Carbohydrate Polymers 92 (2013) 691–696

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Comb-like ionic complexes of hyaluronic acid with alkyltrimethylammonium surfactants

Ainhoa Tolentino, Abdelilah Alla, Antxon Martínez de Ilarduya, Sebastián Muñoz-Guerra*

Department d'Enginyeria Química, Universitat Politècnica de Catalunya, ETSEIB, Diagonal 647, Barcelona 08028, Spain

ARTICLE INFO

Article history:
Received 5 August 2012
Received in revised form
12 September 2012
Accepted 22 September 2012
Available online 28 September 2012

Keywords:
Ionic polymer complex
Comb-like polymer
Hyaluronic acid
Polyuronic acid
Surfactant polyuronic complex

ABSTRACT

Stoichiometric complexes of hyaluronic acid with alkyltrimethylammonium surfactants bearing octadecyl, eicosyl and docosyl groups were prepared by ionic coupling in aqueous solution. The complexes were non soluble in water but soluble in organic solvents. In the solid state they self-assembled in a biphasic layered structure with the alkyl side chains forming a separate phase that melted in the 50–60 °C range. They were stable to heating up to above 200 °C.

1. Introduction

Hyaluronic acid (HyalA) is a linear polysaccharide ubiquitous in the human body. It is composed of repeating disaccharide units of β-1,3-N-acetyl glucosamine and β-1,4-glucuronic acid with a molecular weight up to 6 million Da. With excellent viscoelasticity, high moisture retention capacity, high biocompatibility and non-immunogenicity, HyalA finds a wide-range of applications in surgery, cosmetology, veterinary science, and hygiene medicine, where it has been used for over 30 years (Kogan, Soltes, Stern, & Gemeiner, 2007; Necas, Bartosikova, Brauner, & Kolar, 2008). When chemically modified by reaction of the pendant reactive groups, HyalA can be transformed into a variety of new biomaterials with properties suitable for tissue repair and regeneration (Allison & Grande-Allen, 2006; Burdick & Prestwich, 2011; Schante, Zuber, Herlin, & Vandamme, 2011). HyalA has been recently explored for its use in novel drug delivery systems with increasing enthusiasm because HyalA-binding receptors are believed to be involved in cancer metastasis (Yadav, Mishra, & Agrawal, 2008). Traditionally HyalA was extracted from rooster combs but now it is increasingly produced through microbial fermentation, which has enhanced its application interest and commercial value (Izawa et al., 2009; Liu, Liu, Li, Du, & Chen, 2011).

In these last years it has been revealed that coupling of polyelectrolytes with ionic surfactants is a convenient method for the preparation of ionic complexes with remarkable structure and properties (Macknight, Ponomarenko, & Tirrell, 1998; Ponomarenko, Waddon, Tirrell, & Macknight, 1996). Specifically, coupling of polyacids with tetraalkylammonium surfactants bearing long alkyl chains is known to lead to amphiphilic comb-like systems displaying a layered biphasic structure (Fig. 1a) able to lodge agents with chemical or biomedical activity (Portilla-Arias, García-Alvarez, Martínez de Ilarduya, Holler, & Muñoz-Guerra, 2006a, 2006b; Pérez-Camero et al., 2004; Portilla-Arias, García-Alvarez, Martínez de Ilarduya, & Muñoz-Guerra, 2007a). In this communication we wish to report on the preparation, structure and thermal behavior of complexes made of hyaluronic acid with alkyltrimethylammonium surfactants, abbreviated as *n*ATMA·HyalA, with alkyl chains containing 18, 20 and 22 carbon atoms (Fig. 1b). Recently we have reported on similar complexes made of polyuronic acids (*n*ATMA·PUR), specifically, polygalacturonic and alginic acids (Tolentino, Alla, Martínez de Ilarduya, & Muñoz-Guerra, 2011; Tolentino, Martínez de Ilarduya, Alla, & Muñoz-Guerra, 2010) and the binding of certain amphiphilic drugs to HyalA was investigated with regards to the flexibility of the polyanion (Caram-Lelham, Hed, & Sundelöf, 1997). The choice of HyalA as polyacid made in this work obeys to two reasons, to extend the ionic coupling method to this polysaccharide in order to broaden its potential as biomaterial, and to appraise how the alternating ionic structure of Hyal may affect the formation of these comb-like ionic complexes.

---

* Corresponding author. Tel.: +34 934016680; fax: +34 934017150.
E-mail address: sebastian.munoz@upc.edu (S. Muñoz-Guerra).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.09.042

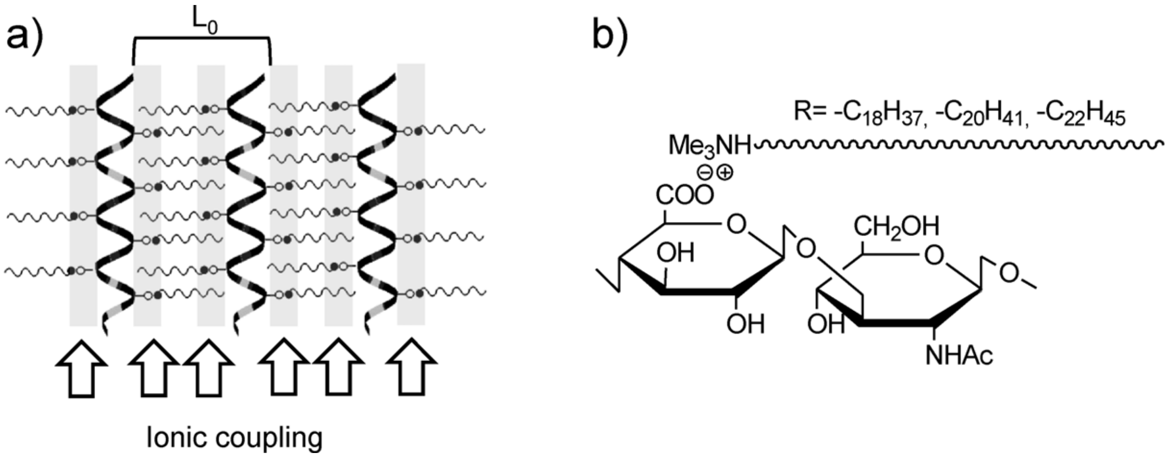

Fig. 1. (a) Side view of the layered structure usually adopted by comb-like ionic polymer complexes and (b) chemical structure of *n*ATMA·HyalA ionic complexes.

2. Experimental part

2.1. Materials

The sodium salt of hyaluronic acid (Na·HyalA), poly(sodium-β-D-glucuronate-[1-3]-β-*N*-acetyl-D-glucosamine-[1–4]), with a weight-average molecular weight of about 50,000 g mol^{-1} used in this work was purchased from Enze Chemicals. Linear alkyltrimethylammonium bromide surfactants of general formula RMe_3NBr were purchased from Sigma–Aldrich (octadecyl, $R = —C_{18}H_{37}$) or synthesized by us according to a procedure described in the literature (eicosyl, $R = —C_{20}H_{41}$ and docosyl, $R = —C_{22}H_{45}$) (Hendrix & Von Rosenberg, 1976).

2.2. Complexes preparation

The complexes were prepared by following the methodology initially reported by Ponomarenko et al. (1996) for poly(α-glutamate) complexes and later applied by us with some minor modifications to the preparation of other complexes (Pérez-Camero et al., 2004; Portilla-Arias et al., 2006a, 2006b; Tolentino et al., 2011). In brief, an aqueous solution of *n*ATMA·Br surfactant was added dropwise to a solution of Na·HyalA in water under stirring at temperatures between 40 and 60 °C depending on the surfactant. Equimolecular amounts of hyaluronic acid and surfactant were used in all cases. The complex precipitated upon standing for a few hours as a white fine powder that was isolated by centrifugation, washed several times in water and dried under vacuum.

2.3. Structure and thermal characterization methods

^1H NMR spectra were recorded on a Bruker AMX-300 NMR instrument with samples dissolved in deuterated water (sodium hyaluronate) or methanol-d_4 (complexes) using TMS as internal reference. Calorimetric analysis was performed under a nitrogen atmosphere in the −30 to 120 °C temperature range with a Perkin-Elmer Pyris 1 DSC instrument provided with an Intracooler device and calibrated with indium and zinc. Thermogravimetric analysis was performed at a heating rate of 10 °C min^{-1} within the 30–800 °C interval under inert atmosphere using a Perkin-Elmer TGA6 thermobalance. X-ray diffraction studies were carried out using synchrotron X-ray radiation (WAXS and SAXS at A2 Hasylab beam line of DESY in Hamburg (Germany) with an energy corresponding to a 0.15 nm wavelength.

3. Results and discussion

3.1. Synthesis of complexes

The complexes *n*ATMA·HyalA with $n = 18$, 20 and 22, were synthesized by mixing aqueous solutions of equimolecular amounts of polyacid and surfactant at the minimum temperature required to dissolve the surfactant. As a result of ionic coupling between the polyacid and the surfactant, complexes precipitated from the mixed aqueous solution after several hours of standing with release of sodium bromide. Complexes were readily isolated by centrifugation as white hygroscopic powders that were soluble in methanol but non-soluble either in chloroform or ethyl ether. The ^1H NMR spectra of the complexes displayed resolution enough as to ascertain the presence of the two counterparts. The spectra of Na·HyalA and 18ATMA·HyalA complex are comparatively depicted in Fig. 2 with full assignment of their respective signals (Blundell, Reed, & Almond, 2006). The composition of the complexes was accurately determined by ^1H NMR on the basis of the area ratio of the hyaluronic acid methyl protons signal (CH_3, $\delta = 2.0$ ppm) to the alkylammonium interior methylenes accumulative signal ($^{3-17}CH_2$, $\delta = 1.2–1.5$ ppm). The results afforded by this analysis revealed that all the *n*ATMA·HyalA complexes had essentially a stoichiometric composition showing an ATMA to HyalA ratio between 1 and 1.1.

3.2. Thermal properties

The thermal behavior of *n*ATMA·HyalA complexes was examined by both TGA and DSC, and data afforded by these analyses are gathered in Table 1. The TGA traces recorded from the complexes in the 25–800 °C range are plotted in Fig. 3 with their respective derivative curves displayed in the inset. These results indicated that *n*ATMA·HyalA complexes have a thermal stability similar to complexes of polygalacturonic and alginic complexes with onset temperatures near below 210 °C (Tolentino et al., 2011). In all cases, decomposition took place along a multi-stage process involving at least three successive stages with maximum rate decomposition temperatures in the 215–225 °C, 265–300 °C and in the proximities of 400 °C, respectively. The weight lost in these three steps was 30–35%, 40–50% and 10–15% of the original mass; no residue was left after heating at 800 °C. The thermal decomposition mechanism of comb-like ionic complexes made from polyglutamic and polymalic acids has been studied by us in some detail (Portilla-Arias et al., 2006a, 2006b; Portilla-Arias, García-Alvarez, Martínez de

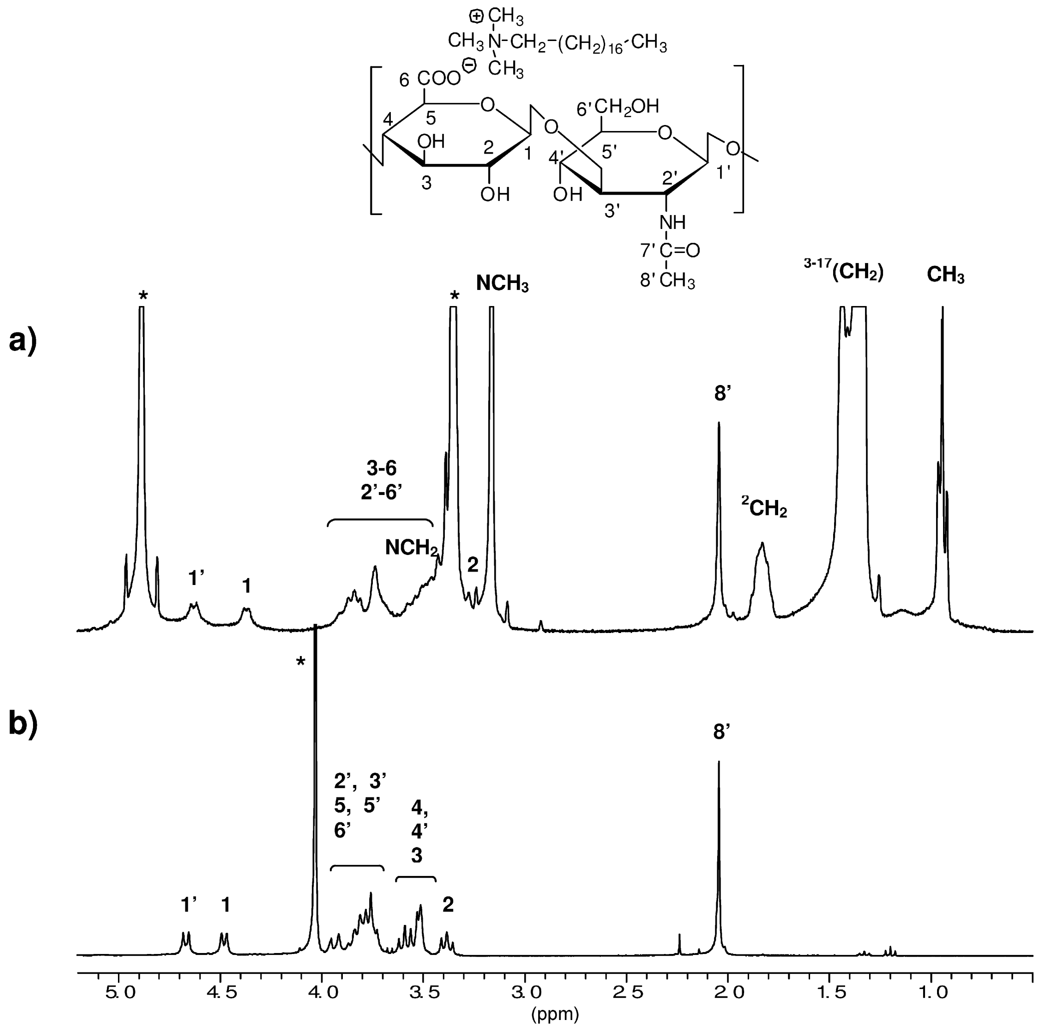

Fig. 2. ^{1}H NMR spectra of 18ATMA·HyalA (in MeOH-*d*) (a) and Na·HyalA (in D_2O) (b). * residual protons of deuterated solvents.

Ilarduya, & Muñoz-Guerra, 2007b); in these complexes decomposition started by depolymerisation of the polypeptide chain along with dissociation of the complex at temperatures slightly above 200 °C followed by degradation of the alkyltrimethylammonium moiety in amines. The thermal decomposition of complexes of polyuronic acids *n*ATMA·PUR seems to happen through a rather more complex mechanism involving degradation of the polysaccharide chain in two stages within the 200–250 °C followed by

Table 1
Thermal properties and XRD data of *n*ATMA·HyalA complexes.

*n*ATMA·HyalA	TGA			DSC		SAXS[e]			WAXS[e]		
	T_d[a] (°C)	T_d[b] (°C)	W^c (%)	T_m[d] (°C)	ΔH_m[d] (kcal mol^{-1})	$L_0^{25\,°C}$ (nm)	$L_0^{T_m}$ (nm)	$L_0^{25\,°C}$ (nm)	$d^{25\,°C}$ (nm)	d^{T_m} (nm)	$d^{25\,°C}$ (nm)
18	209	215–225	68	57	1.7	4.4	4.4	4.4	0.41	0.45	0.41
		265	21			*4.0*	*4.1*	*4.1*	*0.44*		*0.44*
		380–400	10								
		>800	~0								
20	208	215–225	67	66	1.2	4.5	4.6	4.5	0.41	0.45	0.41
		285	22			*4.1*	*4.1*	*4.1*	*0.44*		*0.44*
		380–400	7								
		>800	~0								
22	209	215–225	65	69	2.1	4.5	4.4	4.4	0.42	0.45	0.42
		300	27			*4.1*	*4.1*	*4.1*			*0.44*
		380–400	11								
		>800	~0								

[a] Onset decomposition temperature calculated by the tangent method.

[b] Maximum rate decomposition temperature for each decomposition step.

[c] Remaining weights at the end of the respective decomposition stages.

[d] Melting temperature and enthalpy recorded by DSC from pristine samples.

[e] Long (L_0) and short (d) spacings arising from the repeating interlayer distance and from the interplanar distance of the paraffinic phase at 25 °C, at the melting temperature (70, 75 and 80 °C for n = 18, 20 and 22, respectively) and at 25 °C after cooling. (In italics, spacings arising from the initially disordered phase.)

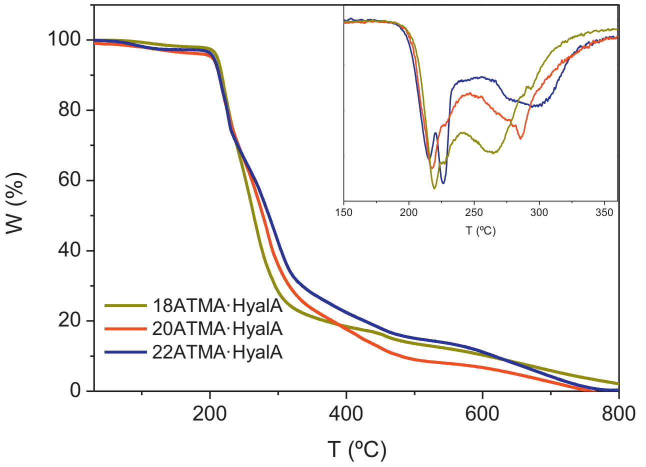

Fig. 3. TGA traces of *n*ATMA·HyalA complexes. Inset: derivative curves showing the maximum rate decomposition temperatures.

decomposition of the complex with release of alkylamines at temperatures steadily increasing with the length of the alkyl side chain.

The DSC analysis revealed that *n*ATMA·HyalA complexes display melting peaks below 80 °C (Fig. 4a). By analogy with *n*ATMA·PUR complexes such peaks should be attributed to the melting of the paraffinic phase constituted by the long alkyl chains of the surfactant. Annealing of the complexes for a few hours at temperatures nearly below melting improved the crystallinity of the material as revealed by DSC (Fig. 4b); peak multiplicity probably arising from the occurrence of crystallite size population heterogeneity, disappeared and the remaining melting peak became sharper and placed at temperatures steadily increasing with the value of *n* (Table 1). The melting enthalpy associated to melting also increased with the length of the alkyl side chain but with a slope much smaller than in the case of *n*ATMA·PUR. Furthermore the evolution of the enthalpy does not follow a monotonous trend as it happens in other complexes series with higher crystallinity previously studied by us (Pérez-Camero et al., 2004; Portilla-Arias et al., 2006a, 2006b; Tolentino et al., 2011). The small differences in enthalpy (less than 1 kcal mol^{-1}) observed in this case may be critically affected by experimental errors probably distorting an actual monotonous trend. Nevertheless, it can be concluded from these DSC results that the thermal behavior of *n*ATMA·HyalA complexes essentially fits in the same pattern as *n*ATMA·PUR complexes which leads to infer that a similar structure must be adopted by all these complexes upon crystallization.

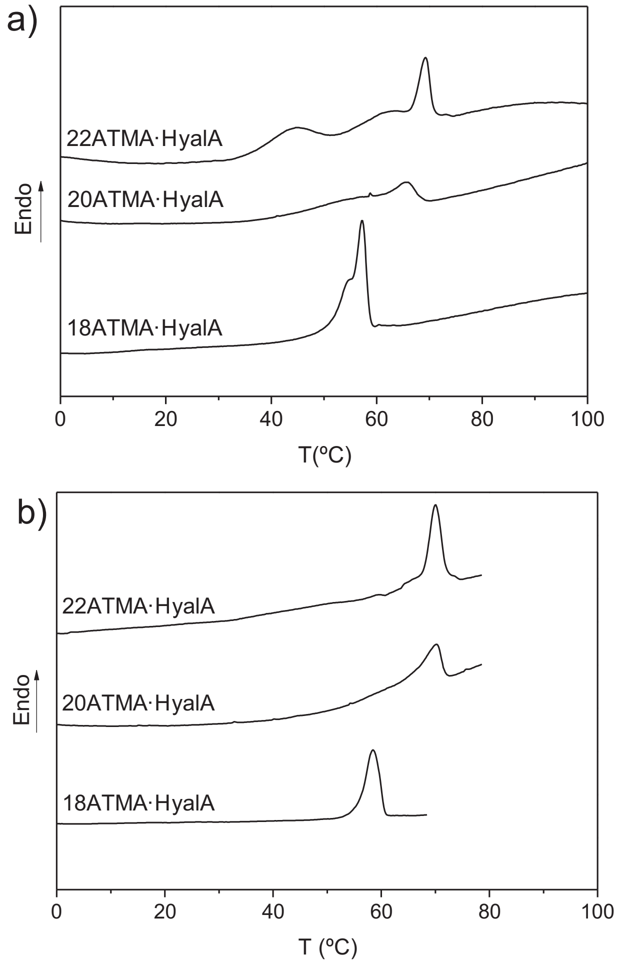

Fig. 4. Heating DSC traces of *n*ATMA·HyalA complexes. (a) Samples coming from synthesis and (b) samples annealed as indicated in the text.

3.3. Structural analysis

Films of these complexes prepared by casting were examined by X-ray diffraction in both wide and small angle regions at different temperatures in order to collect information from both the supramolecular structure and the packing of the alkyl side chains and to evaluate the effect of heating on the structure. The structural spacings recorded from this analysis are gathered in Table 1 and the SAXS profiles recorded from the whole series at 25 °C are compared in Fig. 5a. It was found that the three *n*ATMA·HyalA complexes generated discrete scattering patterns which have in common the presence of a strong peak with a maximum at ~4.4 nm together with a second peak ($n = 18$) or shoulder ($n = 20$ and 22) at ~4.0 nm. In the WAXS angle range, all the complexes show a sharp peak at 0.41–0.42 nm accompanied by another broader one at ~0.45 nm for $n = 18$ and 20 (Figures afforded as SI). According to diffraction data available for other comb-like ionic complexes previously studied by us, and for *n*ATMA·PUR in particular, the 4.4 nm peak should be associated to the periodical spacing L_0 of a biphasic layered structure consisting of sheets made of side-by-side arranged polyuronic chains and separated by a paraffinic phase made of the alkyl side chains. The 0.41–0.42 nm peak appearing in the WAXS region is assigned to d_{100} spacing of the lattice generated by the hexagonal packing of the alkyl side chains arranged in an almost fully extended conformation and oriented with the *c*-axis more or less normal to the plane of the sheets. Although the L_0 spacing in the comb-like ionic complexes usually increases almost linearly with the length of the polymethylene segment, in the present case the interlayer distance appears to be almost constant along the whole series (comparative plot in SI). Nonetheless, this rather exceptional behavior is close to that observed for the complexes of alginic acid, which have been reported to display an increase in L_0 of only 0.2 nm when *n* increases from 18 to 22 (Tolentino et al., 2011).

The evolution of the scattering profile of 20ATMA·HyalA complex with temperature in both SAXS and WAXS regions is shown in Fig. 5b and c. No significant changes were detected in the SAXS profiles after heating up to 75 °C, a temperature well above melting. In fact, the 4.5 nm peak retained its position and intensity, and also its shape remained essentially unaltered. On the contrary, the 0.41 nm peak appearing in the WAXS region vanished at about 70 °C and only the broad peak centered at 0.45 nm was present above that temperature. This process was reversed at cooling with the 0.41 nm peak reappearing at temperatures slightly below 70 °C. The same diffraction pattern was displayed by 18ATMA·HyalA and 22ATMA·HyalA complexes with transition

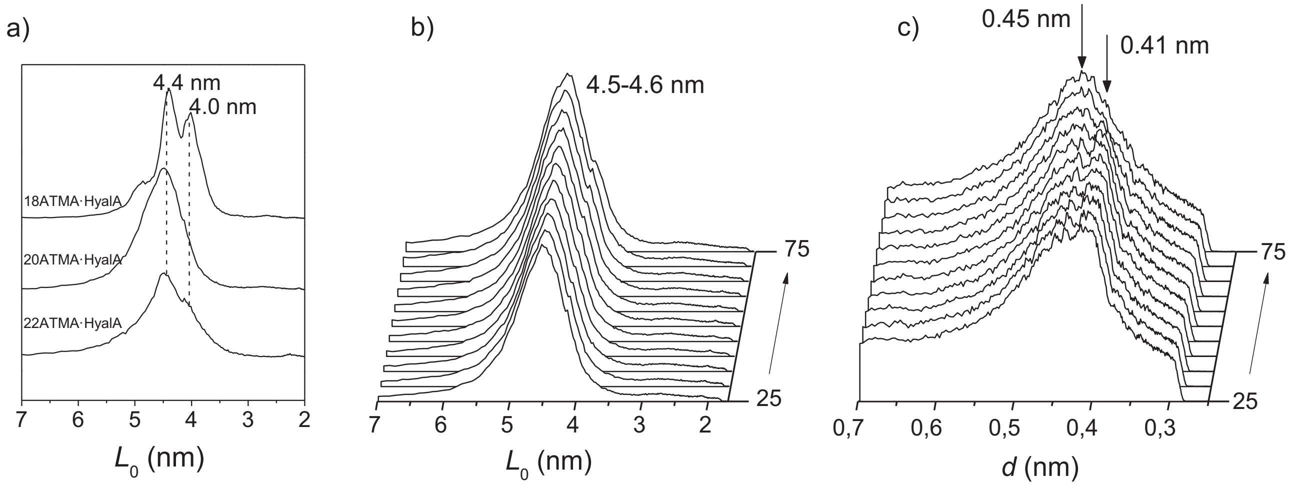

Fig. 5. (a) Compared SAXS profiles of complexes at 25 °C. (b) and c) Evolution of SAXS and WAXS profiles of 20ATMA·HyalA at heating from 25 °C up to above melting.

temperatures correspondingly changed (see SI material), the small deviations in spacings seen in Table 1 falling within the experimental error margins.

In agreement with the DSC results described above and according to previous studies made on other comb-like systems bearing long alkyl side chains, the changes taking place in WAXS region reflect the fusion of the paraffinic pseudohexagonal crystal lattice into a liquid phase in which the polymethylene chains, although disordered, are side-by-side packed with an average chain-to-chain distance of 0.45 nm. The enthalpy of the melting peak observed within the 50–70 °C interval of the DSC traces is an indication of the methylene units fraction that is crystallized in the complex. The recorded melting enthalpy values were in the 1–2 kcal mol^{-1} range, which are much lower than those reported for *n*ATMA·PUR which were in the 2–7 kcal mol^{-1} range corresponding to 3–10 crystallized methylene units (Tolentino et al., 2011). It can be concluded therefore that the fraction of crystallized alkyl side chains in *n*ATMA·Hyal must be very small, which would explain the invariance observed in the L_0 spacing upon heating. The low crystalline order attained in these complexes compared to others similar complexes like those made of polyglutamic, polymalic, polygalacturonic or alginic acids (Pérez-Camero et al., 2004; Portilla-Arias et al., 2006a, 2006b; Tolentino et al., 2011) is likely due to the lower density of anionic charges in HyalA. Although no experimental data are available to ascertain what can be the conformation of HyalA in the complexes, it can be reasonably assumed that the polyacid chain must be in a more or less extended arrangement with the carboxylate ions openly exposed to the ammonium cations. The presence of the non-charged acetylglucosamine units in HyalA implies a larger separation between the carboxylate groups that will hinder the lateral packing of the alkyl chains attached to the coupled ammonium groups.

4. Conclusions

Stoichiometric ionic complexes of hyaluronic acid with cationic alkylammonium surfactants with alkyl side chains containing 18, 20 and 22 carbon atoms could be readily prepared. The complexes were non-water soluble but they dissolved in organic solvents and are stable to heating up to above 200 °C. These complexes self-organized in a biphasic layered structure characteristic of comb-like amphiphilic systems with the polyacid and the alkyl side chains phases alternating periodically with a repeating distance of ~4.5 nm. They displayed melting of the paraffinic phase in the 50–70 °C range without appreciable changes in the layered spacing. The degree of order attained by the alkyl chains in these complexes is significantly lower than usually found in other similar comb-like ionic complexes.

Acknowledgements

XRD work was carried out at the A2 beamline of light source DORIS III at DESY (Hamburg, Ge); authors are indebted to Dr. S. Funari for his invaluable assistance. Thanks also to the MCINN of Spain for Grant MAT2009-14053-C02-01 and the Basque Government for the Ph.D. grant awarded to Ainhoa Tolentino.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.09.042.

References

Allison, D. D., & Grande-Allen, K. J. (2006). Hyaluronan: A powerful tissue engineering tool. *Tissue Engineering*, *12*, 2131–2140.

Blundell, C. D., Reed, M. A. C., & Almond, A. (2006). Complete assignment of hyaluronan oligosaccharides up to hexasaccharides. *Carbohydrate Research*, *341*, 2803–2815.

Burdick, J. A., & Prestwich, G. D. (2011). Hyaluronic acid hydrogels for biomedical applications. *Advanced Materials*, *23*, 41–56.

Caram-Lelham, N., Hed, F., & Sundelöf, L.-O. (1997). Adsorption of charged amphiphiles to oppositely charged polysaccharides. A study of the influence of polysaccharide structure and hydrophobicity of the amphiphile molecule. *Biopolymers*, *41*, 765–772.

Hendrix, W. T., & Von Rosenberg, J. L. (1976). The mechanism of the rearrangement of the hydrocobalt carbonyl catalyzed isomerization of 3-phenylpropene. *Journal of the American Chemical Society*, *98*, 4850–4852.

Izawa, N., Hanamizu, T., Iizuka, R., Sone, T., Mizukoshi, H., Kimura, K., & Chiba, K. (2009). *Streptococcus thermophilus* produces exopolysaccharides including hyaluronic acid. *Journal of Bioscience and Bioengineering*, *107*, 119–123.

Kogan, G., Soltes, L., Stern, R., & Gemeiner, P. (2007). Hyaluronic acid: A natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology Letters*, *29*, 17–25.

Liu, L., Liu, Y. F., Li, J. Y., Du, G. C., & Chen, J. (2011). Microbial production of hyaluronic acid: Current state, challenges, and perspectives. *Microbial Cell Factories*, *10*, 99.

Macknight, W. J., Ponomarenko, E. A., & Tirrell, D. A. (1998). Self-assembled complexes in nonaqueous solvents and in the solid state. *Accounts of Chemical Research*, *31*, 781–788.

Necas, J., Bartosikova, L., Brauner, P., & Kolar, J. (2008). Hyaluronic acid (hyaluronan): A review. *Veterinarni Medicina*, *53*, 397–411.

Ponomarenko, E. A., Waddon, A. J., Tirrell, D. A., & Macknight, W. J. (1996). Structure and properties of stoichiometric complexes formed by sodium poly(alpha,L-glutamate) and oppositely charged surfactants. *Langmuir*, *12*, 2169–2172.

Pérez-Camero, G., García-Alvarez, M., Martínez de Ilarduya, A., Fernández, C., Campos, L., & Muñoz-Guerra, S. (2004). Comblike complexes of bacterial poly(gamma,D-glutamic acid) and cationic surfactants. *Biomacromolecules*, *5*, 144–152.

Portilla-Arias, J. A., García-Alvarez, M., Martínez de Ilarduya, A., Holler, E., & Muñoz-Guerra, S. (2006a). Nanostructurated complexes of poly(beta,L-malate) and cationic surfactants: Synthesis, characterization and structural aspects. *Biomacromolecules*, *7*, 161–170.

Portilla-Arias, J. A., García-Alvarez, M., Martínez de Ilarduya, A., Holler, E., & Muñoz-Guerra, S. (2006b). Thermal decomposition of fungal poly(beta,L-malic acid) and poly(beta,L-malate)s. *Biomacromolecules*, *7*, 3283–3290.

Portilla-Arias, J. A., García-Alvarez, M., Martínez de Ilarduya, A., & Muñoz-Guerra, S. (2007a). Ionic complexes of biosynthetic poly(malic acid) and poly(glutamic acid) as prospective drug-delivery systems. *Macromolecular Bioscience*, *7*, 897–906.

Portilla-Arias, J. A., García-Alvarez, M., Martínez de Ilarduya, A., & Muñoz-Guerra, S. (2007b). Thermal decomposition of microbial poly(gamma-glutamic acid) and poly(gamma-glutamate)s. *Polymer Degradation and Stability*, *92*, 1916–1924.

Schante, C. E., Zuber, G., Herlin, C., & Vandamme, T. F. (2011). Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications. *Carbohydrate Polymers*, *85*, 469–489.

Tolentino, A., Alla, A., Martínez de Ilarduya, A., & Muñoz-Guerra, S. (2011). Comblike ionic complexes of pectinic and alginic acids with alkyltrimethylammonium surfactants. *Carbohydrate Polymers*, *86*, 484–490.

Tolentino, A., Martínez de Ilarduya, A., Alla, A., & Muñoz-Guerra, S. (2010). Ionic complexes of polyacids and cationic surfactants. *Macromolecular Symposia*, *296*, 265–271.

Yadav, A. K., Mishra, P., & Agrawal, G. P. (2008). An insight on hyaluronic acid in drug targeting and drug delivery. *Journal of Drug Targeting*, *16*, 91–107.