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Development and characterization of chitosan/hyaluronan film for transdermal delivery of thiocolchicoside

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ABSTRACT

The objective of this study was the development of chitosan/hyaluronan transdermal films to improve bioavailability of thiocolchicoside. This approach offers the possibility to elude the first-pass metabolism and at the same time it is able to provide a predictable and extended duration of activity. Films were prepared by casting and drying of aqueous solutions containing different weight ratios of chitosan and hyaluronan and characterized for their physico-chemical and functional properties. In accordance with polymeric composition of films and, therefore, with the amount of the net charge after the complexation, films containing the same weight ratio of chitosan and hyaluronan showed lower water uptake ability with respect to films containing only one polymeric species or an excess of chitosan or hyaluronan. Moreover, the lower the hydration of the polymeric network, the lower is the drug diffusion through the films and its permeation through the skin. This study clearly confirmed that the selection of a suitable polymeric weight ratio and appropriate preparative conditions allows the modulation of film functional properties, suggesting that these formulations could be used as a novel technological platform for transdermal drug delivery.

1. Introduction

Chitosan and hyaluronan are receiving a great deal of attention for biomedical applications due to their interesting chemical and biological properties. Chitosan, a natural derivative of chitin, is a polysaccharide consisting of copolymers of glucosamine and N-acetylglucosamine connected by β(1–4) glucosidic bonds. It is a weak base with an intrinsic pK_a near 6.3 and a low charge density. Hyaluronan is also a naturally occurring linear polysaccharide with a high molecular weight consisting of copolymer of N-acetyl-D-glucosamine and D-glucuronic acid connected by alternating β(1–3) and β(1–4) glucosidic bonds. It is a weak polyacid with an intrinsic pK_a near 2.9 and a very low charge density as only one charge can be present for every two residues (Luo & Wang, 2014). In pharmaceutical and medical field, chitosan and hyaluronan are widely used as a component in hydrogels, that are basically three-dimensional hydrophilic or amphiphilic polymer networks formed by chemical or physical crosslinking and capable of retaining large amounts of water or biological fluids yet remaining insoluble in physiological conditions. On the basis of the type of interactions (entanglement, physical and chemical interactions), chitosan and hyaluronan can produce networks with chemico-physical and functional properties different from those of the starting materials, and at the same time preserving interesting original properties, such as nontoxicity, biocompatibility, biodegradability and hydrophilicity (Berger, Reist, Mayer, Felt, & Gurny, 2004; Berger, Reist, Mayer, Felt, Peppas, et al., 2004; Muzzarelli, 2010; Muzzarelli, El Mehtedi, & Mattioli-Belmonte, 2014; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Moreover, hyaluronan is able to produce very strong polyelectrolyte complexes with chitosan both in its acidic or salt form (Denuziere, Ferrier, & Domard, 1996; Lee, Lee, Song, & Park, 2003; Luppi et al., 2009). Since chitosan/hyaluronan complexes are quite stable whatever the pH (Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004) and not easily dissolved in organic or aqueous

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solvent, it is interesting to observe that they cannot be easily used. Nevertheless in the last decade, the research focused on the use of chitosan/hyaluronan based polyelectrolyte complexes as a biomaterial for drug delivery and tissue engineering applications. The delivery systems are mainly in the particulate forms, including microparticles and nanoparticle colloidal systems and in other forms such as composite films, membranes and scaffolds (Luo & Wang, 2014). In the present work, chitosan and hyaluronan are intended to be used for formulation of films able to guarantee systemic delivery of thiocolchicoside through the skin. Thiocolchicoside is a semi-synthetic sulfur derivative of colchicoside, acting as agonist of the GABA receptors in the central nervous system and showing muscle-relaxant, anti-inflammatory, and analgesic properties. It is traditionally administered orally (tablets or capsules), parenterally (i.m.) and topically (creams, ointments and foams). Even if the physicochemical properties of thiocolchicoside (relatively high MW, 563.3; low octanol/water partition coefficient, $\log P = -2.71$) are not ideal for the permeation of the drug (Aguzzi et al., 2008), its transdermal administration has also been studied (Artusi et al., 2004; Artusi, Santi, Colombo, & Junginger, 2003) with the aim to overcome disadvantages such as the low systemic availability (approximately 25%) associated to oral administration (Trellu et al., 2004). It is known that this approach offers the possibility to elude the first-pass metabolism and the gastrointestinal incompatibility, and at the same time the choice of an appropriate dosage form (*e.g.* polymeric film) is able to provide a predictable and extended duration of activity, eliminate multiple dosing schedules, reduce side effects due to the optimization of hematic profiles and improve patient compliance.

The objective of this study was the development of chitosan/hyaluronan transdermal films to improve bioavailability of thiocolchicoside. Initially polymeric films were prepared by polymer solution casting method and characterized in terms of physico-chemical properties, morphology and water uptake ability; then *in vitro* release and permeation studies were carried out to evaluate drug release from films and its permeation through skin.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (molecular weight 1650 kDa) was purchased from ACEF (Piacenza, Italy). Low-viscosity chitosan (molecular weight 150 kDa; deacetylation degree 97%) was purchased from Fluka (Buchs, Switzerland). Thiocolchicoside (molecular weight 563.60 g/mol) was a kind gift of Indena (Milan, Italy). Methanol and acetonitrile (both HPLC grade) were purchased from Carlo Erba (Milan, Italy). All other chemicals (99% formic acid, 85% orthophosphoric acid, glacial acetic acid, ethanol 96°, potassium dihydrogen phosphate, sodium chloride) were of analytical grade and were purchased from Carlo Erba (Milan, Italy). Type 2 water quality (corresponding to analytical-grade water) was obtained by means of an Elix Advantage Water Purification System by Millipore (Billerica, MA, USA).

2.2. Preparation of chitosan/hyaluronan films

Chitosan and sodium hyaluronate were separately dissolved (1%, w/w) in acetic acid solution (1%, w/w) and water, respectively. 0, 2.24, 4.48, 6.72, and 8.96 mL of hyaluronan solution, previously added with 2.24 mL of formic acid (30%, w/w), were added dropwise by means of a buret (tolerance ±0.02 mL) to 8.96, 6.72, 4.48, 2.24, and 0 mL of chitosan solutions, respectively (total volume 11.20 mL) and stirred at room temperature for 24 h, thus obtaining different chitosan/hyaluronan weight ratios (4:0, 3:1, 1:1, 1:3, and 0:4). Loaded films were prepared by adding 1 mL of thiocolchicoside aqueous solution (16 mg/mL) to the different polymeric solutions. About 8 g of the mixture were placed in a Petri-dish (diameter of 57 mm and height of 10 mm) and oven-dried at 50 °C for 8 h (heating oven FD series, Binder, Tuttlingen, Germania). To determine the optimal drying time, equal amounts of mixture were poured in the Petri dishes and weighed every 8 h up to 72 h until a constant weight was achieved.

Different films were named in this work as follows: $F_{CS:HA4:0}$, unloaded films based on chitosan (CS); $F_{CS:HA0:4}$, unloaded films based on hyaluronan (HA); $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$, unloaded films based on chitosan/hyaluronan 3:1 (w/w), 2:2 (w/w), and 1:3 (w/w), respectively; while loaded films were named as follows: $LF_{CS:HA4:0}$, $LF_{CS:HA0:4}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, and $LF_{CS:HA1:3}$ (Table 1).

Table 1
Composition of the mixtures used for loaded film preparation (%, w/w, on wet basis) and thiocolchicoside content in the dried products (theoretical data).

	$LF_{CS:HA4:0}$	$LF_{CS:HA3:1}$	$LF_{CS:HA2:2}$	$LF_{CS:HA1:3}$	$LF_{CS:HA0:4}$
Chitosan	0.73	0.55	0.37	0.18	0.00
Hyaluronan	0.00	0.18	0.37	0.55	0.73
Formic acid	5.51	5.51	5.51	5.51	5.51
Thiocolchicoside	0.13	0.13	0.13	0.13	0.13
Water	93.63	93.63	93.63	93.63	93.63
Thiocolchicoside content in the dried products:					
% (w/w)	15.15				

2.3. Physico-chemical characterization

Fourier-transform infrared (FT-IR) spectroscopy is useful for the chemical characterization of the polymers and for the exploration of the nature of the interaction between them in the film. Mid-IR (650–1800 cm^{-1}) spectra were recorded on powder samples (chitosan and sodium hyaluronate) and unloaded polymeric films ($F_{CS:HA4:0}$, $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$, and $F_{CS:HA0:4}$) using a Spectrum One Perkin-Elmer FT-IR spectrophotometer (resolution 4 cm^{-1}, Perkin-Elmer, Wellesley, MA, USA) equipped with a MIRacle ATR device (PIKE Technologies, Madison, WI, USA).

Thermogravimetric analysis (TGA) was used to measure the degradation temperature of film forming polymers alone ($F_{CS:HA4:0}$ and $F_{CS:HA0:4}$) and complexed ($F_{CS:HA2:2}$). TGA was performed using a STA 409 PC Luxx® apparatus (Netzsch-Gerätebau GmbH, Selb, Germany). Samples of 2–3 mg (non-drug loaded) were analyzed in open aluminum pans from 25 °C to 500 °C at 10 °C min^{-1} under a nitrogen atmosphere.

X-ray powder diffraction (XRPD) was performed to characterize the physical forms (crystalline or amorphous) of the various components present in the loaded polymeric films ($LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$). X-ray powder diffractograms were collected on a Panalytical X'Pert Pro automated diffractometer (Almelo, The Netherlands) equipped with X'Celerator, CuKα, using glass sample holder. Tube voltage and amperage were set at 40 kV and 40 mA, respectively. The program used for data collection was set to record only the data points within the range 3–40° 2θ.

Differential scanning calorimetry (DSC) experiments were performed on loaded polymeric films ($LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$) to identify possible phase transitions (from crystalline to amorphous forms) of drug during the film formulation process. Calorimetric measurements were performed using a DSC 200 F3 Maia® (Metzsch, Germany) differential scanning calorimeter equipped with an intra-cooler. The samples were placed in aluminum pierced pans, and the heating was carried out at 10 °C min^{-1} in a N_2 atmosphere. The films were analyzed after

the preparation and measurements were repeated after 6 months (the films have been maintained at ambient conditions).

2.4. Scanning electron microscopy (SEM)

SEM analysis was performed to evaluate the topographic characteristics and morphology of the loaded and unloaded films. Films were cut with a razor blade, fixed on supports and coated with gold–palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Samples were then observed with LEO 420 (LEO Electron Microscopy Ltd., Cambridge, UK) using secondary electron imaging at 15 kV in order to examine their surface morphology and their internal structure.

2.5. Film thickness and drug content

Circles with a surface area of 1.54 cm^2 were cut from each film and measured for thickness (Mitutoyo pocket thickness gauge; Mitutoyo Mfc. Co. Ltd, Tokyo, Japan). Then each circle was dissolved in 50 mL of sodium chloride (NaCl) solution (0.9%, w/w) and the solutions obtained were analyzed in order to determine the amount of thiocolchicoside contained in the film. The results were expressed as milligrams of drug for square centimeter (mg/cm^2).

In these tests as well as in subsequent experiments the thiocolchicoside concentration was determined by HPLC method. The chromatographic system was composed of a Shimadzu (Milan, Italy) LC-10ATVP chromatographic pump and a Shimadzu SPD-10AVP UV-Vis detector set at 260 nm. Separation was obtained on a Phenomenex (Torrance, CA, USA) Synergi Fusion-RP 80A (150 mm × 4.6 mm I.D., 5 μm) coupled to a Phenomenex (Torrance, CA, USA) SecurityGuard C18 guard cartridge (4 mm × 3.0 mm I.D., 5 μm). The mobile phase was a mixture of pH 7.0 phosphate buffer/acetonitrile/methanol 50:25:25 (v/v/v). The flow rate was 0.4 mL/min, and manual injections were made using a Rheodyne 7125 injector with a 20-μL sample loop. Data processing was handled by means of a CromatoPlus computerized integration system (Shimadzu Italia, Milan, Italy).

2.6. Water uptake ability

Water uptake ability was studied to investigate the maximum time required for films to hydrate and the maximum capacity of swelling in physiological saline solution at room temperature. To measure the water uptake capacity, accurately weighed films (loaded and unloaded samples; surface area = 1.54 cm^2) were placed on filter (MF™ – Membrane Filters, pore size = 0.45 μm, d = 47 mm; Millipore, Billerica, MA, USA) soaked in pH 5.5 NaCl solution (pH of healthy skin surface), and positioned on top of a sponge (5 cm × 5 cm × 2 cm) previously soaked in the hydration medium and placed in a Petri dish filled with the same solution to a height of 0.5 cm. Water uptake (WU) was determined, as weight increase of the film for 90 min, according to the following equation: $WU\ (\%) = (W_{Hff} - W_{Hf} - W_{Df}) \times \frac{100}{W_{Df}}$ where W_{Hff} is the weight of hydrated film and wet filter, W_{Hf} is the weight of wet filter and W_{Df} is the initial weight of the dry film.

2.7. In vitro *release and permeation studies*

In vitro release studies were performed in order to evaluate the drug amount released from films, while *in vitro* skin permeation studies were performed in order to evaluate transdermal absorption of drug from films. These studies were carries out introducing single film (surface area = 1.54 cm^2) in the donor compartment of a Franz-type static glass diffusion cell (15 mm jacketed cell with a flat ground joint and clear glass with a 12 mL receptor volume, diffusion surface area: 1.77 cm^2), equipped with a V6A Stirrer (PermeGear Inc., Hellertown, PA, USA). The film was placed on a test membrane, positioned between the donor and receptor compartments. The membranes were a cellulose filter pre-hydrated for 1 h (MF-Millipore Membrane, mixed cellulose esters, pore size = 0.45 mm) and pig ears skin for release studies and permeation studies, respectively. The pig ears were obtained from a local butcher and the skin from the inner face was excised from the ear using a surgical blade and stored at −20 °C until use. The receptor compartment was filled with NaCl solution (0.9%, w/w), maintained at 32 ± 0.5 °C and continuously stirred at 100 rpm. Samples of the receptor solution were withdrawn at predetermined time intervals of over 6 h and analyzed by HPLC system for the determination of drug permeated. Sink conditions were maintained at any time. About 100 μL of an aqueous saturated solution of thiocolchicoside (6 mg/mL) was also prepared and its permeation ability was analyzed at the same conditions of films. The results of the release experiments are shown as cumulative drug amount released (expressed as fractional amount) plotted as a function of time, while the results of permeation studies are shown as cumulative drug amount (mg) permeated per unit of surface area (cm^2) *versus* time.

2.8. Statistical analysis

All experiments were done in triplicate, while transport experiments were done with five replicas. Results are expressed as mean ± SD. ANOVA and t-test were used to determine statistical significance of studies. The criterion for statistical significance was $p < 0.05$.

3. Results and discussion

In this study, we prepared polymeric thin films based on polyelectrolyte complexes by means of a very simple and easily reproducible preparative method and without the addition of crosslinkers. The only addition to the preparative mixture was formic acid that was chosen for its high solubilizing capacity toward chitosan/hyaluronan complexes and for its high volatility, useful in the film drying process (Vasconcelos, Freddi, & Cavaco-Paulo, 2008). Indeed chitosan/hyaluronan complexes were prepared at low acidic pH value in agreement with the assumption that hyaluronan is able to form strong polyelectrolyte complexes with chitosan whether its carboxylic groups are in salt or acidic form. In this latter case, polyelectrolyte complex formation can be related to the acidity of carboxylic functions that are able to be deprotonated during the complexation process (Denuziere et al., 1996).

3.1. Physico-chemical characterization

Fig. 1a shows FT-IR spectra of chitosan, $F_{CS:HA4:0}$, $F_{CS:HA0:4}$, and sodium hyaluronate. The FT-IR spectrum of chitosan powder presented two bands at 1652 and 1585 cm^{-1} which are the amide I vibration ($\nu_{C=O}$) and N—H bending vibration (δ_{N-H}) from amine overlapping the amide II vibration ($\delta_{N-H} + \nu_{C-N}$), respectively. The FT-IR spectrum of $F_{CS:HA4:0}$ displayed a large band in the 1450–1700 cm^{-1} region representing an envelope of (at least) five bands in close proximity: the amide I vibration, the antisymmetric $-NH_3^+$ deformation (1571 cm^{-1}), the amide II vibration, the N—H bending vibration as well as the $-NH_3^+$ symmetric deformation (Lawrie et al., 2007). FT-IR spectrum of sodium hyaluronate powder presented an intense group of overlapped bands in the region of the carbonyl stretching vibration (1500–1700 cm^{-1}) derived from the vibration of acetamide and carboxylate groups present in the D-N-acetylglucosamine and D-glucuronic acid units, respectively. The highest peak (1605 cm^{-1}) is assigned to the antisymmetrical

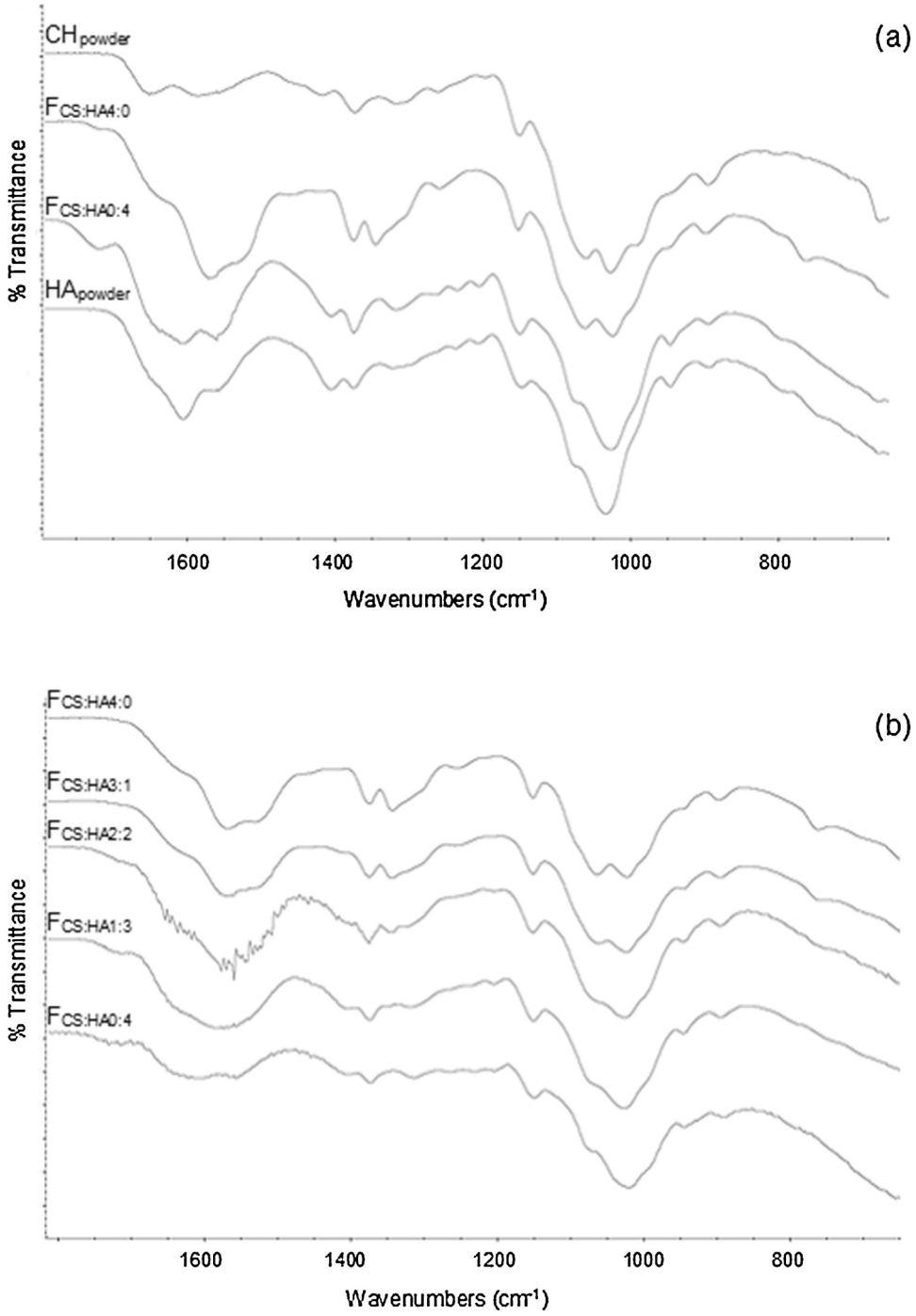

Fig. 1. FT-IR spectra of chitosan powder, $F_{CS:HA4:0}$, $F_{CS:HA0:4}$, and sodium hyaluronate powder (a); FT-IR spectra of $F_{CS:HA4:0}$, $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$, and $F_{CS:HA0:4}$ (b).

stretching vibration in the carbonyl of the carboxylate group ($\nu_{C=O}$), while the shoulder at 1650 cm^{-1} and 1558 cm^{-1} are assigned to the amide I band ($\nu_{C=O}$) and amide II band (δ_{N-H}), respectively. Moreover, at 1405 cm^{-1} the spectrum displayed a band derived from the symmetric stretching vibration in the carboxylate group (ν_{C-O^-}). Comparing the spectrum of sodium hyaluronate powder with that of a hyaluronan film ($F_{CS:HA0:4}$), it is possible to observe that the main difference is the presence of the peak (1719 cm^{-1}) corresponding to the stretching vibration in the carbonyl of the carboxylic group ($\nu_{C=O}$) (Haxaire, Maréchal, Milas, & Rinaudo, 2003; Servaty, Schiller, Binder, & Arnol, 2001).

Fig. 1b shows FT-IR spectra of $F_{CS:HA4:0}$, $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$, and $F_{CS:HA0:4}$. Spectra of $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, and $F_{CS:HA1:3}$ combine the bands associated with hyaluronan ($F_{CS:HA0:4}$), and chitosan ($F_{CS:HA4:0}$). In fact the $F_{CS:HA3:1}$ spectrum showed an intense band peak at 1571 cm^{-1} associated with the excess of chitosan protonated form ($-NH_3^+$) that decreased with the increasing of hyaluronan amount in the complexes ($F_{CS:HA2:2}$ and $F_{CS:HA1:3}$). Contrariwise, peaks associated with the excess of hyaluronan (1719 cm^{-1} and 1405 cm^{-1}) decreased with the increasing chitosan amount in the complexes.

Fig. 2 shows thermograms of $F_{CS:HA4:0}$, $F_{CS:HA2:2}$, and $F_{CS:HA0:4}$. Chitosan and hyaluronan films degraded at 281.59 °C and 214.75 °C, respectively. The thermal degradation of $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$ occurred at lower temperatures than that of chitosan and hyaluronan films (data not shown for $F_{CS:HA3:1}$ and $F_{CS:HA1:3}$)

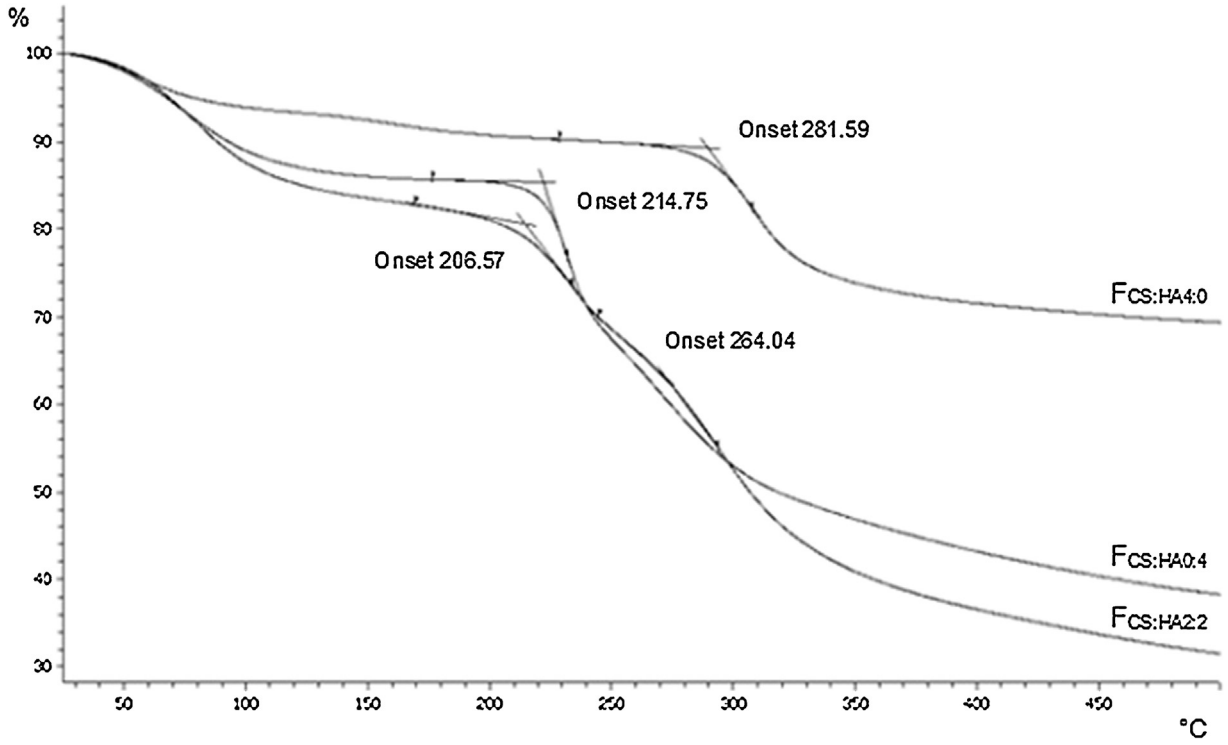

Fig. 2. TGA curves of $F_{CS:HA4:0}$, $F_{CS:HA2:2}$, and $F_{CS:HA0:4}$.

and in particular $F_{CS:HA2:2}$ degraded at 264.04 °C and 206.57 °C. The shift to a lower temperature in the thermal degradation of $F_{CS:HA2:2}$ indicated that the film formulation process changed the physico-chemical properties of the original compounds due to the interaction between the starting polymers.

Fig. 3 shows diffractograms of $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$. All films gave amorphous diffractograms. Thus, we can conclude that in this solid state, chitosan, hyaluronan, chitosan/hyaluronan mixture are not in an organized form. Moreover, the diffractograms do not show even the peaks of the known crystalline forms of thiocolchicoside (Joshi & Gupta, 2013).

The absence of crystalline peaks related to known crystalline forms of thiocolchicoside was confirmed also by the DSC profiles (Fig. 4). In fact thiocolchicoside showed an endothermic peak corresponding to its degradation toward 275 °C, while no peaks were observed in DSC analysis of $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$. Supposedly the molecular state of drug was changed from the crystalline to the amorphous during film preparation. In theory, the amorphous form of the drug represents its most energetic solid state and thus it should produce the biggest advantage in terms of solubility and bioavailability. Finally, DSC analysis performed after 6 months of manufacturing the films

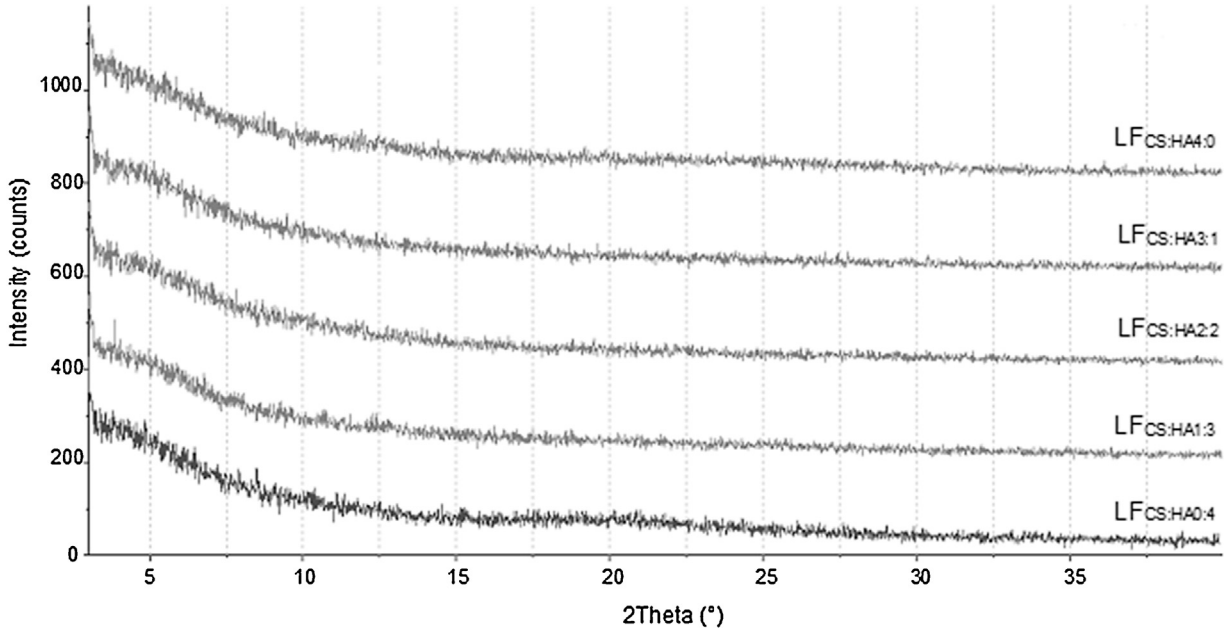

Fig. 3. XRPD patterns of $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$.

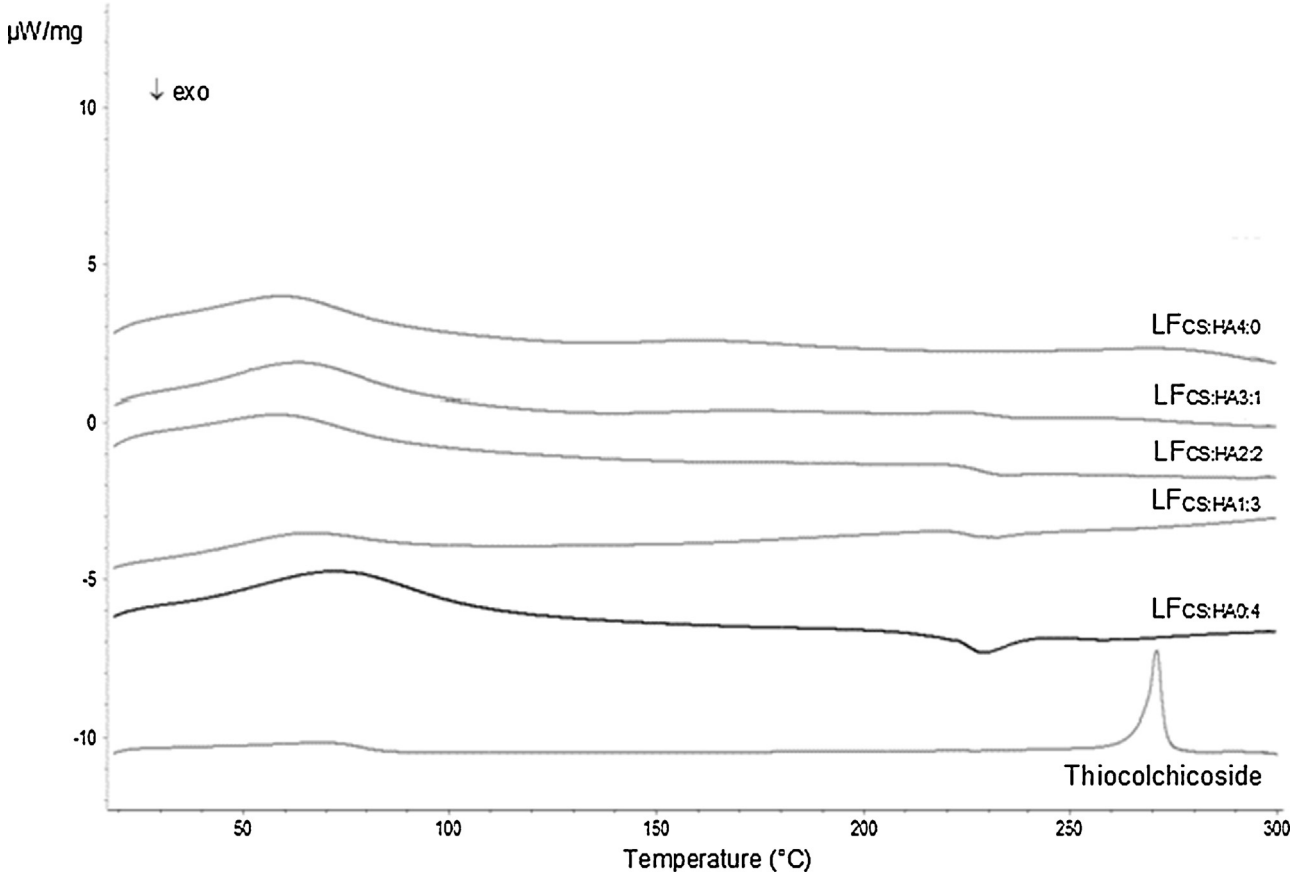

Fig. 4. DSC thermograms of $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, $LF_{CS:HA0:4}$., and thiocolchicoside.

(graph non-reported) does not show any modifications of thermal behavior of the films.

3.2. Scanning electron microscopic (SEM) studies

Unloaded films are transparent and colorless, while loaded films are transparent and slightly yellow due to the presence of thiocolchicoside. The morphology of the different films was analyzed by SEM (Fig. 5). All unloaded films exhibited a uniform and smooth surface. Moreover, they showed a dense and smooth cross-section that indicates homogeneous structure. The presence of drug in the loaded films has given a heterogeneous and grainy structure. The different polymeric composition (chitosan, hyaluronan or chitosan/hyaluronan mixtures) did not affect the morphology of loaded and unloaded films.

3.3. Film thickness and drug content

Physicochemical properties of polymeric films such as thickness and drug content were examined and reported in Table 2. The thiocolchicoside amount experimentally measured in the different films was very close to the amount used for their preparation (high loading efficiency) and there are no remarkable differences among the different samples.

3.4. Water uptake ability

Table 2 showed the water uptake behavior of $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$ in NaCl solution (0.9%, w/w). All the films absorbed water rapidly, reaching equilibrium within 20 min (except $LF_{CS:HA4:0}$ that reached equilibrium within 60 min). Water uptake ability changed in relation to polymeric composition of film. As can be seen, at experimental conditions (pH 5.5) films containing only one polymeric species showed greatest water uptake and in particular $LF_{CS:HA4:0}$ absorbed more water than $LF_{CS:HA0:4}$. This behavior can be attributed to the presence of a high amount of quaternary ammonium groups (NH_3^+) and carboxylate groups (COO^-) in $LF_{CS:HA4:0}$ and $LF_{CS:HA0:4}$, respectively. Moreover, in $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$ and $LF_{CS:HA1:3}$ the amount of net charge as well as the water uptake ability was lower with respect to $LF_{CS:HA4:0}$ and $LF_{CS:HA0:4}$, due to the ionic interactions between positively charged fraction of chitosan and

Table 2
Properties of polymeric films.

CS:HA weight ratio	Thickness[a] (μm)	Drug content[a] (%, w/w)	Drug content[a] (mg/cm^2)	$WU_{after\ 20\ min}$[a] (%)	$WU_{after\ 20\ min}$[b] (%)	Flux[a] (μg/cm^2 h)
4:0	29 ± 2	12.15 ± 2.21	0.33 ± 0.06	53.7 ± 6.8	37.7 ± 4.9	12.9 ± 2.0
3:1	30 ± 3	13.99 ± 2.94	0.38 ± 0.08	29.7 ± 3.1	18.8 ± 2.2	22.7 ± 3.1
2:2	31 ± 2	16.20 ± 0.73	0.44 ± 0.02	8.2 ± 1.2	6.5 ± 0.4	37.9 ± 6.5
1:3	27 ± 4	16.20 ± 1.84	0.44 ± 0.05	20.4 ± 2.8	13.0 ± 1.4	25.0 ± 6.3
0:4	19 ± 1	13.99 ± 1.84	0.38 ± 0.05	40.2 ± 5.8	26.6 ± 1.9	17.7 ± 2.2

[a] Data obtained from loaded films ($LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, $LF_{CS:HA0:4}$). Data expressed as mean ± SD, $n = 3$.
[b] Data obtained from unloaded films ($F_{CS:HA4:0}$, $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$, $F_{CS:HA0:4}$). Data expressed as mean ± SD, $n = 3$.

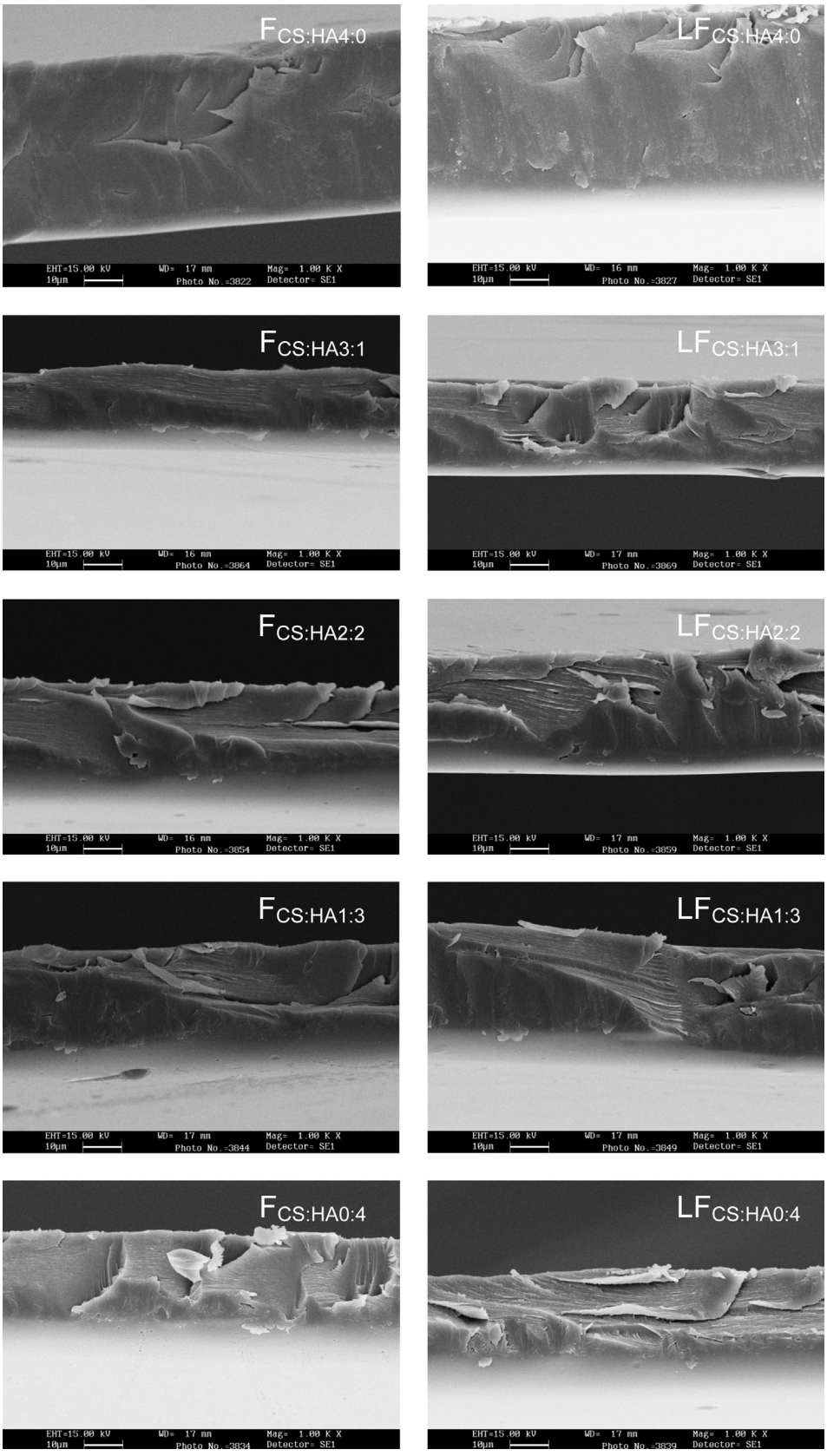

Fig. 5. Scanning electron micrographs of unloaded (left: $F_{CS:HA4:0}$, $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$, and $F_{CS:HA0:4}$) and loaded (right: $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$) films.

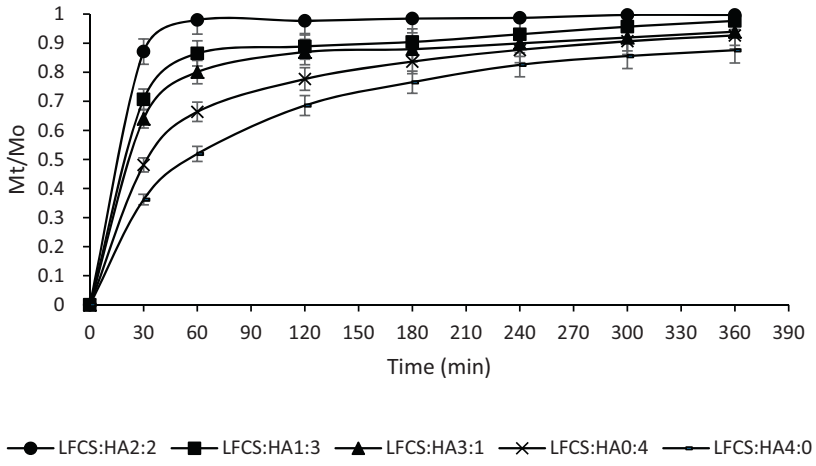

Fig. 6. *In vitro* release profiles of thiocolchicoside from $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$.

negatively charged fraction of hyaluronan. In fact, when the complexes hydrated in the pK_a interval of the two polysaccharides, the interactions between negative and positive charges in the polymeric network reduced free charges, resulting in a lower water uptake (Kim, Lee, & Kim, 2004; Kim, Shin, Lee, Park, & Kim, 2004). Table 2 also showed water uptake results of unloaded films ($F_{CS:HA4:0}$, $F_{CS:HA3:1}$, $F_{CS:HA2:2}$, $F_{CS:HA1:3}$, and $F_{CS:HA0:4}$). As can be seen, the water uptake percentage of each unloaded film was lower than of the corresponding loaded film, probably due to the presence of the drug that enhances the hydrophilic performance of the hydrogel.

3.5. In vitro *release and permeation studies*

Release profiles of thiocolchicoside from $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$ are shown in Fig. 6. Films containing only one polymeric species ($LF_{CS:HA4:0}$, $LF_{CS:HA0:4}$) released lower cumulative amounts of drug with respect to films containing polyelectrolyte complexes ($LF_{CS:HA3:1}$, $LF_{CS:HA1:3}$, $LF_{CS:HA2:2}$) and extended for 5 h. Moreover, films containing an excess of chitosan or hyaluronan ($LF_{CS:HA3:1}$, $LF_{CS:HA1:3}$) did not show difference in their release behavior ($p > 0.05$) and they released higher cumulative amounts of drug with respect to $LF_{CS:HA2:2}$ (complete drug release within 1 h). This release behavior could be explained by the fact that higher hydration produced higher viscosity of the polymeric network in the gelled state, limiting drug diffusion. Likewise, the high degree of interaction between chitosan and hyaluronan in $LF_{CS:HA2:2}$, and therefore the limited presence of free charges, limited complex water uptake and produced a less hydrated and viscous network in the gelled formulation thus improving drug release. The kinetic analysis of release was conducted according to the general equation $Mt/Mo = kt^n$, where Mt/Mo is the fractional drug release, k is a kinetic constant, t is the release time and n is the diffusional exponent that can be related to the drug transport mechanism. Data obtained from this analysis showed n values lower than 0.5, confirming that the diffusion is the drug release mechanism for these thin hydrogel films and mainly by Fickian diffusion for $LF_{CS:HA4:0}$ ($n = 0.521$) and $LF_{CS:HA0:4}$ ($n = 0.465$) (Piai, Lopes, Fajardo, Rubira, & Muniz, 2010; Serra, Doménech, & Peppas, 2006). Moreover, it is possible to hypothesize that thiocolchicoside, a weak base with pK_a value of 10 (Kumar, Shukla, Subudhi, & Ganure, 2012), is able to interact with the carboxylate groups of hyaluronan, reducing its release from films containing an excess of negatively charged groups ($LF_{CS:HA0:4}$) with respect to those containing an excess of chitosan ($LF_{CS:HA4:0}$).

Permeation profiles of thiocolchicoside from $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$ are shown in Fig. 7. As can be observed, the higher drug permeation across pig ear skin was obtained with $LF_{CS:HA2:2}$, followed by films containing an excess of CH or HA ($LF_{CS:HA3:1}$, $LF_{CS:HA1:3}$; differences were not significant, $p > 0.05$) and finally by $LF_{CS:HA0:4}$ and $LF_{CS:HA4:0}$. This behavior could be explained by the fact that higher drug release from films was able to produce higher permeation across the skin. Moreover, when thiocolchicoside was dissolved in water, the

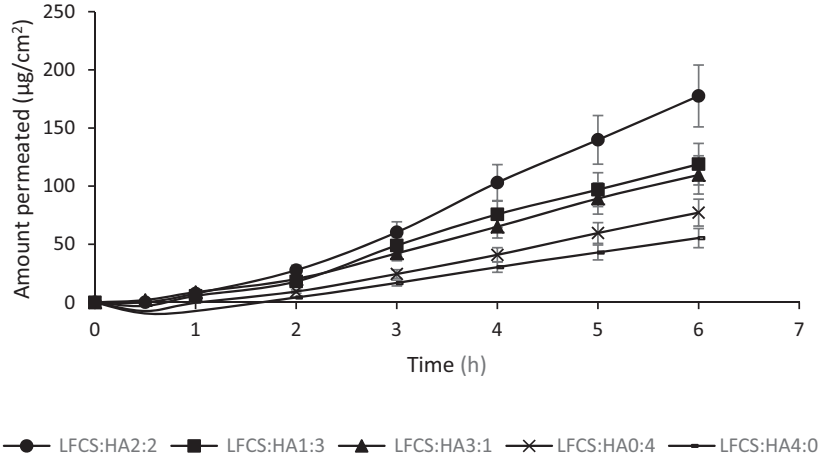

Fig. 7. Permeation profiles of thiocolchicoside through pig ear skin from $LF_{CS:HA4:0}$, $LF_{CS:HA3:1}$, $LF_{CS:HA2:2}$, $LF_{CS:HA1:3}$, and $LF_{CS:HA0:4}$.

permeation profile across skin (graph not reported) was higher than that of films (302.89 μg after 6 h).

In an attempt to overcome the thiocolchicoside low oral bioavailability, other authors have studied formulations able to use alternative administration routes such as transdermal permeation from a topical formulation. Considering that the physicochemical properties of thiocolchicoside are not favorable for its permeation across the skin (relatively high molecular weight and low octanol/water partition coefficient), different strategies were evaluated to enhance its bioavailability. Ceschel (Ceschel et al., 2002) formulated a topical foam able to avoid contact with afflicted area during the spreading phase and added with propylene glycol dipelargonate/ethanol able to increase drug permeability. The importance of the use of permeation enhancers was also described by Artusi et al. (2004) who studied the effect of lauric acid and iontophoresis on thiocolchicoside permeation across skin, confirming that the permeation of this drug across the skin can be enhanced using chemical or physical enhancers. More recently, Kumar, Ali, and Baboota (2014) prepared omega 3 fatty acid-enriched nanoemulsion able to increase *in vitro* permeation of thiocolchicoside fivefold over the control (drug aqueous solution). This behavior was attributed to the nano-size of globules and to the component of nanoemulsions which itself acts as permeation enhancers.

Our study proposed a valid alternative to the use of permeation enhancers. In fact, despite the success of this strategy, there is a need to produce formulations which minimize irritancy typical of enhancers and ensure high patient compliance. Polymeric films described in this study have been shown to be highly efficient, releasing a high percentage of the active. Additionally the selection of a suitable polymeric weight ratio can modulate drug delivery and permeation, thus improving the versatility of the product.

4. Conclusions

Chitosan and hyaluronan are two natural polysaccharides able to produce in solution polyelectrolyte complexes without any chemical cross-linker and they have received significant interest especially for their pharmaceutical and biomedical applications including drug delivery. Ours results confirmed the importance of chitosan/hyaluronan polyelectrolyte complexes as new materials to develop flexible dosage forms able to allow minimal dosage and frequency, characterized by minimal impact on lifestyle, easy and reliable administration. In fact, the selection of a suitable polymeric weight ratio, and appropriate preparative conditions allowed the modulation of film functional properties such as drug release and permeation through the skin, suggesting that these formulations could be used as a novel technological platform for transdermal drug delivery.

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