutralization of CIP hydrochloride salt (Parafarm®, USP grade, Bs.As., Arg.) with 1.0 N NaOH solution, after which the precipitate was washed, filtered and dried at 100 °C until a constant weight was achieved. Chitosan (Ch) (85% deacetylation, low molecular weight, Sigma-Aldrich®); polyvinylpyrroli-

done (PVP, Parafarm®); di-t-butyl-4-[2-(t-butoxycarbonyl)ethyl]-4-isocyanato-1,7-heptanedicarboxylate (Weisocyanate, W) (Frontier Scientific Inc.); dibutyltin dilaurate (Sigma-Aldrich®); dimethylacetamide (Sintorgan®); glacial acetic acid (Cicarelli®); KH_2PO_4 p.a. (Anedra®), NaCl p.a. (Parafarm®), 1 N NaOH and HCl solutions (Anedra®) were used as received. Mucine type III partially purified from porcine stomach was purchased from Sigma-Aldrich (Buenos Aires, Argentina). All experiments were carried out with distilled and purified water.

Staphylococcus aureus ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were stored in our lab at −20 °C in Trypticase Soy Broth (Britania, Argentina) supplemented with 10% glycerol. Fresh cultures were prepared before each experiment.

Six *New Zealand* male albino white rabbits weighing 2–2.5 kg were used. These rabbits were provided with food and water *ad libitum* in a temperature-controlled room (21 ± 5 °C) and exposed to 12 h light:dark cycles. Animal management procedures complied with ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research from the European Communities Council Directive (86/609/EEC). The Institutional Animal Care and Use Committee of the Faculty of Chemical Sciences, National University of Córdoba, Córdoba, Argentina, reviewed and approved the protocols. After a week of adaptation in the facilities, animals were admitted to the experimental sessions.

2.1. Preparation of non-dendronized and dendronized films

As described in previous report (Aldana, Toselli et al., 2012), the dendronized polymer was prepared by a two-step synthesis: Ch film and one-side dendronization. The Ch film was obtained by the casting solvent method. Briefly, 1.0 g of Ch was dissolved in 1.5% acetic acid solution (100 mL) and homogenized at room temperature overnight. PVP (0.180 g) and CIP (0.500 g) powders were subsequently added to the Ch solution, and 50 mL of solution was cast on a glass plate (diameter 10 cm) and gradually dried in air at room temperature. The films were carefully removed from the Petri dishes and analyzed by ATR-FTIR spectroscopy. For dendronization, a special 25 mL nitrogen flask designed to hold a film in its base and equipped with a magnetic stirring bar was charged with Ch-CIP film (0.100 g) in 10-mL DMAc. The W (0.150 g, 0.15 mmol) and dibutyltin dilaurate (0.02 mL) were added and the mixture was stirred at 60 °C for two days. Unreacted dendron on the film was washed with $CHCl_3$ (10 mL × 3), and then dendronized Ch films (ChW-CIP) were carefully removed from the flask and dried under vacuum at room temperature. The same procedure was followed to obtain Ch and ChW films without CIP (Fig. 1).

2.2. Attenuated Total Reflectance Fourier Transform Infrared

The non-dendronized and dendronized films were characterized chemically by analysis of ATR-FTIR interferograms, acquired using an FT-IR spectrometer equipped with an Attenuated Total Reflectance accessory. A 45 ZnSe crystal was used to monitor samples. All spectra were obtained with 32 scans at a 4.0 cm^{-1} resolution in a range between 4000 and 650 cm^{-1}.

2.3. CIP content uniformity in the films

Analysis of the uniformity of dosage units was performed to ensure the consistency of dosage units along the extended area of the films.

The total diameter for each circular film obtained was 5 cm. From these, film discs of 4.8 mm diameter were taken at random and immersed in glass vessels with 10 mL 1 N HCl solution until complete dissolution. These vessels were incubated at 25 °C with magnetic stirring at 100 rpm for 48 h, covered with aluminum foil to protect from photo-degradation. The amount of CIP from drug-loaded films was evaluated by UV–vis spectrophotometry at 272 nm using an Evolution 300 BB spectrophotometer (Termo Sci. Corp., USA). All determinations for each film were performed in triplicate. The amounts of CIP determined by these assays were used in drug release studies as a reference of the total amount of CIP in an area of 4.8 mm diameter discs of film. The results obtained were analyzed statistically by determination of coefficient of variation and expressed as the percentage of this ($\%C_V$). A $\%C_V$ inter-assay ≤5% was considered acceptable (Reed, Lynn, & Meade, 2002).

2.4. Swelling studies

These studies were performed considering our previous work (Aldana, Toselli et al., 2012). The sorption capacity of CIP-unloaded ChP film, and CIP-loaded films (Ch-CIP and ChW-CIP) was evaluated by swelling of the films in USP phosphate buffer solution pH 7.4 at (37.0 ± 0.5) °C. Briefly, film discs of 4.8 mm diameter were placed in the medium for 6 h. The swollen films were collected at different times, superficially dried with tissue paper and weighted on an analytical microbalance. The percentage swelling (%*Sw*) of the films in the medium at each time was calculated according to Eq. (1).

$$\%Sw = \frac{W_S - W_O}{W_O} \times 100 \quad (1)$$

where W_S is the weight of the films at the equilibrium of swelling at each time evaluated, and W_S denotes the initial weight of the films. The swelling experiments were carried out in triplicate for each film. The results are showed as %*Sw* (%*Sw* average at each time with its respective standard deviation) versus time.

2.5. In vitro *bioadhesion assays*

The bioadhesion assays for both CIP-loaded (Ch-CIP and ChW-CIP) and unloaded-CIP (ChP) films were performed by two methodologies: mucin particle method (Takeuchi et al., 2005) and scanning electron microscopy (SEM) for the investigation of mucin interaction with the films (Onnainty, Onida et al., 2016).

2.5.1. Mucine particle method

In this study, pig gastric mucin was used. The mucin was suspended in USP phosphate buffer solution pH 7.4 at a concentration of 0.1 mg/mL and 3 mL of this suspension was placed in contact with (45.2 ± 0.7) mg of films at room temperature. After incubation for 48 h, the zeta potentials of mucin suspension in the absence of films, and mucin suspension in contact with ChP, Ch-CIP and ChW-CIP were measured without dilution, by electrophoretic light scattering using a NanoZetasiser (Nano-ZS, Malvern®, UK) and change values of the mucin particles due to the presence of the films was an indication of bioadhesion (Takeuchi et al., 2005).

2.5.2. Scanning electron microscopy

As it was detailed in Section 2.5.1, mucin from porcine stomach was used. Suspensions of mucin in USP phosphate buffer solution pH 7.4 at a concentration of 0.1 mg/mL (at this concentration the materials obtained present elastic dominant gel like properties) were used. The absence or presence of ChP, Ch-CIP and ChW-CIP films were evaluated after 48 h equilibration at room temperature. Then, aliquots of 10 μL of the suspensions obtained were dried by vacuum and sputtered with gold before imaging in the SEM. Imaging was carried out using and accelerating voltage of 3 kV and the secondary electron detector on an electronic microscope (SUPRA 40 (ZEISS) SEM).

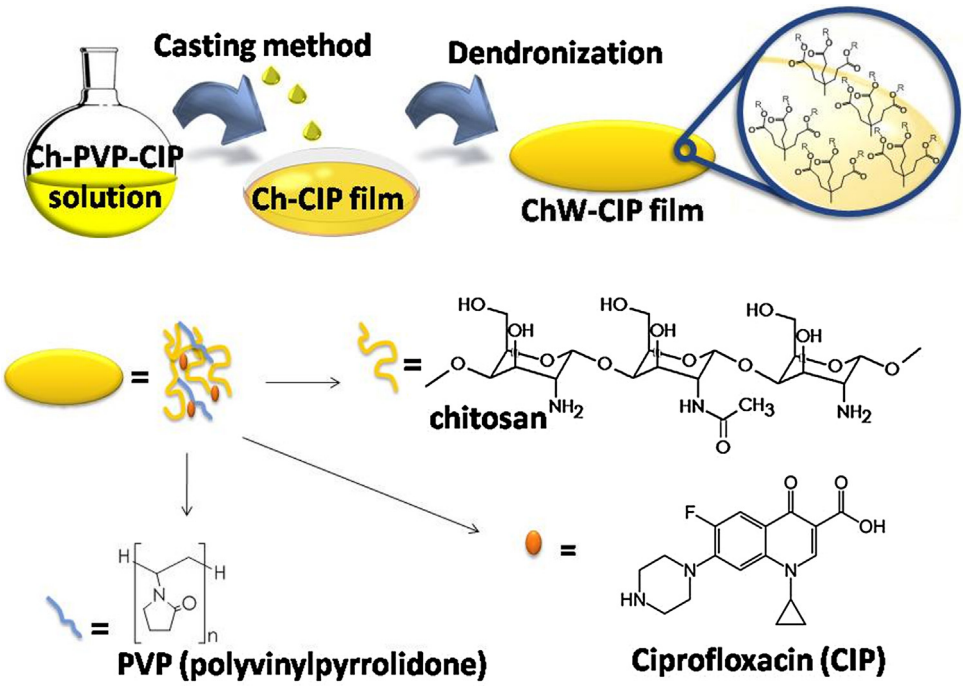

Fig. 1. Synthetic scheme for obtaining ChW-CIP. Preparation of films from native chitosan, crosslinker (PVP), dendron (W) to the loaded of the antibacterial drug (CIP).

2.6. Drug release studies

Drug release from both films was performed in a bicompartmental diffusion device (Franz cells) mounted with a semisynthetic cellulose membrane (12 kDa, Sigma-Aldrich, USA). In the donor compartment was carefully placed a film disc of 4.8 mm diameter, exposing the dendronized face of the film towards the diffusion membrane, close to 0.4 mg of CIP in an area of 0.18 cm^2 (effective diffusion area), and kept in contact with 15 mL of receptor medium at 37 °C. USP buffer solutions of pH 5.8 and 7.4 were used as receptor media. Samples of 1.0 mL of receptor medium were withdrawn at predetermined time intervals and replaced with equal quantities of fresh medium. The concentration of released drug was assayed spectrophotometrically at 272 nm (Evolution 300 BB, Termo-Sci Corp., USA). All experiments were carried out in triplicate and sink conditions were maintained.

The mean release profiles were fitted according to following equations (Eqs. (2)–(4)) (Costa & Lobo, 2001; Siepmann & Peppas, 2001).

For zero-order kinetics, the cumulative drug release is a function only of time and is the ideal method of drug release in order to achieve a prolonged pharmacological action. The following relation (Eq. (2)) can in a simple way express this model:

$$M_t = M_0 + k_0 \times t \tag{2}$$

where M_0 is the initial amount of drug in the donor compartment, M_t is the amount of drug permeated at time t, and k_0 is the zero-order release constant.

The Higuchi model describes drug release as a diffusion process based on Fick's law, *i.e.* square-root-time-dependent. Eq. (3) represents the generally known simplified Higuchi model:

$$\frac{M_t}{M_0} = k_H \times t^{0.5} \tag{3}$$

From linear analysis of the release profiles, plotting percentage of CIP release with respect to the square root of time, k_H, intercept values (a) and determination coefficients (R^2) were calculated.

The semi-empirical diffusion model (Eq. (4)), proposed by Peppas (Peppas, 1985), relating exponentially the drug release to the elapsed time, can be used to characterize different release mechanisms:

$$\frac{M_t}{M_0} = k_P \times t^n \tag{4}$$

where M_t is the amount of drug permeated at time t, M_0 is the initial amount of drug in the donor compartment and k_P is a constant reflecting the structural and geometric characteristics of the device, and n is the release exponent characterizing the diffusional mechanism. For a slab, when $n = 0.5$ the fraction of drug released is proportional to the square root of time (Higuchi Eq. (2)) and the drug release is purely diffusion controlled; and when $n = 1$ drug release is swelling controlled (zero order release kinetics or case-II transport). Values of n between 0.5 and 1 indicate anomalous transport and a superposition of both phenomena. Eq. (3) is valid for the release interval between 5 and 60% of the cumulative drug released. Plots of ln $\frac{M_t}{M_0}$ versus ln$t$ were drawn using the swelling kinetic data and n and k_P values were calculated from the slopes and intercepts of the lines, respectively.

The correlation coefficient values (R^2) were used to compare the fit of the profiles using these kinetic models and the best regression was taking as representative of the kinetic release mechanism of the drug.

In addition, the release profiles of CIP from the films were compared statistically using the similarity factor (f_2, Eq. (5)). According to this methodology, two release profiles were declared to be similar if the value of f_2 was between 50 and 100 (Costa & Lobo, 2001; Food and Drug Administration, 1997).

$$f_2 = 50 \cdot \log\left\{\left(1 + \left(\frac{1}{n}\right)\Sigma_{t=1}^{n}(R_t - P_t)^2\right)^{-0,5} \cdot 100\right\} \tag{5}$$

Where log is the logarithm to base 10, n is the number of sampling time points, $\sum$ is the summation over all time points and R_t and P_t are the cumulative percentages of drug released at each of the n time points of the reference and test product, respectively.

Table 1
Score for potential eye irritation made from observation of eye of the rabbit after the contact with the Ch-CIP and ChW-CIP films.

Score Value	Effects observed
0–2	No irritation
3–7	Mild irritation
8–12	Moderate irritation
12–20	Severe irritation

Adapted from Quinteros, Tártara, Palma, Manzo, and Allemandi (2014).

2.7. In vitro *antimicrobial activity: inhibition zone assay*

An inhibition zone assay was used to evaluate the antimicrobial activity of CIP-unloaded films (ChP) and CIP-loaded (Ch-CIP and ChW-CIP) films against *S. aureus* and *P. aeruginosa.*

Overnight cultures were suspended in 0.9% NaCl sterile solution and adjusted to achieve an absorbance (580 nm) = 0.5. One hundred μL of each microbial suspension was distributed over the surface of the Mueller–Hinton agar (Britania, Argentina) to obtain a uniform growth. After 10–15 min, disks of Ch-CIP and ChW-CIP films (3, 4.8 and 7 mm diameter) were placed onto the inoculated agar. The performance of Ch-CIP and ChW-CIP was compared in each plate. Five replicates were assayed. Furthermore, CIP unloaded Ch films (ChP) were also evaluated. Plates were incubated at 37 °C for 24 h. Antibacterial activity was indicated for the presence of inhibition zone around the disks. The diameters were determinate with caliper.

2.8. In vivo *tolerability test and bioadhesive assessment*

The potential ocular irritancy and/or damaging effects of films were evaluated using a slightly modified version of the Draize method (Palma et al., 2009). The assay was carried out in six right eyes of male albino white rabbits. A circular film of 4.8 mm diameter was collocated into the conjunctiva sac of each eye. For each animal, left eyes were used as control.

Pre- and post-exposure evaluations of the eyelids, conjunctiva, cornea, and iris were performed by external observations under adequate illumination, with additional information being provided by examination using slit-lamp biomicroscopy (Topcon Japan). For each observation, one drop of fluorescein salt (0.25%) was instilled to reveal the potential corneal injury. The rating of ocular irritation or damage was scored (Table 1) at 0, 30, 60, 120, 180, 240, 360 min for each observation.

To assess bioadhesion, the permanence of films into the conjunctiva sac of each eye was evaluated at the same times and it was expressed as residence time.

3. Results and discussion

3.1. *Preparation of dendronized chitosan films*

Physical crosslinking between Ch and PVP has been proposed to improve mechanical strength and chemical stability of the natural polymer in acidic media, thereby providing a material suitable for use in the biomedical field. The miscibility of Ch and PVP in the films has been reported (Li, Zivanovic, Davidson, & Kit, 2010) and it is considered that carbonyl groups in the pyrrolidone rings of PVP interact with amino and hydroxyl groups in Ch by forming hydrogen bonds. We chose PVP as the crosslinker because it is a highly biocompatible material, recognized as safe, and is extensively used in pharmaceutical, cosmetic and food products (Rowe, Sheskey, & Weller, 2006; US Food and Drug Administration, 1980). Physical blending is one of the most effective methods for acquiring materials suitable for use in the field of biomedicine, and the films were made by casting. Thus, Ch and Ch-CIP films were obtained. After crosslinking, dendronization was carefully designed to modify one side of the cross-linked polymer, leaving unmodified Ch on the opposite side. The dendronized Ch films (ChW and ChW-CIP) were successfully prepared by a covalent union with di-t-butyl-4-[2-(t-butoxycarbonyl)ethyl]-4-isocyanato-1,7-heptanedicarboxylate (Weisocyanate, W) dendron. Fig. 2 shows the atomic force microscopy of the surface dendronized and non-dendronized films without CIP, where the bright dots on indicate raised areas forming a rough surface and so the organization of the dendron on the surface. The degree of modification (8.67×10^{-5} W mol/g of the film) (Aldana, Barrios et al., 2016) was calculated by titration of the amino group (ninhydrin test) (Leane, Nankervis, Smith, & Illum, 2004).

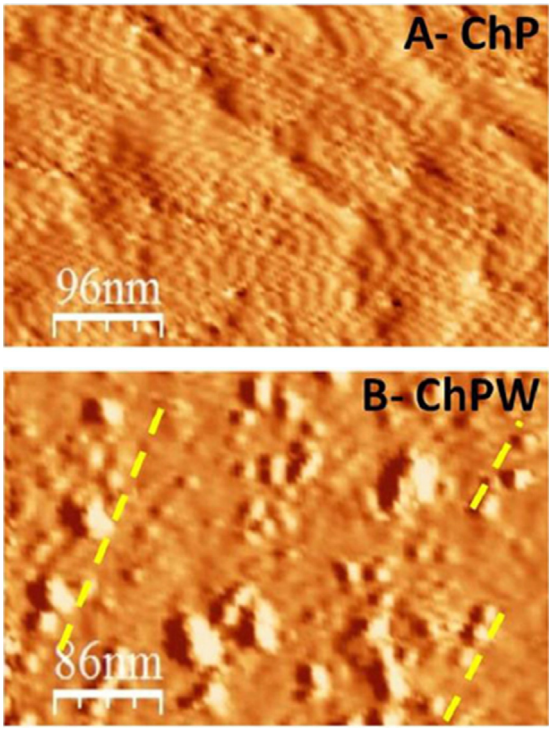

Fig. 2. AFM images of CIP-unloaded A) dendronized and B) non-dendronized films (ChP and ChPW, respectively).
Reprinted from Publication Reactive Functional Polymers, Vol. 100, A.A. Aldana, B. Barrios, M. Strumia, S. Correa b, M. Martinelli. Dendronization of chitosan films: Surface characterization and biological activity, Pages 18–25, Copyright (2016), with permission from Elsevier.

3.2. *Characterization of dendronized and non-dendronized chitosan films*

ATR-FTIR analysis of the sample confirmed that dendronization occurred on one surface of the film only, where characteristic peaks of the dendron were observed, such as the tert-butyl group vibrations at 850 and 756 cm^{-1}, the band at 1225 cm^{-1} corresponding to C—O—C ester group bending and at 2968 cm^{-1},assigned to the methyl stretching. Due to vibration overlap, the band at 1729 cm^{-1} can be assigned to C=O stretching of the dendron or urethane or urea bond formation. Further evidence of the disappearance of the isocyanate group is observed in the infrared spectrum of the Ch-W, since the signal between 2260 and 2130 cm^{-1} corresponding to the vibration of the OC=N bond of the isocyanate group is absent. Also, the solid-NMR-^{13}C (CP MAS) spectra of the dendron, the Ch and the dendronized Ch were recorded and the spectrum of ChW shows the disappearance of the signal at approximately 125 ppm correspond-

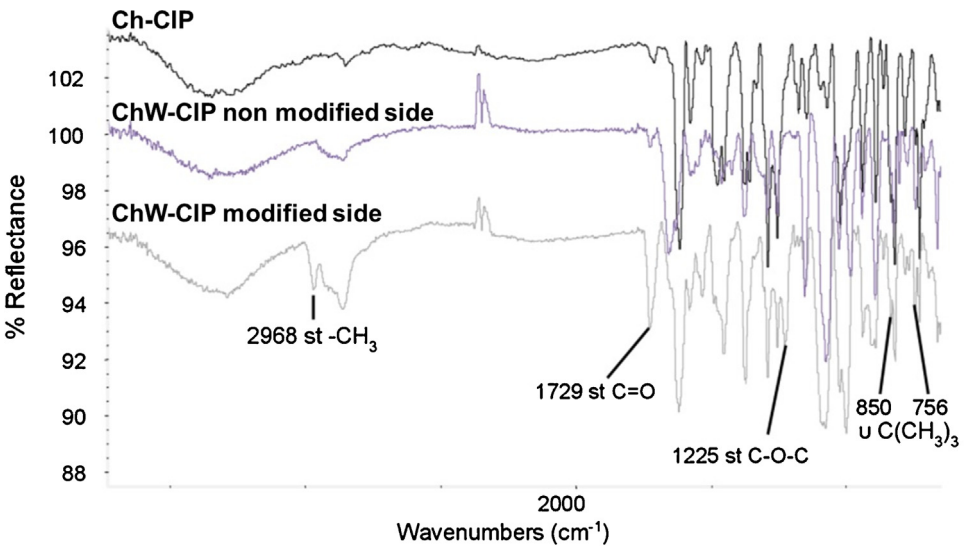

Fig. 3. ATR-FTIR spectra of Ch-CIP and non-modified and dendronized side ChW-CIP films. Main bands and their assignments are indicated.

ing to the carbon atom of the isocyanate group and is highlighted the appearance of a new signal at 156 ppm attributed to the carbon atom of the urea/urethane group (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.carbpol.2017.07.053). The characteristic bands of Ch crosslinking with PVP showed no changes in the spectral profiles of the other side of the film (Fig. 3). Finally, characteristics absorptions bands of CIP (Wang, Dong, Du, & Kennedy, 2007) in the Ch films loaded with this antimicrobial agent were difficult to identify due to the complexity and overlap of bands. However, it is important to denote that CIP can strongly interact with Ch by predominant hydrogen and electrostatic bonds, as it have been previously reported (Semwal, Singh, Archana, Verma, & Dutta, 2012; Wang et al., 2007).

Physical observation revealed that the films possessed smooth surfaces. They were opalescent, uniform yellowish colored and elegant in appearance. Thickness of films ranged from 0.060 to 0.110 mm. With the incorporation of PVP, folding endurance was found to be optimum and the films were flexible and non-brittle, according to previous reports by us (Aldana, González, Strumia, & Martinelli, 2012). We have demonstrated that Ch films crosslinked with PVP showed moderate tensile strength and elongation at breakage, resulting in adequate performance as a bioadhesive system. Whereas the potential application as a wound dressing material, the dendronization process of a Ch film confers improved properties, such as thermal stability, water vapor permeability and mechanical behavior.

The crystalline order over dendronized face of these films (Aldana, Barrios et al., 2016) caused the increase in the thermal stability of the dendronized films. The presence of one dendronized surface polymeric film yielded a material with different hydrophilic/hydrophobic properties on each side and the films could be considered semi-permeable. The hydrophobic nature of the dendron decreases water absorption, and then the dendronized face showed lower water vapor permeability than that of the unmodified face.

The dendronized films are more rigid and resistant up to high tensile stress, due that the elastic modulus increased respect to undendronized films.

Also, we have studied the swelling of the films at different pH: 3.4 and 6.8, which simulates the pH of exuding wound and normal stages (Gethin, 2007), respectively. In general, the swelling behavior was governed by charge of the free amine groups of Ch and was not affected by dendronization. Furthermore, it is important that the films dendronized and non-dendronized were left in contact with each of the buffers for a week and after that period, the films were not degraded (Aldana, González et al., 2012).

Table 2
Uniformity of dosage units of CIP in a disc film of 4.8 mm diameter according measure of UV–vis absorption (λ = 272 nm) of CIP in different sections of the films.

Films	Amount of CIP (mg)	%C_V inter-assay
Ch-CIP	0.39 ± 0.02	5
ChW-CIP	0.34 ± 0.01	3

The cytotoxicity of CIP-unloaded films was previously studied by us and we carried out *in vitro* assays employing fibroblasts and macrophages as essential cells for wound healing, we have reported tests with macrophages (RAW) and fibroblasts (NIH) in order to assess whether contact with the CIP-unloaded dendronized and non-dendronized (ChP and ChPW) films promotes polarization. Macrophages (RAW 264.7 cells) and fibroblasts (NIH 3T3 cells) were seeded on ChP and ChPW films, previously treated. We observed that the inflammatory profile in macrophages was attenuated in fibroblasts and the two metabolic pathways (classic o M1, and alternate o M2) (Gordon, 2003) were not affected by the films.

It is important to highlight that *in vitro* assay that the both CIP-unloaded films did not stimulate or enhance an inflammatory profile in fibroblasts and macrophages, in the contrary the films were able to activate an alternative profile in macrophages, which enhance cell proliferation and wound healing.

In addition, under the assay conditions, the incorporation of CIP in the Ch and ChW network did not produce any alteration of these qualities. Physicochemical characterization, release and *in vivo* evaluations were performed on film discs of 4.8 mm diameter carefully cut using a metal punch. The weights of the film discs were in the range of 0.8–1.7 mg.

3.3. CIP content uniformity in the films

For this assay an adaptation of pharmacopeial specification was considered, because uniformity dosage for this type of formulation is not encoded. In consequence, the amount of CIP in disc films of 4.8 mm diameter randomly cut from different areas of the obtained larger films was determined (Table 2) and the results were expressed as the mean value and the standard deviation of two independent experiments, each one carried out on triplicate.

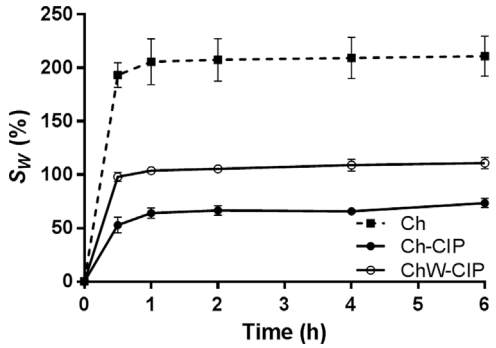

Fig. 4. Percentage swelling (%*Sw*) of CIP-unloaded film (ChP), and CIP-loaded dendronized and non-dendronized films (ChW-CIP and Ch-CIP).

A $\%C_V \leq 5\%$ was considered acceptable as a requirement of quality of the films developed.

Drug content, normalized by unit area, was found to be in the range of (2.2 ± 0.1) and (1.9 ± 0.1) mg cm^{-2} for Ch-CIP and ChW-CIP films, respectively. For the Ch-CIP film, the percentage of C_V was approximately 5%, rather than near 3% for the dendronized film, indicating that the ChW-CIP film achieves better uniformity of CIP along the extended area of film compared to the non-dendronized one.

This pharmaceutical analysis parameter for the quality control of formulation is highly important in the field of pharmaceutical science, because it is required for all dosage forms to ensure a uniformity of drug content. In addition, one of the main challenges in film formation by a casting process is to assure content uniformity in the formulation, since a characteristic of the drying process is frequently the observation of agglomeration or sedimentation of solid particles or air bubbles on the surface, leading to homogeneity problems (Perumal, Govender, Lutchman, & Mackraj, 2008). As is shown in Table 2, the films obtained had excellent CIP content uniformity with very low percentage of C_V, indicating that the casting solvent method employed was success.

3.4. Swelling studies

The swelling behavior of the CIP-loaded dendronized and non-dendronized films (ChW-CIP and Ch-CIP, respectively) changed compared to that of the CIP-unloaded film (ChP). The swelling study was performed in USP phosphate buffer pH 7.4 at 37 °C, because this value of pH refers to exudate produce by skin damage, considering that wound exudate is a normal part of the inflammatory process and the increased capillary permeability allow plasma leakage from the blood vessels (Cameron, 2006).

The percentage swellings (%*Sw*) of the films were determined at each time according to Eq. (1). The results obtained are presented in Fig. 4, where can be appreciated the higher sorption capacity of the CIP-unloaded films in comparison to CIP-loaded ones. Similar results were obtained in our previous work with the non-dendronized Ch film (Aldana, Toselli et al., 2012). In this study, it is possible to observe that an important reduction in the index of swelling is produce due to the presence of CIP, which could physically interact (hydrogen and ionic bonds) with the available amino and/or hydroxyl groups of Ch as crosslinker agent to form less hydrophilic matrix (Semwal et al., 2012; Wang et al., 2007).

Also, the both CIP-loaded films presented different fluid uptake and in both cases is less than the CIP-unloaded film. The Ch-CIP showed higher sorption capacity than ChW-CIP film, which could be related to its increase of the superficial area due to the high roughness of the ChW-CIP film, as it can be seen in Fig. 2.

It is important to highlight that, in the time of assay and after one week in the suspension of mucin, the integrity of Ch-CIP and ChW-CIP is maintained, which is and important characteristic for topical application in skin wounds (Aldana, Toselli et al., 2012).

3.5. In vitro *bioadhesion studies*

The *in vitro* interaction of ChP (CIP-unloaded film), Ch-CIP and ChW-CIP with mucin was characterized by measuring the zeta potential of mucine suspension under equilibrium conditions after the addition of the films. Mucin without films was used a control to study their native behavior in this assay. Fig. 5 shows the zeta potential under the different conditions evaluated. As it can be seen, mucin presented a negative charge due to ionization of carboxyl groups since environmental pH was higher than their pKa (pKa of mucin = 2.6). In presence of the films the zeta potential of mucin changed its value from negative to positive with a light decrease of pH (Fig. 5). The determined changes in the zeta potential values are indications that electrostatic interactions occur between the films and mucin, thus revealing their bioadhesive properties (Onnainty, Schenfeld et al., 2016; Sogias, Williams, & Khutoryanskiy, 2008; Takeuchi et al., 2005).

To extend our investigations further, the effect of the films on the mucin matrix architecture was evaluated by SEM and these results could be associated to the bioadhesive behavior of different pharmaceutical systems (Onnainty, Onida et al., 2016; Onnainty, Schenfeld et al., 2016). To this, pure mucin and mucin after interaction with the films were spread out on a slide and vacuum-dried for observation by SEM. Considering that mucin present hydrocolloid nature the samples were prepared without fixation and dehydration, to avoid disturbing the mucin matrix architecture in the sample and to observe the mucin gel structure as present in its native network in the mucus (Onnainty, Onida et al., 2016; Onnainty, Schenfeld et al., 2016). Fig. 6A shows a swollen network formed by mucin, which occupy the total volume of the slide. Fig. 6B–D shows the mucin after interaction with the films. As can be seen, the microscopic appearance of mucin was modified due to the presence of the films. In these images, filaments of mucin can be observed and this finding reveals that the films can bind tightly to mucin, an event that could have an important role in the release mechanism of the loaded drug and the performance of the films as wound dressing materials. The changes observed in the matrix architecture of mucin could be attributed to the interaction by bioadhesion of the films. Similar results for other types of systems were previously reported (Crowther, Hughes, & Marriott, 1984; Onnainty, Onida et al., 2016; Onnainty, Schenfeld et al., 2016). In addition, a proportion of crystalline CIP can be observed on mucin architecture in the sample obtained by interaction with Ch-CIP film, which could be related to minor interactions between CIP and non-dendronized Ch and the slightly higher pH value observed in this sample in comparison to ChW-CIP film.

3.6. In vitro *drug release studies*

The CIP release behaviors of both films were studied to evaluate the potential for topical drug delivery applications. Buffer solutions at pH 5.8 and 7.4, simulating the physiological conditions of intact skin and plasmatic environment, respectively, were used as receptor media. Figs. 7 and 8 show profiles of CIP release from films to buffer solutions at pH 5.8 and pH 7.4, respectively. A slow and extended release of CIP from films into both receptor media was observed. After 24 h at pH 7.4, the cumulative percent of total CIP released from Ch-CIP film was near to 40%. However, for dendronized film the cumulative percent of CIP released was higher than 80%, showing an increase of release rate between 1.5 to 2 times with respect to non-dendronized polymer based films. The opposite behavior in the CIP release profiles was observed when buffer pH 5.8 was employed as receptor medium, where the cumu-

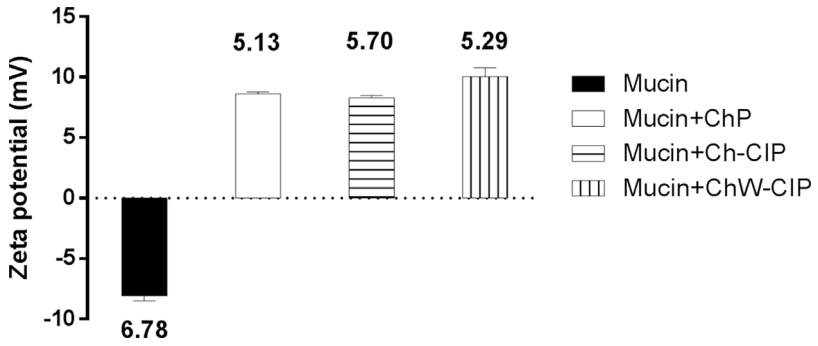

Fig. 5. Interactions of CIP-unloaded film (ChP), and CIP-loaded dendronized and non-dendronized films (ChW-CIP and Ch-CIP) with mucin by dynamic light scattering. The values informed near to the bars correspond to the pH values for each condition evaluated.

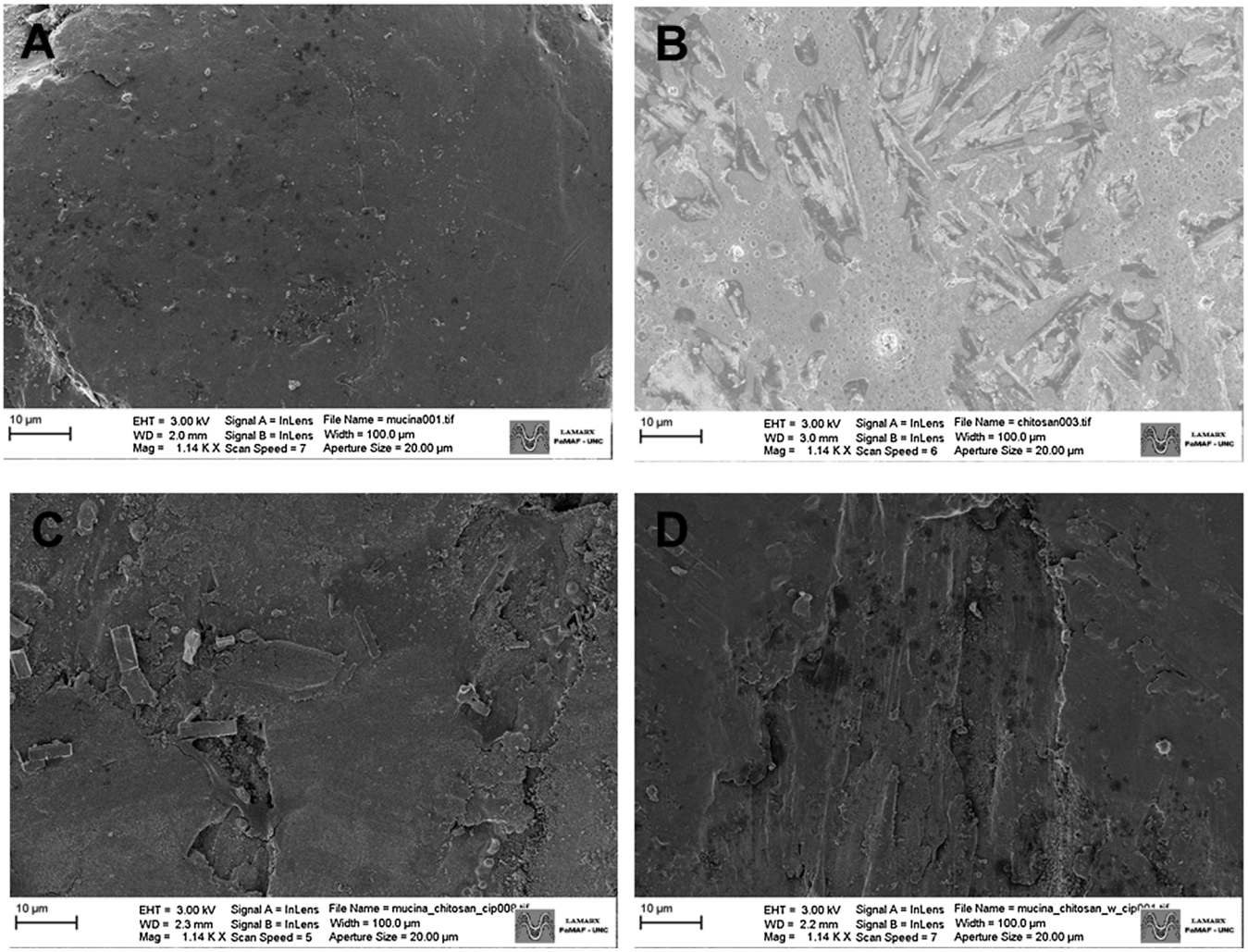

Fig. 6. SEM images of pig gastric mucin after 48 h equilibration at room temperature in the A) absence or presence of B) ChP, C) Ch-CIP and D) ChW-CIP films.

lative percent of drug released was higher from Ch-CIP (~80%) than ChW-CIP (<50%), showing at this pH value a significant decrease of release rate, greater than 2.5 times, attributable to dendronization of Ch. This behavior could arise from the strong interaction between Ch and PVP chains by the formation of hydrogen bonds, mainly at pH 7.4. Moreover, due to the dendronization, entanglement could hinder the removal of CIP from the network. At pH 5.8, the release from non-dendronized film was facilitated because the network is less entangled, since the amine groups are protonated and the ionic crosslinking is less; in this case the dendronization could hinder the diffusion of the drug due hydrophobic interactions between dendron and CIP. Previously, at pH values close to 7, significant differences in the swelling of the modified and non-modified network were not observed, indicating that few free amine groups of Ch are not charged, so the swelling does not change with dendronization (Aldana, Toselli et al., 2012).

The significance of the release profiles differences was confirmed through similarity tests, resulting in $f_2 = 34.9$ and 45.9 at buffers of pH 5.8 and 7.4, respectively. These f_2 values indicated that release profiles of both dendronized and non-dendronized Ch-

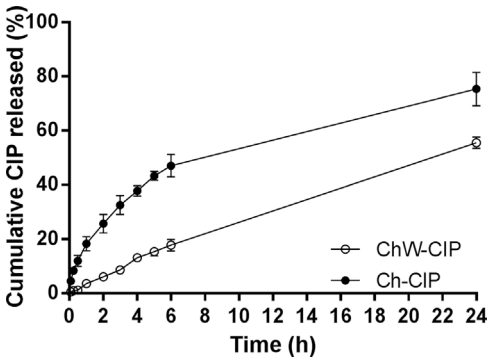

Fig. 7. *In vitro* CIP release profiles from ChW-CIP (empty symbols) and Ch-CIP (filled symbols) films using Franz cells filled with pH 5.8 buffer solution as receptor media.

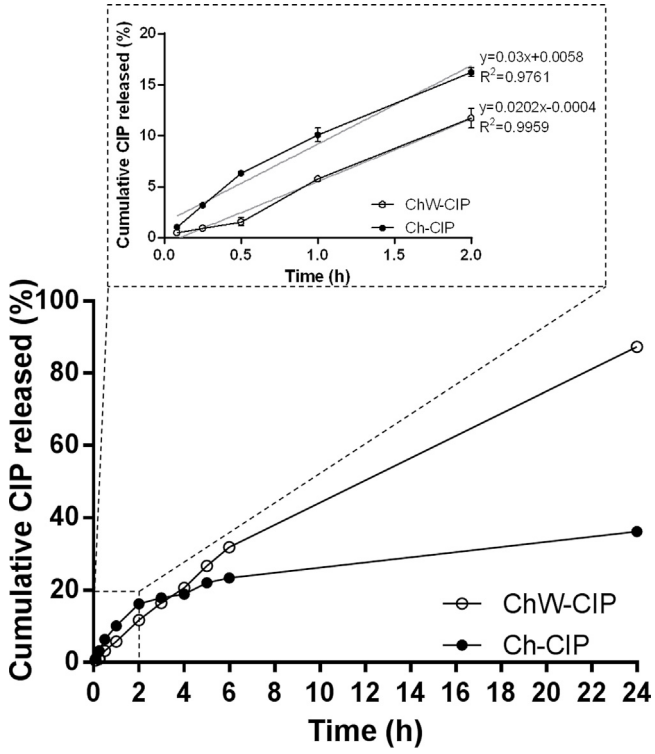

Fig. 8. *In vitro* CIP release profiles from ChW-CIP (empty symbols) and Ch-CIP (filled symbols) films using Franz cells filled with pH 7.4 buffer solution as receptor media. An amplification of the first two hours of release is presented, and the linear regression (grey lines) and the equations calculated are presented.

films presented significant differences at the pH values of the two release media assayed, showing that dendronization can play an important role in the release behavior of CIP.

Kinetic analysis of *in vitro* release data using zero-order, Higuchi diffusion and Peppas power equations were performed in order to evaluate dendron incidence on release behavior and the main mechanism of CIP transport through polymeric film. Results of the kinetic analyses are summarized in Table 3. Drug release data from non-dendronized films plotted as Higuchi diffusion were found to be fairly linear and were well supported by their regression coefficient values. However, data from dendronized films have a better fit with respect to zero-order kinetics, showing high regression coefficient values ($R^2 > 0.99$).

The *n*-values confirmed that the kinetics of drug release from non-dendronized films, slightly above 0.5, indicated an anomalous transport with the preponderant release mechanism controlled by drug diffusion. On the other side, the release data of dendronized films resulted *n*-values close to 1 and confirmed the non-Fickian or anomalous transport, where swelling of the film is the main phenomenon that controls drug release. For non-dendronized films, the hydrophilic character of the system and the ability to form hydrogen bonds between Ch and PVP chains are the determining factors in the release behavior, whereas for the dendronized films the hydrophobic character of the dendron becomes important, since it regulates the release behavior of CIP. Properties such as contact angle, swelling index, and the permeability of both PVP-crosslinked dendronized and non-dendronized Ch support this assertion (Aldana, González et al., 2012; Aldana, Toselli et al., 2012). The hydrophobic nature of the dendronized films was evidenced by contact angle and permeability values being lower for dendronized than for non-dendronized films; in addition the dendronized face showed the most hydrophobic character. This change in the kinetic control of CIP release from dendronized films could be attributed to the orderly structuring of the polymer network and the higher hydrophobicity of the dendritic polymer where the rates of swelling, complex relaxation and ion exchange are the preponderant mechanisms in the release of CIP.

It should be note that *in vitro* tests revealed that the release of CIP from these bioadhesive films was appropriate, both in terms of magnitude and velocity, for the design of topical antimicrobial controlled-release systems. CIP is a fluoroquinolone that presents a pattern of antimicrobial activity classified as Type-I, in which considering their pharmacodynamic properties, the ideal dosing regimen would maximize concentration because the higher the concentration, the more extensive and the faster is the degree of killing (Toutain, Del Castillo, & Bousquet-Mélou, 2002). Release rates of CIP from bioadhesive films were ranged between 56 and 146 $\mu g\,cm^{-2}\,min^{-1}$, using sink conditions. Considering our previous work (García et al., 2016) where *in vitro* CIP release from dendronized hydrogels and bacterial growth inhibition tests were evaluated using similar conditions against both *S. aureus* and *P. aeruginosa* as models of the main opportunist pathogen microorganisms more frequently found in topical and mucosal infections, revealed that hydrogels effectively inhibited bacterial growth at a similar magnitude to a reference solution of CIP. It is important to highlight that, the release rate of CIP from this hydrogel ($12\,\mu g\,cm^{-2}\,min^{-1}$ at pH = 6.8) was significantly lower than that obtained for bioadhesive film in this work. In consequence, we anticipate that the films may have adequate antibacterial activity, which was confirmed with the inhibition zone assays carried out against Gram positive and Gram negative bacteria.

Therefore, it would be expected that films based on dendronized Ch would provide a slow and sustained release of CIP after topical administration, with the capacity of maintaining a suitable level of CIP above the minimal inhibitory concentration values for long periods of time, without altering the biostructures of skin or mucous membranes.

3.7. In vitro *antimicrobial activity: inhibition zone assay*

The antimicrobial activity of the films was investigated by measuring their ability to inhibit *S. aureus* and *P. aeruginosa* growth on agar culture plates. These species were evaluated as potential microorganisms causing wound infections and burns.

Both Ch-CIP and Ch-W-CIP films exhibit good antibacterial activity. Fig. 9 shows the results of the inhibitory zone exposed by 7 mm diameter film discs. The size of the inhibitory zones around the film disks containing CIP increased in a dose-dependent manner, as observed by increasing disc diameter (data not shown).

Antimicrobial activity of Ch has been demonstrated against many bacteria (Kong et al., 2010). However, the drug-free films evaluated in this study have no inhibitory effect (Fig. 9). It is noteworthy that the physical state of Ch (solutions *versus* film) has been

Table 3
Release kinetic data obtained from drug release studies using empirical equations: Zero order, Higuchi and Peppas models.

Film	pH receptor media	Zero order			Higuchi			Peppas		
		k_Z	M_0	R^2	k_H	M_0	R^2	k_P	n	R^2
Ch-CIP	5.8	143.1	217.2	0.974	431.2	−42.7	0.999	384.5	0.54	0.999
	7.4	62.0	163.0	0.910	218.1	−10.3	0.973	211.7	0.51	0.972
ChW-CIP	5.8	56.4	3.8	0.994	165.7	−92.2	0.967	66.0	0.88	0.992
	7.4	98.5	7.7	0.998	289.9	−160.9	0.977	110.1	0.94	0.998

k expressed as ($\mu g\,cm^{-2}\,min^{-1}$), ($\mu g\,cm^{-2}\,min^{0.5}$) and ($\mu g\,cm^{-2}\,min^{-n}$) for Zero order, Higuchi and Peppas model, respectively.

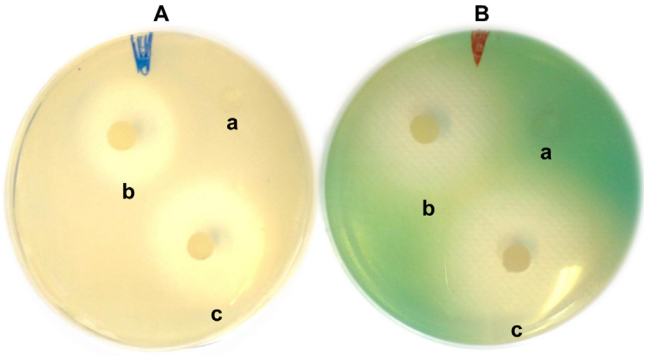

Fig. 9. Photograph of *in vitro* antimicrobial activity evaluated by assay of zones of inhibition of bacterial growth corresponding to a) ChP, b) ChW-CIP and c) Ch-CIP films (7 mm diameter) against A) *Staphylococcus aureus* and B) *Pseudomonas aeruginosa.*

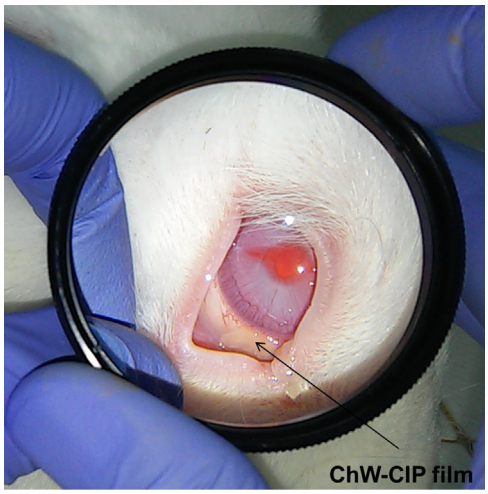

Fig. 10. External observation rabbit' eye by examination using slit-lamp biomicroscopy after adhesion of the ChW-CIP film to the conjunctiva at 3 h of post-administration.

included among the various factors on which its bactericidal efficacy depends (Kong et al., 2010; Leceta, Guerrero, Ibarburu, Dueñas, & De la Caba, 2013). In particular, the behavior observed in this assay can be explained by the limitation of Ch diffusion on agar plate.

The comparison of the zones of inhibition corresponding to each film loaded with CIP shows that ChW-CIP exhibits slightly less activity than Ch-CIP against the microorganisms tested. The average diameter of the inhibition halos generated by ChW-CIP represents between 90 and 95% of those corresponding to Ch-CIP film.

The diameter of the inhibition zone in the *in vitro* bioassay is proportional to the concentration of drug in the sample. However, multiple variables affect the diameter of the zone of inhibition. The rate of release of CIP from the films could condition the diffusion of the same in the agar while the microorganisms grow. As it can be seen in the amplification of Fig. 8, a delay in the release of CIP from the ChW-CIP film during the first part of Franz cell experiments as compared to that observed with Ch-CIP. This behavior could result in lower concentration gradient around the films and to be the cause of the smaller size of the zones of inhibition observed in the bioassay.

3.8. In vivo *tolerability and bioadhesive assessment*

To evaluate the irritant potential and bioadhesion properties of films for wound application, an *in vivo* study in rabbit was performed and the eyes were selected as model organs due to their high sensitivity as a topical surface (Huhtala, Salminen, Tähti, & Uusitalo, 2008). Bearing in mind the similarities between the eye and the skin lesions, it was considered that if the system is non-irritant and bioadhesive to the eye, it would, in consequence, present those properties with skin wounds.

The evaluation of the ocular irritation by the modified Draize method (Palma et al., 2009) was based on an objective medical evaluation of lesions caused by films in different tissues of the eye, using a procedure designed to assign scores to potential ocular irritancy and/or damaging effects. The biomicroscopic evaluation was performed with a slit lamp, an instrument that has several levels of illumination, allowing it to realize optical cuts *in vivo,* and by the coloration of the ocular surface with fluorescein and a cobalt filter, microscopic alterations and/or lesions of the cornea and conjunctiva (such as ulcer, local irritation or inflammation, keratitis) can be identified. Fig. 10 shows the eye of a rabbit with the ChW-CIP film adhered to the conjunctival sac. The time of permanence for both films was 4 h. After that time, the films were removed from the eye, because of palpebral movement of the rabbit. According to the results score, all films, both those that contained CIP and placebo, appeared to be innocuous and non-irritant, because they presented a score lower than 2, at all times evaluated.

In addition, it is important to highlight that the films did not undergo any remarkable swelling and they kept their integrity in contact with physiological fluids, which ensured more predictable *in vivo* release performance, minimizing undesirable effects and improving the patient compliance.

Finally, the results reached with CIP-containing ChW films were very promising for the design of functional systems for topical drug controlled release, not only for their application in skin wounds, but as ophthalmic, buccal or intravaginal mucosal infection treatments as wound dressing material to prevent or treat opportunist infections.

4. Conclusions

Novel CIP controlled-release films with potential application as scaffolds for topical delivery were developed, employing native and dendronized Ch. A dendritic structure was employed as building blocks to development of molecular nanostructures with well-defined particle shapes and sizes. Dendronized Ch films of one face were obtained by a simple and efficient methodology, and then

loaded with antibacterial drug (CIP). The dendronized films preserved the distinctive properties of Ch, such as biodegradability, biocomatibility, non-toxicity and antibacterial activity. In addition, the dendronization controlled de physico-chemical properties of the films and the drug delivery profile.

The CIP load was found to be uniformly distributed across the area of both films. The biocompatibility and bioadhesion over the eyes suggested that these films have good properties for application in topical administration over skin wounds. The controlled release of CIP depended on the nature of dendronization and the receptor media, and would allow reductions in the frequency of administration. Both films exhibit antibacterial activity against the two bacterial species evaluated, indicating that the drug is released from the film upon contact with the agar and diffuses such that it prevents microbial growth

The films developed showed very promising performance to be employed as wound dressing materials for prophylaxis or for treatment of topical and mucosal infections.

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