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Abstract

Hyaluronan (MW: 1.5 MDa) was linked to a phospholipid (dipalmitoyl phosphatidylethanolamine, DPPE) by an amidification procedure to obtain novel macromolecules (HA-DPPE) able to coat liposomes. Liposomes made of dipalmitoyl phosphatidylcholine and cholesterol (DPPC/Chol: 95/5 molar ratio), with a mean size around 100 nm, were incubated with HA-DPPE at 55 ◦C, allowing the insertion of DPPE moieties in the liposomal bilayer and leading to hyaluronan-coated liposomes (HAsomes) as evidenced by several techniques including dynamic light scattering and differential scanning calorimetry. The amount or HA-DPPE coating liposomes was quantified by different methods among which capillary electrophoresis and their stability in serum was finally compared to that of plain liposomes. As a conclusion, we provide insight into the physico-chemical characterization of HA-DPPE and of HAsomes demonstrating that easy coating of phospholipid vesicles can be achieved by post-insertion of a lipid derivative of hyaluronan. This approach represents an innovative strategy for coating vesicular systems to confer them simultaneously with long circulation properties and selective targeting towards HA-receptors.

1. Introduction

Among nanocarriers, liposomes represent the most investigatedexample because of their peculiar structure made up of phospho-lipids that self-assemble into vesicles able to entrap hydrophilic andlipophilic drugs modulating their biopharmaceutical characteris-tics [1]. Many liposomal formulations are already on the marketor under clinical trials [2]. In particular, the systemic administra-tion of liposomes favors the increase of the pharmacological effectand half-life of the entrapped active compound(s) and the decreaseof their side effects, as it is true in the case of Doxil®/Caelyx®.For these reasons, approaches improving the technological prop-erties of liposomal systems are continuously under investigation[3]. The systemic administration of a nanocarrier requires specificcharacteristics such as a mean size around 100 nm, surface proper-ties allowing to avoid destabilization/aggregation and long blood circulation properties. Water-soluble polymers are widely usedas coating agents in nanocarriers avoiding their opsonization inthe blood stream and increasing their blood residence time, thuspromoting the passive targeting of solid tumors by the enhancedpenetration and retention (EPR) effect [4,5]. Polyethylene glycol(PEG) is a coating polymer widely used in pharmaceutical technol-ogy to efficiently modulate the pharmacokinetic profile of activecompounds encapsulated into nanocarriers and avoid nanocarrieropsonization leading to their uptake by the mononuclear phago-cyte system [6,7]. Unfortunately, the accelerated blood clearance (ABC) effect in relation with the hyper-production of IgM antibod-ies from the spleen after the repetitive systemic administrationsof PEG-coated (also called PEGylated) nanocarriers was described[8]. ABC induces a significant decrease of the plasmatic residencetime of PEGgylated nanocarriers reducing the modulation of thebiopharmaceutical properties of the entrapped drug(s) [8,9]. A wayto bypass ABC consists in using other hydrophilic polymers ableto efficiently coat nanocarrier’s surface and keep their plasmatichalf-life stable over multiple administrations [10,11]. Hyaluronic acid (HA), a glycosaminoglycan polymer made up ofrepeating units of D-glucuronic acid and N-acetyl-d-glucosamine

linked together through alternating -1,3 and -1,4 glycosidic bonds, could be a suitable candidate as a consequence of its bio-compatibility and biodegradability [12]. The physiological turnoverof HA is remarkably rapid because it is degraded in lysosomesinto glucuronic acid and N-acetylglucosamin that are metabolizedby hepatocytes into CO2, H2O and urea. HA exists in the bodyat different molecular weights (MW) that display different phys-iological functions. High MW HA is involved in maintaining cellintegrity and water content in extracellular matrix, while low MWHA plays a crucial role in receptor-mediated intracellular signal-ing and is responsible for angiogenesis and inflammation [13,14].These effects are made possible by the numerous HA receptors,such as the cluster determinant 44 (CD44) receptor, the receptorfor hyaluronate-mediated motility (RHAMM), the HA receptor forendocytosis (HARE) and the lymphatic vessel endothelial hyaluro-nan receptor-1 (LYVE-1). All these receptors can be used to obtaina selective targeting of specific cells and tissues [15,16].For these reasons, novel HA-phospholipid conjugates were syn-thesized to coat liposomes either by hydration of the lipidic filmor by post insertion on preformed liposomes. Dipalmitoyl phos-phatidylethanolamine (DPPE) was chosen as lipid anchor to allowthe insertion in the liposomal bilayer, thus avoiding the hindranceof aqueous compartments and a decrease of the entrapment effi-ciency of water soluble drugs as this was shown for PEGylatedliposomes [17]. The conjugates were synthesized by means of anamidic linkage between the activated carboxylic residues of HA(MW 1.5 MDa) and the amino-group of DPPE with the aim of yield-ing several lipid anchors on the polysaccharide backbone. Here,we describe the synthesis and characterization of HA-DPPE conju-gates. The efficacy of liposomes coating either by direct hydrationof the lipidic film or post insertion into preformed liposomes toobtain hyaluronan-coated liposomes (HAsomes) was evaluated andcharacterized. Preliminary serum stability experiments were also carried out.

2. Materials and methods

2.1. Materials

High molecular weight hyaluronic acid (HA) (sodium salt,1500 kDa, purity of 95%) was purchased from Acros organics (Geel,Belgium). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC),1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) wasprovided by Avanti Polar Lipids (USA), while 1-ethyl-3-[3-dimethyl)aminopropyl]carbodiimide (EDC), HNO3(65%), ferricchloride hexahydrate, ammonium thiocyanate, phosphorus stan-dard solution, perchloric acid (70%), ammonium molybdatesolution, amino-naphthyl-sulfonic acid reagent, borate buffer, nin-hydrin, iodine, molybdenum blue and cholesterol were obtainedfrom Sigma Aldrich (Saint Quentin Fallavier, France). Chloroformand methanol were provided by Carlo Erba Reagenti (Milan, Italy).

2.2. Synthesis and characterization of HA-DPPE conjugate

HA-DPPE conjugate was obtained by the synthetic proceduredescribed by Surace et al. [18] with some modifications. Briefly, HA(14 mg) was dissolved into distilled water and pre-activated withEDC at pH 4 adjusted by titration with HCl 0.1 N. Subsequently,DPPE (360 mg) was added to the HA solution and the pH wasadjusted at 8.6 with a 0.1 M borate buffer pH 9.4. The reaction waslet to proceed for 24 h at 37◦C (Fig. 1). The final product was purifiedby ultrafiltration and dialysis (Spectra/Por regenerated cellulosemembrane with a molecular cut-off of 50,000). The reaction wasmonitored by thin layer chromatography (TLC) using F254 silica gelprecoated sheets (Sigma-Aldrich, Saint-Quentin-Fallavier, France).After migration of the mobile phase (CHCl3/CH3OH 2:1 v/v), sheetswere exposed to iodine vapors, solutions of molybdenum blueand ninhydrin (2,2-dihydroxyindene-1,3-dione) solution (1 mg/mLethanol).HA-DPPE conjugate was successively lyophilized and charac-terized by1H NMR (Bruker Avance 300 MHz). The amount of DPPEcovalently coupled to HA was quantified after re-suspension of theconjugate in distilled water by phosphorus quantification using theBartlett assay as well as a modified Stewart method [19,20]. Prior tothis assay, HA-DPPE conjugates were incubated with HNO3(65%)at 70◦C for 10 min to hydrolyze the amide bound and facilitate thequantification of DPPE residues.

2.3. Preparation and characterization of HAsomes

Liposomes were prepared using the thin lipid hydration tech-nique as previously described [1,21]. Briefly, a lipid mixture madeof DPPC/Chol (95/5 molar ratio) was dissolved in a mixture of chloroform/methanol (2:1, v/v) in a Pyrex®tube and then placed ina rotary evaporator for solvent removal. The dry lipidic film washydrated afterwards with a HEPES solution (1 ml) to yield a finallipid concentration of 40 mM. To reduce liposome mean size, thevesicles were extruded at 60◦C using a Lipex Extruder®(Vancouver,Canada) equipped with polycarbonate membrane filters of 100 nmpore size (15 cycles) (Millipore, France).To investigate the possibility to obtain HAsomes, differentamounts of HA-DPPE were added either during the hydration phaseor after the extrusion process, incubating the vesicles with the con-jugate at a temperature of 55◦C for 2 h to allow the insertion of DPPEmoieties in the bilayer. The same procedure was performed usingHA in order to compare the amount of polysaccharide associatedto liposomes with respect to HA-DPPE. Liposomes and HAsomeswere purified by ultracentrifugation at 150,000 × g for 1 h at 4◦C (Optima LE–80 K, Beckman Coulter, USA). Mean size, polydisper-sity index (PDI) and Z-potential were determined with a ZetasizerNano ZS (Malvern Instruments Ltd., Worcestershire, United King-dom), operating at 670 nm with a detection angle of 173◦. Sizemeasurements were performed after dilution in water whereas Zeta potential measurements were performed after dilution in NaCl1 mM. Measurements were carried out in triplicate on three differ-ent batches (3 determinations for each batch) at 20◦C. The resultswere expressed as the mean ± standard deviation.Transmission Electron Microscopy (TEM) was performed ona JEOL JEM-1400 microscope operating at 80 kV with a filamentcurrent of about 55A. The suspension of liposomes (5l of for-mulation) was deposited onto copper grids covered with a formvarfilm (400 mesh) for 1 min. Negative staining (30 s) with phospho-tungstic acid 2% was achieved. The excess solution was blottedoff using filter paper, and the grids were dried before observa-tion. Images were acquired using a post column high-resolution(11 megapixels) high-speed camera (SC1000 Orius; Gatan) and pro-cessed with Digital Micrograph (Gatan) and ImageJ.

2.4. Differential scanning calorimetry (DSC)

DSC analysis was accomplished on the different liposomalformulations by using a differential scanning calorimeter (DSC-Diamond, PerkinElmer, USA). The different formulations werecentrifuged as previously described and resuspended in 100 l of HEPES solution. About 10 mg accurately weighed samples wereloaded into 40 l aluminum pans and analyzed. DSC runs wereperformed from 10 to 70◦C at a rate of 5◦C/min. Calibrationwas achieved using Indium (Tonset= 156.60◦C) as well as n-decane(Tonset= −29.66◦C). Experiments were performed at least in dupli- Enthalpies were normalized with respect to the DPPC weightin the sample.

2.5. Evaluation of HA or HA-DPPE associated to liposomes

The amount of HA or HA-DPPE associated to liposomes was investigated by capillary electrophoresis. Liposomes were washedtwice by ultracentrifugation (see above) before the analysis in orderto eliminate the polysaccharides that were not associated with thevesicles. A Beckman P/ACE System5500 was used with an uncoatedfused silica capillary of 57 cm effective length and internal diameterof 75 cm. Electrophoresis conditions to analyze HA-DPPE conjugatewere derived from Grimshaw et al. [22] for the assay of HA. The cap-illary was first conditioned by 4 successive rinsing steps of 5 mineach with water, 1 M NaOH, 0.1 M NaOH and back to water. Beforeeach analysis, the capillary was washed at 20 psi with successivelywater for 2 min, 0.1 M NaOH for 3 min and finally equilibrated withthe background electrolyte for 5 min. The separation buffer was65 mM sodium tetraborate containing 20 mM of sodium dodecylsulfate (SDS) with pH 9. Sample injection was carried out at 0.5 psifor 5 s. All samples were adjusted to a concentration of 1 mg/mL ofHA or HA-DPPE by evaporation under vacuum using an Eppendorf Concentrator®at 30◦C. This step was validated after verification bydynamic light scattering that no modification of the diameter andzeta potential occurred during the concentration step. Detectionwavelength was 200 nm and the voltage applied was 25 kV. Thecapillary was maintained at 25◦C during electrophoresis. Experi-ments were performed at least in duplicate.To confirm data obtained by capillary electrophoresis, the car-bazole assay, a colorimetric analysis, was performed [23]. Briefly,samples were treated with a H2SO4solution (96%) containing 25 mM Na2B4O710H2O and heated at 100◦C for 10 min. Then,

they were cooled up to room temperature and incubated with anethanol solution containing carbazole (0.125% w/v). Finally, theywere newly heated up to 100◦C, cooled and absorbance at 530 nmwas determined by a spectrophotometer (Perkin Elmer Lambda 25).

*2.6. Serum incubatio*n

Plain liposomes, HA-incubated liposomes and HA-DPPE incu-bated liposomes were incubated in 60% FBS to investigate stabilityand size modification [24]. Briefly, 200 l of liposomal formulationswere added to 1 ml of 60% FBS and incubated at 37◦C for 4 daysupon stirring at 600 rpm. Liposome size was evaluated by DLS aspreviously described after a 1:50 dilution of samples.3.

Results and discussion

Recently, DPPE was conjugated to the HA by means of a reduc-tive amination approach [16,25]. Instead, in this investigation, the DPPE moieties have been linked to HA backbone by theamidification procedure. This approach was previously used toobtain HA-DOPE conjugates applied to formulate novel lipoplexestargeting CD44+ cells [12,15]. The HA-DPPE conjugation was char-acterized in terms of reaction yield and amount of phospholipidmoieties bound to the polysaccharide backbone. The HA-DPPE con-jugate was obtained with a yield of 70–80%.1H NMR analysis ofthe obtained product was carried out to demonstrate the effective linkage of the polysaccharide to phospholipid moieties. In Fig. 2 itis possible to observe the characteristic NMR-peaks of the differentcompounds; in particular, the signal at ∼0.9 ppm was attributed tothe terminal methyl proton of the DPPE moiety, while the peaksat 1.2–1.6 ppm were attributed to the methine protons of phos-pholipid [26]. On the other hand, the peaks at 3.0–4.0 ppm wereassigned to the protons of hydroxyl residues of HA, while thepeaks at 2.0 ppm were attributed to the protons of CH3CO- portionof the polysaccharide [27]. HA-DPPE clearly showed the peaks of both compounds, providing the evidence of the efficacy of the synthetic procedure. The purity of HA-DPPE was confirmed by differentanalytical methods. TLC analysis performed on purified and freeze-dried conjugate evidenced a Rf= 0, while DPPE showed a Rf= 8 thatwas not detectable in the case of HA-

DPPE, thus confirming thatfree DPPE was removed by the purification procedure (data notshown). Moreover, DSC analysis evidenced that the thermogramof HA-DPPE did not show the characteristic main transition peakof DPPE (at ∼64◦C), thus providing a further evidence of the effi-cacy of the purification procedure (Fig. S1). Finally, the amount oflipid associated to HA-DPPE was evaluated by means of two meth-ods. The phosphorus assay evidenced an amount of phospholipidof ∼1.5% wt per molecule of conjugate while the Stewart methodprovided a value of ∼1.2% wt, corresponding to 45–60% graftingefficacy.3.1. Physicochemical characterization of HAsomesThe possibility to efficiently coat liposomes with HA-DPPE wasthen evaluated using the post-insertion technique. For this rea-son, HA-DPPE was added in two different steps during liposomepreparation in order to demonstrate that the lipophilic residuesof the conjugate allow the insertion of the macromolecule inthe bilayer of already formed liposomes (lipid concentration of40 mM). Namely, different amounts of HA-DPPE were added in thehydration medium during the liposomal preparation or after theextrusion process. Simultaneously, the same concentrations wereused to prepare liposomes coated with HA (as free form) even if thepolysaccharide induced the gelification of the system at concentra-tions higher than 2 mg/mL. Naked liposomes displayed a mean sizevalue of about 110 nm, a low polydispersity index (PDI < 0.1) anda surface charge of −3 mV, in good agreement with other authors(Table 1) [28,29]. Introduction of HA induced a significant increaseof liposomal mean diameter as well as an increase of the polydis-persity probably because of the increased aqueous viscosity whichmodified the extrusion process after hydration. When HA was incu-bated with liposomes aggregation might arise from the osmoticeffect of HA (Table 1, Fig. S2). On the contrary, introduction ofHA-DPPE induced little changes in the diameter and polydisper-sity index in the range 0.1–0.25 whether it was introduced duringthe hydration phase or post inserted (Table 1, Fig. S3). The Zeta-potential measurements evidenced a more negativesurface charge of samples containing HA with respect to the nakedliposomes. In addition, in presence of HA, liposomes were morenegatively charged than in presence of HA-DPPE. This could arisefrom the greater number of free carboxylic residues of HA withrespect to the formulations containing HA-DPPE where DPPE wasgrafted onto

COOH groups (Table 1, Fig. S3). Moreover, at highconjugate concentrations, the conformation of HA-DPPE on the sur-face of liposomes may be different from the conformation of HAas DPPE-residues could promote a stronger interaction with theliposomal bilayer, thus masking many carboxylic residues. Similarzeta potential values were obtained when HA-DPPE was intro-duced either in the hydration medium during sample preparationor incubated with liposomes after extrusion. This proves that thenumber of DPPE moieties that can insert into the bilayer is lim-ited by hydrophobic interaction and the spatial configuration ofHA-DPPE. DLS analysis provided the evidence that either the hydra-tion or the post-insertion of HA-DPPE in liposomes allow to obtainnanocarriers with suitable size and zeta potential for a systemicadministration. TEM observations confirmed this trend showingthat the morphological structure of the vesicles was not modifiedafter incubation with various concentrations of HA and HA-DPPE (Fig. 3). Molecular modeling investigations are in progress to eval-uate the rearrangement of HA-DPPE in water and its interactionwith a biomembrane model. In fact, the various lipid anchors of theconjugate could origin a different coating of the liposomal surfaceas a function of the spatial conformation [30]. To provide further evidence that the post-insertion of HA-DPPEallows to obtain HAsomes as a consequence of the interactionbetween the DPPE residues of the conjugate and the liposomalbilayer, DSC analysis was performed. A comparison with additionof 1 mg of DPPE was made. In the case of liposomes and HA, lipo-somes incubated with 1 or 3 mg of HA did not exhibit a change ofthe onset temperature of the DPPC transition nor a drastic changein its enthalpy. For larger amounts of HA (6 mg) the transition tem-perature is shifted towards higher temperature with a decreasedenthalpy (Table 2, Fig. 4). This finding is in agreement with previous results showing adsorption of HA onto DPPC bilayers andinteractions with polar heads [31,32].The incubation of liposomes with HA-DPPE at different concen-trations induced a shift of the onset temperature of DPPC (from aTonset= 42◦C to a Tonset= 38◦C) for the highest amount of HA-DPPE(6 mg) with no change of the transition enthalpy of DPPC (Fig. 4,Table 2). Moreover, the peak was flattened and irregular proba-bly as a consequence both DPPE insertion and HA adsorption. Aliposomal formulation prepared with 1 mg of DPPE induced a

sig-nificant shift of the main transition peak of DPPC that remained welldefined, confirming the peak flattening observed with HA-DPPE isa combination of HA adsorption and DPPE insertion.3.2. Evaluation of the amount of HA-DPPE associated to the liposomes. The amount of HA or HA-DPPE that was associated with the lipo-somes was evaluated by the carbazole assay. As can be observed inFig. 5, the hydration procedure allowed to obtain similar amountsof HA or HA-DPPE associated to the vesicles. Namely, no morethan 200–250 g of HA or HA-DPPE were associated to the vesi-cles probably as a consequence of several phenomena such as i)a physical entrapment in the vesicular aqueous compartment ofthe polysaccharides, ii) an interaction between the polar heads of Fig. 4. DSC thermograms of liposomes incubated with different concentrations ofHA or HA-DPPE.the phospholipids and the HA, and iii) an integration of the DPPEmolecules in the liposomal bilayer in the case of HA-DPPE.The incubation of HA or HA-DPPE with liposomes provided dif-ferent behaviors. In fact, the post-insertion of HA-DPPE in extrudedliposomes favored a significant increase of the amount associatedwith the vesicles (450–550 g/formulation) with respect to the HA(120–250 g/formulation) (Fig. 4). This trend clearly demonstratesthat the DPPE residues were critical to allow a good interactionbetween HA-DPPE and lipid membrane. This effect was promotedby the greater surface area of the extruded liposomes (post-insertion) with respect to that of multilamellar vesicles (obtainedafter lipid hydration). In detail, the hydration of liposomal bilayerwith the aqueous phase containing HA-DPPE and the followingheating at 55◦C promoted the encapsulation of the macromoleculein liposomes and the integration of the DPPE within the phos-pholipid bilayer but favored a saturation of the “binding sites”.On the contrary, the extrusion of liposomes and their incubationwith the HA-DPPE at 55◦C offered a wide surface area allow a verygood interaction of DPPE residues with other phospholipids presentwithin the membranes.Carbazole assay is a test that could be influenced by many inter-ferences and for this reason the same experiments was performedusing capillary electrophoresis. The amount of both HA or HA-DPPEnot interacting with liposomes was evaluated after the prepara-tion of liposomes and the amount of compounds associated to thevesicles was obtained by the difference with the amount initiallyadded. The results obtained

with this technique were in very goodagreement with those aforesaid described (Figs. S4 and S5); forexample, when 1 mg of HA was used in the hydration step, 20%remained adsorbed on vesicles, while less than 10% was detectablein the case of HA-DPPE (Fig. S4). On the contrary, when HA-DPPEwas incubated with liposomes, 50% of the conjugate was detectedon the liposomal surface, while the amount of HA that remainedadsorbed on the vesicles was similar to that obtained by usingthe hydration technique (Fig. S5). This trend clearly demonstratesthat the post-insertion technique could be a useful approach toallow coating of vesicles avoiding the hindrance of liposomal aque-ous compartment. Moreover, the two techniques evidenced that aplateau of HA-DPPE associated to the liposomes was reached at aconcentration of 2 mg when the post-insertion technique was used(Fig. 5).Fig. 5. Evaluation of unconjugated HA and HA-DPPE amount on the liposomal surface by carbazole assay as a function of the concentration and technique used. Each valueis the mean of three different experiments ± standard deviation.

Each value is the mean of three different experiments ± standard deviation. Error bars, if not shown, are smaller than symbol size.3.3. Evaluation of serum stabilityConsidering the physico-chemical characterization of HAsomesobtained by the post-insertion technique of HA-DPPE with lipo-somes, a concentration of conjugate of 1–2 mg was suitable toprovide an efficient coating of the vesicles with a slow varia-tion of their size and polydispersity. The behavior of HAsomesin medium containing serum is a fundamental information to beinvestigated before the encapsulation of drugs in these colloidal systems and their use in in vitro and in vivo experiments. For thisreason, HAsomes obtained by the post-insertion technique of 1 mgof HA-DPPE were incubated in 60% FBS at 37◦C and their size wasmonitored for 4 days and compared to naked liposomes and lipo-somes incubated with 1 mg of HA. The mean sizes of the colloidalformulations have been investigated in absence of serum proteinsin the same conditions as control and no aggregation was observed (Table S1).Fig. 6 evidences a stability of all formulations up to 12 h incuba-tion, while afterwards an increase of the hydrodynamic diameter ofall samples was observed. In particular, after 24 h incubation, nakedliposomes and liposomes incubated with HA exhibit a dramaticincrease of their diameter up to

450 nm, while HAsomes showedhigher but constant sizes (∼300 nm). This trend is probably due tothe lipid composition of the liposomes. Moreover, the hydrophiliclayer surrounding the liposomal surface, promoted by the insertionof HA-DPPE in the vesicular bilayer, is probably more efficient thanthe HA physically associated to the liposomes to prevent the coatingof serum proteins. This results from the strong interaction betweenthe DPPE anchors and the vesicular lipophilic compartment thatfavors the retention of the conjugate. On the other hand, the spa-tial conformation of HA and HA-DPPE, adsorbed and post-inserted within liposomes, respectively, could play a crucial role to explainthis phenomenon. Therefore size increase due to destabilization byserum protein adsorption is limited in the case of HAsomes.Furthermore, naked liposomes showed a decrease of theirsurface charge down to ∼−12 mV while the HA-coated formula-tions did not evidence significant variation of the Zeta-potential (−25/−30 mV). These results are in very good agreement with theexperimental data of Wolfram and coworkers that demonstratedthe role of hydrophilic-coating agents like PEG to preserve the sur-face charge of liposomes after their incubation in FBS [24].4. ConclusionInnovative compounds able to efficiently coat liposomes arenecessary to allow the modulation of their physico-chemicalfeatures and their in vivo behavior. In this investigation, wedemonstrate that hyaluronan conjugated to a phospholipid is ableto interact with the liposomal phospholipid bilayer, generating by post-insertion technique novel HA-coated liposomes namedHAsomes a novel approach in the field of HA-coated vesicles. Thismethod prevents the hindrance of the liposomal aqueous compart-ment and offers the opportunity to present the HA backbone onvesicle surface. In addition, the HA-coating can be used to increasethe plasmatic half-life of liposomes avoiding the appearance of theABC phenomenon due to multiple administrations of the PEGylatedliposomes and to target the vesicles against cells over-expressingthe HA receptors without affecting the possibilities of entrappinghydrophilic drugs in the aqueous compartment.

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