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Hyaluronan as drug carrier. The *in vitro* efficacy and selectivity of Hyaluronan–Doxorubicin complexes to affect the viability of overexpressing CD44 receptor cells

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ABSTRACT

We report Doxorubicin ionic complexes with hyaluronic acid (HiH-Dx) or its sodium salt (HiNa-Dx) as tumor-targeting delivery system. The complexes were prepared *in situ* by mixing aqueous solutions of Dx.HCl with HiH or HiNa. Clear colloidal dispersions with a high degree of counterionic condensation (cc) were obtained. Affinity constants ($\log K_{cc}$) of *HiNa-Dx* and *HiH-Dx* were 7.96 and 8.08, respectively. Delivery rates of Dx from the complexes were measured in a Franz-type bicompartimental device. In line with the high affinity constants, loaded Dx was slowly released from the complexes.

To test the targeting potential of the complexes, carcinogenic A549 cells overexpressing the CD44 receptors were used. HTR8/SVneo cells without overexpression of CD44 were used as control. In A549 cells, cytotoxicity of both *HiH-Dx* and *HiNa-Dx* complexes was 3-fold higher than that of the reference solution. However, no differences were observed between the complexes and free Dx solution in HTR8/SVneo cells. Flow cytometry data suggested successful uptake of Dx in cells, with a greater internalization of Dx in A549 cells than in HTR8/SVneo cells when the complexes were used. Similarly, microscopy imagines revealed a higher concentration of Dx in A549 cells with the complexes.

This work provides more detailed information in order to contribute to more solid bases to evaluate the potentiality of Hi as an antineoplastic drug carrier convenient for being used in specific therapeutic indications with minimal side effects.

1. Introduction

The therapeutic efficacy of most drugs greatly depends on their ability to cross the cellular barrier and reach their intracellular target sites. Due to the lack of specificity and selectivity of anticancer drugs towards the tumor cells, numerous side effects appear as a result of their administration.

To transport anticancer drugs effectively through the cellular membrane and to deliver them into the intracellular environment, several interesting smart carrier systems based on both synthetic or natural polymers have been designed and developed (Choi et al., 2012). Intracellular delivery has been improved by conjugating tumor-interacting moieties such as antibodies, nucleic acids, proteins, and various other ligands, onto the surface of the carriers (Lukyanov et al., 2004; Qian et al., 2002; Eliaz and Szoka, 2001).

In relation to this point, the interactions of hyaluronan (Hi) with CD44 receptors that are overexpressed in several types of cancer were recently described (Knudson et al., 2004).

The development of drug resistance and tumor relapse has been closely associated recently with the accumulation of cancer-initiating or cancer stem cells (CSCs) in tumors following chemotherapy (Baguley, 2010). These CSCs are characterized by overexpression of drug efflux transporters, formation of multicellular spheroids, and overall reduced metabolic activity, which make them resistant to cytotoxic drugs (Jordan et al., 2006).

Hi 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). Hi 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.

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Fig. 1 shows the monomeric unit of the acid form (*HiH*). Moreover, Hi also possess numerous desirable physicochemical and biological properties for drug delivery applications such as biocompatibility, biodegradability and non-immunogenicity.

Hi with different molecular weights (MW) play different roles in the body. Low-MW polymers typically induce receptor-mediated intracellular signaling, while high MW-polymers maintain cell integrity and matrix organization (Stern et al., 2006). This biopolymer has been extensively studied in pharmaceutical and biomedical applications, including cancer therapy, where various drugs have been conjugated to Hi like paclitaxel or Doxorubicin (Luo et al., 2002). Many drug-resistant cancer cells and CSCs display elevated levels of CD44 receptors that bind Hi. Thus, CD44 receptors can be targeted for the treatment of drug-resistant tumors and CSCs (Marhaba et al., 2008). Previously, it was shown that Hi-grafted liposomes have an increased cellular uptake through CD44-mediated endocytosis, which is a highly regulated process of binding, internalization, and ligand transfer through a series of intracellular compartments (Qhattal and Liu, 2011). This interaction has been extensively investigated because of its involvement in a wide variety of cellular functions. In particular, CD44 receptor is closely involved in a variety of significant cellular events, including cell proliferation, cell differentiation, cell migration, and angiogenesis. The interaction between Hi and CD44 receptors is known to mediate signaling for cell survival and endocytosis of Hi for its degradation. For this reason, Hi could be considered an excellent carrier for tumor-targeted delivery of chemotherapeutic agents (Choi et al., 2012; Slomiany et al., 2009; Naor et al., 2002; Kaya et al., 1997).

At present, an interesting amount of research has been devoted to the development of systems containing derivatized Hi either covalently bounded to anti-cancer drugs, or as a derivatization of upper structures (Song et al., 2014; Cai et al., 2010). Then, the obtained products arise from complex processes and multiple synthesis steps. However, reversible polyelectrolyte (PE)-drug complexes have not yet been well described.

Hyaluronan bearing a carboxylic group in each glucuronic unit (p*Ka* 3–4) (Brown and Jones, 2005; Hascall and Lauren, 1997), behaves as an acidic PE able to form complexes with drugs possessing an appropriate basic group.

In a previous work, we showed the Hi capability to form ionic complexes with basic drugs. A detailed knowledge regarding the affinity of protonable drugs for the carboxylic groups of Hi and the release properties of the PE-drug complexes formed under different conditions were reported there (Battistini et al., 2013).

In the present work, we focus on using Hi as a carrier for Doxorubicin (Dx), which is an antineoplastic drug widely used in several types of cancers that like other anthracyclines it works by intercalating with DNA. Then, the main points addressed in this report are:

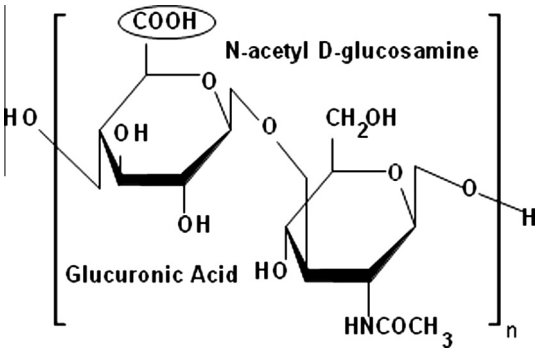

Fig. 1. Structure of the repeating disaccharide unit (N-acetyl-D-glucosamine and D-glucuronic acid) present in hyaluronan.

(i) The ability of commercial *HiNa* and the acid form *HiH* to produce aqueous dispersions of reversible ionic complexes with Dx as shown in Eqs. (1) and (2):

$$\text{R-COOH} + \text{DxH}^+\text{Cl}^- + \text{H}_2\text{O} \rightleftarrows \text{R-COO}^- + \text{DxH}^+ \rightleftarrows [\text{R-COO}^-\text{DxH}^+] + \text{H}_3\text{O}^+ + \text{Cl}^- \quad (1)$$

$$\text{R-COO}^-\text{Na}^+ + \text{DxH}^+\text{Cl}^- + \text{H}_2\text{O} \rightleftarrows \text{R-COO}^- + \text{DxH}^+ \rightleftarrows [\text{R-COO}^-\text{DxH}^+] + \text{NaCl} \quad (2)$$

In which R-COOH represents the acid pending groups of Hi.

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

(iii) The ability of Hi-Dx ionic complexes to internalize Dx via CD44 receptors mediated endocytosis and thus causing an increase in cell death compared to a reference solution of Dx.

(iv) The higher selectivity of Hi-Dx ionic complexes towards cancer cells overexpressing CD44 receptors over those that are not tumor cells.

In this field, Yi Luo et al. (2002) obtained covalent bioconjugates between Dx, Hi and N-(2-hydroxypropyl) methacrylamide for target delivery and for promoting Dx uptake in cancer cells (Luo et al., 2002). Besides, Miao et al. (2013) carried out an exhaustive *in vitro*/in vivo study based on hyaluronyl reduced graphene oxide nanosheets as tumor-targeting delivery systems for anticancer agents (Miao et al., 2013). However, all this system requires multiple synthesis steps.

At the present time, a work in which Hi and Dx are forming ionic complex is not available. Taking advantage of the selectivity of Hi towards CD44 receptors, 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 Hi as an antineoplastic drug carrier convenient for being used in specific therapeutic indications.

2. Materials and methods

2.1. Preparation of complexes

Sodium Hyaluronate (*HiNa*), Parafarm®, Bs. As. Argentina, obtained from bacteria's fermentation, (MW = 1655 kDa). The free acid species (*HiH*) was obtained from *HiNa* by a process previously reported (Battistini et al., 2013). Both *HiH* and *HiNa* were used without any further purification. Doxorubicin hydrochloride (Dx.HCl) pharmaceutical grade was kindly provided by Filaxis S.A, Bs. As., Argentina. All other reactants were of analytical grade. Mili-Q water was used for all the experiments.

Series of complexes, regarded as *HiH-Dx*$_x$ and *HiNa-Dx*$_x$, having x = 25 or 50%, were prepared *in situ*. The subscript "x" refers to the percentage of moles of carboxylic groups of *HiH* or to the carboxylate groups of *HiNa* loaded with Dx.

Complexes to perform species distribution and release rate experiments were prepared *in situ* by mixing aqueous solutions of Dx.HCl and *HiH* or *HiNa* previously acidified with HCl to get a final pH between 2.8 and 3.0 in order to avoid complex precipitation.

Complexes to perform cell viability assays were prepared in the same way but using culture medium instead of water as solvent and without the addition of HCl acid. Concentrations of dispersions are expressed in% w/v which was represented on the basis of the acid form (*HiH*).

The stable and transparent dispersions obtained were stored overnight at 4–8 °C to ensure ionic pair formation, and then allowed to reach room temperature before use. Table 1 summarizes the physicochemical properties of both Hi and Dx.HCl used.

2.2. Species distribution in the dispersions

The proportions of the species *Dx*, DxH^+, and the complexed one *[R-COO⁻DxH⁺]* were determined by dialysis using a tube of cellulose membrane (12.000 Da; Sigma, St Louis, MA, USA)). Exactly 10 mL of *HiNa-Dx*$_{25}$ and *HiH-Dx*$_{25}$ aqueous dispersions at a 0.25% w/v were put into the dialysis tube (donor compartment) which was introduced into a flask containing 500 mL of water (receptor compartment). Previous studies demonstrated that the system reaches the equilibrium after 24 h of dialysis at 25 °C. After that, the pH of donor and receptor phases was recorded and Dx concentration in the receptor compartment was spectrophotometrically assayed at 480 nm to get the apparent Dialysis Ratio (DRapp). Calculations were performed according to the following equations.

$$DR_{app} = \frac{(DxH^+)_r + (Dx)_r}{(Dx)_d + (DxH^+)_d + ([RCOO^-DxH^+])_d} \quad (3)$$

$$Ka = \frac{(Dx)(H^+)}{(DxH^+)} \quad (4)$$

The affinity constant for the counterionic condensation (K_{cc}) was also calculated as previously reported (Battistini et al., 2013).

$$K_{cc} = \frac{([RCOO^-DxH^+])(H^+)}{(RCOOH)Ka(DxH^+)} \quad (5)$$

in which (RCOOH) was calculated from Eq (6).

$$\begin{aligned}(R-COOH) &= [RCOOH]_{st} - (RCOO^-DH^+) - (RCOO^-) \\ &= [RCOOH]_{st} - (RCOO^-DH^+) - (DH^+)\end{aligned} \quad (6)$$

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^+).

2.3. In vitro drug release

The extent and rate of *in vitro* release of Dx from 0.125% w/v aqueous dispersions were determined. Release rate from an aqueous solution with an equivalent concentration of Dx.HCl was used as reference. Experiments were performed in bicompartimental Franz cells equipped with a cellulose membrane (12.000 Da; Sigma, St Louis, MA, USA). Complexes required HCl addition to get a final pH near 3.0 in order to avoid complex precipitation. The donor compartment was filled with an exactly weighted amount, close to 1 ml, of the dispersion to assay, and sealed with Parafilm®. The receptor compartment was filled with 17 ml of water or NaCl 0.9%, both 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 with an equal volume of pre-warmed medium. Data were corrected for dilution. The concentrations of Dx were determined by UV-spectroscopy at 480 nm (Thermo-Electronic Corporation, Evolution 300 BB, England). All the assays were run in triplicate and followed for 180 min.

2.4. Cell culture and Dx treatment

Lung carcinoma cell line (A549, over expressing CD44 receptors) (Wojcicki et al., 2012) was grown in Dulbecco's modified Eagle's medium enriched with glucose, glutamine, pyridoxine, pyruvate and bicarbonate, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin (supplemented DMEM, Gibco® by Life Technologies™, New York.). First trimester human trophoblast cells (HTR8/SVneo, not over expressing CD44 receptors) (Novakovic et al., 2011; Marzioni et al., 2001; Goshen et al., 1996) were grown in supplemented DMEM-F12. In both cases the cells were plated on each well and incubated in an oven at 37 °C and humidified with 5% CO_2.

Each well contained: (i) cells without treatment (control cells), (ii) cells to which *HiH* or *HiNa* polymers were added (polymer references), (iii) cells treated with Dx.HCl (Dx reference), and (iv) cells treated with either *HiH-Dx*$_{50}$ or *HiNa-Dx*$_{50}$ complexes with a Dx concentration equivalent to Dx reference.

2.5. Cell viability/cytotoxicity assay

After determining the optimum cell number to carry out these experiments, 1×10^4 A549 or HTR8/SVneo cells were plated in each well of a 96-well plate. Three plates were used, each one corresponding to a time assayed (24, 48, and 72 h). Dx reference cells and those treated with *HiH-Dx* or *HiNa-Dx* complexes using Dx concentration close to 0.01 μM were assayed. Besides, control cells as well as polymer reference cells were evaluated using the metabolic dye 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) which enters into viable cells, and is metabolized in the mitochondria generating a violet precipitate called formazan. This precipitate is then dissolved in DMSO and its concentration is determined spectrophotometrically. Briefly, at 24 h, MTT solution (5 mg/mL) was 1:10 added to the culture medium and incubated for 2.5 h at 37 °C. After the incubation, the medium was removed and the precipitated formazan was dissolved in 100 μL of DMSO. Absorbance was read at 540 nm, and the results were expressed as% of viable cells relative to control. The same procedure was carried for 48 and 72 h plates. Three independent experiments were conducted in quintuplicate.

Areas over the curves (AOC) between 0 and 72 h were calculated from the plots of viable cells (%) vs. time (h) as an indicator of efficacy in which the higher the AOC obtained, the lower the cell count. AOC were calculated using the trapezoidal method. This includes finding the AOC in a series of trapezoidal portions limited for the values of the dependent variable and the independent one. Microsoft Excel® was used as a processor.

The appropriate concentration of Dx to carry on the experiments was determined through a dose–response curve. Briefly, 1×10^4 A549 or HTR8/SVneo cells were plated in each well of a 96-well plate. Dx concentration between 1×10^{-3} and 1×10^2 μM were used. Treated cells were incubated for 24 h and

Table 1
Structural and physicochemical properties of Hi and Dx.

Compound	MW	Ionisable group	pKa	Log CP	Water solubility
Hyaluronan	1655 KDa	Carboxylic acid	3–4[a]	–	High[a]
Doxorubicin HCl	580	Primary amine	8.22	0.10	39.2 mg/mL

[a] Brown and Jones (2005) and Hascall and Lauren (1997).

revealed with MTT assay. The IC_{50} obtained by dose–response assay through MTT was 4.49 μM and 4.31 μM of Dx for A549 and HTR8/SVneo respectively.

2.6. Fluorescence microscopy

A549 cells suspended in 2 mL of supplemented DMEM were plated in each well of a 6-well plate. Three plates were used, each one corresponding to a time assayed (24, 48, and 72 h). Dx reference solution and *HiH-Dx* or *HiNa-Dx* complexes were assayed at an equivalent concentration of Dx close to 1 μM. At every interval time, each plate was visualized using a Nikon optical microscope (Nikon eclipse TE2000-U, USA) without further treatment. A red fluorescence corresponding to Dx emission was observed in treated cells.

2.7. Flow cytometry

To perform the flow cytometry assay, 4×10^5 A549 cells in 2 mL of supplemented DMEM and HTR8/SVneo cells in 2 mL of supplemented DMEM-F12 were incubated for a period of 24 hs in a 6-well plate. Control cells, polymer references cells, Dx reference and those treated with *HiH-Dx* or *HiNa-Dx* complexes were equally treated. Dx reference solution and Hi-Dx complexes were assayed at an equivalent concentration of Dx close to 1 μM.

Besides, an assay in which A549 cells were pre-treated with the polymers itself (HiNa or HiH) was carried out to evaluate the effect of the pre-occupation of CD44 receptors. Subsequently, pre-treated cells were incubated with either Dx or ionic complexes.

Cells were washed once with phosphate buffer saline pH 7.4 (PBS, prepared with 137 mM of NaCl, 2.7 mM of KCl, 10 mM of K_2HPO_4 and 1.8 mM KH_2PO_4) following trypsinization (0.25%) and finally resuspended in 1 ml of PBS. For fixation, cells were centrifuged at 1000 rpm for 5 min. Then, the pellet was resuspended in 1 mL of cold ethanol 70% and stored at 4 °C for 1 h. For staining procedure, tubes were centrifuged at 1000 rpm for 5 min and the ethanol was removed. The pellet was resuspended in 100 μL of PBS. Dx fluorescence intensity was measured at excitation and emission wavelengths of $\lambda_{ex} = 496$ nm and $\lambda_{em} = 592$ nm, respectively. Fluorescence of the cells was read immediately by flow cytometer (FACS Canto II equipped with three lasers, a blue one (488 nm), a red one (633 nm, 17 mW HeNe) and a violet one (405 nm, 30 mW)) and analyzed with FCS express software trial version.

2.8. Statistical analysis

The data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using t test for independent samples ($n = 5$; $^*p < 0.05$). InfoStat® software was used.

3. Results and discussion

3.1. Species distribution in the dispersions

Fig. 2 shows the high proportion of Dx that is ionically condensed (higher than 78%) with Hi, in both *HiNa-Dx*$_{25}$ and *HiH-Dx*$_{25}$ complexes, yielding counterionic condensation constants (K_{cc}) of the order of 1×10^8. Owing to the process of preparation, *HiH* chains are shorter than those of *HiNa* (Battistini et al., 2013). However, the complexing capacity of the original salt form is maintained. Moreover, the higher proportion of counterionic condensation in *HiH-Dx*$_{25}$ respect to *HiNa-Dx*$_{25}$ is in accordance with the effect produced on equilibria by the *NaCl* formed *in situ* in the last one, as depicted in Eq. (2).

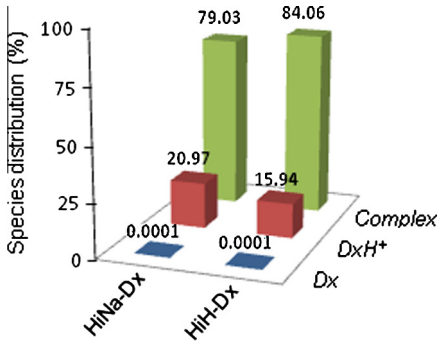

Fig. 2. Species distribution at equilibrium (percentage of Dx, DxH^+ and Ionic Complex) after dialysis of *HiH-Dx* and *HiNa-Dx* complexes. Each complex contained the equivalent of 25 mol% of Dx.

Table 2 shows the proportions of species (*D*, DH^+ and $[R\text{-}COO^- DH^+]$), the K_{cc} and pHs of the dispersion before and after dialysis.

3.2. In vitro drug release

The knowledge of the affinity of the basic group of Dx towards the carboxylic groups of *HiH* or *HiNa* to yield ionic complexes provides solid basis to understand the kinetics of drug release from the resulting carriers.

Figs. 3a and b, show the slow release of Dx from the ionic complexes when water was placed as a receptor medium. Notice that under the experimental conditions assayed and the presence of Cl^- in the dispersion, the diffusion should occur essentially trough DxH^+Cl^- due to the very low concentration of neutral Dx. As NaCl 0.9% was placed as a receptor medium instead of water, an increase in the release rate of the drug was observed, which according to Eqs. (1) and (2), may be associated with the diffusion of Cl^- and Na^+ from the receptor to the donor compartment. This phenomenon has been previously described in our group using both, anionic or cationic polymers complexed with contraionic drugs (Ardusso et al., 2010; Battistini et al., 2013; Guzmán et al., 2012; Jiménez Kairuz et al., 2005, 2003, 2002; Quinteros et al., 2008; Ramirez-Rigo et al., 2009, 2006). The systems assayed behave as a reservoir of Dx 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 the drug for the carboxylic groups of Hi.

These results provide valuable information regarding the expected interactions between the complexes and the biological fluids.

Taking into account the acid-base interaction described in Eqs. (1) and (2), the pKa of the basic group of Dx (8.22) and that of the acid groups of Hi (3–4) it is expected that the release at pH close to 7 has nearly the same kinetic performance than that observed at more acidic pHs. Moreover, at neutral pH essentially all the acidic groups of Hi will be dissociated and available to interact with protonated Dx species.

3.3. Cell viability/cytotoxicity assay

In order to develop *in vitro* assays with a lung carcinoma-derived A549 and HTR8/SVneo cell lines, dispersions of the complexes at 0.01 μM of Dx in supplemented DMEM (pH close to 7) were used.

Fig. 4 shows the effect over time produced on the A549 or HTR8/SVneo cell counts when *HiH-Dx*$_{50}$, *HiNa-Dx*$_{50}$ complexes or Dx reference solution were added into the culture.

Table 2
Species distribution at equilibrium and logK_{cc} obtained by dialysis of *HiH-Dx* and *HiNa-Dx* complexes at 25 °C.

Complex	[D] water	pH (donor)		Species distribution (%)			logK_{cc}[a]
		Initial	Equilibrium	(*D*)	(DH^+)	($RCOO^-DH^+$)	
HiNa-Dx_{25}	20.33	2.75	3.03	$1 \times 10^{-4} \pm 1 \times 10^{-4}$	20.97 ± 0.98	79.03 ± 2.00	7.96
HiH-Dx_{25}	21.62	2.81	3.07	$1 \times 10^{-4} \pm 2 \times 10^{-4}$	15.93 ± 1.08	84.06 ± 2.82	8.08

[a] K_{cc} calculation was performed according to Battistini et al., 2013.

As can be seen in Fig. 4 a, although *HiH-Dx*, *HiNa-Dx* and Dx reference lowered the A549 cell counts compared with the control, the complexes caused a significant decrease in cell viability respect to the Dx reference solution. Such difference in activity between the complexes and the reference was observed at 24, 48, and 72 h.

In HTR8/SVneo cells (Fig. 4b), both Hi-Dx complexes and Dx reference solution produced a decrease in cell viability compared with control cells. However, there is no significative difference between the lowering effect of Hi-Dx complexes compared with Dx reference solution.

Last, in line with the intrinsic properties of Hi, which is regarded as a non immunogenic, non toxic and biodegradable polymer, there was no difference in cell viability respect to control when *HiH* or *HiNa* was added to either A549 or HTR8/SVneo cells.

Areas over the curves (AOC) between 0 and 72 hs were calculated from the plots of Fig. 4 as an indicator of efficacy, in which the higher the AOC obtained, the lower the cell count. Table 3 shows AOC of all conditions assayed and the ratios $AOC_{Hi\text{-}Dx}$/$AOC_{Dx.HCl}$. Therefore, a differentiated activity of *HiH-Dx* or *HiNa-Dx* complexes towards A549 and HTR8 cells was observed. On the one hand, in A549 cells, both *HiH-Dx* and *HiNa-Dx* complexes yielded areas 3-fold higher than the reference Dx solution. On the other hand, in HTR8/SVneo cells, there was no difference between AOC of Hi-Dx complexes and Dx.HCl solution.

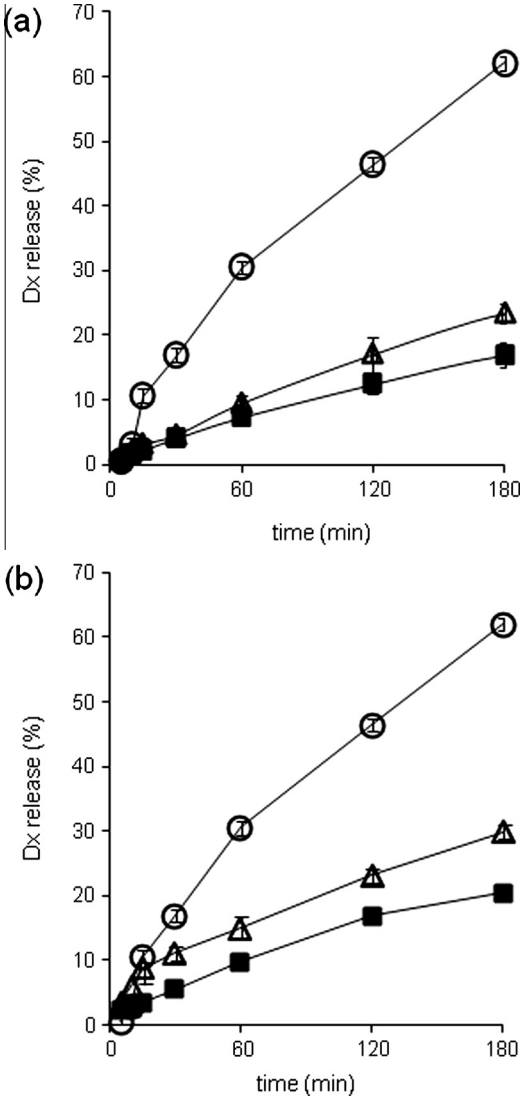

Fig. 3. Cumulative release of Dx as a function of time using a side-by side diffusion chamber for (a) *HiH-Dx* and (b) *HiNa-Dx*. Each formulation contained the equivalent of 25 mol% of Dx. Dx reference solution (O), Hi-Dx towards water (■), Hi-Dx towards NaCl 0.9% (Δ).

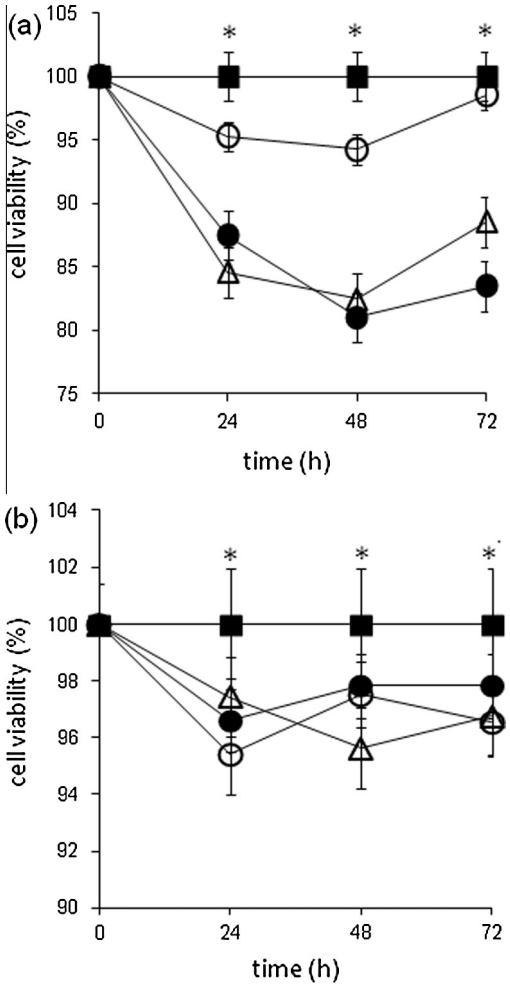

Fig. 4. Cell viability (%) versus time (h) for: (a) A549 cells and (b) HTR8/SVneo cells. All treated with equivalent concentrations of Dx 0.01 μM. Control cells (■), Reference Solution (O), *HiH-Dx* (Δ), *HiNa-Dx* (●). ($n = 5$; $^*p < 0.05$).

Table 3
Areas over curve for Dx, polymers and Hi-Dx complexes. Results are expressed as a coefficient between the areas obtained in each case and the area of Dx solution.

Compound	Cell Line	Areas	Hi-Dx/Dx rate
Dx	A549	278	1.00
	HTR8	240	1.00
HiH	A549	140	0.50
	HTR8	127	0.52
NaHi	A549	180	0.65
	HTR8	134	0.55
HiNa-Dx	A549	845	3.03
	HTR8	251	1.05
HiH-Dx	A549	837	3.01
	HTR8	249	1.03

3.4. Fluorescence microscopy

The natural fluorescence of Dx is a useful property to drive uptake indicative fluorescence assays. In this line, Fig. 5 shows the fluorescence of A549 treated cells with Dx reference solution and *HiH-Dx* or *HiNa-Dx* complexes. The intensity of the fluorescence examined by microscopy is indicative of the amount of Dx inside the cells. After 24 h of incubation with Dx solution, strong fluorescence of Dx was observed mostly in cell nuclei in addition to a weak fluorescence in the cytoplasm, suggesting rapid intercalation of intracellular Dx to the chromosomal DNA after passive diffusion into the cells. In contrast, *HiH-Dx* and *HiNa-Dx* fluorescence exhibited speckled red dots throughout the cytoplasm as well as in nuclei, indicating that complexes were initially trapped within endosomal compartments after cellular uptake. These data not only demonstrated that Hi-Dox was an efficient vehicle to transport Dx into the cytoplasm, but also suggested that the internalization mechanism of polymersomes was different from that of free Dx.

In samples of 48 and 72 h, higher fluorescence intensity was observed in A549 cells treated with both Hi-Dx complexes in comparison with those treated with the Dx reference solution. Besides, those cells treated with Hi-Dx complexes exhibited spherical shapes, which suggest that were apoptotic cells, while many of the cells treated with the Dx reference solution retain their morphology which indicates that were still viable.

This phenomenon was previously observed by Upadhyay et al. (2010) who obtained polymersomes composed of poly (g-benzyl L-glutamate)-b-hyaluronan showing a greater internalization of Dx loaded via CD44 receptors in breast cancer cells (MCF-7) that overexpress these receptors compared to Dx solution (Upadhyay et al., 2010).

3.5. Flow cytometry

A complementary flow cytometry assay was performed with A549 and HTR8/SVneo cells subjected to 24 h of contact with either Dx reference solution or Hi-Dx complexes. Fig. 6a and b, shows the number of events versus fluorescence intensity of the fluorophore (Dx). As can be seen in both figures, neither HiH nor HiNa polymers themselves produced any difference in the shifts compared with the control cells. In all cases, the fluorescence observed with either the Dx reference solution or Hi-Dx complexes should be ascribed to the Dx placed into the cells. In Fig. 6a, if Dx reference solution and *HiH-Dx* or *HiNa-Dx* ionic complexes are compared, no significative difference is observed in their shifts. These results may be associated with the absence of overexpression of CD44 receptors in HTR8/SVneo cell line, which would produce a greater internalization of Dx.

Besides, both *HiH-Dx* and *HiNa-Dx* complexes produce a shift towards higher fluorescence intensities than that of the Dx reference solution (Fig. 6b). These results are also indicative of an increased cellular uptake into cells of Dx when it was complexed with Hi due to the presence of overexpressed CD44 membrane

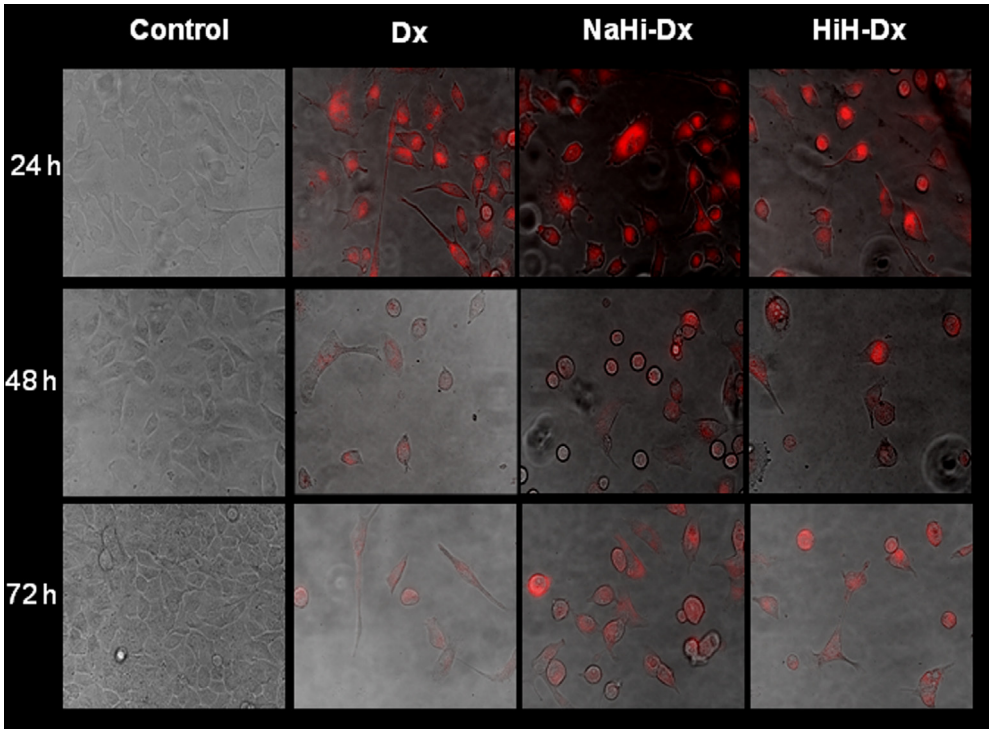

Fig. 5. Fluorescence microscopy versus time (h) of A549 cells treated with equivalent concentrations of Dx 1 μM. The images are the result of the superposition of the optical image and of the fluorescence one.

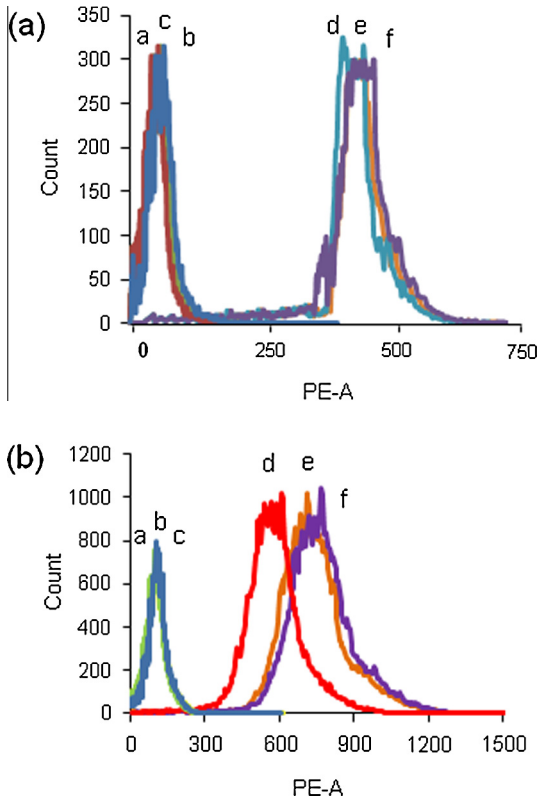

Fig. 6. Number of events versus fluorescence intensity of fluorophore (Dx) for: (a) HTR8/SVneo cells and (b) A549 cells both treated with equivalent concentrations of Dx 1 μM. a-Control, b-HiH, c-HiNa, d-Dx, e-*HiH-Dx* and f-*HiNa-Dx*. (c) A549 cells pre-treatment with Hi: a-Control, b-Dx, c-Dx$_{(\text{pre-treated with HiH})}$, d-Dx$_{(\text{pre-treated with HiNa})}$, e-*HiH-Dx*$_{(\text{pre-treated with HiH})}$ and f-*HiNa-Dx*$_{(\text{pre-treated with HiNa})}$.

receptors, which would favor the entrance of Dx into the cell by endocytosis of the ionic complex.

When CD44 receptors in A549 cells were pre-occupied with either HiNa or HiH (Fig. 6c), no differences were observed between cells treated with the Dx solution or Hi-Dx ionic complexes.

Therefore, it appears that the pre-treatment of A549 cells with Hi produces the internalization of CD44 receptors, not allowing them to be available to produce the later endocytosis of Hi-Dx iconic complex. This result reinforces the point view about the role of CD44 receptors in the increased concentration of Dx in the target cells when is carried by Hi.

The mechanism involved in this process, wherein the Hi-Dx complex binds to the CD44 membrane receptors of cancer cells was previously described (Ludwig, 2005). This binding produces an intracellular signaling cascade that results in the internalization of the receptor bound to the membrane, and thus a greater amount of *Dx* is internalized into the cell.

Upadhyay et al. (2010) showed that polymersomes of hyaluronan loaded with Doxorubicin produced shifts towards higher fluorescence intensities in MCF-7 cells overexpressing CD44 receptors. This phenomenon was attributed to a higher internalization of the drug compared to a Dx reference solution (Upadhyay et al., 2010).

4. Conclusions

Owing to its versatile physicochemical and biological properties such as biocompatibility, biodegradability, non-immunogenicity, and selective uptake by specific cancer cells, Hi has been widely utilized as an important constituent in the development of diverse carrier systems for cancer diagnostics and therapeutics.

From the results reported here, we envisioned that Hi conjugated to anticancer drugs would be useful for: (1) targeted delivery of the drug to tumor cells overexpressing the CD44 receptor, and (2) for producing greater internalization of the drugs into the tumor cell because of the endocytosis of the PE-D complex by CD44 receptors for its degradation.

This work provides more detailed information in order to contribute to more solid bases to evaluate the potentiality of Hi as an antineoplastic drug carrier convenient for being used in specific therapeutic indications.

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References

Ardusso, M.S., Manzo, R.H., Jimenez-Kairuz, A.F., 2010. Comparative study of three structurally related acid polyelectrolytes as carriers of basic drugs: Carbomer, Eudragit L-100 and S-100. Supramol. Chem. 22 (5), 289–296.
Baguley, B.C., 2010. Multiple drug resistance mechanisms in cancer. Mol. Biotechnol. 46, 308–316.
Battistini, F.D., Olivera, M.E., Manzo, R.H., 2013. Equilibrium and release properties of hyaluronic acid–drug complexes. Eur. J. Pharm. Sci. 49, 588–594.
Brown, M.B., Jones, S.A., 2005. Hyaluronic acid: a unique topical vehicle for the localized delivery of drugs to the skin. J.E.A.D.V. 19, 308–318.
Cai, S., Thati, S., Bagby, T.R., et al., 2010. Localized doxorubicin chemotherapy with a biopolymeric nanocarrier improves survival and reduces toxicity in xenografts of human breast cancer. J. Control. Release 146, 212–218.
Choi, K.Y., Saravanakumar, G., Park, J.H., Park, K., 2012. Hyaluronic acid-based nanocarriers for intracellular targeting: interfacial interactions with proteins in cáncer. Colloid Surface B. 99, 82–94.
Eliaz, R.E., Szoka Jr., F.C., 2001. Liposome-encapsulated doxorubicin targeted to CD44: a strategy to kill CD44-overexpressing tumor cells. Cancer Res. 61, 2592–2601.
Goshen, R., Ariel, L., Shuster, S., et al., 1996. Hyaluronan, CD44 and its variant exons in human trophoblast invasion and placental angiogenesis. Mol. Hum. Reprod. 2 (9), 685–691.
Guzmán, M.L., Manzo, R.H., Olivera, M.E., 2012. Eudragit E 100 as a carrier of drugs: the remarkable affinity of phosphate ester group for the dimethylamine groups. Mol. Pharm. 4 (9), 2424–2433.
Hahn, S.K., Kim, S.J., Kim, M.J., Kim, D.H., Lee, Y.P., 2005. Development of a novel sustained release formulation of recombinant human growth hormone using sodium hyaluronate microparticles. J. Control. Release 104, 323–335.
Hascall, V.C., Lauren, T.C., 1997. Hyaluronan: structure and physical properties. In: Glycoforum, Science of Hyaluronan.
Hirakura, T., Yasugi, K., Nemoto, T., Sato, M., Shimoboji, T., Aso, Y., Morimoto, N., Akiyoshi, K., 2010. Hybrid hyaluronan hydrogel encapsulating nanogel as a protein nanocarrier: new system for sustained delivery of protein with a chaperone-like function. J. Control. Release 142, 483–489.
Jiménez Kairuz, A.F., Allemandi, D.A., Manzo, R.H., 2002. Mechanism of lidocaine release from carbomer–lidocaine hydrogels. J. Pharm. Sci. 91, 267–272.
Jiménez Kairuz, A.F., Allemandi, D.A., Manzo, R.H., 2003. Equilibrium properties and mechanism of kinetic release of methoclopramide from carbomer hydrogels. Int. J. Pharm. 250, 129–136.
Jiménez Kairuz, A.F., Llabot, J.M., Allemandi, D.A., Manzo, R.H., 2005. Swellable Drug – Polyelectrolyte Matrices (SDPM): characterization and delivery properties. Int. J. Pharm. 288, 87–99.
Jordan, C.T., Guzman, M.L., Noble, M., 2006. Cancer stem cells. N. Engl. J. Med. 355, 1253–1261.
Kaya, G., Rodriguez, I., Jorcano, J.L., Vassalli, P., Stamenkovic, I., 1997. Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. Genes Dev. 11, 996–1007.
Knudson, W., Peterson, R., et al., 2004. The hyaluronan receptor: CD44. In: Garg, H.G., Hales, C.A. (Eds.), Chemistry and Biology of Hyaluronan, Boston, USA, pp. 83–123.
Lee, F., Chung, J.E., Kurisawa, M., 2009. An injectable hyaluronic acid–tyramine hydrogel system for protein delivery. J. Control. Release 134, 186–193.
Ludwig, A., 2005. The use of mucoadhesive polymers in ocular drug delivery. Adv. Drug Deliv. Rev. 57, 1595–1639.

Lukyanov, A.N., Elbayoumi, T.A., Chakilam, A.R., Torchilin, V.P., 2004. Tumor-targeted liposomes: doxorubicin-loaded long-circulating liposomes modified with anti-cancer antibody. J. Control. Release 100, 135–144.

Luo, Y., Bernshaw, N.J., Lu, Z.R., Kopecek, J., Prestwich, G.D., 2002. Targeted delivery of doxorubicin by HPMA copolymerhyaluronan bioconjugates. Pharm. Res. 19, 396–402.

Marhaba, R., Klingbeil, P., Nuebel, T., Nazarenko, I., Buechler, M.W., Zoeller, M., 2008. CD44 and EpCAM: cancer-initiating cell markers. Curr. Mol. Med. 8, 784–804.

Marzioni, D., Crescimanno, C., Zaccheo, D., et al., 2001. Hyaluronate and CD44 expression patterns in the human placenta throughout pregnancy. Eur. J. Histochem. 45, 131–140.

Miao, W., Shim, G., Kang, C.M., et al., 2013. Cholesteryl hyaluronic acid-coated, reduced grapheme oxide nanosheets for anticancer drug delivery. Biomaterials 34, 9638–9647.

Naor, D., Nedvetzki, S., Golan, I., Melnik, L., Faitelson, Y., 2002. CD44 in cancer. Crit. Rev. Clin. Lab. Sci. 39, 527–579.

Novakovic, B., Gordon, L., Wong, N.C., et al., 2011. Wide ranging DNA methylation differences of primary trophoblast cell populations and derived-cell lines: implications and opportunities for understanding trophoblast function. Mol. Hum. Reprod. 17 (6), 344–353.

Qhattal, H.S., Liu, X., 2011. Characterization of CD44-mediated cancer cell uptake and intracellular distribution of hyaluronan-grafted liposomes. Mol. Pharm. 8, 1233–1246.

Qian, Z.M., Li, H., Sun, H., Ho, K., 2002. Targeted drug delivery via the transferring receptor-mediated endocytosis pathway. Pharmacol. Rev. 54, 561–587.

Quinteros, D.A., Rigo Ramírez, M.V., Jimenez Kairuz, A.F., Manzo, R.H., Allemandi, D.A., 2008. Interaction between a cationic polymethacrylate (Eudragit E100) and anionic drugs. Influence of the physical–chemical properties of drugs on selectivity and affinity coefficients. J. Pharm. Sci. 33, 72–79.

Ramirez-Rigo, M.V., Allemandi, D.A., Manzo, R.H., 2006. Swellable Drug-Polyelectrolyte Matrices (SDPM) of alginic acid. Characterization and delivery properties. Int. J. Pharm. 322, 36–43.

Ramirez-Rigo, M.V., Allemandi, D.A., Manzo, R.H., 2009. Swellable drug-polyelectrolyte matrices of drug-carboxymethylcellulose complexes: characterization and delivery properties. Drug Deliv. 16, 108–115.

Slomiany, M.G., Dai, L., Bomar, P.A., et al., 2009. Abrogating drug resistance in malignant peripheral nerve sheath tumors by disrupting hyaluronan-CD44 interactions with small *Hyaluronan Oligosaccharides*. Cancer Res. 69 (12).

Song, S., Qi, H., Xu, J., et al., 2014. Hyaluronan-based nanocarriers with CD44-overexpressed cancer cell targeting. Pharm. Res. http://dx.doi.org/10.1007/s11095-014-1393-4.

Stern, R., Asari, A.A., Sugahara, K.N., 2006. Hyaluronan fragments: an information-rich system. Eur. J. Cell Biol. 85, 699–715.

Upadhyay, K.K., Bhatt, A.N., Mishra, A.K., et al., 2010. The intracellular drug delivery and anti tumor activity of doxorubicin loaded poly(g-benzyl L-glutamate)-b-hyaluronan polymersomes. Biomaterials 31, 2882–2892.

Wojcicki, A.D., Hillaireau, H., Nascimento, T.L., et al., 2012. Hyaluronic acid-bearing lipoplexes: physico-chemical characterization and in vitro targeting of the CD44 receptor. J. Control. Release 162, 545–552.