Ionic coupling of hyaluronic acid with ethyl *N*-lauroyl L-arginate (LAE): Structure, properties and biocide activity of complexes

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Abstract

Ethyl $^{\alpha}$*N*-lauroyl L-arginate hydrochloride (LAE) was coupled with hyaluronic acid (HyA) to form ionic complexes with LAE to HyA ratios of 1:1 and 1:2. The complexes were extensively characterized by FTIR and NMR spectroscopies and their thermal properties evaluated by thermogravimetry and calorimetry. Thin films prepared from these complexes by casting displayed a smectic-like structure based on an ordered arrangement of LAE and HyA layers with a nanometric periodicity of 3.8-3.9 nm. Films immersed in water at pH 7.4 and 5.5 dissociated to deliver free LAE to the environment and reaching the equilibrium in few hours. The biocide activity of these films against both Gram-positive and Gram-negative bacteria was preliminary assessed by the liquid medium method, and shown to be notable in both cases. The antibacterial property of the complexes was found to increase with the content of LAE and to be particularly efficient against Gram-negative *S. enterica* bacteria.

1. Introduction

Complexation of polyelectrolytes with counter-ions of low molecular weight is a relatively simple approach recurrently used for creating materials that largely differ in properties from the parent polymer. This is so because coupling between complementary ionic building-blocks usually leads to molecular arrangements able to self-assemble in well-organized nanostructures (Macknight, Ponomarenko, & Tirrell, 1998a). The complexes made of ionic polypeptides and tetraalkylsurfactants constitute a representative example of these systems (Hanski et al., 2006; Macknight, Ponomarenko, & Tirrel, 1998b; Pérez-Camero et al., 2004; Ponomarenko, Waddon, Tirrell, & MacKnight, 1996). These complexes tend to adopt a layered biphasic structure with crystallinity depending on the length of the surfactant alkyl chain and a periodical spacing sensitive to temperature. Negatively charged polysaccharides, and in particular polyuronic acids, have been demonstrated to behave in a way not far from that described for anionic polypeptides. Thus coupling of alginic, pectinic and hyaluronic acids with alkyltrimethylammonium surfactants has been recently reported to render stable ionic complexes arranged in layers showing order at the nanometric scale (Tolentino, Alla, Martínez de Ilarduya, & Muñoz-Guerra, 2011, 2013).

In this paper we wish to report on ionic complexes made from hyaluronic acid and an arginine-based compound, namely ethyl $^{\alpha}$*N*-lauroyl L-arginate hydrochloride (LAE), which are able to display antimicrobial properties. Due to the increasing resistance of bacteria to antibiotics, bactericide polymers start to be considered today as an attractive antiseptic alternative (Engler et al., 2012). In such systems, the polymer acts as a matrix for holding and controlling the release of the antimicrobial agent so that the possible toxicity associated to the biocide is largely repressed and the period of activity increased when compared to the neat biocide. During the last decade the attention given to polymeric materials with antimicrobial activity has been very noticeable (Santos et al., 2016) and some hyaluronic acid-based materials incorporating antimicrobial agents may be found in the recent literature. HyA-silver

nanoparticles and HyA coupled with polyhexanide (Baier et al, 2013; Kemp et al., 2009), and nisin polypeptide (Lequeux, Ducasse, Jouenne, & Thebault, 2014) are representative examples of such materials. To our knowledge, no study addressed to examine the ionic LAE·HyA complexes including their antimicrobial activity has been described so far.

Hyaluronic acid (HyA) is a high molecular weight mucopolysaccharide composed of repeating disaccharide units of β-1,3-*N*-acetyl D-glucosamine and β-1,4-D-glucuronic acid with a great capacity to retain moisture and to form hydrogels with excellent viscoelastic properties. Furthermore HyA is high biocompatible, non-immunogenic and susceptible to biodegradation by human hyaluronidases. HyA is always present in the human body in small amounts, and it is widely used in medicine, cosmetic and veterinary surgery (Necas, Bartosikova, Brauner, & Kolar, 2008; Stern, Kogan, Jedrzejas, & Šoltés, 2007). The polysaccharide may be chemically modified to render biomaterials with properties suitable for tissue engineering (Allison & Grande-Allen, 2006; Burdick & Prestwich, 2011; Schanté, Zuber, Herlin, & Vandamme, 2011). Rooster combs were the traditional source of HyA but today it is mainly produced by microbial fermentation which has boosted its applications and raised its commercial value (Liu, Liu, Li, Du, & Chen, 2011). On the other hand, LAE is a synthetic surfactant consisting of an ethyl esterified arginine head with a lauroyl tail attached to the α-amino group. LAE is widely recognized as a highly powerful preservative agent for a large variety of food-borne bacteria (Becerril, Manso, Nerin, & Gómez-Lus, 2013; Otero et al., 2014) a property that is due to its ability for altering the microorganisms metabolism without producing cellular disruptions (Rodriguez, Seguer, Rocabayera, & Manresa, 2004). LAE has been assessed to be nontoxic and it has been classified by FDA (Food and Drug Administration) as GRAS (Generally Recognized as Safe) at concentrations up to 200 ppm. According to their biological properties, ionic coupling of LAE with HyA is envisaged as a very convenient approach to build antimicrobial films with potential use in food preservation and design of medical antiseptic devices.

64 The working hypothesis for this research is that LAE·HyA complexes will be readily
65 formed with prefixed compositions, and that they will show strong activity against pathogens
66 because LAE will be released under control to the wet environment by water-mediated
67 dissociation of the ionic pair. LAE and HyA are largely expected to combine ionically because
68 both compounds have been reported to form stable ionic complexes when they enter in contact
69 with diverse counter-ions. Thus LAE is known to interact positively with the anionic
70 polysaccharides present in the food (Asker, Weiss, & McClements, 2009; Loeffler et al., 2014),
71 and complexes of HyA with a diversity of organocationic compounds have been also described
72 (Battistini et al., 2017; Bračič, Hansson, Pérez, Zemljič, & Kogej, 2015; Chytil, Trojan, &
73 Kovalenko, 2016; Tolentino, Alla, Martínez de Ilarduya, & Muñoz-Guerra, 2013). Complexes of
74 LAE with poly(γ-glutamic acid) (LAE·PGGA) with antibacterial properties have been recently
75 described by us (Gamarra-Montes, Missagia, Morató, & Muñoz-Guerra, 2017).
76 In this paper cationic LAE is coupled with the polyanionic hyaluronic acid to obtain the
77 ionic complexes abbreviated as LAE·HyA with LAE to HyA ratios of either 1:1 or 1:2. These
78 complexes are extensively characterized by different techniques (FT-IR, NMR, TGA, DSC, XRD
79 and POM) and paying detailed attention to the nanostructure. Then the release rate of LAE from
80 complexes is estimated under different conditions and the antimicrobial properties of the films
81 are evaluated in vitro experiments against both Gram-positive (*L. monocytogenes* and *S.*
82 *aureus)* and Gram-negative bacteria (*S. enterica* and *E.coli).*

83 **2. Experimental**

84 *2.1. Materials*

85 The sample of sodium salt of hyaluronic acid (Na·HyA) with a weight-average of 50,000
86 Da was purchased from Enze Chemicals. The ^{1}H NMR spectrum ascertaining the identity of this
87 sample is provided in the Supplementary Material file (SM) associated to this article (Figure S1).

The sample of Ethyl $^{\alpha}$*N*-lauroyl L-arginate hydrochloride (LAE) used in this work was a kind gift from Vedeqsa Grupo LAMIRSA (Terrassa, Barcelona).

2.2. Measurements

FTIR spectra within the 4000-600 nm range were recorded from powder samples on a Perkin Elmer Frontier equipment provided with an ATR accessory. ^{1}H NMR spectra were recorded on a Bruker AMX-300 NMR instrument operating at 300.1 MHz. Samples were dissolved in deuterated methanol and TMS was used as internal reference. Thermogravimetric analysis (TGA) was performed over the 30 to 600 ºC interval at a heating rate of 10 ºC·min^{-1} under an inert atmosphere on a Mettler-Toledo TGA/DSC 1 Star System thermobalance. Sample weights of 10-15 mg were used for this analysis. Differential scanning calorimetry (DSC) was carried out on a Perkin-Elmer DSC 8000 instrument calibrated with indium and zinc. Heating-cooling cycles at a rate of 10 ºC·min^{-1} under a nitrogen atmosphere within the -30 to 200 ºC temperature range were applied for the analysis using sample weights of 2-5 mg. X-ray diffraction studies were performed using X-ray synchrotron radiation at the BL11 beamline (NCD, Non-Crystalline Diffraction) of ALBA synchrotron in Cerdanyola del Vallès, Barcelona. Simultaneous SAXS and WAXS were taken in real time from powder samples subjected to heating-cooling cycles at a rate of 10 ºC·min^{-1}. The radiation energy employed corresponded to 0.1 nm wavelength, and spectra were calibrated with silver behenate (AgBh) and Cr_2O_3 for small and wide angle diffraction, respectively. Optical microscopy was carried out on an Olympus BX51 polarizing optical microscope (POM) equipped with a digital camera and a Linkam THMS-600 hot stage provided with nitrogen gas circulating system. Samples for POM observation were prepared by evaporation at room temperature of a 3 % (w/w) solution of the complex in methanol placed between microscope cover slides.

2.3. Complexes formation and film preparation

The methodology used by (Ponomarenko, Waddon, Tirrell, & MacKnight, et al.,1996) for the preparation of ionic complexes from poly(α-amino acids) and ionic surfactants was applied in this work. This methodology with some slight modifications has been used by us for the synthesis of complexes made from either PGGA or polyuronic acids and quaternary tetraalkylammonium salts bearing long linear alkyl chains containing from 12 to 22 carbon atoms (Pérez-Camero et al., 2004; Tolentino, Alla, Martínez de Ilarduya, & Muñoz-Guerra, 2011a, 2013) and also for the preparation of LAE·PGGA complexes (Gamarra-Montes, Missagia, Morató, & Muñoz-Guerra, 2017). The procedure applied here is essentially as follows: A solution of LAE hydrochloride in water was slowly poured into a solution of NaHyA in water under mild stirring at a temperature around 35 ºC. The complex precipitated as a white powder after several hours of standing. The precipitate was recovered by centrifugation and repeatedly washed with water and finally dried under vacuum for at least 48 h. Complexes were prepared from mixtures containing both 1:1 and 1:2 molar ratios of LAE to HyA, and they are abbreviated as LAE·HyA-1 and LAE·HyA-0.5, respectively.

Consistent films made of LAE·HyA complexes were prepared by casting a solution of the complex in methanol on 3x3 cm^2 Petri plates and leaving it to dry at room temperature for 24 h. Films were cut in 1x1 cm^2 squares and further dried under vacuum for 24 h. Film thickness were estimated to be 105 ± 5 μm as measured by using a Mitutoyo micrometer (Osaka, Japan).

2.4. Complex dissociation and bactericide activity

For evaluation of the dissociation of LAE·HyA complexes upon incubation in aqueous medium, LAE·HyA complex films were suspended in 2 mL of buffer and the suspension placed into cellulose membrane tubes (2000 Da cut-off) and dialysis performed against 10 mL of the same buffer for a week. Assays were carried out at pH= 7.4 and 5.5 at 25 ºC, and the amount of LAE released to the environment was determined by measuring the absorbance of the dialysate

at 220 nm at prefixed incubation times. Aqueous buffers used for incubation were prepared from mixtures of disodium hydrogen phosphate and potassium hydrogen phosphate according to the European Pharmacopea 5.0.

The antimicrobial activity of LAE·HyA complexes over time was tested in vivo against both Gram-negative and Gram-positive bacteria in liquid culture media. Cultures of *S. enterica* CECT 4594, *E. coli* NCTC 9001, *L. monocytogenes* ATCC 19115 and *S. aureus* ATCC 6538 were obtained from the National Collection of Type Cultures (NCTC, Public Health England, UK), the Spanish Type Culture Collection (CECT, Valencia, Spain) and the American Type Culture Collection (ATCC, USA), respectively. The organisms were stored at -20°C in tryptic soy broth (TSB; Merck, Darmstadt, Germany) containing 50% (v/v) glycerol until used. To activate them, a *loopful* of each bacterium was spread to give single colonies on tryptic soy agar (TSA; Difco Laboratories) petri dishes, and incubated at 37 °C for 24 h. Representative colonies were then picked out and suspended into 10 mL tubes of TSB pH 7.0, and incubated at 37 °C for 24 hours to obtain early stationary phase cells (optical density of 0.9 at 600 nm). The cultures were then inoculated (100 µL) into fresh TSB, and incubated at 37 °C for 18 h to reach the exponential phase (optical density of 0.2 at 600 nm). At this stage the bacteria cultures were diluted to obtain a concentration of 10^5 CFU·mL^{-1}. 100 µL of these bacterial suspensions were then transferred to sterile tubes containing an approximate 1 cm^2-film piece of HyA (negative control), LAE·HyA-1, or LAE·HyA-0.5 in 10 mL of fresh TSB. The tubes were placed in a thermostated oven at 37 °C and 100 µL aliquots were removed from the suspension at selected periods of time (2, 8 and 24 hours), diluted with peptone water (1% v/v), and plated in petri dishes with 15 mL of TSA culture medium. A bacterial suspension prepared in the same way but without film added was used as blank. Quantification of colonies was made in triplicates and data are represented as logarithm of colony forming units (Log(CFU)). Formulae used for logarithm reduction value (LRV) and percent reduction calculation were the following (Durán, Marcato, De Souza, Alves, & Esposito, 2007),

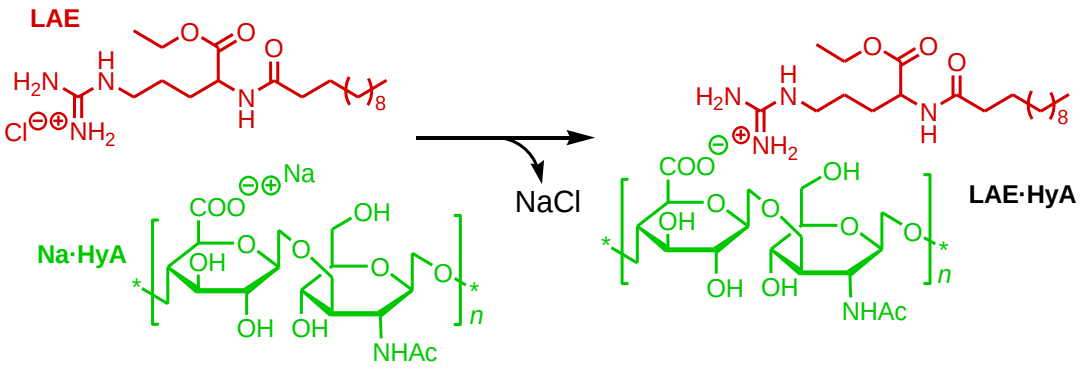
Log reduction value = $\log_{10}(A/B)$ Percentage reduction = $[(A-B)/A]\cdot 100$

where A and B are the number of viable bacteria in the negative control and after treatment with either LAE·HyA-1 or LAE·HyA-0.5.

3. Results and discussion

3.1. Synthesis of complexes

The preparation of LAE·HyA complexes was performed according to Scheme 1. Mixing at 35 ºC of the two aqueous solutions containing LAE and Na·HyA, respectively, rendered the complex as a white precipitated that could easily recovered by centrifugation. Two LAE·HyA molar ratios, *i.e* 1:1 and 1:2, were used at mixing with the purpose of obtaining complexes with equal or half molar amount of LAE respect to HyA. The ^{1}H NMR analysis revealed that the actual compositions of the complexes were very close to those used for feeding with a slight deficiency in the cationic component. Data related with the synthesis are given in Table 1.



Scheme 1. Formation of LAE·HyA complexes by coupling reaction of LAE with hyaluronic acid. LAE to HyA ratios of 1:1 and 1:2 were used.

3.2. Chemical characterization

The FTIR spectra recorded from the LAE·HyA complexes are shown in Figure 1 together with those of Na·HyA and LAE and their interpretation was supported by previously reported

data of the two components (Gilli et al., 1994; Gamarra, Missagia, Morató, Muñoz-Guerra, 2017). The spectra of the complexes include the bands characteristic of the two counterparts with relative intensities according to composition. In fact, the digital region of these spectra over the 1800-650 cm^{-1} may be made to correspond to the overlapping of those observed for HyA and LAE with their contributions being proportional to their contents in the complexes. In particular the bands characteristic of LAE appearing at 1526 and 1176 cm^{-1} are detected in the complexes with intensity increasing with the LAE:HyA ratio. Also the bands at 2917 and 2840 cm^{-1} due to the stretching of C-H bonds, which are shared by the two components but with much higher intensity in LAE, increase in parallel to the content of this compound in the complex. On the other hand, the strong band displayed by HyA at c.a. 1030 cm^{-1} and arising from C-O-C stretching keeps the same features in the spectra of the complexes. Finally just to notice that the broad absorption displayed by HyA in the 3500-3200 cm^{-1} region, which is attributed to hydroxyl groups, is fully retained in the complexes impeding the visualization of the weak NH stretching peaks expected to arise from the LAE counterpart.

Table 1. Synthesis, thermal properties and structural data.

Compound	Synthesis results				Thermal properties				X-ray diffraction data (nm)					
					TGA[d]			DSC[e]	SAXS[f]			WAXS[f]		
	c^a (M)[a]	T (ºC)[b]	Yield (%)	LAE:HyA[c]	$^{o}T_d$ (ºC)	$^{max}T_d$ (ºC)	W (%)	T_m (ºC)	$L_o^{10ºC}$	$L_o^{120ºC}$	$L_o^{10ºC}$	$d^{10ºC}$	$d^{120ºC}$	$d^{10ºC}$
HyA	-	-	-	-	200	228	35	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
LAE·HyA-0.5	0.02	35	70	0.4 :1.0	217	228/328	19	-	3.8	3.8	3.7	0.45	0.45	0.45
LAE·HyA-1	0.01	35	80	0.9 :1.0	228	259/328	11	-	3.9	3.9	3.8	0.45	0.45	0.45
LAE	-	-	-	-	245	275/311	10	62	3.0	-	-	m[g]	-	-

[a] Concentration of the two solutions mixed to form the complex.
[b] Mixing temperature selected according to the LAE solubility in water.
[c] Molar ratio of LAE to HyA in the complex estimated by ^{1}H NMR.
[d] $^{o}T_d$ and $^{max}T_d$: onset (5% weight loss) and maximum rate decomposition temperatures; W: remaining weight after heating at 600 ºC.
[e] T_m is the melting temperature recorded by scanning calorimetry.
[f] Small angle (SAXS) and wide angle (WAXS) X-rays scattering recorded at 10 ºC, 120 ºC and 10 ºC (after cooling) as indicated.
n.d.: not determined.
[g] Many peaks observed.

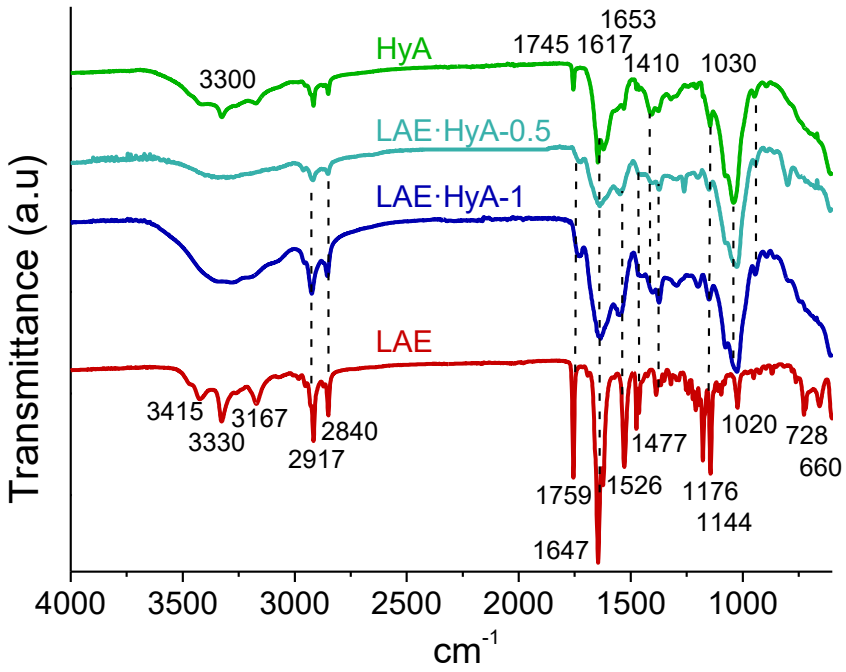


Figure 1. Comparison of FTIR spectra of LAE, HyA and their complexes with indication of most characteristic bands.

The ^{1}H NMR spectrum recorded from LAE-HyA-1 in methanol solution is reproduced in Figure 2 with all signals consistently assigned to the chemical structure of the complex. The spectra of LAE, HyA, and the LAE-HyA-0.5 complex may be inspected in the SM file (Figures S1 and S2). In the complexes' spectra, signals due to of the HyA counterpart are according to that reported for the complexes made from HyA and alkyltrimethylammonium surfactants (Tolentino, Alla, Martínez de Ilarduya & Muñoz-Guerra, 2013), and those arising from the surfactant counterpart coincide almost exactly with those observed in the spectrum of pristine LAE. In general signals appear well resolved although those arising from the inner methylenes of the dodecyl group of LAE appear overlapped (k,l), and those due to the protons of the pyranose rings are grouped in two undifferentiated broad signals (2-5 and 2'-6').

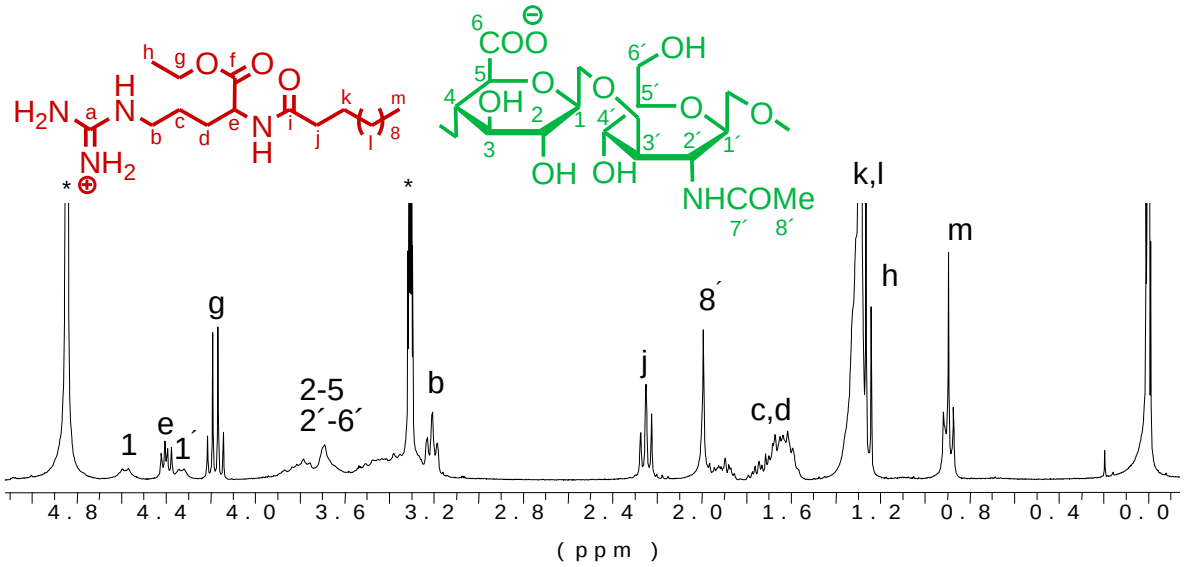


Figure 2. ^{1}H NMR spectrum of LAE·HyA-1 recorded in MeOD. *Signals arising from the solvent.

Signals arising from the anomeric proton of the glucuronic unit of HyA and the α-methylene protons of the lauroyl moiety of LAE (signals 1 and j) were used to determine the composition of the complexes. The relative area quantification of these signals revealed that the complexes have compositions very close to those used for their synthesis (Table 1), with deviations within the range that could be expected according to results obtained for other ionic complexes of HyA previously studied by us (Tolentino, Alla, Martínez de Ilarduya & Muñoz-Guerra, 2013; Gamarra, Missagia, Morató, Muñoz-Guerra, 2017).

3.2. Thermal properties and structure

Firstly the thermal stability of the complexes was evaluated by TGA. The traces registered for both LAE-HyA-1 and LAE-HyA-0.5 over the 20-600 ºC were compared with those of HyA and LAE in Figure 3a, and their respective derivative curves are represented in Figure 3b. It should be noted that the trace of HyA shows a weight loss of about 5% at temperatures around 100 ºC, which is interpreted as due to the release of the remaining adsorbed water that could not be removed by the drying treatment applied to the sample. Nevertheless, all compounds

started to decompose above 200 ºC at an onset temperature that increases with the content in LAE. Furthermore, the maximum decomposition rate in LAE took place around 50 ºC higher than for HyA. These differences are reflected in the complexes which show $^{max}T_d$ of 230 ºC and 260 ºC for LAE-HyA-0.5 and LAE-HyA-1, respectively. It can be stated on the basis of these results that the LAE·HyA complexes could be comfortably handled in the case that usual heating processes were applied for film manufacture.

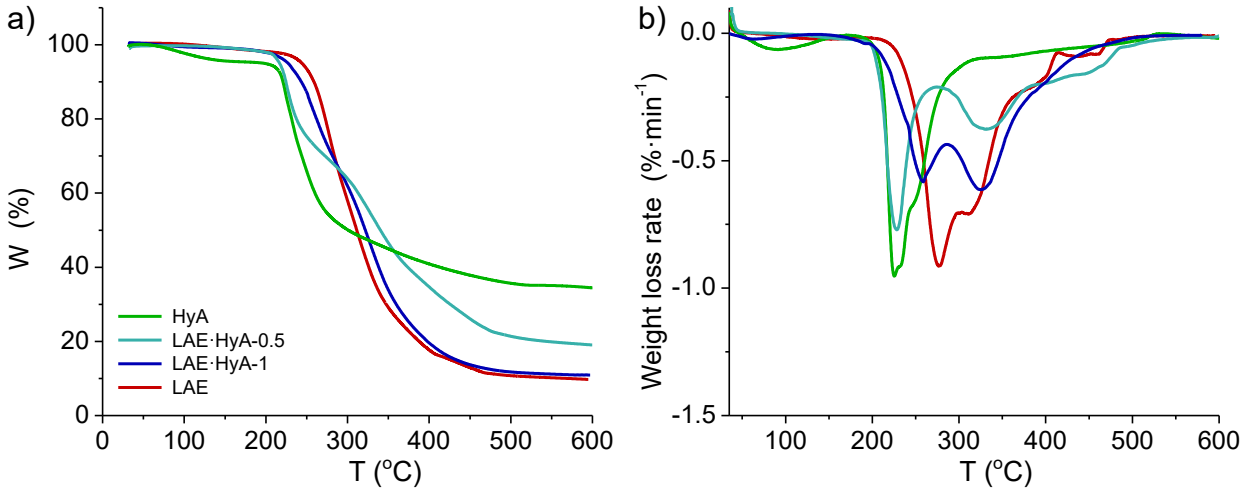


Figure 3. TGA traces of HyA, LAE and the LAE·HyA complexes recorded under an inert atmosphere (a), and their respective derivative curves (b).

The DSC analysis (Figure 4a) revealed that only LAE shows melting transition whereas almost plain traces were registered for HyA and their complexes (the slight endotherm appreciated on the trace of HyA in the 80-120 ºC interval is very likely due to water evaporation). The crystalline nature of LAE was clearly evidenced in the spherulitic texture that is seen in films of this compound prepared by casting when they are observed by POM (Figure 4b left). Accordingly, it can be concluded that the crystallinity of LAE is lost when this compound is attached to HyA. However, POM of the LAE·HyA-1 film showed a fan-like texture typical of a smectic mesophase (Figure 4b right) indicating that complexes are ordered in a crystal liquid

structure. A similar appearance was observed for the LAE·HyA-1 complex (Figure S3 in SM file).

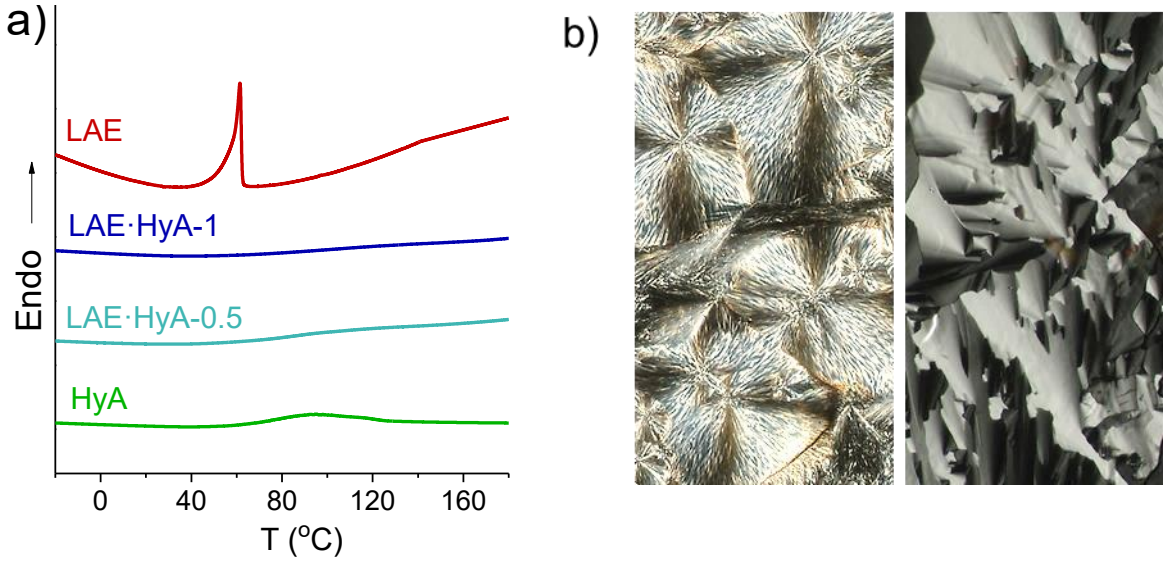


Figure 4. a) First heating DSC traces recorded from LAE and its ionic complexes. b) POM micrographs taken from LAE (left) and LAE·HyA-1 (right) thin films at 25 ºC.

The XRD scattering recorded in the WAXS region for LAE and the complexes (Figure 5a) was in full agreement with DSC results. The trace recorded for LAE contains a large number of well-defined peaks, as it should be expected for a crystalline organic compound, whereas a broad hill centered around 0.45 nm is the only alteration appearing on the traces of the complexes. In the SAXS region (Figure 5b), spectra of both LAE and complexes show an apparent sharp peak corresponding to a repeat distance of 3.0 nm for the former and around 3.9-3.8 nm for the latter. These results confirm the occurrence of an ordered arrangement at the nanometric level in the complexes in spite that they are not crystallized.

With the purpose of assessing the influence of temperature on these structures, samples of LAE and LAE·HyA-1 were subjected to a heating-cooling cycle over the 10-120 ºC range at a rate of 10 ºC·min^{-1}, and the changes taking place in the X-ray scattering were registered over

the whole temperature range at real time (Figure 6). Both SAXS and WAXS signals observed for LAE fully disappeared when temperature reached 60-65 ºC, which is clear evidence that the structure melted and all the order initially present in the sample vanished. The cooling trace did

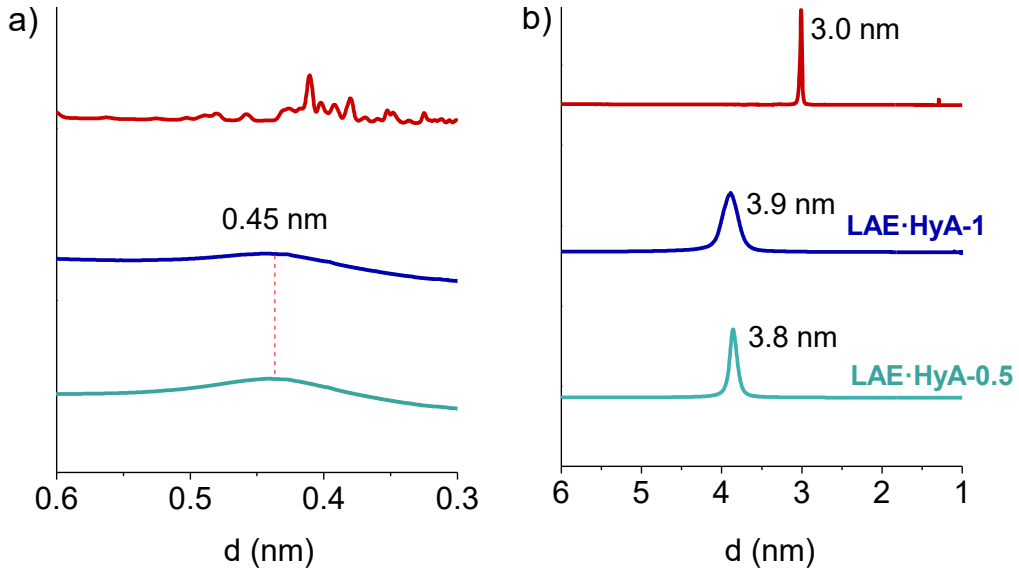


Figure 5. XRD profiles recorded at 10 ºC from LAE and their complexes LAE·HyA in the WAXS (a) and SAXS regions (b).

not show any discrete scattering (see Figure S4 in the SM file) indicating that the initial structure present in LAE was not recovered. The behavior observed for LAE·HyA-1 was noticeably different. The 3.9 nm peak remained unchanged along the whole heating treatment bringing into evidence the stability of the mesophase up to temperatures close to 200 ºC. This is consistent with the observations made by POM in which the birefringence displayed by the films of LAE·HyA-1 did not vanished when subjected to heating at similar temperatures. Similar results were obtained in the XRD/POM analysis of LAE·HyA-0.5 complex (Figures S5 in SM). It seems reasonable to assume therefore that higher temperatures are needed to destroy the liquid crystal phase adopted by these complexes, an assumption that unfortunately cannot be tested since their thermal decomposition starts to be noticeable at 215-230 ºC. As much expected, the

WAXS profiles arising from the average interatomic distances present in the disordered phase made of lauroyl chains of the LAE moiety remained unperturbed along the heating treatment.

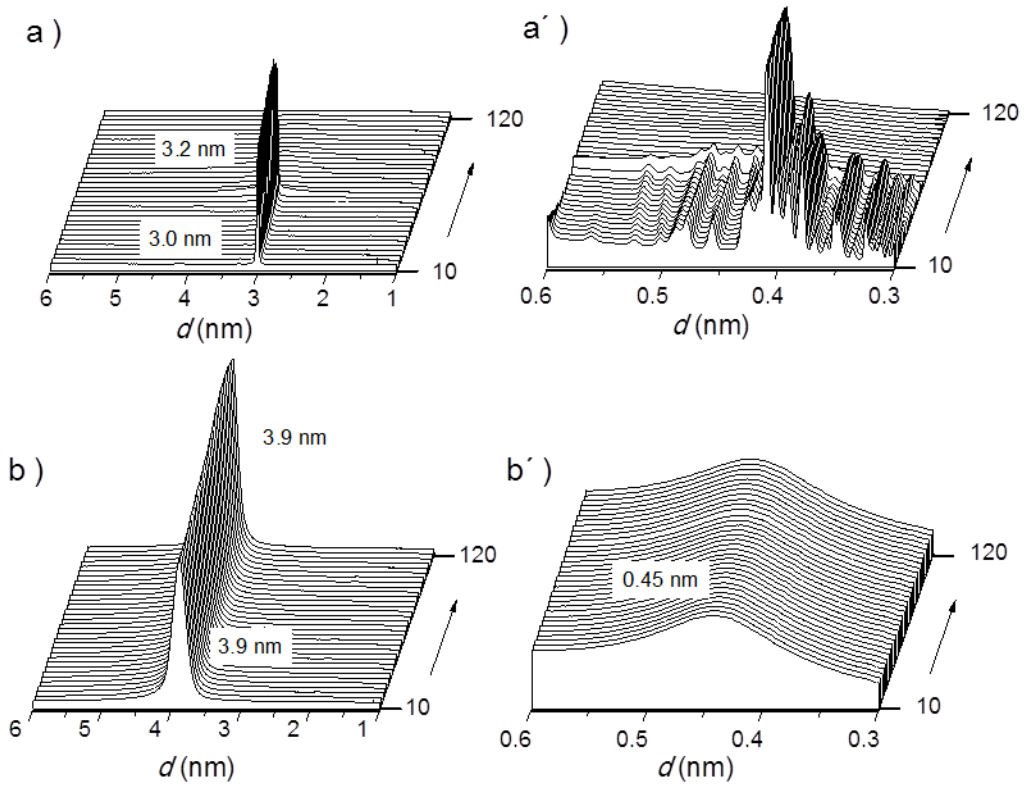


Figure 6. Evolution of the SAXS and WAXS profiles of LAE (a and a') and LAE·HyA-1 (b and b') at heating from 10 to 120 °C.

3.3. *LAE release and antimicrobial properties*

According to the hypothesis formulated for this work, the biocide activity expected for the LAE·HyA complexes should arise from the free LAE that is released to the environment as a consequence of the dissociation undergone by the ionic pair upon incubation in an aqueous medium. To evaluate the capacity of these complexes to deliver LAE under conditions commonly found in food environments, the amount of LAE that is released from both LAE·HyA-1 and LAE·HyA-0.5 and accumulated in the incubation medium at pH 7.4 and 5.5 was

measured by UV absorption at 220 nm, and results are compared in Figure 7. In all cases, the dissociation equilibrium was reached within an incubation period of 4 to 8 h. The concentration of LAE attained at that time was much higher at pH 7.4 than at pH 5.5 and, as largely expected, the accumulated amount of free LAE noticeably increased with the LAE:HyA ratio in the complex. An additional experiment carried out at pH 7.4 but at a temperature of 4 ºC revealed that cooling delayed the release of LAE in an extent similar to that observed for decreasing the pH down to 5.5 (Figure S6 in SM).

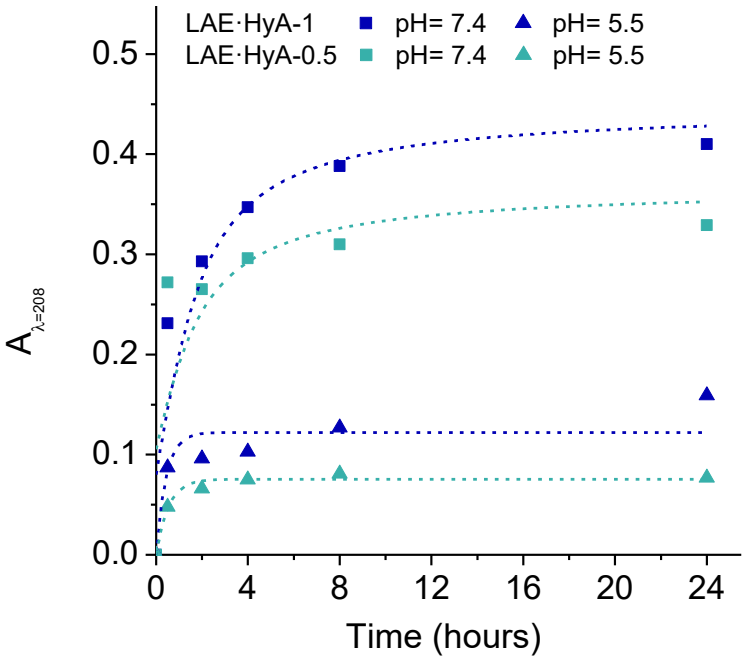
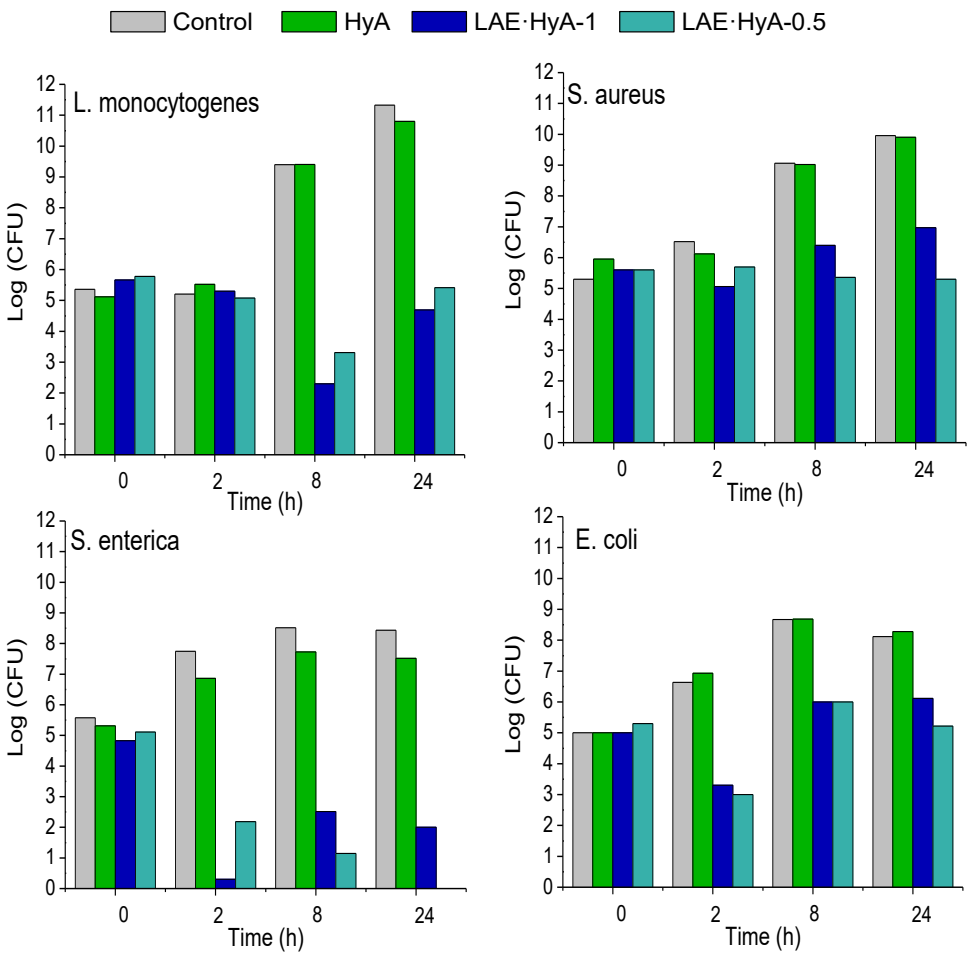


Figure 7. Dissociation of LAE·HyA complexes upon incubation in aqueous medium at pH= 7.4 and 5.5 at 25 ºC.

The bactericide activity of films made of LAE·HyA-1 and LAE·HyA-0.5 complexes at pH 7.4 and 37 ºC was evaluated by in vitro assays applying the liquid medium method and using as substrates two pairs of bacteria representatives of the Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative (*S. enterica* and *E. coli*) groups. A fast appraisal of the bactericidal effect could be made by visual inspection of the tubes which revealed disappearance of the

initial turbidity for all the cases after 24 h of incubation (Figure S7, SM file). Quantitative results of these preliminary assays expressed as Log(CFU) (colony forming units), Log(RV) (reduction value) and PR (percentage of reduction) are compared in the bar graphs of Figure 8 for the four bacteria, and numerical values of these results are collected in the Table S1 of the SM file. As it can be inferred from the graphs, the antimicrobial activity of both, LAE·HyA-1 and LAE·HyA-0.5, is noticeable from the earlier incubation stages so they inhibited the growth of all bacteria just after 2 h of contact. This activity was found especially important against Gram-negative bacteria so that Log(RV) values of 6.6 and 4.7 for *S. enterica,* and 3.6 and 3.9 for *E. coli* was measured for LAE·HyA-1 and LAE·HyA-0.5, respectively. This result is remarkable since Gram-negative bacteria are usually less sensible to cationic surfactants than Gram-positive bacteria due to the outer lipopolysaccharide coat that surrounds the cell wall (Vaara, 1992).

The antimicrobial activity of the complexes against Gram-positive bacteria was found to be lower with Log(RV) of 0.2 and 0.4 observed for *L. monocytogenes* and 1.1 and 0.4 for *S. aureus* for LAE·HyA-1 and LAE·HyA-0.5, respectively, after 2 h of incubation. The higher antimicrobial efficacy observed for the complex containing more LAE is quite reasonable and according to observations reported on antimicrobial films based on LAE loaded chitosan (Higueras, López-Carballo, Hernández-Muñoz, Gavara, & Rollini, 2013). However, it was interesting to see that the antimicrobial activity of the LAE·HyA-0.5 complex against *S. aureus* became higher than that of LAE·HyA-1 when long incubation times (8 and 24 h) have elapsed. This behaviour may be convincingly explained by taking into account the observations reported by Baier et al (Baier et al., 2013) on the biocide activity of HyA-based nanocapsules containing the antimicrobial agent polyhexanide. They found that *S. aureus* was particularly sensitive to the biocide agent incorporated in such systems because its release was favored by the biodegradation of HyA, a process that happened due to the action of the hyaluronidases provided by the bacteria.



340 **Figure 8.** Antimicrobial activity of LAE·HyA-1 and LAE·HyA-0.5 films over time against Gram-positive and
341 Gram-negative bacteria.

342 ## 4. Conclusions

343 The well recognized biocide guanidinium-based compound LAE was coupled with
344 hyaluronic acid to render ionic complexes with two compositions, *i.e* LAE:HyA = 1 and 0.5.
345 These complexes were non-soluble in water and stable to heat up to temperatures above 200
346 ºC. Although the genuine crystallinity of LAE was lost when it was incorporated in the complex,
347 a smectic liquid crystal arrangement was adopted by both LAE·HyA-1 and LAE·HyA-0.5. The

nanostructure of the complexes is characterized by a periodicity of about 3.8-3.9 nm corresponding to the repeating distance of an alternating array of HyA and LAE layers in which the undecyl tail of the lauroyl group of LAE is in the disordered state. These complexes dissociated upon incubation in an aqueous medium in a range of hours providing free LAE at equilibrium concentrations depending on pH and complex composition. Films prepared from these complexes displayed significant biocide activity against both Gram-positive and Gram-negative bacteria with the highest efficiency being shown by LAE·HyA-1 against *S. enterica*.

Acknowledgements

This work received financial support from MCINN (Spain) with Grant MAT2012-38044-C03 and MAT-2016-77345-CO3-03. XRD analyses realized in this research were carried out at the BL11 line of ALBA synchrotron (Cerdanyola del Vallès, Barcelona, Spain) with the invaluable support of Dr. Christina Kamma-Lorger. Thanks to Dr. Marta Fernández García from CSIC (Madrid) for her valuable comments on the methodology followed for biocide activity estimation. Authors acknowledge the LAE sample kindly gifted by Vedeqsa (LAMIRSA GROUP, Terrassa, Barcelona, Spain). Thanks also to the MICINN for the Ph.D. grant awarded to Ana Gamarra Montes.

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