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Equilibrium and release properties of hyaluronic acid–drug complexes

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ABSTRACT

With the aim to provide more rational basis about the potentiality of hyaluronic acid (or hyaluronan) as drug carrier a set of ionic complexes of its acid form (HA) and its sodium salt (NaHA) with three model drugs (D) (atenolol, propranolol and lidocaine) were prepared. Besides NaHA subjected to hyalurodinase depolimerization ($NaHA_d$) was also used. Transparent dispersions were obtained. They exhibited negative electrokinetic potential and a high degree of counterionic condensation with affinity constants ($\log K_{cc}$) in the range of 5.8–6.1 for propranolol complexes (pK_a 9.45) and 4.0–4.6 for lidocaine ones (pK_a 7.92).

Delivery rates of D from the complexes were measured in a Franz-type bicompartimental device. Loaded D were slowly released from the three types of complexes, even when a neutral salt was added to the dispersion placed in the donor compartment, revealing the high affinity between the protonated drugs and the ionisable groups of the polymer.

Complex dispersions based on HA or on $NaHA_d$ exhibited lower viscosity than those of NaHA but their complexing ability remained unaltered.

The results reported on equilibrium and release properties of Hyaluronan-model D complexes contribute to expand the use of HA and NaHA as drug carriers for different routes of administration.

1. Introduction

Hyaluronic acid also known as hyaluronan is a naturally occurring polysaccharide belonging to the glycosaminoglycan family, composed by repeated D-glucuronic acid-β (1,3)-N-acetyl-D-glucosamine disaccharide units linked together through β (1,4) glycosidic bonds. Fig. 1 shows the monomeric unit of the acid form (HA). This biopolymer is widely distributed in body tissues being the major constituent of the extracellular matrix of vertebrates. It is involved in many biological processes, such as cellular adhesion, mobility and differentiation (Leach and Schmidt, in press; Delpech et al., 1997; Rooney et al., 1995; Laurent, 1987). Sodium hyaluronate (NaHA) is the high molecular weight fraction of purified natural sodium salt of hyaluronan (European Pharmacopoeia 5.0, 2005). It is widely used as a parenteral and ophthalmic viscoelastic agent, applied in the joints and instilled in the bladder for the treatment of interstitial cystitis (Rah, 2011; Gomis et al., 2009; Iavazzo et al., 2007; Ludwig, 2005; Akira, 2004; Ghosh and Guidolin, 2002).

Hyaluronan has attracted the attention of many scientists to use it in the design of parenteral delivery systems (Hirakura et al., 2010; Lee et al., 2009; Hahn et al., 2005). In relation to this point, the interactions of hyaluronan with CD44 receptors that are overexpressed in several types of cancer (Choi et al., 2012; Slomiany et al., 2009) were recently described.

However, it is well known that hyaluronan exhibits a short biological half-life since it is subjected to degradation by hyaluronidase enzymes (Oh and Kim, 2010; Necas et al., 2008; Fraser and Laurent, 1997; Rooney et al., 1995). This degradative process has been considered as a severe shortcoming that would affect its performance as a drug carrier. In connection with this point, a number of chemical derivatives, in which hyaluronan reactive groups are covalently bonded to other moieties, has been proposed (Young et al., 2012; Akira, 2004; Yuna et al., 2004).

However, hyaluronan bearing a carboxylic group in each glucuronic unit (pK_a 3–4) (Brown and Jones, 2005; Hascall and Lauren, 1997), behaves as an acidic polyelectrolyte (PE) able to form complexes with drugs (D) possessing an appropriate basic group. The strategy relating to the use of different PE–D ionic complexes as drug carriers is currently proposed in the area of drug delivery (Guzmán et al., 2012; Ramirez Rigo et al., 2009; Quinteros et al., 2008; Jiménez Kairuz et al., 2005; Jiménez Kairuz et al., 2003). In this field, Doherty et al. (1995) obtained stable and reversible ionic complexes between lidocaine and medium molecular weight hyaluronan that allowed the prolongation of epidural analgesia when injected into the epidural space in rabbits, although such effect was not observed in dogs (Doherty et al., 1996). Saettone et al. (1991),

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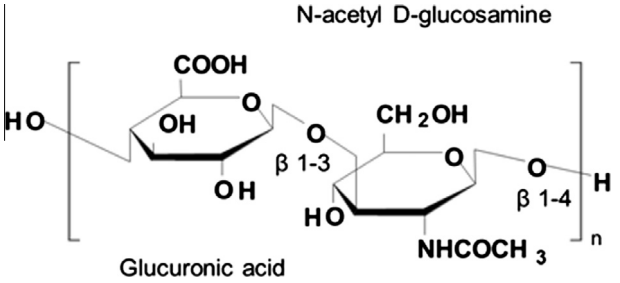

Fig. 1. Structure of the repeating disaccharide unit (N-acetyl-D-glucosamine and D-glucuronic acid) present in hyaluronan.

Table 1
Structural and physicochemical properties of selected drugs.

Drug	MW	Amlne	pK_a	Log	Solubility (mg/mL)
Lidocaine	234.34	Ternary	7.92	2.26	3.98
Atenolol	266.34	Secondary	9.55	0.16	12.8
Propranolol	259.34	Secondary	9.45	3.03	0.12

Saettone et al. (1989) reported some ionic drug complexes for topic ophthalmic formulations. In addition, a patent covering ophthalmic formulations based on drug-hyaluronan salts was also registered (Della Valle et al., 1995). However, at present a detailed knowledge regarding the affinity of protonable D for the carboxylic groups of hyaluronan under different conditions is not available. Therefore, it is of our interest to provide more detailed information concerning this point in order to contribute with more solid bases to evaluate the potentiality of hyaluronan as a drug carrier convenient for being used in specific therapeutic indications.

Then, the main points of concern addressed in this report are:

(i) The ability of commercial NaHA, its hyaluronidase-depolymerized product ($NaHA_d$) and the acid form HA to produce ionic reversible complexes with model basic D according to the following equations:

$$\text{R-COOH} + \text{D} \rightleftarrows \text{R-COO}^- + \text{DH}^+ \rightleftarrows (\text{R-COO}^-\text{DH}^+) \quad (1)$$

$$\text{R-COO}^-\text{Na}^+ + \text{D} + \text{H}_2\text{O} \rightleftarrows \text{R-COO}^- + \text{DH}^+ + \text{OH}^- + \text{Na}^+ \rightleftarrows (\text{R-COO}^-\text{DH}^+) + \text{NaOH} \quad (2)$$

in which R-COOH represents the acid pending groups of hyaluronan, and D and DH^+ represent the unprotonated and protonated species of D.

(ii) How much the equilibrium and release properties of the complexes are affected by environmental conditions such as pH and inorganic ions.

For this purpose, three D that were previously used to describe PE–D interactions (Lidocaine (Li), Atenolol (At) and Propranolol (Pr)) were selected based on their lipophilicity and basicity (Table 1).

2. Materials and methods

2.1. Materials

NaHA, Parafarm®, Bs. As. Argentina, obtained from bacteria's fermentation, (MW = 1655 kDa), was used. Bovine testicular hyaluronidase (HAse), with a specific activity of 801 USP IU/mg, was purchased from Sigma Chemical Co., St Louis, USA. Both, NaHA and HAse, were used without any further purification. At, Li and Pr hydrochloride, all pharmaceutical grade, were obtained from Parafarm®, Bs. As., Argentina. Pr was obtained by neutralization of its hydrochloride salt with 1 N NaOH. The solid product obtained, that was filtered, washed with distilled water and dried in oven to 50 °C to constant weight, melts at 91.21 °C. Phosphate Buffer Saline (PBS, 10 mM, pH 6.80) was prepared according to USP 34-NF 29 (2011). Cyclohexane pharmaceutical grade was purchased from Cicarelli SA (Argentina). All other reactants were of analytical grade. Mili-Q water was used for all the experiments.

2.2. Preparation and characterization of the free acid form HA

HA was obtained after neutralization of NaHA with an ionic exchange resin. Briefly, 100 mL of a 0.5% w/v aqueous dispersion of NaHA (pH = 6.80) was passed through a glass column (4.2 cm diameter and 21 cm high) containing the sulfonic acid resin Amberlite® IR 120 in hydrogen form (Sigma–Aldrich). After that, several 20 mL-water portions were added to the column to get the complete drainage of the HA generated (pH = 2.82). Solid HA was obtained by lyophilization of this solution under a vacuum of 10×10^{-3} mBar after initial freezing with liquid air. Besides, HA was titrated with HCl and NaOH respectively to determine the equivalents of carboxylic groups per gram. They were also subjected to differential scanning potentiometry (DSP) according to Manzo et al. (1991) to assess acidic or basic purity. In order to evaluate the N-acetyl-D-glucosamine, reducing ends of NaHA and HA, 0.1% dispersions of both solids were subjected to the experimental procedure described by Reissig et al. (1995). In this experiment, the reaction between the Ehrlich's reagent (p-Dimethylamino benzaldehyde, DMAB) and N-acetyl-D-glucosamine reducing ends of the hyaluronan chains gave a pink colour (maximum wavelength 585 nm). Briefly, a borate solution was prepared by dissolving 4.94 g boric acid and 1.98 g potassium hydroxide in 100 mL of Milli-Q water. In addition, a 0.1 g/mL DMAB solution was prepared by dissolving 5 g DMAB in 6.25 mL of HCl 12 N and made up to a final volume of 50 mL with glacial acetic acid. The latter solution was 10-fold diluted with glacial acetic acid just before use (and at least 15 min before use). A 200 μL-aliquot of NaHA or HA dispersions was added to 50 μL of the borate solution in a glass tube. The solution in the tube was immediately vortexed, heated in a boiling water bath for exactly 3 min, and then placed in a cold water bath at approximately 10 °C for 1 min. Then, 1.5 mL of the diluted DMAB solution was added to each of these tubes, which were vortexed and placed at 37 °C for exactly 15 min. This was transferred to a plastic cuvette of 1 cm pathlength and immediately scanned by UV–Vis spectroscopy between 400 and 700 nm, using water as reference.

The Fourier Transformed Infrared (FTIR) spectra of 1% solid HA and NaHA dispersed in KBr discs were recorded in a NICOLET FTIR (360 FTIR ESP, Thermo Nicolet, Avatar) spectrometer.

The viscosity of 1% HA and NaHA dispersions as well as that of the HA dispersion added with enough NaOH to neutralize all its carboxylic groups, were measured at 37 °C and 100 RPM in a Haake (Karlsrube, Germany) viscometer VT500 equipped with a software VT500/VT 3.01, and a MV2 sensor.

2.3. Depolymerization of NaHA by HAse

The device shown in Fig. 2 was used to determine the depolymerization of NaHA by action of bovine HAse. Since depolymerization produces a lowering in viscosity, this parameter was selected as a kinetic indicator. Fifteen mL of a 0.66% w/v dispersion of NaHA in PBS was introduced in the reaction vessel provided with a magnetic stirrer and thermostatized at 37 °C. This concentration was selected because its viscosity was appropriate to be followed through time. A solution of HAse (2.5 mg/5 mL) was prepared the day of the experiment by dissolving the enzyme in PBS. An aliquot of 200 μL corresponding to 160.2 IU of HAse, previously incubated for an hour at 37 °C, was added in the NaHA dispersion. A 2 mL pipette provided

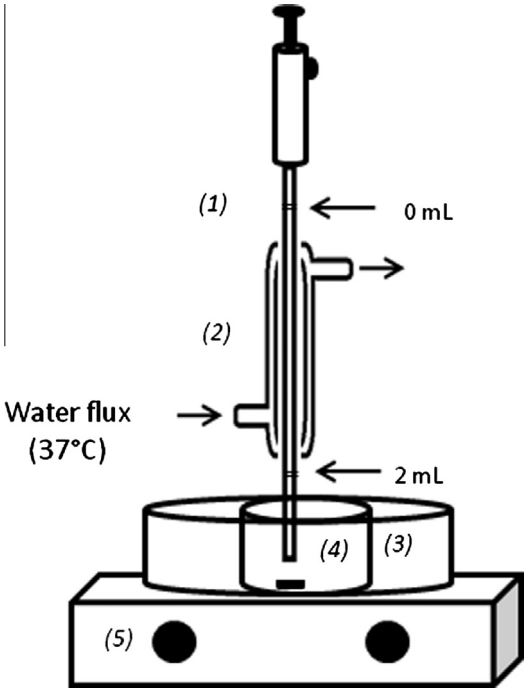

Fig. 2. Device used to determine the lowering in viscosity of NaHA dispersions. (1) 2-mL pipette assembled to a pipette pump thorough which each dispersion was let to drain by gravity; (2) thermostatized jacket, (3) water bath thermostatized at 37 °C, (4) vessel containing NaHA dispersion, and (5) magnetic stirrer and heater.

with a thermostatized jacket was used to pick up an exact volume of the reacting dispersion. This volume was let to drain by gravity and the draining time from 0 to 2 mL was monitored as a function of time. The draining time taken immediately after adding the HAse solution was regarded as t_0 and those measured at selected time intervals for 150 min as t_t. Each point was the mean value of two independent determinations. Results are expressed as percentage of viscosity loss (V%) calculated as $V\% = (t_t/t_0) \times 100$. At the end, the reaction vessel containing depolymerised hyaluronan ($NaHA_d$) was immersed in an ice bath and further stored at 4–8 °C. The amount of N-acetyl-D-glucosamine reducing ends in $NaHA_d$ was assayed by the experimental procedure described in Section 2.2.

2.4. Preparation of the complexes

Series of complexes were prepared by addition of water to a mixture of adequate amounts of D (as the solid free base) and HA or NaHA to obtain HA–D_x and NaHA–D_x complexes having x = 25%, 50%, 75% or 100%. The subscript "x" refers to the percentage of moles of carboxylic groups of HA or to the carboxylate groups of NaHA loaded with D. A mixed complex was obtained by addition of water to a mixture of D plus an equimolar amount of NaHA and HA. This complex was regarded as NaHA–HA–D_x. A 0.5% w/v complex dispersion of $NaHA_d$–At_{75} was prepared to perform a comparative release experiment with undepolymerized complexes.

The transparent dispersions obtained were used at concentrations of 0.15 and 0.5% w/v to perform the species distribution at equilibrium and release experiments, respectively. The % w/v was calculated on the acid form (HA) basis. The dispersions were stored overnight at 4–8 °C, and then allowed to reach room temperature before use.

2.5. Species distribution at equilibrium

The proportions of the species (D), (DH^+), and the complexed one ([R-COO^-DH^+]) were determined through the selective extraction of D with cyclohexane (CH) according to the procedure described by Jimenez Kairuz et al. (2002), Ardusso et al. (2010). Such determinations were performed on Li and Pr complexes since At does not have an appropriate partition coefficient to be determined by this method. Aqueous dispersions of NaHA–Li_x, NaHA–Pr_x and HA–Li_x, whose pHs were regulated to nearly 7 by dropwise addition of 1 M NaOH or HCl, were shake flask-partitioned at a CH/dispersion ratio of 2:1. Concentrations of Li or Pr in CH were spectrophotometrically assayed at 262 and 283 nm, respectively, to get the apparent partition coefficient (PC_{app}). The pH of the aqueous phase was recorded before extraction and at partition equilibrium. Besides, the partition equilibrium of Li and Pr was measured to get the true partition coefficients (PC_t). Data were processed through Eqs. (3)–(5), based on Eqs. (1) and (2).

$$PC_{app} = (D)_{CH}/(D)_w + (DH^+)_w + ([R\text{-}COO^-DH^+])_w \quad (3)$$

$$PC_t = (D)_{CH}/(D)_w \quad (4)$$

$$K_a = (D)_w \cdot (H^+)/(DH^+) \quad (5)$$

The affinity constant for the counterionic condensation (K_{cc}) was calculated as:

$$K_{cc} = (RCOO^-DH^+)/(RCOOH) \cdot (D) = (RCOO^-DH^+) \cdot (H^+)/(RCOOH) \cdot K_a.(DH^+) \quad (6)$$

in which (RCOOH) was calculated from Eq. (7).

$$(RCOOH) = [RCOOH]_{st} - (RCOO^-DH^+) - (RCOO^-) = [RCOOH]_{st} - (RCOO^-DH^+) - (DH^+) \quad (7)$$

Since the sum of the negative species ($RCOO^-$) plus (OH^-) equals to (DH^+) plus (H^+), and considering that $(RCOO^-) \gg (OH^-)$ and $(DH^+) \gg (H^+)$; then $(RCOO^-)$ approaches to (DH^+).

2.6. Release rates from HA–At_{75}, NaHA–At_{75} and $NaHA_d$–At_{75} complexes

At was selected as model loading D since it is more water soluble than Pr and Li. The extent and rate of *in vitro* release of At from a set of 0.5% w/v aqueous dispersions of HA–At_{75}, NaHA–At_{75} and $NaHA_d$–At_{75} were determined. For comparison purposes, HA–At_{75} dispersion was added with enough 1 M NaOH solution to produce the neutralization of 100% of the carboxylic groups of HA. Release rate from an aqueous solution with an equivalent concentration of At was used as reference. Experiments were performed in bicompartimental Franz cells equipped with an acetate cellulose membrane (12.000 Da cut-of; Sigma, St Louis, MA, USA). The donor compartment was filled with an exactly weighed amount of the dispersion, close to 1 mL, to assay and sealed with Parafilm®. The receptor compartment was filled with 17 mL of water maintained at 37.0 ± 0.1 °C. Samples of 0.9 mL of the receptor medium were withdrawn at predetermined time intervals and immediately replaced by an equal volume of pre-warmed water. Data were corrected for dilution. The concentrations of At were determined by UV spectroscopy at the maximum wavelength (Thermo-Electronic Corporation, Evolution 300 BB, England). The pH of the donor compartment was recorded at the beginning of the experiment.

In addition, a set of experiments in which NaCl or Glycine (Gly) was added to the donor compartment and in which the receptor was filled with water were carried out. In these case, a 0.5% w/v dispersions of NaHA–At_{75} were added with 0.25 mL of a 0.020% w/v NaCl or Gly solution, to get a ratio NaCl/At or Gly/At of 0.5.

All the assays were run in triplicate and followed for 3 h.

2.7. Dynamic light scattering (DLS)

Diffusion coefficients (DC) and electrokinetic potential (ξ) were determined using Delsa Nano C instrument (Beckman Coulter, Osaka, Japan) equipped with a 658 nm laser diode, scattering angle set at 165° and temperature controller. Measurements were performed in triplicate at 25 °C allowing the instrument to automatically optimize signal intensity of the sample. The instrument software is Delsa Nano 2.20, Beckman Coulter, Osaka, Japan. For DC determinations 1% of NaHA and HA dispersions were used. For ξ determinations 0.1% dispersiones of NaHA, HA and NaHA–HA, alone or complexed with 25%, 50% and 100% of At were used. Adittionally, the pH of the dispersions was recorded.

3. Results and discussion

3.1. Preparation and characterization of HA

Titrimetric experiments indicated that HA contains 2.44 meq/g of carboxylic groups, which is in accordance with its monomeric structure. Besides, the negative area of DSP profile observed after titration of HA (Fig. 3a) means that all carboxylic groups are in its undissociated acid form (R-COOH) while those of starting NaHA (Fig. 3b) are all in its carboxylate form (R-COO$^-$). FTIR spectrum also confirms these results since HA exhibited a strong band at1732 cm^{-1} ascribed to the C=O st of the R-COOH. This band is absent in NaHA spectra which showed bands at 1458 and 1638 cm^{-1} ascribed to the symmetric and asymmetric stretching mode of R-COO$^-$ groups.

As expected, aqueous dispersions of NaHA exhibited a concentration-dependent rise of viscosity (data not shown). However, dispersions of HA did not significantly increase the water viscosity even after addition of enough NaOH to neutralize it. This behaviour has been reported previously (Doherty et al., 1994) and was associated with same degree of depolymerization during the preparation process

Depolymerization of HA produces an increase of N-acetyl-D-glucosamine at the end of the resulting segments. The increase of N-acetyl-D-glucosamine end moieties in HA was confirmed using the classical colorimetric assay as a qualitative indicator. As Fig. 4 shows, the dispersion of NaHA exhibited a significant base line due to turbidity but did not develop a specific light absorption. However, the dispersion of HA developed specific light absorption at the visible region and a lower base line that revealed some degree of depolymerization. In agreement with these findings, DLS showed that the DC of HA was about ten times higher than that of NaHA (7.45×10^{-9} and 7.64×10^{-10} respectively), which was associated with a reduction in the MW. This is in agreement with the increase in the N-acetyl-D-glucosamine end moieties and the reduction in the viscosity above informed.

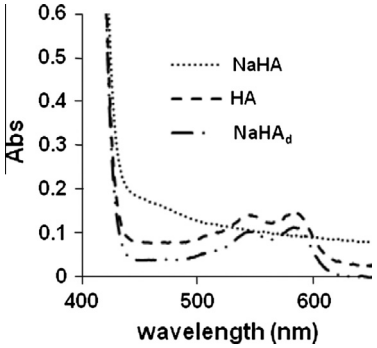

Fig. 4. Determination of N-Acetyl reducing ends. Absorption spectra of the colour developed with Reissig et al method.

3.2. Complexation of HA and NaHA with basic D

In all cases, regardless of the different lipophilicity of the three D, transparent systems were obtained. They exhibited a unimodal DC and negative ξ. Fig. 5a and b report a representative set of data. It shows the effects of the progressive addition of At on pH and ξ of HA and NaHA. As can be seen there, HA exhibits a low negative ξ due to a modest dissociation of its carboxylic groups. The addition of At raises the pH and also increases ξ from −5 to −10 mV, as expected from the equilibria depicted in Eq. (1). In contrast, NaHA exhibits a high negative ξ and nearly neutral pH. The addition of At increases pH but progressively changes ξ towards a value close to that of HA–At$_{100}$. The decrease of ξ would be associated with the counterionic condensation depicted in Eq. (2). Lastly, the addition of At to a mixture of equivalent amounts of NaHA and HA also increases pH and lowers ξ, which is in accordance with the above results. It should be noted that the composition HA–At$_{50}$ Na$_{50}$ has a pH close to 7, being this an appropriate composition to assay in biological systems.

3.3. Species distribution at equilibrium

In order to know the degree of PE–D complexation, the proportions of free species D, DH$^+$ and the complexed one [R-COO$^-$DH$^+$] were determined through the selective extraction of D with CH. Table 2 reports the proportions of reactants and pH of the dispersions used.

It should be noted that the addition of one or both inorganic ions on equilibrium 1 or 2 generates a depletion of the counterionic condensation according to one or more of the following mechanisms:

$$(\text{R-COO}^-\text{DH}^+) + \text{NaOH} \rightleftarrows \text{RCOO}^- + \text{Na}^+ + \text{D} + \text{H}^2\text{O} \quad (8)$$

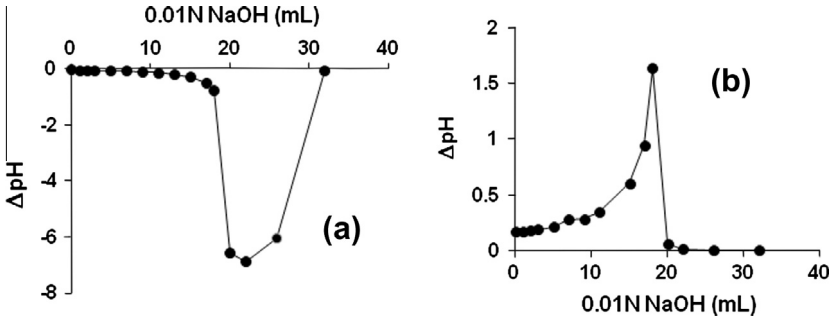

Fig. 3. Areas described by the pH differences obtained after titration by DSP of HA (a) or NaHA (b) with the reference solution.

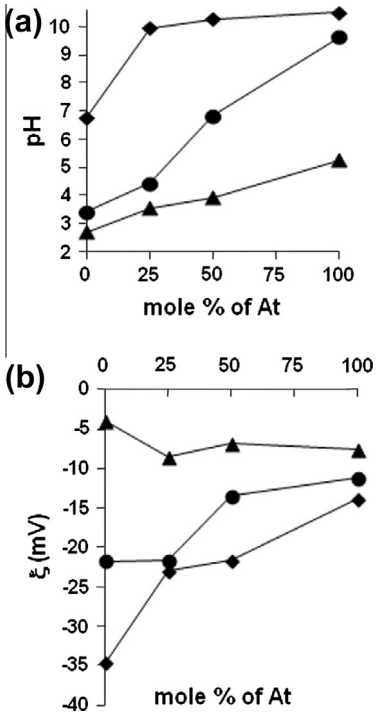

Fig. 5. Changes in (a) pH and (b) electrokinetic potential (ξ) of NaHA (♦), HA (▲) or on a mixture of equivalent amounts of NaHA and AH (●) by the progressive addition of At.

$$(\text{R-COO}^{-}\text{DH}^{+}) + \text{HCl} \rightleftarrows \text{RCOOH} + \text{DH}^{+} + \text{Cl}^{-} \quad (9)$$

$$(\text{R-COO}^{-}\text{DH}^{+}) + \text{NaCl} \rightleftarrows \text{RCOO}^{-} + \text{Na}^{+} + \text{DH}^{+} + \text{Cl}^{-} \quad (10)$$

Table 3 reports the species distribution after the partition extraction of D with CH.

Said table also shows the stoichiometric concentration of [D], the ratios (Na^{+})/[D] and (Cl^{-})/[D], the pH of the dispersions and $\log K_{cc}$.

As can be seen in the table:

(a) According to Eqs. (1) and (2), the rise in the molar proportion of D increases the proportion of ionic pairs ($R\text{-}COO^{-}DH^{+}$).
(b) The comparison between the complexes of Li with HA and NaHA revealed that the former maintains the complexing capacity of the original salt form. Indeed, HA–Li dispersions that have a lower proportion of inorganic ions exhibited higher ($RCOO^{-}LiH^{+}$) and lower (LiH^{+}) proportions. Li also complexed with the mixture of equivalent amounts of NaHA, and HA exhibited the expected average proportion of a complexed drug. In accordance with these results, $\log K_{cc}$ of both series of Li complexes did not exhibit significant differences.
(c) The series of Pr complexes exhibited higher K_{cc} than those of Li, which is in agreement with the higher basicity of Pr ($pK_a = 9.45$) respect to Li ($pK_a = 7.92$).

3.4. Depolymerization of NaHA by HAse

In order to know if NaHA subjected to the action of HAse retains the original ability of complexing basic D, 0.66% w/v dispersions were used to follow the reaction under the conditions already described. The NaHA viscosity was selected as a kinetic indicator since it is known that it decreases after depolymerization by HAse (Girish and Kemparaju, 2007; Asteriou and Deschrevel, 2001). The profile of viscosity against time reported in Fig. 6 allows determining the time of 120 min to obtain the depolymerized product regarded as $NaHA_d$ (in which viscosity decreased more than 90%).

Table 2
Proportions of reactants and pH of the dispersions used to determine species distribution. Either HCl or NaOH was added in order to get a nearly neutral pH.

Compicx	Stoichiometric composition			PH	
	[D] (M)	[NaOH] (M)	IHCQ(M)	Initial	Equilibrium
NaHA–Li_{25}	9.08E−04		7.50E−04	7.02	6.24
NaHA–Li_{50}	1.82E−03		1.50E−03	7.14	6.24
NaHA–Li_{100}	3.63E−03		3.00E−03	7.24	6.29
NaHA–Pr_{25}	9.00E−04		8.00E−04	6.58	6.28
NaHA–Pr_{50}	1.80E−03		1.60E−03	6.68	6.46
NaHA–Pr_{100}	3.60E−03		3.40E−03	6.72	6.49
HA–Li_{25}	9.18E−04	2.00E−03		7.35	6.79
HA–Li_{50}	1.84E−03	1.50E−03		7.42	6.80
HA–Li_{100}	3.67E−03	5.00E−04		7.19	6.51
NaHA–HA–Li_{50}	1.84E−03	5.00E−04		7.71	6.67

Table 3
Species distribution after the selective extraction of D with CH.

Complex	[D] water[a] (mole %)	Species distribution (%)			$[Na^{+}]$/[D]eq[b]	$[Cl^{-}]$ [D]eq[b]	$\log K_{cc}$
		(D) water	(DH+) water	(RCOO DH+) water			
NaHA–Li_{25}	14.02	0.94 ± 0.02	42.99 ± 0.96	56.07 ± 1.00	7.21	1.47	4.251
NaHA–Li_{50}	31.12	0.77 ± 0.14	36.99 ± 4.68	62.23 ± 4.82	3.25	1.33	4.361
NaHA–Li_{100}	64.61	0.80 ± 0.004	34.58 ± 0.42	64.61 ± 0.42	1.56	1.28	4.362
NaHA–Pr_{25}	15.08	0.02 ± 0.00122	39.64 ± 1.6	60.33 ± 1.59	6.76	1.47	5.898
NaHA–Pr_{50}	30.75	0.03 ± 0.0016	38.47 ± 2.2	61.49 ± 2.21	3.32	1.45	5.78
NaHA–Pr_{100}	78.56	0.02 ± 0.0014	21.42 ± 3.54	78.56 ± 3.53	1.30	1.20	6.14
HA–Li_{25}	18.42	1.89 ± 0.12	26.6 ± 2.90	71.61 ± 3.02	2.95		4.04
HA–Li_{50}	39.84	1.51 ± 0.11	19.4 ± 0.73	79.08 ± 0.83	1.02		4.18
HA–Li_{100}	83.20	0.72 ± 0.13	17.57 ± 1.98	81.71 ± 2.1	0.16		4.56
NaHA–HA–Li_{50}	34.25	1.61 ± 0.08	28.24 ± 2.24	70.14 ± 2.32	1.85		4.05

[a] Mole% of D remaining in the aqueous phase.
[b] Na+/D and Cl^{-}/D ratios after partition.

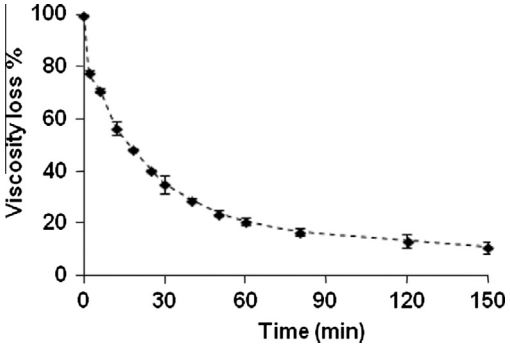

Fig. 6. In vitro depolymerization of a 0.66% NaHA dispersions after addition of HAse.

This product shows the increase in the N-Acetyl reducing ends of the polymer (Fig. 4).

3.5. Drug release

The knowledge of the affinity of basic D towards the carboxylic groups of hyaluronan to yield ionic complexes provides solid basis to understand the kinetics of drug release from the resulting carriers. Table 4 displays the set of 0.5% w/v dispersions, in which 75 mol% of At was incorporated into NaHA, HA, their mixture and $NaHA_d$.

A model of the mechanism of D release from PE–D complexes was previously proposed (Jiménez Kairuz et al., 2003, 2002). Briefly, according to Eqs. (1) and (2), release towards water as a receptor medium occurs essentially through the Fickian diffusion of the neutral species D since diffusion of DH^+ is mainly prevented by the electrostatic gradient produced by the polyanion. Consequently, diffusion rate of D is by far higher than that of DH^+. As D diffuses away from the complex environment, equilibria 1 or 2 quickly respond to provide fresh free D.

On the other hand, the incorporation of an external anion, as described by Eqs. (4) and (5), provides a counterion for DH^+ allowing its free diffusion with a consequent rise in release rate. In relation to this point, it should be noted that the saline composition of biological fluids promotes the D release from these PE carriers.

Figs. 7 and 8 show that the complexation of At modulates its release rate in relation to the same concentration of the reference solution of free At. The following asseverations can be made from Fig. 7:

(a) According to Eq. (1), the complex obtained from HA, which has an acidic pH, exhibited the lowest release rate.
(b) As the pH of the dispersion was raised due to the addition of NaOH, the release rate was increased. In accordance with Eq. (5), the higher pH provides a higher proportion of neutral species D, which is able to freely diffuse together with a proportion of Na^+ that competes with DH^+ to interact with $R\text{-}COO^-$ groups.

Table 4
Stoichiometric composition and pH of the dispersions used in drug release experiments.

Complexes	PH
$HA–At_{75}$	4.13
$NaHA–At_{75} + Gly_{25}$	9.45
$NaHA–At_{75} + NaCl_{25}$	9.83
$HA–At_{75} + NaCl_{25}$	3.89
$NaHA–At_{75}$	10.11
$HA–At_{75} + NaOH_{100}$	10.36
$NaHA_d–Al_{75}$	9.17

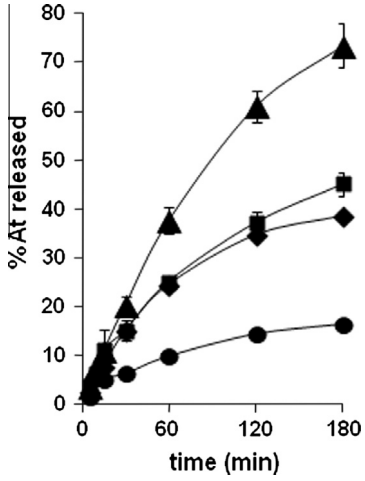

Fig. 7. Cumulative release of At as a function of time towards water using a side-by side diffusion chamber. Each formulation contained the equivalent of 75 mol% of At. At reference solution (▲), $HA–At_{75}$ (●), $HA–At_{75}$ + NaOH (♦), $HA–At_{75}$ + NaCl (■).

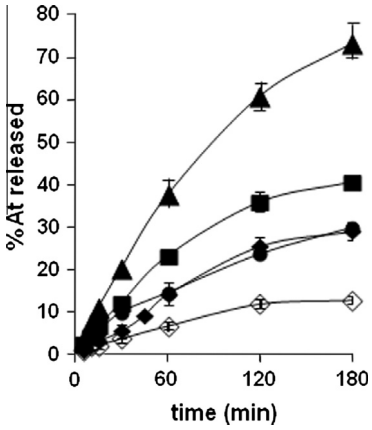

Fig. 8. Cumulative release of At as a function of time towards water using a side-by side diffusion chamber. Each formulation contained the equivalent of 75% mole of At. At reference solution (▲), $NaHA–At_{75}$ (●), $NaHA–At_{75}$ + Gly (♦), $NaHA–At_{75}$ + NaCl (■), $NaHA_d–At_{75}$ (◇).

(c) A similar release-increasing effect was produced by the addition of NaCl, in accordance with Eq. (8).

Fig. 8 shows that:

(a) The release profile of At obtained from the complex $NaHA–At_{75}$ (pH 10.11) was similar to that of $HA–At_{75}$ + NaOH, which reveals that the partial depolymerization of HA produced during the preparation process does not affect its complexing ability, as already observed under equilibrium conditions.
(b) Its release rate was not modified by the addition of glycine since it does not shift equilibrium 2, while addition of NaCl increased it in the same way as noted in $HA–At_{75}$.

Nevertheless, under such unfavourable conditions, the release rates from the complexes remained strongly modulated in relation to the At solution used as reference.

(c) The figure also shows that the complex $NaHA_d–At_{75}$ that has a lower pH than $NaHA–At_{75}$ also exhibited a slower release rate, which reveals that its complexing ability remains, at least, at the same level.

The block of results concerning the release properties of the set of complexes clearly reveals that the equilibrium described by the Eqs. (2), (3), 5, 6, and 7 exerts the kinetic control of drug delivery.

These results are in agreement with the behaviour of other acid polyelectrolytes such as carbomer (Jiménez Kairuz et al., 2005, 2003) and alginic acid (Ramirez Rigo et al., 2006) and with those early reported by Doherty et al. (1995).

4. Conclusions

The three forms of hyaluronic acid, NaHA, HA and $NaHA_d$ exhibited high ability to form complexes with model D that has a protonable group.

Their aqueous dispersions are physically stable and optically isotropic systems. They have a negative electrokinetic potential ξ and a high degree of counterionic condensation with affinity constants ($\log K_{cc}$) in the range of 5.8–6.1.

The three systems assayed behave as a reservoir of D that is slowly released from the complexes. The release control is maintained even though a neutral salt is added to the dispersion, which reveals the high affinity of D for the carboxylic groups of HA.

The results already discussed provide a more rational basis to expand the use of HA and NaHA as a drug carrier for different ways of administration.

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