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## **Polysaccharide-coated liposomes by post-insertion of a hyaluronan-lipid conjugate**

Donato Cosco<sup>a,b</sup>, Nicolas Tsapis<sup>a</sup>, Thais L. Nascimento<sup>a,c</sup>, Massimo Fresta<sup>b</sup>, David Chapron<sup>a</sup>, Myriam Taverna<sup>a</sup>, Silvia Arpicco<sup>d</sup>, Elias Fattal<sup>a,\*</sup>

<sup>a</sup> Institut Galien Paris-Sud, CNRS, Univ. Paris-Sud, Université Paris-Saclay, 92296 Châtenay-Malabry, France

<sup>b</sup> Department of Health Sciences, University “Magna Græcia” of Catanzaro, Campus Universitario “S. Venuta”, Viale S. Venuta, Germaneto, I-88100 Catanzaro, Italy

<sup>c</sup> CAPES Foundation, Ministry of Education of Brazil, Brasília DF 70040-020, Brazil

<sup>d</sup> Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Facoltà di Farmacia, Via Pietro Giuria 9, 10125 Torino, Italy

## **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 of 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 investigated example because of their peculiar structure made up of phospho-lipids that self-assemble into vesicles able to entrap hydrophilic and lipophilic drugs modulating their biopharmaceutical characteristics [1]. Many liposomal formulations are already on the market under clinical trials [2]. In particular, the systemic administration of liposomes favors the increase of the pharmacological effect and half-life of the entrapped active compound(s) and the decrease of their side effects, as it is true in the case of Doxil®/Caelyx®. For these reasons, approaches improving the technological properties of liposomal systems are continuously under investigation [3]. The systemic administration of a nanocarrier requires specific characteristics such as a mean size around 100 nm, surface properties allowing to avoid destabilization/aggregation and long blood circulation properties. Water-soluble polymers are widely used as coating agents in nanocarriers avoiding their opsonization in the blood stream and increasing their blood residence time, thus promoting the passive targeting of solid tumors by the enhanced penetration and retention (EPR) effect [4,5]. Polyethylene glycol (PEG) is a coating polymer widely used in pharmaceutical technology to efficiently modulate the pharmacokinetic profile of active compounds encapsulated into nanocarriers and avoid nanocarrier opsonization leading to their uptake by the mononuclear phagocyte system [6,7]. Unfortunately, the accelerated blood clearance (ABC) effect in relation with the hyperproduction of IgM antibodies from the spleen after the repetitive systemic administration of PEG-coated (also called PEGylated) nanocarriers was described [8]. ABC induces a significant decrease of the plasmatic residence time of PEGylated nanocarriers reducing the modulation of the biopharmaceutical properties of the entrapped drug(s) [8,9]. A way to bypass ABC consists in using other hydrophilic polymers able to efficiently coat nanocarrier's surface and keep their plasmatic half-life stable over multiple administrations [10,11]. Hyaluronic acid (HA), a glycosaminoglycan polymer made up of repeating 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 turnover of HA is remarkably rapid because it is degraded in lysosomes into glucuronic acid and N-acetylglucosamine that are metabolized by hepatocytes into CO<sub>2</sub>, H<sub>2</sub>O and urea. HA exists in the body at different molecular weights (MW) that display different physiological functions. High MW HA is involved in maintaining cell integrity and water content in extracellular matrix, while low MW HA plays a crucial role in receptor-mediated intracellular signaling 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 receptor for hyaluronate-mediated motility (RHAMM), the HA receptor for endocytosis (HARE) and the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1). All these receptors can be used to obtain a selective targeting of specific cells and tissues [15,16]. For these reasons, novel HA-phospholipid conjugates were synthesized to coat liposomes either by hydration of the lipidic film or by post insertion on preformed liposomes. Dipalmitoyl phosphatidylethanolamine (DPPE) was chosen as lipid anchor to allow the insertion in the liposomal bilayer, thus avoiding the hindrance of aqueous compartments and a decrease of the entrapment efficiency of water soluble drugs as this was shown for PEGylated liposomes [17]. The conjugates were synthesized by means of an amide linkage between the activated carboxylic residues of HA (MW 1.5 MDa) and the amino-group of DPPE with the aim of yielding several lipid anchors on the polysaccharide backbone. Here, we describe the synthesis and characterization of HA-DPPE conjugates. The efficacy of liposomes coating either by direct hydration of the lipidic film or post insertion into preformed liposomes to obtain hyaluronan-coated liposomes (HASomes) was evaluated and characterized. 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) was provided by Avanti Polar Lipids (USA), while 1-ethyl-3-(3-dimethyl)aminopropyl]carbodiimide (EDC), HNO<sub>3</sub>(65%), ferric chloride hexahydrate, ammonium thiocyanate, phosphorus standard solution, perchloric acid (70%), ammonium molybdate solution, amino-naphthyl-sulfonic acid reagent, borate buffer, ninhydrin, iodine, molybdenum blue and cholesterol were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Chloroform and 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 procedure described by Surace et al. [18] with some modifications. Briefly, HA (14 mg) was dissolved into distilled water and pre-activated with EDC at pH 4 adjusted by titration with HCl 0.1 N. Subsequently, DPPE (360 mg) was added to the HA solution and the pH was adjusted at 8.6 with a 0.1 M borate buffer pH 9.4. The reaction was let to proceed for 24 h at 37°C (Fig. 1). The final product was purified by ultrafiltration and dialysis (Spectra/Por regenerated cellulose membrane with a molecular cut-off of 50,000). The reaction was monitored by thin layer chromatography (TLC) using F254 silica gel pre-coated sheets (Sigma-Aldrich, Saint-Quentin-Fallavier, France). After migration of the mobile phase (CHCl<sub>3</sub>/CH<sub>3</sub>OH 2:1 v/v), sheets were exposed to iodine vapors, solutions of molybdenum blue and ninhydrin (2,2-dihydroxyindene-1,3-dione) solution (1 mg/mL ethanol). HA-DPPE conjugate was successively lyophilized and characterized by <sup>1</sup>H NMR (Bruker Avance 300 MHz). The amount of DPPE covalently coupled to HA was quantified after re-suspension of the conjugate in distilled water by phosphorus quantification using the Bartlett assay as well as a modified Stewart method [19,20]. Prior to this assay, HA-DPPE conjugates were incubated with HNO<sub>3</sub>(65%) at 70°C for 10 min to hydrolyze the amide bond and facilitate the quantification of DPPE residues.

### *2.3. Preparation and characterization of HASomes*

Liposomes were prepared using the thin lipid hydration technique as previously described [1,21]. Briefly, a lipid mixture made of DPPC/Chol (95/5 molar ratio) was dissolved in a mixture of chloroform/methanol (2:1, v/v) in a Pyrex<sup>®</sup> tube and then placed in a rotary evaporator for solvent removal. The dry lipidic film was hydrated afterwards with a HEPES solution (1 ml) to yield a final lipid concentration of 40 mM. To reduce liposome mean size, the vesicles were extruded at 60°C using a Lipex Extruder<sup>®</sup> (Vancouver, Canada) equipped with polycarbonate membrane filters of 100 nm pore size (15 cycles) (Millipore, France). To investigate the possibility to obtain HASomes, different amounts of HA-DPPE were added either during the hydration phase or after the extrusion process, incubating the vesicles with the conjugate at a temperature of 55°C for 2 h to allow the insertion of DPPE moieties in the bilayer. The same procedure was performed using HA in order to compare the amount of polysaccharide associated to liposomes with respect to HA-DPPE. Liposomes and HASomes were purified by ultracentrifugation at  $150,000 \times g$  for 1 h at 4°C (Optima LE-80 K, Beckman Coulter, USA). Mean size, polydispersity index (PDI) and Z-potential were determined with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom), operating at 670 nm with a detection angle of 173°. Size measurements were performed after dilution in water whereas Zeta potential measurements were performed after dilution in NaCl 1 mM. Measurements were carried out in triplicate on three different batches (3 determinations for each batch) at 20°C. The results were expressed as the mean  $\pm$  standard deviation. Transmission Electron Microscopy (TEM) was performed on a JEOL JEM-1400 microscope operating at 80 kV with a filament current of about 55 A. The suspension of liposomes (51 of formulation) was deposited onto copper grids covered with a formvar film (400 mesh) for 1 min. Negative staining (30 s) with phosphotungstic acid 2% was achieved. The excess solution was blotted off using filter paper, and the grids were dried before observation. Images were acquired using a post column high-resolution (11 megapixels) high-speed camera (SC1000 Orius; Gatan) and processed with Digital Micrograph (Gatan) and ImageJ.

### *2.4. Differential scanning calorimetry (DSC)*

DSC analysis was accomplished on the different liposomal formulations by using a differential scanning calorimeter (DSC-Diamond, PerkinElmer, USA). The different formulations were centrifuged as previously described and resuspended in 100 l of HEPES solution. About 10 mg accurately weighed samples were loaded into 40 l aluminum pans and analyzed. DSC runs were performed from 10 to 70°C at a rate of 5°C/min. Calibration was achieved using Indium (Tonset= 156.60°C) as well as n-decane (Tonset= -29.66°C). Experiments were performed at least in duplicate. Enthalpies were normalized with respect to the DPPC weight in 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 washed twice by ultracentrifugation (see above) before the analysis in order to eliminate the polysaccharides that were not associated with the vesicles. A Beckman P/ACE System 5500 was used with an uncoated fused silica capillary of 57 cm effective length and internal diameter of 75 µm. Electrophoresis conditions to analyze HA-DPPE conjugate were derived from Grimshaw et al. [22] for the assay of HA. The capillary was first conditioned by 4 successive rinsing steps of 5 min each with water, 1 M NaOH, 0.1 M NaOH and back to water. Before each analysis, the capillary was washed at 20 psi with successively water for 2 min, 0.1 M NaOH for 3 min and finally equilibrated with the background electrolyte for 5 min. The separation buffer was 65 mM sodium tetraborate containing 20 mM of sodium dodecylsulfate (SDS) with pH 9. Sample injection was carried out at 0.5 psi for 5 s. All samples were adjusted to a concentration of 1 mg/mL of HA or HA-DPPE by evaporation under vacuum using an Eppendorf Concentrator® at 30°C. This step was validated after verification by dynamic light scattering that no modification of the diameter and zeta potential occurred during the concentration step. Detection wavelength was 200 nm and the voltage applied was 25 kV. The capillary was maintained at 25°C during electrophoresis. Experiments were performed at least in duplicate. To confirm data obtained by capillary electrophoresis, the carbazole assay, a colorimetric analysis, was performed [23]. Briefly, samples were treated with a H<sub>2</sub>SO<sub>4</sub> solution (96%) containing 25 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O and heated at 100°C for 10 min. Then,



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

### 2.6. Serum incubation

Plain liposomes, HA-incubated liposomes and HA-DPPE incubated liposomes were incubated in 60% FBS to investigate stability and size modification [24]. Briefly, 200  $\mu$ l of liposomal formulations were added to 1 ml of 60% FBS and incubated at 37°C for 4 days upon stirring at 600 rpm. Liposome size was evaluated by DLS as previously described after a 1:50 dilution of samples.

## Results and discussion

Recently, DPPE was conjugated to the HA by means of a reductive amination approach [16,25]. Instead, in this investigation, the DPPE moieties have been linked to HA backbone by the amidification procedure. This approach was previously used to obtain HA-DOPE conjugates applied to formulate novel lipoplexes targeting CD44<sup>+</sup> cells [12,15]. The HA-DPPE conjugation was characterized in terms of reaction yield and amount of phospholipid moieties bound to the polysaccharide backbone. The HA-DPPE conjugate was obtained with a yield of 70–80%. <sup>1</sup>H NMR analysis of the obtained product was carried out to demonstrate the effective linkage of the polysaccharide to phospholipid moieties. In Fig. 2 it is possible to observe the characteristic NMR-peaks of the different compounds; in particular, the signal at ~0.9 ppm was attributed to the terminal methyl proton of the DPPE moiety, while the peaks at 1.2–1.6 ppm were attributed to the methine protons of phospholipid [26]. On the other hand, the peaks at 3.0–4.0 ppm were assigned to the protons of hydroxyl residues of HA, while the peaks at 2.0 ppm were attributed to the protons of CH<sub>3</sub>CO- portion of 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 different analytical methods. TLC analysis performed on purified and freeze-dried conjugate evidenced a R<sub>f</sub> = 0, while DPPE showed a R<sub>f</sub> = 8 that was not detectable in the case of HA-

DPPE, thus confirming that free DPPE was removed by the purification procedure (data not shown). Moreover, DSC analysis evidenced that the thermogram of HA-DPPE did not show the characteristic main transition peak of DPPE (at  $\sim 64^{\circ}\text{C}$ ), thus providing a further evidence of the efficacy of the purification procedure (Fig. S1). Finally, the amount of lipid associated to HA-DPPE was evaluated by means of two methods. The phosphorus assay evidenced an amount of phospholipid of  $\sim 1.5\%$  wt per molecule of conjugate while the Stewart method provided a value of  $\sim 1.2\%$  wt, corresponding to 45–60% grafting efficacy.

### 3.1. Physicochemical characterization of HASomes

The possibility to efficiently coat liposomes with HA-DPPE was then evaluated using the post-insertion technique. For this reason, HA-DPPE was added in two different steps during liposome preparation in order to demonstrate that the lipophilic residues of the conjugate allow the insertion of the macromolecule in the bilayer of already formed liposomes (lipid concentration of 40 mM). Namely, different amounts of HA-DPPE were added in the hydration medium during the liposomal preparation or after the extrusion process. Simultaneously, the same concentrations were used to prepare liposomes coated with HA (as free form) even if the polysaccharide induced the gelification of the system at concentrations higher than 2 mg/mL. Naked liposomes displayed a mean size value of about 110 nm, a low polydispersity index ( $\text{PDI} < 0.1$ ) and a surface charge of  $-3$  mV, in good agreement with other authors (Table 1) [28,29]. Introduction of HA induced a significant increase of liposomal mean diameter as well as an increase of the polydispersity probably because of the increased aqueous viscosity which modified the extrusion process after hydration. When HA was incubated with liposomes aggregation might arise from the osmotic effect of HA (Table 1, Fig. S2). On the contrary, introduction of HA-DPPE induced little changes in the diameter and polydispersity index in the range 0.1–0.25 whether it was introduced during the hydration phase or post inserted (Table 1, Fig. S3). The Zeta-potential measurements evidenced a more negative surface charge of samples containing HA with respect to the naked liposomes. In addition, in presence of HA, liposomes were more negatively charged than in presence of HA-DPPE. This could arise from the greater number of free carboxylic residues of HA with respect to the formulations containing HA-DPPE where DPPE was grafted onto

COOH groups (Table 1, Fig. S3). Moreover, at high conjugate concentrations, the conformation of HA-DPPE on the surface of liposomes may be different from the conformation of HA as DPPE-residues could promote a stronger interaction with the liposomal bilayer, thus masking many carboxylic residues. Similar zeta potential values were obtained when HA-DPPE was introduced either in the hydration medium during sample preparation or incubated with liposomes after extrusion. This proves that the number of DPPE moieties that can insert into the bilayer is limited by hydrophobic interaction and the spatial configuration of HA-DPPE. DLS analysis provided the evidence that either the hydration or the post-insertion of HA-DPPE in liposomes allow to obtain nanocarriers with suitable size and zeta potential for a systemic administration. TEM observations confirmed this trend showing that the morphological structure of the vesicles was not modified after incubation with various concentrations of HA and HA-DPPE (Fig. 3). Molecular modeling investigations are in progress to evaluate the rearrangement of HA-DPPE in water and its interaction with a biomembrane model. In fact, the various lipid anchors of the conjugate could originate a different coating of the liposomal surface as a function of the spatial conformation [30]. To provide further evidence that the post-insertion of HA-DPPE allows to obtain HASomes as a consequence of the interaction between the DPPE residues of the conjugate and the liposomal bilayer, DSC analysis was performed. A comparison with addition of 1 mg of DPPE was made. In the case of liposomes and HA, liposomes incubated with 1 or 3 mg of HA did not exhibit a change of the onset temperature of the DPPC transition nor a drastic change in its enthalpy. For larger amounts of HA (6 mg) the transition temperature is shifted towards higher temperature with a decreased enthalpy (Table 2, Fig. 4). This finding is in agreement with previous results showing adsorption of HA onto DPPC bilayers and interactions with polar heads [31,32]. The incubation of liposomes with HA-DPPE at different concentrations induced a shift of the onset temperature of DPPC (from a  $T_{onset} = 42^{\circ}\text{C}$  to a  $T_{onset} = 38^{\circ}\text{C}$ ) 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 probably as a consequence both DPPE insertion and HA adsorption. A liposomal formulation prepared with 1 mg of DPPE induced a

significant shift of the main transition peak of DPPC that remained well defined, confirming the peak flattening observed with HA-DPPE is a 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 liposomes was evaluated by the carbazole assay. As can be observed in Fig. 5, the hydration procedure allowed to obtain similar amounts of HA or HA-DPPE associated to the vesicles. Namely, no more than 200–250 g of HA or HA-DPPE were associated to the vesicles probably as a consequence of several phenomena such as i) a physical entrapment in the vesicular aqueous compartment of the polysaccharides, ii) an interaction between the polar heads of the phospholipids and the HA, and iii) an integration of the DPPE molecules in the liposomal bilayer in the case of HA-DPPE. The incubation of HA or HA-DPPE with liposomes provided different behaviors. In fact, the post-insertion of HA-DPPE in extruded liposomes favored a significant increase of the amount associated with the vesicles (450–550 g/formulation) with respect to the HA (120–250 g/formulation) (Fig. 4). This trend clearly demonstrates that the DPPE residues were critical to allow a good interaction between HA-DPPE and lipid membrane. This effect was promoted by the greater surface area of the extruded liposomes (post-insertion) with respect to that of multilamellar vesicles (obtained after lipid hydration). In detail, the hydration of liposomal bilayer with the aqueous phase containing HA-DPPE and the following heating at 55°C promoted the encapsulation of the macromolecule in liposomes and the integration of the DPPE within the phospholipid bilayer but favored a saturation of the “binding sites”. On the contrary, the extrusion of liposomes and their incubation with the HA-DPPE at 55°C offered a wide surface area to allow a very good interaction of DPPE residues with other phospholipids present within the membranes. Carbazole assay is a test that could be influenced by many interferences and for this reason the same experiments were performed using capillary electrophoresis. The amount of both HA or HA-DPPE not interacting with liposomes was evaluated after the preparation of liposomes and the amount of compounds associated to the vesicles was obtained by the difference with the amount initially added. The results obtained

with this technique were in very good agreement with those aforesaid described (Figs. S4 and S5); for example, when 1 mg of HA was used in the hydration step, 20% remained adsorbed on vesicles, while less than 10% was detectable in the case of HA-DPPE (Fig. S4). On the contrary, when HA-DPPE was incubated with liposomes, 50% of the conjugate was detected on the liposomal surface, while the amount of HA that remained adsorbed on the vesicles was similar to that obtained by using the hydration technique (Fig. S5). This trend clearly demonstrates that the post-insertion technique could be a useful approach to allow coating of vesicles avoiding the hindrance of liposomal aqueous compartment. Moreover, the two techniques evidenced that a plateau of HA-DPPE associated to the liposomes was reached at a concentration 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 value is the mean of three different experiments  $\pm$  standard deviation.

Each value is the mean of three different experiments  $\pm$  standard deviation. Error bars, if not shown, are smaller than symbol size.

### 3.3. Evaluation of serum stability

Considering the physico-chemical characterization of HASomes obtained by the post-insertion technique of HA-DPPE with liposomes, a concentration of conjugate of 1–2 mg was suitable to provide an efficient coating of the vesicles with a slow variation of their size and polydispersity. The behavior of HASomes in medium containing serum is a fundamental information to be investigated before the encapsulation of drugs in these colloidal systems and their use in *in vitro* and *in vivo* experiments. For this reason, HASomes obtained by the post-insertion technique of 1 mg of HA-DPPE were incubated in 60% FBS at 37°C and their size was monitored for 4 days and compared to naked liposomes and liposomes incubated with 1 mg of HA. The mean sizes of the colloidal formulations have been investigated in absence of serum proteins in the same conditions as control and no aggregation was observed (Table S1). Fig. 6 evidences a stability of all formulations up to 12 h incubation, while afterwards an increase of the hydrodynamic diameter of all samples was observed. In particular, after 24 h incubation, naked liposomes and liposomes incubated with HA exhibit a dramatic increase of their diameter up to

450 nm, while HASomes showed higher but constant sizes (~300 nm). This trend is probably due to the lipid composition of the liposomes. Moreover, the hydrophilic layer surrounding the liposomal surface, promoted by the insertion of HA-DPPE in the vesicular bilayer, is probably more efficient than the HA physically associated to the liposomes to prevent the coating of serum proteins. This results from the strong interaction between the DPPE anchors and the vesicular lipophilic compartment that favors the retention of the conjugate. On the other hand, the spatial conformation of HA and HA-DPPE, adsorbed and post-inserted within liposomes, respectively, could play a crucial role to explain this phenomenon. Therefore size increase due to destabilization by serum protein adsorption is limited in the case of HASomes. Furthermore, naked liposomes showed a decrease of their surface charge down to  $\sim -12$  mV while the HA-coated formulations did not evidence significant variation of the Zeta-potential ( $-25/-30$  mV). These results are in very good agreement with the experimental data of Wolfram and coworkers that demonstrated the role of hydrophilic-coating agents like PEG to preserve the surface charge of liposomes after their incubation in FBS [24].

#### 4. Conclusion

Innovative compounds able to efficiently coat liposomes are necessary to allow the modulation of their physico-chemical features and their in vivo behavior. In this investigation, we demonstrate that hyaluronan conjugated to a phospholipid is able to interact with the liposomal phospholipid bilayer, generating by post-insertion technique novel HA-coated liposomes named HASomes a novel approach in the field of HA-coated vesicles. This method prevents the hindrance of the liposomal aqueous compartment and offers the opportunity to present the HA backbone on vesicle surface. In addition, the HA-coating can be used to increase the plasmatic half-life of liposomes avoiding the appearance of the ABC phenomenon due to multiple administrations of the PEGylated liposomes and to target the vesicles against cells over-expressing the HA receptors without affecting the possibilities of entrapping hydrophilic drugs in the aqueous compartment.

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