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Hyaluronan nanocapsules as a new vehicle for intracellular drug delivery

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ABSTRACT

Here we report the development of new drug nanocarriers – named hyaluronan nanocapsules – for the intracellular delivery of hydrophobic anticancer drugs. These nanocapsules are composed of a lipid core and a shell of hyaluronic acid (HA). Nanocapsules were produced by a modified solvent displacement technique, which allows the formation of the polymer shell around the oily core using a cationic surfactant as an interphase bridge. The resulting nanocapsules have a size of ~200 nm, a negative zeta potential and a spherical shape. The model drug docetaxel could be efficiently encapsulated within their core. The in vitro cell culture studies (NCI-H460 cancer cell line) showed that the cytotoxicity of docetaxel could be significantly enhanced due to its encapsulation within the nanocapsules. Interestingly, the nanocapsules were stable during storage and they could also be transformed into a powder by freeze-drying. These novel nanostructures hold promise as intracellular drug delivery systems.

1. Introduction

Currently, it is known that the use of nanoscale drug delivery vehicles represents a very promising strategy for improving the biodistribution and intracellular delivery of anticancer drugs. Taxanes (paclitaxel, docetaxel) are good examples of potent chemotherapeutic agents, which could greatly benefit from these delivery carriers. Indeed, despite their efficacy, these drugs display significant draw-backs related to their indiscriminate biodistribution and the necessity to use toxic solubilizers for their intravenous administration (Ten Tije et al., 2003). Besides the marketed formulation of albumin nanoparticles (Abraxane®), a number of nanocarriers have been disclosed in the literature for the delivery of these specific compounds. Particularly attractive for this purpose are the nanocapsules, which can easily accommodate hydrophobic drugs (Bae et al., 2007; Heurtault et al., 2002a,b; Lozano et al., 2013, 2008). For example, the group of Benoit and co-workers has reported the potential of PEG-coated lipid nanocapsules loaded with paclitaxel in different cancer animal models. Overall, the authors observed that PEGylated nanocapsules have long circulating properties as well as the ability to improve the intracellular accumulation of drugs in the tumor cells (Garcion et al., 2006; Lacoeuille et al., 2007). An alternative nanocapsule-type carrier was recently reported by our group for the delivery of docetaxel (DCX) (Lozano et al., 2013, 2008). These nanocapsules are made of chitosan and polyarginine and display a number of properties, among which it is important to highlight: (i) their capacity to be internalized by different cell lines, such as the breast cancer (MCF-7) and lung cancer (A-549) cell lines, (ii) the improved efficacy of DCX-loaded nanocapsules in MCF-7, A-549 and NCI-H460 (non-small cell lung cancer) cell lines compared to DCX alone; and (iii) the possibility to functionalize them with ligands such as anti-TMEFF-2 in order to target them to TMEFF-2, a transmembrane protein, that is overexpressed in non-small cell lung tumors (Torrecilla et al., 2013).

Following this experience, we found it important to modify the polymer corona of the nanocapsules using hydrophilic negatively charged polymers, such as hyaluronic acid (HA). This polysaccharide attracted our attention because of its reported ability to prolong the plasma circulation time of liposomes (Peer et al., 2007; Peer and Margalit, 2004a,b), polymeric nanoparticles (Choi et al., 2009), nanoparticle like-clusters (Rivkin et al., 2010) and solid lipid nanoparticles (Yang et al., 2012). Additionally, HA offers the possibility of targeting, and promotes the intracellular access by endocytosis, of the drug-loaded nanocarrier to the cancer cells that overexpress the endogenous receptor for this polymer (called CD-44 receptors) (Choi et al., 2012; Toole, 2004). As a consequence of these properties, HA-based nanocarriers have resulted in an improved tumor growth inhibition and a decreased systemic toxicity, when compared to the free drug (Akima et al., 1996; Auzenne et al., 2007; Eliaz et al., 2004; Eliaz and Szoka, 2001; Hyung et al., 2008; Luo and Prestwich, 1999; Peer and Margalit, 2004a,b; Rivkin et al., 2010; Rosato et al., 2006).

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The most popular HA-anticancer drug delivery nanocarriers described so far involve the use of covalent linkages. Examples of these nanocarriers include: drug-HA complexes (Auzenne et al., 2007; Bassi et al., 2011; Galer et al., 2011; Journo-Gershfeld et al., 2012; Lee et al., 2012, 2008; Luo and Prestwich, 1999; Rosato et al., 2006; Saravanakumar et al., 2010), HA-covalently modified liposomes (Eliaz et al., 2004; Eliaz and Szoka, 2001; Peer and Margalit, 2004a,b), PLGA (Hyung et al., 2008), histidine (Wu et al., 2012), superparamagnetic iron oxide nanoparticles (El-Dakdouki et al., 2012), and nanoparticle-like clusters (Rivkin et al., 2010).

As an alternative approach, very recently Yang et al. (2012) reported the development of solid lipid nanoparticles ionically coated with HA and loaded with paclitaxel. The behavior of these new nanocomposition was shown to be very promising either in vitro (cell cultures) or in vivo (Yang et al., 2012).

Taking this background information into account, our aim in this work has been to develop a new and alternative HA-based anticancer drug delivery nanocarrier, named HA nanocapsules. Differently from most HA-based nanocarriers, our approach does not involve chemical linkages but simply the ionic interaction between HA and a conveniently stabilized cationic nanoemulsion. According to the results presented here, such nanocarrier exhibits a number of interesting pharmaceutical properties, namely: (i) it is based on the combination of a number of biomaterials with an acceptable safety record, (ii) because of its oily container, it is ideal for the encapsulation of lipophilic drugs, (iii) it is able to enter cancer cells providing intracellular drug delivery, (iv) it can be produced according to a simple and easily scalable nanotechnology, and, finally, (v) there is a preliminary evidence of its potential adequate stability profile.

2. Experimental section

2.1. Materials

Chemicals: docetaxel (DCX, from Fluka), poloxamer (Pluronic F-68®), benzalkonium chloride (BKC) and hexadeciltrymethylammonium bromide (CTAB) were purchased from Sigma–Aldrich (Spain). Miglyol 812®, which is a neutral oil formed by esters of caprylic and capric fatty acids and glycerol, was donated by Sasol Germany GmbH (Germany). The surfactant Epikuron 145V, which is a phosphatidylcholine-enriched fraction of soybean lecithin, was donated by Cargill (Spain). Hyaluronic acid of 29 kDa was purchased from Imquiaroma (Barcelona, Spain) and that of 160 kDa was kindly donated by Bioiberica (Barcelona, Spain). Milli-Q water was used throughout the experiments and organic solvents were HPLC degree.

2.2. Preparation of HA nanocapsules

HA nanocapsules were prepared following two different procedures that have been previously described by our group (Lozano et al., 2008; Prego et al., 2005). The first, called "two-stage procedure" consists of adding 125 μl of Miglyol 812 to an organic phase comprising 30 mg of lecithin, and 4 mg of BKC or 1.8 mg of CTAB, dissolved in 0.5 ml of ethanol and 9.5 ml of acetone. This organic phase was added to an aqueous phase (20 ml). The formation of the cationic nanoemulsions was instantaneous, which was evident due to the milky appearance of the mixture (interestingly, the cationic nanoemulsion was also obtained after the addition of the organic phase to a homogenizer/sonicator, data not shown). The above solution was rotaevaporated until a volume of 10 ml, and then incubated with an aqueous solution of HA (0.1–50 mg) in a volume ratio 4:1.5 (cationic nanoemulsion:HA solution); when the anionic HA interacts with the cationic nanoemulsion, it forms a polymer corona at the oil/aqueous interface thus originating HA nanocapsules. The second procedure, called "one-stage procedure" involves adding 125 μl of Miglyol 812 to an organic phase comprising 30 mg of lecithin, and 4 mg of BKC or 1.8 mg of CTAB, dissolved in 0.5 ml of ethanol and 9.5 ml of acetone. This organic phase was added to an aqueous phase (20 ml) that contains the polymer HA (0.1–50 mg). The above solution was rotaevaporated until a volume of 10 ml; here the formation of HA nanocapsules occurs concomitantly to the evaporation of the solvent due to the adsorption of the polymer onto the nanocarrier-surface. In both cases, the incorporation of DCX, required the previous dissolution of this molecule in ethanol to obtain a final concentration of 1 mg/ml. Next, an aliquot of the stock solution was added to the organic phase and the same procedure was followed. The final DCX concentration obtained in HA nanocapsules carriers was 7.27 μM.

2.3. Physicochemical characterization of HA nanocapsules

The size and zeta potential of the colloidal systems were determined by photon correlation spectroscopy and laser Doppler anemometry, with a Zetasizer Nano-ZS (Malvern Instruments, United Kingdom). Each batch was analyzed in triplicate.

The morphological examination of the systems was performed by transmission electron microscopy (TEM) (CM12 Phillips, Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid and immobilized on copper grids with Formvar® for viewing by TEM.

2.4. Encapsulation efficiency of DCX-loaded HA nanocapsules

The encapsulation efficiency of DCX in the nanocarriers was determined indirectly by the difference between the total amount of DCX and the free drug recovered in the continuous phase. The total amount of drug was estimated by dissolving an aliquot of non-isolated DCX-loaded HA nanocapsules with acetonitrile. This sample was centrifuged during 20 min at 4000×*g* and the supernatant was measured using a high performance liquid chromatography (HPLC) system. The non-encapsulated drug was determined by the same method following separation of the nanocapsules from the aqueous medium by ultracentrifugation (27,500*g*, 60 min). DCX was assayed following a method originally developed and validated by Lee et al. (1999), and subsequently adapted by our group with slight modifications (Lozano et al., 2012, 2008). The HPLC system consisted of an Agilent 1100 series instrument equipped with a UV detector set at 227 nm and a reverse phase Zorbax Eclipse XDB- C8 column (4.6 × 150 mm i.d., pore size 5 μm Agilent, U.S.A.). The mobile phase consisted of a mixture of acetonitrile and 0.1% v/v orthophosphoric acid (55:45, v/v) and the flow rate was 1 ml/min. The standard calibration curves of DCX were linear ($r^2 > 0.999$) in the range of concentrations between 0.3 and 2 μg/ml.

2.5. In vitro release studies

The release studies of DCX from HA carriers were performed by incubating a sample of the formulation in a slightly acid medium by using milli-Q water (pH 5.5–5.8) at an appropriate concentration to ensure sink conditions. The vials were placed in an incubator at 37 °C for horizontal shaking. A total of 3 ml of the suspension were collected and ultracentrifuged (27,500*g*, 60 min) using Herolab high speed centrifuge labware tubes (Herolab GmbH, Germany) at different time intervals. The DCX released was calculated indirectly by determining how much was left in the system by processing the isolated HA nanocapsules with acetonitrile before HPLC analysis.

2.6. Cellular assays

Cell viability assay and IC_{50} estimation: Human non-small cell lung cancer cell line NCI-H460 was cultured in RPMI 1640 medium (ATCC), supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% carbon dioxide. Tetrazolium salt 3-(4,5-dimewthylthiazol-2-yl)2,5 diphenyltetrazolium bromide (MTT, Acros Organics) was used for mytocondrial activity evaluation. Briefly, cells were plated onto 96-well plates, with a seeding density of 15×10^3 cells/well in 100 μl of culture medium. After 24 h, the medium was renewed but contained the following three treatments: DCX, DCX-loaded HA nanocapsules and blank HA nanocapsules. Finally, after 48 h cell survival was measured by the MTT assay (Mossman, 1983). In brief, medium was removed and the cells were washed twice with 100 μl Hank's Balanced Salt Serum (HBSS). Then 20 μl of a MTT solution (5 mg/ml in PBS) and 100 μl HBSS were added to the wells and maintained at 37 °C in an atmosphere with 5% CO_2 for 4 h. Afterwards, buffers were replaced by 100 μl DMSO per well and maintained at 37 °C in an atmosphere with 5% CO_2 overnight. Absorbance (λ = 515 nm) was measured in a spectrophotometer (Tecan, Ultra evolution) removing any background absorbance (λ = 630 nm). Moreover, short incubation times of 2 h were assayed in order to determine the efficacy of HA nanocapsules to quickly interact with the cells and deliver the drug intracellularly. Thus, after a 2 h incubation with the three treatments, the medium was replaced by a fresh one and cells were grown for 48 h. Finally, cell viability was measured as described.

The percentage of cell viability was calculated by the absorbance measurements of control growth in the presence of the formulations at various concentration levels. IC_{50} values were obtained by fitting the data by non-linear regression, with Prism 2.1 software (GraphPad, San Diego, CA).

2.7. Stability study at storage conditions

The suspension stability of HA nanocapsules prepared with the surfactants BKC and CTAB was evaluated according to terms of time and temperature of storage. Therefore, aliquots of non-diluted DCX-loaded HA nanocapsules were placed in sealed tubes at 4 and 37 °C for storage. Size and polydispersity index of the nanocarriers were measured for a period of 3 months, meanwhile zeta potential values were controlled at the end of the study. Each sample corresponds to a different HA nanocapsules batch.

2.8. Freeze-drying studies of HA nanocapsules

Concentrations of HA nanocapsules (0.5 and 1% w/v) and of the cryoprotectant trehalose (5% and 10%) were considered the variables for the lyophilization study. Therefore, 2 ml dilutions of HA NCs were transferred into 5 ml volume glass vials and frozen at −20 °C. The lyophilization procedure was done in a Labconco equipment (Labconco Corp., USA) and consisted in a primary drying step for 60 h at −35 °C, followed by a secondary drying at 0 °C for 24 h. The vacuum was obtained by using a high vacuum pump (50 mTorr). Finally, the temperature was slowly increased (6–8 h) until room temperature (the end of the lyophilization process was fixed by selecting the conditions that provided a final product with a constant weight). HA nanocapsules were recovered by adding 2 ml of ultrapure water to the freeze-dried powders followed by manual resuspension and were characterized as explained above.

2.9. Statistical analysis

Cell culture results were evaluated in order to determine the statistical significance between the different formulations studied. The statistical evaluation of the cell viability results was performed by an ANOVA test followed by the post hoc Tukey test comparison analysis (SigmaStat Program, Jandel Scientific, version 3.5).

3. Results and discussion

The main aim of this work was the development of a new anticancer drug nanocarrier consisting of a lipid liquid core surrounded by a shell of HA. The conceptual bases of the system were: simplicity (minimum amount of components and easy to produce), acceptability (from the regulatory stand point) and efficacy (improvement of cytoxicity in cancer cells). Thus, DCX was chosen as a model compound and its efficacy was evaluated in the lung cancer cell line NCI-H460. As indicated in the introduction, the biopolymer HA was chosen because of its interesting biopharmaceutical properties (Choi et al., 2009; Ossipov, 2010; Surace et al., 2009; Yang et al., 2012).

3.1. Development and characterization of HA nanocapsules

HA nanocapsules were prepared by a number of simple techniques, which involved the formation of a cationic nanoemulsion and the attachment of the outer HA corona. One of the techniques was the solvent displacement technique, which allowed the emulsification process to occur simultaneously to the attachment of the polymer corona. Other techniques involved two steps, i.e. an emulsification process either by the solvent displacement technique, homogenization or sonication, followed by coating with the polymer. In all cases, the formation of the polymer capsule is driven by the ionic interaction of positively charged surfactants with the negatively charged HA. Following an initial screening of different experimental approaches, we chose the two-step solvent diffusion technique previously used for the formation of chitosan nanocapsules (Prego et al., 2005), and investigated the influence of different formulation parameters on the physicochemical properties of the resulting systems. The formulation parameters were the type and concentration of the cationic surfactant, the concentration of HA, and the molecular weight of HA. The cationic surfactants, selected on the basis of their acceptable toxicological profile, were benzalkonium chloride (BKC) and hexadecyltrimethylammonium bromide (CTAB) (Rowe et al., 2012). On the other hand, the quantity of the surfactants used was the minimum that allowed the formation of stable systems. In the case of CTAB this amount was 1.8 mg, and in the case of BKC 4.0 mg.

Table 1a and b show the characteristics of the nanocapsules prepared by the two-stage procedure using the surfactants BKC or CTAB, respectively. As can been noted, the use of adequate concentrations of HA with the cationic nanoemulsions results in the formation of homogenous populations of nanocapsules of around 250 nm. The results also show a shielding of the original positive zeta potential of the nanoemulsion, which leads to an inversion to negative values, as the concentration of HA increases. This dependency of the zeta potential with the amount of HA evidences the surface localization of HA molecules and indicates the necessity of using a minimum amount of HA in order to obtain stable nanocapsules. The formation of the HA corona results in a minor increase in the size of the nanocapsules with respect to the nanoemulsion, a result that supports the idea that HA is tightly attached to the oily droplets.

Table 1a and b also indicate that the viability of the system is compromised under certain conditions. More specifically, we observed a precipitation of the system due to the neutralization of the positive surface charge caused by the addition of increasing amounts of HA. After the inflection point, the addition of subsequent amounts of HA leads to the inversion of the zeta potential

Table 1
Physicochemical properties of the HA nanocapsules prepared following a two-stage procedure, with different quantities of HA (29 kDa) and a fixed amount of the surfactants (a) BKC (4.0 mg) or (b) CTAB (1.8 mg) (mean ± S.D., $n \geqslant 3$).

HA 29 kDa (mg)	Size (nm)	Polydispersity index	Zeta potential (mV)
(a)			
0.0	223 ± 10	0.1	+27.1 ± 3.5
0.5	250 ± 10	0.2	+9.6 ± 2.5
1.0	pp.	–	–
3.1	pp.	–	–
6.2	249 ± 10	0.2	−42.3 ± 2.1
12.5	235 ± 13	0.1	−45.6 ± 4.7
25.0	252 ± 19	0.1	−46.7 ± 2.9
50.0	pp.	–	–
(b)			
0.0	215 ± 7	0.1	+35.3 ± 2.1
0.1	232 ± 8	0.1	+18.3 ± 2.2
0.5	pp.	–	–
1	pp.	–	–
3.1	238 ± 2	0.1	−35.3 ± 2.4
6.2	238 ± 3	0.1	−37.6 ± 2.2
12.5	260 ± 20	0.2	−31.3 ± 3.2
25.0	pp.	–	–
50.0	pp.	–	–

pp.: Precipitation.

and the subsequent stabilization of the system. Finally, there is another precipitation point that is related to the excess of the HA in the formulation. Here, the precipitation phenomenon could be attributed to a combination of factors such as the high ionic strength in the medium (due to the high concentration of sodium ions added together with the hyaluronate) (Santander-Ortega et al., 2011), combined with the increase in the viscosity, which might modify the kinetics of adsorption of the polymer onto the cationic nanoemulsion.

Table 2a and b display the results of size and zeta potential of the HA nanocapsules prepared according to a single stage procedure. Overall, the conclusion is that the incorporation of HA either during or after the emulsification process does not affect the formation of the HA corona (both show similar zeta potential values and precipitation conditions). However, the size of the nanocapsules prepared by a single stage procedure was smaller than that of nanocapsules obtained by a two-stage procedure (~180 nm and ~250 nm, respectively). This difference in size has also been observed for chitosan nanocapsules prepared following a single (Lozano et al., 2008) or two-stage procedure (Prego et al., 2005) and could be attributed to the stabilizing role of the polymer during the emulsification process.

Table 2
Physicochemical properties of the HA nanocapsules prepared following a single stage procedure with different quantities of HA (29 kDa) and a fixed amount of the surfactants (a) BKC (4.0 mg) or (b) CTAB (1.8 mg) (mean ± S.D., $n \geqslant 3$).

HA 29 kDa (mg)	Size (nm)	Polydispersity index	Zeta potential (mV)
(a)			
0.0	223 ± 10	0.1	+27.1 ± 3.5
0.5	251 ± 5	0.1	+27.2 ± 1.4
1.0	pp.	pp.	pp.
3.1	pp.	pp.	pp.
6.2	pp.	pp.	pp.
12.5	185 ± 5	0.1	−44.2 ± 2.3
25.0	186 ± 7	0.1	−47.4 ± 2.3
50.0	pp.	pp.	pp.
(b)			
0.0	216 ± 7	0.1	+35 ± 2.5
0.1	229 ± 17	0.1	+33.7 ± 3.0
0.5	pp.	pp.	pp.
1.0	pp.	pp.	pp.
3.1	168 ± 3	0.1	−35.9 ± 2.7
6.2	170 ± 6	0.1	−35.6 ± 1.0
12.5	273 ± 13	0.2	−35.7 ± 2.8
25.0	pp.	pp.	pp.
50.0	pp.	pp.	pp.

pp.: Precipitation.

The size and appearance of the nanocapsules has also been observed by transmission electron microscopy (TEM). As an example, Fig. 1 illustrates the size and structure of those prepared with the BKC surfactant. Overall, the nanocapsules exhibited a round shape and the core-corona structure typically observed for polymeric nanocapsules (Lozano et al., 2008; Prego et al., 2005).

In a second part of the nanocapsules development process, we evaluated the influence of the HA MW (160 kDa vs. 29 kDa used in the original study) in the characteristics of the nanocapsules prepared by the two-stage solvent diffusion technique. Table 3 shows the characteristics of the formulations prepared by incubation of the preformed emulsion containing BKC with high MW HA. Overall, for the range of mass of 6.2–25 mg, the size of the nanocapsules was larger and their zeta potential higher than the corresponding ones obtained with the low MW HA (Table 1a). This HA MW-dependent behavior, has been previously reported for HA-coated solid lipid nanoparticles (Yang et al., 2012) and could be attributed, to the different external projection of the HA molecules towards the surface of the oily core, together with viscosity differences in the aqueous medium (Yang et al., 2012). On the other hand, the increase in the zeta potential values observed for the high MW HA nanocapsules could be a consequence of the greater number of carboxylic groups of HA at the shell surface. Taking into account their size distribution profile, but also the reported enhanced tumor accumulation and lack of immunological reactions of low MW HA-based nanocarriers (Mizrahy et al., 2011), we chose HA nanocapsules containing 12.5 mg of 29 kDa HA for further experiments.

3.2. Encapsulation and release of docetaxel (DCX) from HA nanocapsules

The high efficacy of DCX in the treatment of a wide range of solid tumors (Crown and O'Leary, 2000), together with its hydrophobic character makes this molecule an attractive candidate for inclusion in the developed nanocapsules. As shown in Table 4, DCX could be efficiently encapsulated in the HA nanocapsules prepared with the surfactants BKC or CTAB. These data correlate well with those previously reported for other types of lipid core-based nanocapsules (Khalid et al., 2006; Lozano et al., 2008).

In an additional experiment, we evaluated the release pattern of the encapsulated DCX upon incubation of highly diluted nanocapsules prepared with the surfactants BKC or CTAB in aqueous medium. The results showed similar drug release profiles for nanocapsules containing BKC or CTAB with only critical differences in the initial time-release point (Fig. 2). The release follows a biphasic profile, characterized by a rapid initial release, followed by a second stage in which no further changes were observed. The initial release stage, which is typically observed in oily systems, (Lozano et al., 2013, 2008; Prego et al., 2006) has been attributed to the dilution of the nanocapsules in the incubation medium and the subsequent partition of the drug between the oily core and the external aqueous phase. The different drug partition observed for BKC nanocapsules as compared to CTAB nanocapsules could be related to a distinct restrictive release behavior of the surfactant-HA complex. The absence of release in the second stage confirms the high affinity of the DCX by the oil core. While these data provide us with information about mechanistic details, in their interpretation it is important to point out that the release behavior is not necessarily expected to correlate with the in vivo behavior. In the in vivo situation, the presence of physiological

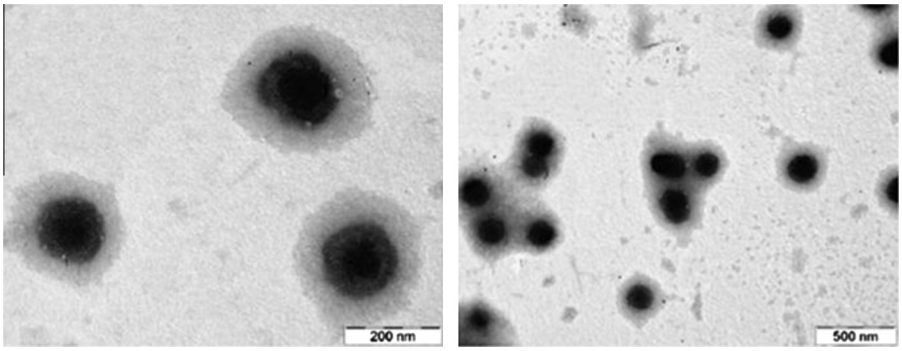

Fig. 1. Transmission electron microscopy images of HA nanocapsules (HA = 29 kDa, 12.5 mg) prepared with and the surfactant BKC (4 mg).

Table 3
Physicochemical properties of the nanocapsules prepared following a two-stage procedure with different quantities of HA (160 kDa) and the surfactant BKC (4 mg) (mean ± S.D., $n \geqslant 3$).

HA 160 kDa (mg)	Size (nm)	Polydispersity index	Zeta potential (mV)
0.0	208 ± 6	0.1	+27.3 ± 3.1
0.5	235 ± 10	0.2	+27.3 ± 3.6
1.0	271 ± 54	0.5	+3.9 ± 1.8
3.1	pp.	–	–
6.2	257 ± 11	0.5	−52.4 ± 8.8
12.5	295 ± 41	0.4	−62.3 ± 8.1
25.0	411 ± 70	0.5	−59.9 ± 6.1
50.0	pp.	pp.	pp.

pp.: Precipitation.

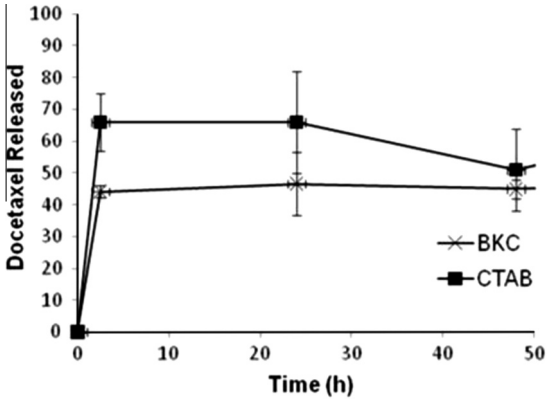

Fig. 2. In vitro DCX release from DCX-loaded HA nanocapsules using BKC (X) or CTAB (■) as surfactants (mean ± S.D., $n = 3$).

occurring macromolecules and ions could significantly influence the release profile (Ahmed and Ayres, 2011).

3.3. In vitro efficacy of the DCX-loaded HA nanocapsules in human lung cancer cell line

Cell viability studies were performed in order to assess the efficacy of the HA nanocapsules in the non-small cell lung cancer NCI-H460 cell line. These cells were selected because over-express CD44 receptors that have demonstrate to interact avidly with HA (Coradini et al., 2004). Fig. 3a and b shows the cellular viability profiles of DCX-loaded HA nanocapsules (formulation containing 1.8 mg of CTAB and 12.5 mg of 29 KDa HA) upon cell exposure for up to 2 and 48 h respectively. The results indicate that the encapsulation of DCX in HA nanocapsules lead to a significant increase of its cytotoxicity. The toxicity values were multiplied by a factor of 3.3 depending upon the incubation time. The results also show that blank nanocapsules did not cause any noticeable damage to the cells. These results are summarized in Table 5, which illustrates the cytotoxicity values of DCX-loaded nanocapsules measured by their IC_{50}. Overall, the potency of DCX was increased up to more than 3 times upon its encapsulation into HA nanocapsules.

Fig. 4a and b shows the cytotoxicity profiles of the cells exposed to the HA nanocapsules prepared with the surfactant BKC for up to 2 and 48 h, respectively. The cellular behavior observed for 2 h of exposition is similar to that observed for nanocapsules prepared with the surfactant CTAB. Accordingly, the results in Table 5 show that DCX-loaded nanocapsules are 3.6 times more efficient than the free drug in terms of the IC_{50} values. This improved efficacy was also observed after 48 h incubation (Fig. 4b), leading to a 2.4-fold reduction in the IC_{50} value (Table 5). In this case, a certain reduction of cell viability was also observed at high concentrations of the blank nanocapsules, a result that was attributed to the higher toxicity of BKC over CTAB (Rowe et al., 2012), and the necessity of using a greater amount of BKC vs. CTAB for the formation of stable nanocapsules.

The significant improvement in cytotoxicity observed for HA nanocapsules loaded with DCX is in agreement with the results observed for HA-coated lipid nanoparticles (Yang et al., 2012) and also for other types of nanocapsules, such as lipid-PEG (Garcion et al., 2006), chitosan (Lozano et al., 2008) or polyarginine nanocapsules (Lozano et al., 2013) tested in a variety of cell lines, including: glyoma cells (9L and F98 cell lines), human breast carcinoma (MCF-7), human lung cancer (A-549 and NCI-H460), murine melanoma (B16), mouse colon cancer (CT26) and human colon cancer (HCT116). The improvement in cytotoxicity has been attributed to an improved interaction (mediated by CD44 receptors),

Table 4
Characteristics of the selected blank and DCX-loaded HA nanocapsules prepared with the surfactants BKC or CTAB. (mean ± S.D., $n \geqslant 3$).

Formulation	Size (nm)	Polydispersity index	Zeta potential (mV)	Association efficiency (%)
HA NCs (BKC)	234 ± 13	0.1	−45.0 ± 4.3	–
DCX-loaded HA NCs (BKC)	250 ± 21	0.1	−52.3 ± 11.3	65.5 ± 3.1
HA NCs (CTAB)	267 ± 23	0.1	−31.1 ± 3.4	–
DCX-loaded HA NCs (CTAB)	276 ± 10	0.1	−36.7 ± 1.4	65.1 ± 3.8

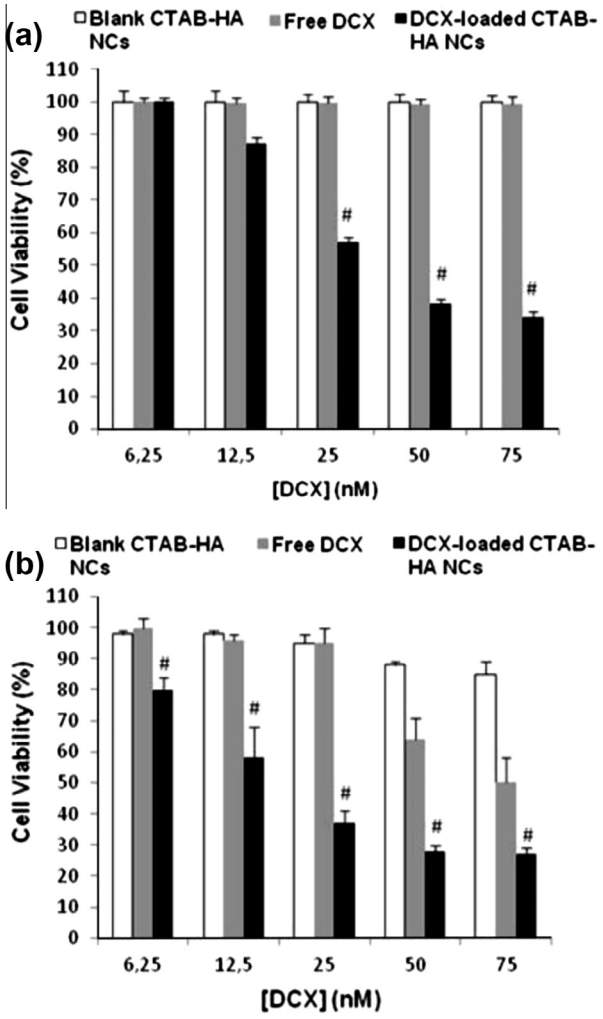

Fig. 3. Effect on NCI-H460 cell viability of DCX-loaded HA nanocapsules (HA = 29 kDa, 12.5 mg) prepared with the surfactant CTAB (1.8 mg) after (a) 2 or (b) 48 h of contact with the cells. Blank HA NCs (white bars), free DCX (gray bars) and DCX-loaded HA NCs (black bars) ($n = 4$). # $p < 0.005$: DCX-loaded HA NCs vs. free DCX.

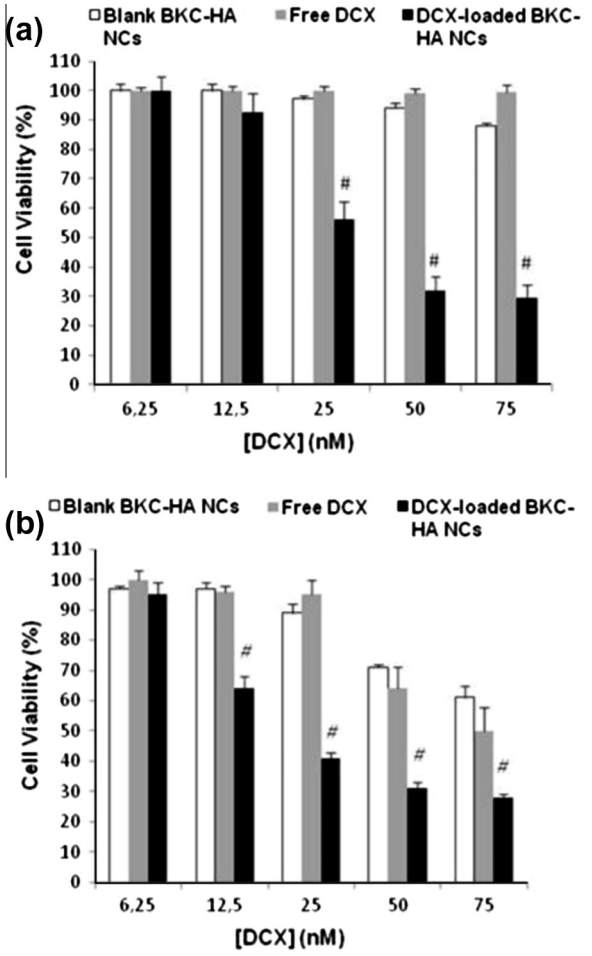

Fig. 4. Effect on NCI-H460 cell viability of DCX-loaded HA nanocapsules (HA = 29 kDa, 12.5 mg) prepared with the surfactant BKC (4 mg) after (a) 2 or (b) 48 h of contact with the cells. Blank HA NCs (white bars), free DCX (gray bars) and DCX-loaded HA NCs (black bars) ($n = 4$). # $p < 0.005$: DCX-loaded HA NCs vs. free DCX.

Table 5
IC_{50} values (drug concentration resulting in a 50% of cell viability) expressed in nM. Mean values of 4 independent experiments. n.d: none of the concentrations tested resulted in a significant reduction in the viability.

Formulation	2 h exposure	48 h exposure
Blank HA CTAB NCs	n.d.	n.d.
Blank HA BKC NCs	n.d.	[a]
DCX	105.0 ± 9.0	36.4 ± 4.0
DCX-loaded HA CTAB NCs	31.5 ± 2.1[b]	10.8 ± 1.1[b]
DCX-loaded HA BKC NCs	29.1 ± 1.8[b]	15.3 ± 2.0[b]

[a] The maximum reduction in cell viability was 39.0 ± 8.0%.
[b] $p < 0.01$ with respect to IC_{50} of DCX (One-way ANOVA test, post hoc Tukey test).

internalization and intracellular drug delivery capacity of the nanocapsules in association with a potential reversal of the multidrug resistance effect.

Some of the above indicated nanocarriers and those functionalized with a monoclonal antibody (anti TMEFF-2) have also been tested in a variety of animal models of cancer, such as: glyoma (Garcion et al., 2006), hepatocellular carcinoma (Lacoeuille et al., 2007), colon adenocarcinoma (Khalid et al., 2006), melanoma (Yang et al., 2012), and lung cancer (Torrecilla et al., 2013). The results obtained so far have shown an improved or similar efficacy of the encapsulated drug compared to that obtained for the free drug.

3.4. Stability studies of HA nanocapsules during storage

Stability is a critical issue in the development of a nanocarrier formulation. Variations on temperature are known to significantly compromise the stability of colloidal systems, and show evident importance during storage (Freitas and Müller, 1998). The size increase of the formulations could be attributed to the weakening/breaking-down of linkers between the molecules that form the nanocarriers, and may alter their drug release and induce destabilization of the formulations. A decrease in size could also occur due to the detachment of components from the nanocarriers or to an increase on the interaction strengths between linkers, potentially affecting the desired efficacy of the nanocarrier. In the present study we evaluated, for a period of 3 months, the stability of the DCX-loaded nanocapsules prepared with the surfactant CTAB under storage at conditions that represents refrigeration (4 °C) and also under extreme room conditions (37 °C) that could accelerate destabilization of the formulation. Fig. 5 shows that nanocapsules were generally stable during the storage irrespective of the temperature conditions. The overall trend of prolonged stability is justified by the electrostatic repulsive effect due to the high zeta

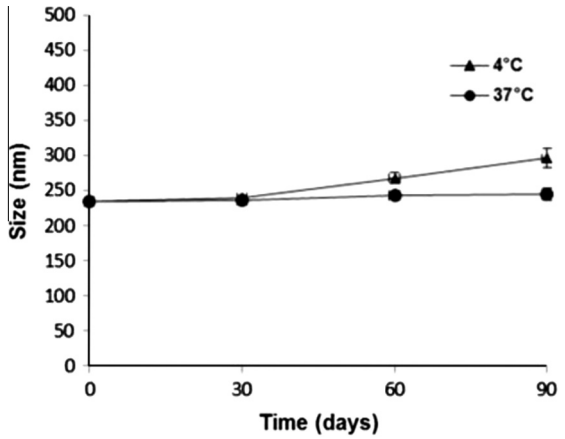

Fig. 5. Stability study of DCX-loaded HA nanocapsules during 3 months of storage. Nanocapsules were prepared with CTAB and tested at 4 °C (▲) and 37 °C (●). (Mean ± S.D.; $n = 3$).

potential values of nanocapsules (around −40 mV). Similar results were obtained when the surfactant BKC was used for the preparation of the nanocapsules (data not shown). This acceptable stability profile has been previously observed for other polymer nanocapsules (Lozano et al., 2013; Prego et al., 2005) and attributed to the polymer corona surrounding the oily nanodroplets.

3.5. Development of freeze-dried HA nanocapsules

Lyophilization is one of the most frequent and efficient methods of preserving the properties of nanoparticulate systems during storage. Nevertheless, this process becomes quite complex in the case of nanocapsules, due to the fluidity of the polymer shell and also to the presence of the oil core, which is susceptible of leakage (Choi et al., 2004). In order to facilitate the lyophilization of the nanocarriers and avoid their collapse, the use of cryoprotectant agents is necessary (Abdelwhaed et al., 2006). The first evidence that polymeric nanocapsules were effectively protected, by isotonic concentrations of cryoprotectants derived from sugars, during a lyophilization process was carried out by Calvo et al. (1997). Subsequent works (Lozano et al., 2013, 2012); have indicated that trehalose is an adequate agent for preserving the stability of fluid nanosystems during lyophilization. The main arguments, that support the use of this agent over other cryoprotectants, is its less hygroscopicity together with its higher glass transition temperature.

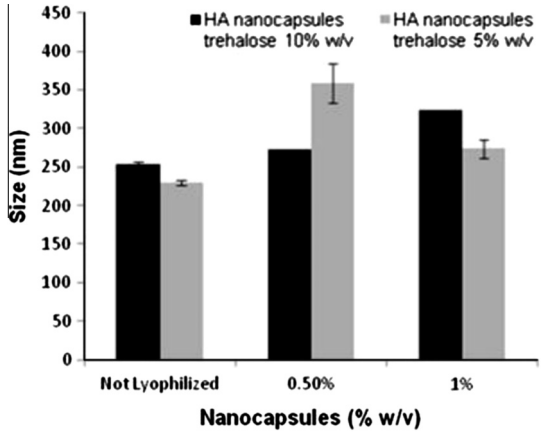

Fig. 6. Particle size of the reconstituted freeze dried HA nanocapsules using BKC as surfactant. Different concentrations (w/v) of nanocapsules were lyophilized using trehalose at 10% (black bars) or 5% w/v (gray bars). (Mean ± S.D.; $n = 3$).

Fig. 6 shows the size of HA nanocapsules containing BKC upon reconstitution of the freeze-dried product. Overall the results indicate that at relatively high concentrations of nanocapsules (1% w/v) it is possible to achieve an adequate resuspension of the dried product without altering the size of the nanocapsules.

4. Conclusions

In this paper we report the formation and characterization of a new type of drug nanocarriers named as HA nanocapsules. These nanocarriers are easy to prepare, do not involve the formation of covalent linkages, and are able to successfully encapsulate the hydrophobic drug docetaxel because of their hydrophobic core. In addition, thanks to their HA hydrophilic coating, these nanocarriers have the capacity to interact with NCI-H460 cancer cells and improve the pharmacological effect of the model drug docetaxel. Finally, pharmaceutical parameters such as drug release, stability during storage and reconstitution of freeze-dried HA nanocapsules render these novel systems promising platforms for the intracellular delivery of anticancer drugs.

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